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Plant Biotechnology: Tool for Sustainable Agriculture

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Abstract

Agriculture has been the backbone of the human food supply directly and indirectly and global agricultural productivity must increase due to availability of limited agricultural land. Therefore, must increase in order to meet the increasing food demands. The agriculture was earlier practiced manually followed by modernization that allowed an increase in agricultural productivity. The cumulative recognition of biotechnology as an economic and social growth factor has stimulated countries to provide financial support to their local biotechnology companies to nurture research, development, and commercialization of ideas and products that have boosted biotechnological innovations and improvement in the quality and services. In this chapter the thrust will be laid on usefulness of plant biotechnology for increasing the diversity of genes and germplasm available for incorporation into crops and by significantly shortening the time required for the production of new cultivars, varieties and hybrids vis a vis contribution towards agricultural sustainability. In the last part of chapter, the conservation techniques for agriculture and sustainable development are documented with some case studies.

Keywords

Agriculture · Conservation · Genetechnology · Sustainability

1.1 Concept and Importance of Biotechnology in Agriculture

Any method that uses living organisms or products from these organisms to make or modify a product for increasing food production, and making agriculture more sustainable is called as agricultural biotechnology (FAO 2004; Hansson and Joelsson 2013). Genetic engineering technique is used to advance crop varieties that manage

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drought and salinity and disease resistant (Wang et al. 2003; Fuchs 2010) besides nutrients are taken more efficiently. And are desirable in a varying climatic conditions with increasing population and competitions for other land resources. The agricultural biotechnology is a contentious, and reviewers refer to the ecological and health risks complicated and to the negative effects of GM crops on small-scale traditional agricultural farming (Peters 2000). The biotechnology is contemplated eiher an important part of or a severe threat for sustainable agricultural production systems. However for assessing the implications of potential biotechnology sustainability it deals with the continuance of agricultural production systems over time and hardly anyone agreed-on description of agricultural sustainability instead, academic and policy related definitions vary (Dash et al. 2016).

The miscellany devoted to the concept of agricultural sustainability has steered authors to contend that it is fundamentally a challenged concept (Thompson 2010) and one such arena is concerns regarding the role of biotechnology in creating sustainable agricultural production systems (Connelly 2007). Further the increasing recognition of biotechnology as an economic and social growth factor has enthused governments in several countries to provide financial sustenance to their local biotechnology companies to foster research, development, and commercialization of ideas and products (Awais et al. 2010); But on the other side, predictions of malnutrition, famine, untreatable diseases and unresolved environmental problems have boosted biotechnological improvements and enhancement in the quality and services specified by various companies (Boccia and Sarnachiaro 2015) likewise production of GM crops designed at weed and pest control resulting in increased crop yields (Dash et al. 2016).

The efforts to alleviate poverty and inequality have established to be insufficient, mostly in rural and semi-urban areas notwithstanding the great biotechnological advances in agricultural production accomplished over the years. Contemplating the advances in technology, hunger has been translated into a preventable harm. In the past decade, consumer behavior concerning food products has been influenced by quality and safety (Boccia and Sarnachiaro 2015). Presently, highly processed foods with a high content of chemical products are not accepted among the population, and there is a clear tendency towards consuming fresh food. Biotechnology via genetic manipulation of crops, has offered a means to guarantee food production or crop nutritional improvement (Fernández-Suaréz 2009). However, GM crops have developed the center of debate due to the difference of opinions that arise around the aftermaths that the consumption of these types of food may have on human and environmental health. In this chapter, biotechnological improvements at different periods of history and their efforts have focused on trying to alleviate the shortage of food supply and associated malnutrition besides to discuss controversy around the application of various biotechnological tools for improving agricultural produce and production.

1.1.1 Biotechnological Advancement of Agriculture

Agriculture has been the mainstay of the human food supply via direct as well as indirect ways. Though, agricultural land is limited, due to the increasing world population consequently, global agricultural productivity must increase in order to meet the increasing food demands (FAO 2013). Agriculture is contemplated as one of the oldest activities experienced by humankind. The Industrial Revolution, which bridged from 1875 to 1885, permitted an accelerated economic development that became strategic for the growth of countries (FAO 2012), which in turn prompted the migration of people from rural areas to industrialized cities. The use of machinery on farms was imperative, progressing through remarkable improvements for increasing the agricultural production to meet consumer and producer needs, The overview of chemical fertilizers about the same period of time permitted crop protection against disease and attainment of higher yields.

Agriculture became a science by performing novel experiments aimed at improving agricultural methods in various developed countries (Cubero 1993), which eventually led to important innovations such as crop rotation and new discoveries such as the ability of some legumes to convert atmospheric N to NO_3 (Overton 1996). The earliest records of plant hybrids were given by Cotton Mather (1716) followed by development of plant hybridization in 1776. The Experiments on plant hybridization (Persley 1991), marked the beginning of new technologies intended to recuperate vegetable species. However, plant hybridization occurred long before Mendel's experiments; most likely it was unintentional at first and could have occurred at any stage in the crop domestication process (Adenle 2011). In circumstance, crosses for both floral morphology and hybrid vigor were greatly expanded by many researchers during the next 100-year (Teranishi 1978). In 1960, the new concept of Green Revolution for the rapid increase in food production, especially in underdeveloped and developing nations, via the introduction of high-yield crop varieties and the application of modern agricultural techniques changed agricultural farming (FAO 2004). The technologies developed during this period, which usually involve bioengineered seed that worked in conjunction with chemical fertilizers and heavy irrigation had an enormous influence on three main cereals (viz., maize, wheat and rice). Further in the 1960s, the discovery how the biological molecule of DNA was responsible for inheritance resulted into a predominantly imperative findings and the genetic code was cracked, and subsequent studies began the transfer of genetic material from one organism to another through genetic engineering techniques (Dash et al. 2016). And the intersection between genetic engineering and biotechnology remained the key factor in the creation of GMOs. International Maize and Wheat Improvement Center (CIMMYT), is one of the first agricultural research centers created in Mexico in the 1960s, with the help of the Rockefeller Foundation (FAO 2000). Today, wheat and maize produced from research at CIMMYT are planted in millions of hectares around the word. However, one constraint regarding

basic and applied agrarian research that is usually is carried out in advanced countries with stated climate conditions, and inventions and improvements in crop yield can only be acquired in similar conditions and paying way to adapt the new technologies and discoveries to warmer or more arid climates that prevail in underdeveloped countries.

The Itanca or soybean MON87701, was one of the first GE crops worldwide which articulated the crystal insecticide protein CytAc1derived from *Bacillus thuringiensis* (Bt toxin) that stipulated protection against feeding damage caused by some lepidopteran pests (EFSA 2011). Further, Monsanto introduced the GMO soybean MON89788, a crop expressing the cp4-epsps gene from the soil bacterium *Agrobacterium tumefaciens*, which encodes the EPSPS enzyme, providing resistance to the herbicide glyphosate. Monsanto subsequently engineered a stacked trait soybean using MON89788 x MON87701 (Then and Bauer-Panskus 2017), combining into one GMO the expression of the insecticidal Bt toxin, Cry1Ac, with resistance to glyphosate. GM soybean has made rapid advances in recent decades, and its cultivation area has been increasing yearly. The genetic modification has been efficacious in maize, tomato, rice and cotton plantations (Tabashnik et al. 2011), mainly by inserting exogenous genes that encode a protein that is toxic to specific pests.

It has been pragmatic that some microorganisms, including PGPR, fungi and cyanobacteria, have shown biofertilizer-like activities in the agricultural sector (Mahanty et al. 2016). The utilization of microbes as biofertilizers is currently being considered as an alternative to chemical fertilizers for crop production. Besides role of nanotechnology can boost agricultural production, considered as important tools in modern agriculture. Nanotechnology specifies new agrochemical agents and new delivery mechanisms to improve crop productivity, and it promises to reduce pesticide use through nano-formulations of agrochemicals. Nanotechnology also allows the application of nanosensors or nano-biosensors in crop protection (Bhupinder 2014).

1.1.2 Biotechnological Improvement of Food

Biotechnology has been employed in the food sector through the production of additives and ingredients as well as the improvement of more resourceful and less costly operations for the food production. In addition, biotechnological interventions have been focused on modifying or enhancing taste, aroma, shelf life, texture and nutritional value of food products, employing fermentation, enzyme technology, nanotechnology and molecular biology. Now it is observed that, technology would become a tool that would help solve the problems of the worldwide supply of food though it befell. However, it is convenient to note the great achievements that were carried out thanks to the discovery of microorganisms and the development of biological, biochemical and molecular techniques that allowed the progress of this branch of science.

Currently, the fermentation is nevertheless a very beneficial technique for food processing. Fermented food from different sources such as milk, cereal, fruits,

vegetables and meat have subsidized to the livelihood of large populous. For example, how lactic acid bacteria could accomplish the novel role of efficient cell factories for the production of functional biomolecules and food ingredients to enrich the quality of cereal-based beverages (Waters et al. 2015). As we know that enzymatic technology has been a gismo used for food biotechnology for optimizing and accelerating bioprocesses. A large number of enzymes are used in the food industry such as baking, juice processing, starch, dairy and other related industries, wherein enzymes play a crucial role as biocatalysts in the biotransformation process. Likewise in the bakery industry, proteases act on the protein of wheat flour, reducing the gluten elasticity and therefore reducing the shrinkage of dough (Dash et al. 2016).

1.1.3 Transgenic Food

Transgenic foods or genetically modified or engineered foods are produced/processed from organisms that have had changes introduced into their DNA using the methods of genetic engineering (Fernández-Suárez 2009; PALT 2014). Further the transgenic foods can include the following-

(a) Crops with genetic modification and can be edible e.g., Pest resistant corn crops.
(b) Food with an ingredient or an additive derived from a GMO.
(c) And those foods that uses a supplementary GMO product for their production, for example cheese made from recombinant chymosin obtained from a strain of the fungus *Aspergillus niger*.

Since it is well documented that GM foods are produced from GMOs and characteristically, GM foods are transgenic plant products. A tomato, called Flavr Savr, was the first commercial GM food, which was modified to ripen without softening by the Californian company Calgene (Bagwan et al. 2010). Baring from low price rates the production problems and competition for a conventionally bred variety with a longer shelf life prohibited the product from flattering profitable. Besides some disease preventive tomatos with three times more lycopene than conventional varieties has been developed (Awais et al. 2010). Genetic engineering plays a noteworthy role in enhancing proteins, vitamins as well as iron and zinc components by gene insertion. For example Golden Rice, variety of *Oryza sativa* was designed to produce β- carotene, a precursor of vitamin A, in rice (Bagwan et al. 2010). It was developed as a stimulated food to be used in areas with shortage of dietary vitamin A. thereafter one more new variety called Golden Rice 2 which produces up to 23 times more beta-carotene than the original version was developed. Bt corn is a variant of GM maize that expresses the bacterial Bt toxin, which is poisonous to the European corn borer. However, there are several foods from GM crops that are resistant to herbicides (glyphosate) and are resistant to insects (using Bt toxin), including crops such as soybean, canola, sweet corn and sugar beet

(Bagwan et al. 2010). Nonetheless, despite the distinctive biotechnological achievements, the consumption of transgenic food is still associated with their impacts on the Human environment and health (Boccia and Sarnacchiaro 2015).

The Biotechnology has played an imperative role in the progression of the health sector with numerous benefits to the human race. Biotechnological applications in health have taken advantage of the chemistry of living organisms for molecular biology or cell manipulation to develop new or alternative methods aimed at finding more effective ways of producing conventional products. The recombination of deoxyribonucleic acid (rDNA) is one of the common genetic engineering techniques used for the treatment and prevention of diseases. Similarly developing the transgenic food, which can also be valuable to human health, or agricultural biotechnology, which can produce more food to meet population demands. However the debatable point is regarding the distrust surrounding the production and consumption of GM foods. And the fact is due to uncertainty regarding the methods used in their development. GM food may have objectionable properties; transgenic seeds and plants may amend the microbial flora of the soil and could contaminate nearby crops (Bagwan et al. 2010) affecting the natural balance. Transferred genes may unenviably contaminate another organism, and organisms with mixed genes could arise between organisms that are evolutionarily distant, such as plants, animals, bacteria and even viruses (Chamas 2000).

In the economic sector, the use of GMOs in Europe has fortified agricultural biotechnology resulted in more prolific agriculture, increasing the incomes of farmers with a minor impact on the environment by reducing pesticide treatments (Zamora 2016). Transgenic Bt corn has saved 193 million euros in corn imports in Spain (Fundación Antama 2016). Further, 28 countries have planted 179.7 million ha of biotech crops; 20 of them are developing countries, and 8 of them are industrialized ones up to 2015. The United States is the largest producer, with 70.8 million ha, and Brazil is the top developing country, having planted 44.2 million ha of biotech crops (Fundación Antama 2016). Rural areas with developing economies absolutely stand to benefit from crops formed with biotechnology, whether by importing grain or seeds from foreign countries or developing their own GM crops (Delaney 2015).

1.2 Benefits of Genetic Technology in Agriculture

In the coming time, the recombinant DNA technology application to plant biology and crop production has the prospective impact on world agriculture. The general concept of agricultural technology is to isolate genes, direct their expression, monitor inheritance and re-insertion into plants. Plant biotechnology accompaniments plant breeding efforts by increasing the diversity of genes and germplasm accessible for incorporation into crops and shortening the time required for the production of new cultivars, varieties and hybrids as well. As for as economic perspective is concerned, the plant biotechnology offers noteworthy potential for the seed, agrochemical, food processing, and specialty chemical and pharmaceutical

industries to develop new products and manufacturing processes. Conceivably the most convincing attribute of the application of plant biotechnology to agriculture is its significance both to helping ensure the availability of environmentally sustainable supplies of safe, nutritious and affordable food for developed countries and to providing a readily accessible, economically viable technology for adopting primary food production needs in the developing world. The need for new agricultural technologies, in general, is driven by two distinct, and at times contradictory societal requirements confirming a safe, nutritious, and reasonable food supply for the planet and similarly minimizing the negative environmental impacts of food production itself. As per reports the world population will be doubling in the next 40 years up to 10 billion. The amalgamation of population increase, the decline in the availability of arable land, and the need for improvements in the quality of dietary intake in many developing countries means that agricultural production will have to be doubled or even tripled, on a per acre basis to meet this need. Simply put, farmers will have to produce more calories during the next 40 years than they have done in the entire history of agriculture.

Together, the societal concerns over the environmental impact of assured agricultural practices will increasingly check the types of tools used for crop production. There are some basic questions regarding the sustainable agricultural production regarding the increase the industrious competence of existing cultivated land without irrevocably damaging the planet. The answer is illusorily forthright investment in and development of new agricultural technologies is categorically critical for a future sustainable agriculture. Contemporary agricultural technologies such as plant breeding and agrochemical research and development will continue to play a chief role in promising a plentiful and safe food supply, ecologically sensitive and economic farm management practices. Plant biotechnology is exclusively important tool, which can significantly impact crop productivity and that is compatible with sustainable, environmentally sound agricultural practices. Besides it is a technique that is definitely the source of value-added genes and traits for increasing the farmer productivity and profitability. There are numerous technological applications for the improvement of productivity and production that is documented hereunder:

Plant genetic engineers currently have in hand a large battery of regulatory sequences that provide for both constitutive expression as well as highly accurate targeting of gene expression to specific tissues within transgenic plants (Benfey and Chua 1989). Moreover, established differential screening methods allow for ready isolation of regulatory sequences that may be required for even more sophisticated expression requirements (Shewmaker et al. 1994). The ability to decrease endogenous gene expression in plants represents a remarkably powerful tool, and striking phenotypic alterations have been observed by selective inactivation of genes using antisense technology (Oeller et al. 1991). Achieving even higher levels of gene expression in selected plant organs would increase opportunities for more economic specialty chemical or pharmaceutical production in plants, and site-specific insertion could minimize the variability of gene expression among transformants. However, current expression systems appear sufficient for meeting immediate crop improvement needs.

Extraordinary development has been made in the progress and application of gene technology transfer to crops. More than 80 species of crop plants have been genetically manipulated using available *Agrobacterium tumefaciens* or a variety of free DNA delivery transformation systems including all the major dicotyledonous crops and other monocotyledonous crops as well. Further it is likely that routine gene transfer systems will exist for nearly all crops within the years to come. It is obvious that technical developments will lead to further upsurges in transformation efficiency, extend transformation to elite profitable germplasm and lower transgenic plant production cost baring any significant barrier to the application of plant transformation technique in crop improvement.

Advancement in identification and isolation of new gene coding sequences are of unlimited significance to the improved engineered plants. The interspecies-specific use of transposons and T-DNA insertion has permitted the tagging and isolation of novel genes from several plant sources (Chuck et al. 1993). The availability of high resolution physical maps in Arabidopsis and tomato has previously led to mapping of several novel loci and new methods will allow direct testing of the isolated DNA for its ability to accompaniment the mutation of interest. Advances in the redesign of coding sequences for plant expression allow for foreseeable, high-level expression of a variety of non-plant genes in crop plants (Adang et al. 1993). An ongoing research efforts will certainly and severely increase the probability and efficiency of gene discovery and isolation, it would emerge that even with today's methods the most genes might be identified and isolated. The gene discovery will not be a limiting element for very long although only meager advancement in gene discovery and sequence analysis has been made.

It appears almost firm that plant biology is entering a unique period where both basic research and commercial applications will be limited only by the ingenuity of the researcher and by funding levels. There are no substantial technical hurdles remaining although there is an understandable need for extensive expansion of our considerate of basic plant biochemistry and physiology for exploiting scientific advances. The progress in the field has been exceedingly rapid, and genes conversing these new traits have already been successfully introduced into several significant crop species.

1.2.1 Insect Resistance

The plant production with natural insect control is clearly an important implication for crop improvement in seed and agrochemical industries. *Bacillus thuringiensis* (Bt) genes were frequently used in emerging of insect control in transgenic plants (Pigott and Ellar 2007). Most strains of Bt. are toxic to lepidopteran (caterpillar) larvae, though some strains with toxicity to coleopteran (beetle) or dipteran (fly) larvae. The mode of action of the B.t. insect control protein involves disruption of K^+ ion transport across brush border membranes of susceptible insects. Transgenic plants like tomato, tobacco, cotton and maize comprises Bt. gene (Koziel et al. 1993). A novel approach for increasing expression of Bt genes in plants, which

involves restructuring of the DNA coding sequence without altering the encoded amino acid sequence, has managed to significant enhancement in insect control (Sanahuja et al. 2011). Similarly the cotton plants with a high level of resistance to boll damage by caterpillars have been established (Perlak et al. 1990). In numerous field studies that confirm exceptional protection from bollworm, budworm and pink bollworms. Excellent protection from defoliation by Colorado potato beetle has also been observed in greenhouse and field experiments with potato plants containing the novel coleopteran active Bt tenebrionis gene (Perlak et al. 1993). The insect resistant plants sustained no damage from Colorado beetles through the growing season under circumstances of high insect pressure. Widespread efforts are under way to identify other microbial and plant insecticidal proteins for protection from insect pests. The genetically engineered plants express different proteinase inhibitor genes to enhance resistance to a range of insect pests (Boulter et al. 1989); *in vitro* studies indicate the α-amylase inhibitor protein has broad-spectrum insecticidal action (Huesing et al. 1991). It is well documented that introduced genes will provide an enormous percentage of insect control in annual crops in the next 2-3 decades.

1.2.2 Weed Control

The engineered plants provide an alternative approach to for crop protections to specific herbicides. The RandD efforts by private companies have so far focused only on those herbicides with minimal environmental impact, with emphasis on properties such as high unit activity, low toxicity and rapid biodegradation (CAST 1991). Further it was ensured that herbicide-tolerant genes would not be introduced into crops which could become ”volunteer” weeds in ensuing crop rotations or which outcross eagerly with weed species. The improvement of crop plants, which are tolerant to such herbicides, would offer more effective, less costly and more environmentally sound weed control options. There is two general methods followed in engineering herbicide tolerance: altering the level and sensitivity of the target enzyme for the herbicide and integrating a gene encoding enzyme, which can deactivate the herbicide. The tolerance to Roundup® herbicide has been engineered into soybean, cotton and maize by introducing genetic constructions for the overproduction of herbicide resistant EPSPS enzymes (Shah et al. 1986). Confrontation to gluphosinate, the active ingredient in Basta®, and bromoxynil has been accomplished by the alternate approach of introducing bacterial genes encoding enzymes that deactivate the herbicides by acetylation or nitrile hydrolysis (de Block et al. 1987; Stalker et al. 1988) respectively.

The existing crop targets for engineered herbicide tolerance comprise soybean, cotton, maize, rapeseed and sugarbeet. While choosing a particular weed control system for the farmers, many factors that is weed spectrum, herbicide performance, environmental impact, seed and chemical cost, application timing and flexibility have to be contemplated. The obtainability or accessibility of herbicide tolerance in annual crops over the next decade will give farmers more suppleness

in choosing effectual and less costly options for weed control. Herbicide-tolerant plants will have the constructive impact of shifting overall herbicide usage through replacement of more efficient and environmentally tolerable products. Such developments in chemical weed control will also allow for higher implementation of minimum tillage practices, and encourage crop rotations to further reduce soil erosion (CAST 1991).

1.2.3 Disease Resistance

Momentous resistance to a variety of plant viral diseases has been accomplished by coat protein-mediated protection involving expressing the coat protein gene of a particular virus in transgenic plants (Teh and Hofius 2014). The coat protein mediated cross protection process is likely to encompass interference with the un-coating of virus particles in cells prior to translation and replication. The results have been obtained satisfactory for transgenic tomato, alfalfa, tobacco, potato, melon and rice against a broad spectrum of plant viruses, including alfalfa mosaic virus, cucumber mosaic virus, potato virus X (PVX), potato virus Y (PVY) and potato leaf roll virus (Beachy et al. 1990). Outstanding tolerance has been perceived in field tests of Russet Burbank potatoes containing coat protein genes to both PVY and PVX (Kaniewski and Thomas 1993). Lately, very significant resistance to TMV in tobacco plants has also been achieved by an expression of a subgenomic viral replicase component (Schulze-Lefert and Panstruga 2011). Likewise the resistance to the bacterial pathogen *Pseudomonas syringae*, which causes wildfire in tobacco, has been introduced in transgenic tobacco by expressing a tabtoxin resistance gene that codes for an acetyltransferase (Anzai et al. 1989) that proves a successful method to engineering disease resistance in plants by detoxification of pathogenic toxins (Schweiger and Schwenkert 2014). Similarly the chitinase gene from *Serratia marcescens* was sturdily expressed in transgenic tobacco (Lee et al. 2016). The preliminary results indicated that the expression of the bacterial chitinase in transgenic tobacco leaves resulted in suggestively reduced sternness of disease produced by a brown-spot pathogen, *Alternaria longipes*. The plants were reported to have significantly reduced fungal lesions as well as delayed vulnerability to the pathogen. A bean chitinase gene driven by a high level, constitutive promoter has been articulated in tobacco plants (Swaminathan et al. 2016). These plants exhibit increased resistance to the pathogenic fungus *Rhizoctonia solani*, resulting in expressively reduced root damage and enhanced ability to survive in infested soil. Genes conferring fungal resistance based on the plant's own defense response are being cloned as one of these proteins, termed osmotin, has been revealed to have potent *in vitro* activity against *Phytophthora infestans*, the causal agent of late blight disease in potato (Swaminathan et al. 2016).

1.2.4 Stress Resistance

Many abiotic stresses involving water, temperature and soil composition are known to impact crop productivity. Although the complication of plant stress responses has eluded early validation of improved phenotypes using plant biotechnology methods, that are applied to dissect and comprehend the molecular basis for plant response. A number of plant genes induced by exposure to heat, cold, salt, heavy metals, phytohormones, nitrogen etc., have been acknowledged (Benfey and Chua 1989). Additionally, rapid progress is being made in identifying ion transport pumps and proteins, which regulate transport of molecules through channels and plasmodesmata. Further some metabolites like proline and betaines have been associated in stress tolerance in both bacteria and plants and to evaluate the potential of these metabolites to alleviate stress in engineered plants and comprehend their mode of action is under way (Van Camp et al. 1994).

1.3 Genetic Contributions Towards Agricultural Sustainability

Yield potential has not reached an asymptote even in the most extensively improved crops such as maize, wheat, rice, soya bean and cotton. The biological potential of these crops has continued to be increased by plant breeding systems aimed at increasing harvest index, water use efficiency, nutrient acquisition and genetic protection against biotic and abiotic challenges. Protection against pathogens and pests often enables a crop to continue to be produced in an area where the entry of a virulent pathogen or the evolution of a new strain of an endemic pathogen would otherwise have made production uneconomic (Frisvold and Reeves 2011). For example, rust devastated wheat yields in regions of Australia until breeders produced varieties resistant to the pathogen challenge. There are similar examples for all crops and their accompanying pathogens and pests.

Over the past 100 years most crop yields in agriculture have been increasing steadily. Agronomic management and plant improvement programmes have contributed significantly to these increases (Aerni 2010). In recent years, these two components of yield improvement have become more intimately intertwined with inbuilt genetic traits delivered in the seed being able to replace some management inputs, particularly in pest control. The gap between best farm yield and yield potential of the crop for a range of input regimes (Fedoroff 2010) have been closed because of improved management. In parallel, the average farm yields have approached best farm yields as a consequence of better extension services, accessible computer decision support tools and increased abilities of farmers to recognize and adopt best industry practice (Hess et al. 2013). In the most extensively improved crops such as maize, wheat, rice, soya bean and cotton, Yield potential has not reached an asymptote. Plant breeding systems aimed at increasing harvest index,

water use efficiency, nutrient acquisition and genetic protection against biotic and abiotic challenges have increased as a result of biological potential of these crops. Protection against pathogens and pests often enables a crop to continue to be produced in an area where the entry of a virulent pathogen or the evolution of a new strain of an endemic pathogen would otherwise have made production uneconomic (Frisvold and Reeves 2011).

Identifying sources of resistance genes or more correctly, resistance alleles in wild relatives of crop species and in the extensive germplasm collections available for most major crops that can act as gene donors through sexual reproductive methods (Varshney and Dubey 2009), plant breeders have been remarkably skilled. To access alleles from distantly related species genetic system manipulations and Embryo rescue are frequently needed. Rarely the introduced genes have been unrelated to the genes of the crop species and overall they are usually accommodated by the metabolic and cellular pathways already existing in the crop species (Varshney and Tuberosa 2007). By the introduction of single genes in different allelic forms, not all breeding goals have been met. In many cases, the breeder has had to cope with introducing alleles of several loci, often unlinked, which have products that interact to produce the desired phenotype. Breeders have also faced another hurdle of gene product interaction where the entry of the new allele produces the needed phenotype, but has pleiotropic, sometimes negative, effects on other traits (Araki and Ishii 2015). Polygenic inheritance with pleiotrophic have been dealt with by various strategies in the construction of breeding systems, mostly without any obvious understanding of what is happening at the molecular, cellular or tissue levels of plant function and in polygenic inheritance these complications have caused major obstacles in achieving breeding system objectives (Thompson 2011).

The power and efficiency of plant improvement programmes has increased as result of recent advances that have increased our understanding as how plants function and develop (Forster et al. 2015). The molecular and cellular mechanisms and pathways behind plant architecture, development and function, the regulation of gene expression and the knowledge of gene and genome sequences have provided new opportunities for breeders to rationally design improvement programmes providing for more homeostasis in the environmental responses of a crop and to better mould the phases and components of plant development within the constraints set by the crop environment. Owing to heterogeneity in field-based bioassays and the uncertainty of environmental pressures (Brown et al. 2014) breeders are confronted with difficulties in their selection programmes. DNA sequence has provided sequence markers for desired alleles and, in some cases, these enable a breeder to bypass bioassays and environmental assays that previously had introduced major constraints and unreliability into breeding programmes. Robust and stable phenotype for different genetic traits has also facilitated the stacking of duplicate systems of protection (Bado et al. 2015). Gene interactions and pleiotropic effects are now frequently understood at a molecular and cellular level with breeders being able to specifically avoid some of the negative interactions, e.g. selection for a subset of functions of certain transcription factors or selection for more specific phenotypes with a high level of understanding of the feedback loops in metabolic

pathways and their impacts on phenotype. The global population increase has demanded more powerful and more efficient plant breeding for our major food plants. More food needs to be produced but on the available land to full fill the needs of next 30–40 years. In fact, since a significant proportion of the lands on which we now have agricultural production is already marginal, with continued agriculture leading to increasing damage, then the challenge is to produce the required additional food on rather less land than we use now.

The way ecosystems are working in the world and the increase in the knowledge about this, our societies are demanding that our agricultural production systems work with empathy to the environment and not in opposition. The removal of nutrients from the soil is an inevitable process but these nutrients must be replaced at the same time, the agricultural production lands should not be seen to be damaging the adjacent non-agricultural production ecosystems. In addition the natural resources like soil, water and air should not be put under threat as a consequence of agricultural practices. If this approach is followed then we will surely approach sustainability needed for the food production systems of the world. Human nutrition has highlighted many food products and processes food products are less than optimal for human health. There are clear deficiencies in our food supply in both developed and developing countries as evidenced by major non-infectious and non-communicable diseases, such as heart disease and diabetes. When one major food commodity forms a major staple of the diet nutritional deficiencies are particularly evident in developing countries. In developing countries there is ample choice for food, the fault lies with both dietary habits and lifestyle choices. With the increased knowledge of human nutritional requirements and our increasing abilities to modify the characteristics of our food plants, we have a clear expectation that modern plant breeding should be able to enhance the nutritional qualities of our major food plants so that they approach the optimal composition for human health regardless of lifestyle.

The other main common objective that plant breeding can address includes the rising global energy needs and the reduction of petroleum-based resources. The renewable resources are thus gaining the greater attention across the world. In addition to the production of ethanol during fermentation of plant sugars, or methane production from waste plant products as well as diesel from plant oils, it is extremely apparent that the crops can also produce important pharmaceutical and other industrial products, which are having the benefit of sustainable supply chain that is based on agriculture. With the introduction of new technologies for genetic alteration and also the new levels of knowledge regarding plant genomes, the requirement of modification to our crop plants has been increased enormously. But there still exists a hitch to deal with such developments as many social communities of the world do not agree to accept the food and other goods that are produced from transgenic crops, or those crops whose few traits/characters have been developed by means of certain laboratory technology and not through the usual process of sexual reproduction. The majority of the countries across the world readily accept the products in medicine that are engineered genetically like genetically engineered human insulin as well as human growth hormone, as these products are proved to

improve the quality of life thus such products are used easily by the customers throughout the world. On the other hand, the genetically modified or transgenic crops receive a low level of acceptance may be due to downright fear and animosity. A large number of people also believe that these modified crops are only a means of success for big business and therefore does not provide any means to improve the quality of life of an individual. However, this view changed with the introduction of first generation Genetically modified crops that provided both the economical as well as environmental benefits to the society in general and the farmers in particular without posing the overwhelming impacts with respect to both ecological and health as these technologies deliver crops that produce better foods as well as improved drugs and vaccines that provide effortless mechanisms for delivery and thereby improving the health quality in developing countries of the world. The one main reason responsible for meager public image regarding the benefits of these genetically modified crops is the propaganda and the inaccurate information that is being provided by the media across the world. But now days, the scientists have gained a success by the use of new technologies for accomplishing a significant advancement in understanding about the development and functioning of plants. We have greatly enhanced the abilities to offer suitable genetic information regarding our main crops so that they can optimally execute in a wide range of environment and also present us with those food products that are personalized to nutritional necessities of humans. But as researchers we have been unsuccessful in effectively combating the campaign of inaccurate information that is being provided by different activist groups who for their personal benefits speak ill about the transgenic crops as well as about the whole food production systems.

1.3.1 Function and Regulation of Plant Genes- A Firm Foundation for the New Genetics in Crop Improvement

The controlled expressions of approximately 30,000 genes that are present in the whole genome of plants represent the different ways in which the plants can develop and thus respond to environmental conditions thereby producing the best possible yield of food and fibre. The role of genomics is to define the role of these genes, determination of how these genes are regulated and the interaction among the gene products. Thus, these findings can help in improvement of the crops. The genome of a dicot, *Arabidopsis thaliana*, which is related to canola and cabbage, and the genome of a monocot, like rice, had been entirely sequenced (Goff et al. 2002). However, the sequencing of large number of species including maize, lotus, *Medicago truncatula*, grape, poplar and tomato is in progress (www.ncbi.nlm.nih.gov/genomics). The sequenced genomes of both *Arabidopsis* in case of dicots and rice in case of monocots provide a basic reference for all crop plants. The genetic content of these two extensively different species is extremely related and it is believed that further sequencing of the genome will strengthen the resemblance of genetic makeup of all the flowering plants. The association of genetic content of the diverse species is not necessarily seen through the way in which these genes are

being regulated and by the association of their products in regulatory networks as these properties can vary noticeably. Thus in the differentiation of species, the distinction in patterns of gene expression plays an important role. The genes that signify the secondary metabolites or even the particular characteristic like structural property have been developed from a common gene pool. The opportunity that provides a variation to be acted on by means of natural selection is provided by gene duplication process as occurs in polyploidy and the attainment of separate roles and patterns of expression by individual duplicated genes (Adams and Wendel 2005). In order to know which genes are involved in identifying a particular character of a plant, the genomics plays a critical role. For identifying the function of a particular gene we need to compare its nucleotide or amino acid sequence with the sequences present in the databases derived from genomes of the other species. The function may perhaps be allocated through resemblance to other genes whose function is known, thus genomes help to identify the function as it can have utility across species or even across the kingdom. Genome-wide mutagenesis by means of transposable elements like Ac/Ds, Tos17, which is an endogenous retro-transposon of rice or T-DNA insertions, have resulted in production of populations of several lines, in which each line in a population contains an insert in a particular gene. As the sequence of the DNA of insert is well known, therefore it is easy to find out the gene cloning that has been disrupted by the flanking sequence. There is a freely available set of Arabidopsis lines containing inserts that are present in around 80% of the genes and similar proportion has been tagged in case of rice also (www.arabidopsis.org/abrc/ecker_frank.jsp) and (Hirochika et al. 2004). Such tagged lines could be made homozygous and we can determine their phenotypes in order to relate a gene with an exact phenotype. Thus the tagged genes can become candidates for crop development as markers of DNA or directly in transgenic breeding. Interlude of the gene activity may also be produced by RNAi, the supplementary type of mutagenesis, with the help of which a construct initiated into a plant gives rise to a double-stranded RNA that stimulates a degradation mechanism which is sequence-specific that interrupt the mRNA of the target gene that may produce a phenotype (Wang and Waterhouse 2002). For functional genomics the advantages of RNAi is that the RNA constructs that are targeted to a gene proceed in a dominant way, and thus we can target any gene present in the background such as a mutant background. Currently, different kinds of synthetic microRNA are used for gene silencing. Such type of microRNA plays an important role in controlling genes that are involved in development of plants and stress response so that the new technologies offer the supplementary choice for gene silencing. Since the microRNAs are smaller around 21 nucleotides in comparison to 200–300 bp that are typically targeted by RNAi, conserved areas in the gene families may be targeted, thereby silencing several genes at the same time (Alvarez et al. 2006; Schwab et al. 2006).

The fundamental feature of using genomics to improve crops is that there should be the capability to use high-throughput technologies to monitor the changes in the phenotype. However, the phenotyping involves the programmed growth measurements and imaging under different stress conditions of the environment. It also involves field-based screening like characters, which are valuable in glasshouse

and are sometimes not maintained in the field. Therefore, such a high-throughput approach can form the foundation for the large number of international consortia to describe alterations or silenced lines in the specific classes of *Arabidopsis* genes for example Agrikola, to categorize the utility of specific kind of transcription factor genes (Hilson et al. 2004). Gene array or microarray is the second type of resource that expands and complements the genome sequences and consists of different number of oligonucleotides or cDNAs that are displayed on the slides. In case of the sequenced genomes, all the predicted genes can be incorporated on the array. Then the array can be hybridized to RNA extracted from the tissue of particular origin or from the developmental stage of mutant or a plant that is subjected to environmental stress or any disease, in order to verify the genes that are expressed in comparison to expression in control wild-type plants. The fundamental principal for microarray is that if a particular gene is expressed in certain circumstances, it can play an important role in that specific condition for example the genes that are induced by salt offers protection in saline conditions. However, for those genomes that are not sequenced, anonymous cDNAs or expressed sequenced tags (ESTs) could be arrayed and hybridized in the same manner and thus afterward the candidate genes can be sequenced. Microarrays can be chosen on the basis of similarity in gene expression patterns when compared to known genes. Cost reduction in sequencing ha lead to the enhanced development of higher throughput sequencing of cDNA gene expression libraries indicating gene expression levels. In order to EST to its gene sequence, parallel signature strategies of sequencing are a better point of start for determination of gene expression levels in a gene present in the particular tissues etc. from which the libraries are made. Conserving biological processes and genes between the species can conserve expression patterns. Thus investigation of the Arabidopsis expression databases in which different microarray experiments have been gathered for public access (www.genevestigator.ethz.ch/) provided information about the functional role of a gene by comparing it with gene sequence from other plant (Baker et al. 2006).

After identification of a candidate gene from comparison of sequences and expression patterns i.e. increase or decrease in activity level can be used for confirmation of importance in a specific gene pathway. Gene specific Knockout and over expression lines can be passaged through a microarray experiment. Complete genome sequence availability allows the tilling arrays production in which not only the coding regions of the gene but all the genomic bases are arrayed in an overlapping manner. Tilling arrays allow assaying of both transcripts that are associated with coding region and those that are not associated with coding regions for example small regulatory RNAs that are known to play role in gene expression. Epigenetic mechanisms like methylation or histone modification of DNA determine the changes in gene expression by using whole genomic arrays. Further these epigenetic controls con be probed by immune-precipitation of protein bound to DNA which act as transcription factors or other regulatory proteins that allows in mapping regulatory regions in the genome and helps in identification of transcription factors that regulate different genes. In addition these arrays assist in rapid sequencing of related species, help in understanding the association between phenotypes and sequences and define

the gene structures and linkages conserved in evolution and speciation. Wu et al. (2006) carried studies on cotton in order to understand the genes that control the physiological processes and compared the gene expression at early stages of fibre initiation with those expressed by fibreless mutants. he concluded that genes play an important role in fibre initiation and quality by identifying the unexpressed genes in mutants. Differential expression of many genes has been demonstrated by monitoring the changes in changes in gene expression. These differential changes depend upon the paternal or maternal gene i.e. genes are imprinted. The imprinting of genes acts as an example of epigenetic regulation controlled by DNA architecture rather than sequence (Autran et al. 2005). In plants the developmental transitions like flowering, seed development and vernalisation are controlled by Polycomb group proteins (a repressive protein complex) that modify chromatin mediated through epigenetic control (Kohler and Grossniklaus 2002). Crop improvement can be achieved by decrease in gene activity called as gene silencing that is done using RNAi technology. This technology is now used widely used to achieve novel crop phenotypes. The sensitiveness of gene expression to developmental and environmental factors poses a challenge in controlling the expression of genes for plant improvement programmes. Changes in single gene expression can dramatically affect in multiple pathways due to complexity in gene interaction networks. Modeling of Interactions to understand gene regulatory networks is an emerging field in genomics. This approach will greatly enhance the ability to harness gene activity for plant improvement.

1.3.2 Improving the Essential Amino Acid Balance in Plant Proteins Used for Food and Feed

A large population and farm animals around the world depend on seeds for their dietary protein. However, the protein in seeds can have a skewed amino acid composition due to the high abundance of a limited number of individual seed storage proteins. Amino acids are classified as non-essential and essential. Animals are unable to synthesize essential amino acids and thus obtain these from the diet. Due to the insufficiency in amino acids, malnutrition can be caused and thus reducing the efficiency of animal production. In spite these deficiencies can be counterbalanced by combining two or more seeds, animal feeds are still added with synthetic amino acids for maximizing nutrient content (Habben and Larkins 1995). In developing countries, upto 90% of food uptake can be derived by balancing amino acids of individual seeds of a single species of crop. In recent years, mutation Plant breeding and modifications in genome has been successfully used in modifying composition of amino acids in cereals and legumes. This section is focused on modification of mainly grain legumes to improve their content of the essential, sulphur-containing amino acid, methionine. Three approaches have been used: genetic modification to increase methionine biosynthesis; genetic modification to increase methionine storage in protein; and selection of mutants with increased methionine.

1.3.3 Engineering the Methionine Biosynthetic Pathway in Plants

Sulphates taken from soil are reduced in plastids of plant cells and then incorporated an amino acid backbone derived from serine via the action of the enzyme serine acetyltransferase and producing cysteine. Cysteine is the first stable S- metabolite in the cell. It acts as substrate for other biochemical pathways. Sequential action of three enzymes viz; cystathionine γ-synthase (CGS), combines O-phosphohomoserine from the aspartate amino acid pathway and cysteine result in the synthesis of methionine (Leustek and Saito 1999). Various studies have been carried in the field of gene manupulated enzymatics of reductive sulphur assimilation and biosynthesis of amino acid (Amir and Tabe 2006). In genetically modified plants, leaves have an increase in methionine as well as cysteine, sometimes at specific growth stages. Protein bound amino acids are abundant in plants than free amino acids. However, minor effect has been observed on total methionine content due to gene manipulations. For example, constitutive expression of a CGS enzyme from A. thaliana in GM tobacco or GM alfalfa increased free methionine in the leaves but had no significant effect on protein-bound methionine (Hacham et al. 2002; Bagga et al. 2005). Contrary to this generalization large increase in both free and protein bound methionine in the leaves of GM tobacco have been obtained by expression of mutated form of CGS showing an abnormal phenotype (Hacham et al. 2002). In summary, in most studies, increasing flux through the methionine biosynthetic pathway seems to have produced little increase in the methionine content of endogenous plant protein. A natural maize mutant was identified by screening for germination on media containing lysine plus threonine, a combination that inhibits flux through the aspartate amino acid biosynthetic pathway, leading to methionine starvation. The mutant maize seeds showed high levels the sulphur-rich δ-zein, a methionine-rich seed storage protein. The further analysis of the mutant revealed a lesion in a post-transcriptional control mechanism that normally suppressed δ-zein transcript levels (Swarup et al. 1995). The same high-methionine phenotype was subsequently engineered in GM maize by mutation of the δ-zein gene to remove the target site for negative regulation by the *dzr1* locus. The modified maize had methionine levels theoretically high enough to obviate the need for synthetic methionine in animal feed formulations containing the GM seed (Lai and Messing 2002). Both genetic modifications and mutation breeding have been successfully used to improve the nutritionally important sulphur-containing amino acid methionine in these plants. In both cases, these modified plant products with improved seed storage protein composition will be screened for any change in allergenicity prior to commercial release, because many seed proteins are known to elicit allergic responses in some people (Mills et al. 2003). The intention of increasing methionine content, and thus the nutritive value, of plant protein is currently being achieved to a large extent and will continue to develop in future.

1.3.4 Starch Biosynthesis and Functionality

The paradox between the apparent structural simplicity of starch, and its synthetic complexity has fascinated researchers for several decades. The simplicity of the structure arises due to the fact that starch is composed of glucose monomer, that is linked to form the polymer through just two bond types, α-1,4 glycosidic and α-1,6 glycosidic. However, the different functional properties of starch arrise due to the heterogeneity of chain lengths and total molecular weight, heterogeneity in the placement and number of α-1,6 linkages. Further the complexity arise because starches are laid down in granules, and the control of granule size, number and structure adds a further layer through which functional properties are determined. The molecules within a given starch can be classified into amylose and amylopectin. Amylose, is a relatively linear α-1,4 glucan of relatively low molecular weight (degree of polymerization from 500 to 2000) and having less than 1% α-1,6 branch points, however amylopectin, is a highly branched molecule with a relatively high molecular weight (degree of polymerization 5000–50000). The initial committed step in the starch biosynthesis is the formation of ADP–glucose from gluose-1-phosphate and an ATP. This committed step is distinctive to starch biosynthesis, acting as important step for the regulation of flux to starch synthesis as compared with other metabolic needs. In the cereal grain, it has long been recognized that the enzyme catalysing the formation of ADP–glucose is ADP–glucose pyrophosphorylase. This enzyme regulates its activity at three levels. Firstly, the ADP–glucose pyrophosphorylase is present in both cytosolic and plastidic forms (Denyer et al. 1996). In the developing endosperm the majority of the enzyme flux is via the cytosolic form, while in chloroplasts the plastidic form dominates. Secondly, the enzyme is subject to redox control, apparently coordinating activity levels with photosynthetic flux. Thirdly, the enzyme is also subject to allosteric regulation, being activated by 3-phosphoglycerate and inhibited by inorganic phosphate (Ghosh and Preiss 1966). However, the mechanisms how these regulatory mechanisms interact to change the flux through the starch synthesis pathway are yet to be completely understood. The synthesis of amylose requires granule-bound starch synthase (GBSS), this enzyme is principally located within the starch granule. There are evidences that other enzymes also contribute to the amylose synthesis, but GBSS being absolutely required for its synthesis (Ball and Morell 2003). In endosperm, there are separate GBSS genes expressed than in other plant parts and hence providing basis for the differences in amylose content as well as structure between leaf and endosperm starches (Edwards et al. 2002).

The amylopectin synthesis is complex, involving a range of enzymes. Firstly, the enzymes for the elongation of amylopectin chains. Plants contain a family of starch synthases with differing substrate specificities, called isoforms that are responsible for the elongation of amylopectin. Genetic analysis suggests that these isoforms have differing roles in amylopectin synthesis. The synthesis of the short external

chains of amylopectin is thought to be carried by the enzyme Starch synthase (SS) I (Delvalle et al. 2005), whereas SSIIa is responsible for the synthesis of longer chains, from DP12–20. Elimination of this enzyme in barley (Morell et al. 2003), wheat (Yamamori et al. 2000) and rice (Umemoto et al. 2002) led to a very characteristic phenotype having reduced amylopectin external chain length, reduced granule gelatinization temperature and reduced starch swelling properties. The role of the enzyme SSIII is not yet clear but this enzyme, along with GBSS, is known to contribute to the synthesis of longer chains in amylopectin (Zhang et al. 2005). There are at least two other classes of starch synthase genes SSIIb and SSIV present in the genome of rice. Both the genes are primarily expressed in the leaves and the role they play is being defined currently. In monocots, there are three branching enzyme genes known so far, branching enzyme (BE) I, BEIIa and BEIIb. The mutation studies in a number of monocot species indicate that the effects of eliminating BEI activity in a normal background range from undetectable to extremely subtle (Regina et al. 2004). Moreover the effects of BEI mutations are only seen in a background lacking either BEIIa or BEIIb. Mutants in all the three genes in maize have been identified so far and also the double mutants have been constructed. The mutation of BEIIa gene indicates that there is no noticeable effect on the amylose content or the starch structure in the endosperm, however this mutation has indicated a remarkable effect on the leaf starch. The mutations in BEIIb have been already known to result in a phenotype with high-amylose content, in keeping with the observation that this is a major BEII isoform expressed in the endosperm. In wheat Regina et al. (2006) have demonstrated the expression of BEIIa and BEIIb, where BEIIa is more highly expressed than BEIIb and to get increased amylose content, suppression of BEIIa, rather than BEIIb, is important. A puzzle in the starch synthesis research is the role of debranching enzymes. Genomic studies in a wide range of plants have shown four debranching enzyme genes in the plant genome, among the sequenced genes three are isoamylase-like genes (isoamylases 1, 2 and 3) and one is pullulanase- (or limit dextrinase-) type gene (Morell and Myers 2005). Mutation studies in a number of species, including rice (Nakamura et al. 1996), maize (James et al. 1995), barley (Burton et al. 2002), *Arabidopsis* (Zeeman et al. 1998) and Chlamydomonas (Mouille et al. 1996), reveal that mutation in isoamylase 1 leads to a phenotype with low-starch and high-phytoglycogen. More recent data suggest that an identical phenotype is obtained when isoamylase 2 is mutated. This may be because it is suggested that isoamylase 1 and 2 form a complex and their function is abolished if either of the two is absent is absent. The role of isoamylase 3 still remains unclear. Pullulanase mutants have only a subtle direct phenotype (Dinges et al. 2003) but have major effects in an isoamylase 1-deficient background, indicating that there may be some functional overlap between the two debranching enzymes. The role played by these debranching enzymes in starch biosynthesis is yet not absolutely clear. According to one of the views isoamylases are directly involved in the starch biosynthesis, ‘editing’ the

emerging amylopectin molecule such that a crystallization-competent amylopectin is formed in the crystalline lamellae regions of the starch granule (Myers et al. 2000). This view is supported by observations that relate the level of activity of isoamylase in the developing endosperm to corresponding changes in branch point frequency and starch structure in starch granules (Kubo et al. 2004). Other views are that isoamylase plays a role in removing highly branched phytoglycogen from the amyloplast stroma (Zeeman et al. 1998) and disbranching enzymes are involved in starch granule initiation (Burton et al. 2002). Despite this large amount of information present on the starch synthesis pathway, there is still a lot to be discovered to completely understand the pathways. Bacteria contain glycogen and the synthesis of bacterial glycogen involves the same enzyme activities (ADP–glucose pyrophosphorylase, glycogen synthase, branching enzyme and a debranching enzyme) as higher plant starch synthesis, but a very different non-crystalline product is synthesized (Ball and Morell 2003). Studies on many green algae show that a complex set of starch synthesis isoforms is present in even the simplest green algae, indicating the high conservation of function of the various isoforms (Bellaloui et al. 2014). A cyanobacteria with semi-crystalline amylopectin has been interestingly, identified by Nakamura et al. (2006), with a reduction in isoform number. Here too the actual roles of individual isoforms, and their interactions, are yet to be dissected. The information on the events that lead to starch granule initiation is not clearly understood, and little understanding of the control of complex granule developmental processes as seen in wheat and barley starches. The recent research in developing cereal endosperm, on discovering and describing the presence of phosphorylation-dependent complexes of starch biosynthetic enzymes can unlock further secrets in starch biosynthesis (Tetlow et al. 2004a). Complexes between starch biosynthetic enzymes have also been found to act as carbohydrate chaperones' by having the potential to channel substrates to specific structural endpoints (Tetlow et al. 2004b). However, it is clear that further research is essentially required to determine exactly how the various levels of regulation, transcriptional, allosteric and post-translational, interact to control the structure of starch and starch granules. Only when this level of knowledge is achieved, the full potential for the rational design of starches with specific functionality will be possible (Morell and Myers 2005). The main sites of assimilation of sulphur in plants are assumed to be the photosynthetic source leaves. However, in developing soya bean seeds, it has been established that the pathway of reductive sulphur assimilation is active, and the biosynthesis of sulphur-containing amino acids occurs in the developing embryos in the grain legume, *Lupinusangusti folius*. Thus, in the developing seeds, sulphur assimilation itself appears to be the vital source of sulphur containing amino acids for legume seed storage protein synthesis. Recently, it was shown in developing lupin seeds that the manipulation of the cysteine biosynthetic pathway results into the large increases in the free cysteine, though free methionine and total sulphur containing amino acid levels were not increased.

1.3.5 Expression of Methionine-Rich Proteins in Genetically Modified (GM) Plants

For modifying the plant methionine content, the expression of an added gene for a methionine-rich protein or 'methionine sink' has proven to be a successful GM approach. This strategy has been mostly used to improve the amino acid balance of legume seed protein, which can contain less than half the methionine essential for optimal animal nutrition. Early efforts of increasing the methionine content in seeds by the transgenic expression of genes for endogenous storage proteins mutated to add extra methionine residues were unsuccessful (Hoffman et al. 1988). A better approach was the creation of a synthetic gene encoding an artificial protein rich in essential amino acids. Under the control of a seed-specific promoter, the expression of a synthetic protein, containing 31% lysine and 20% methionine residues in genetically modified tobacco seeds, increased the over-all methionine concentration by 30% in the mature seeds (Keeler et al. 1997). An analogous result in a grain legume would give substantial improvement in the nutritive value of the seed protein. The manipulation of the methionine sink has most commonly involved the transgenic expression of the naturally occurring, methionine-rich plant proteins. The S-rich proteins that have been expressed in GM dicots include 2S seed albumins from sunflower, sesame and Brazil nut proteins that contain up to 18% methionine residues (Tai et al. 1999a, b). This approach has mainly been applied to the grain legumes, because of their low-intrinsic methionine concentrations. However, the seeds of other species like canola and maize have also been modified, as a means to provide additional protein methionine in the animal feed formulations containing grain legumes. For example, the sulphur-rich zeins in maize, containing up to 28% methionine residues have been overexpressed in the genetically modified maize (Chui and Falco 1995). In a strategy to enhance the sulphur-containing amino acid content of seed protein in *Lupinusangusti folius*, the 2S seed albumin from sunflower was used. In the genetically modified lupins, the sunflower albumin was expressed under the control of a strong, seed-specific promoter from a pea vicilin gene, which resulted into increases of up to 100% in total seed methionine, when compared with the parental genotype. The availability of the additional methionine to rats and chickens was also verified (Ravindran et al. 2002). Importantly, the methionine also benefitted the sheep owing to the rumen stability of the added methionine-rich sink protein (White et al. 2001). The expression of the Brazil nut 2S albumin in a number of seeds like canola, tobacco, soya bean and narbon bean, has increased the total seed methionine by 30–100%, when compared with wild type (Tabe and Higgins 1998). In the GM narbon beans and soya beans, the seed methionine levels were predicted to be adequate for optimal animal nutrition. However, the commercial usage of the Brazil nut protein has been prevented owing to its potential human allergenicity.

In GM cereals, the expression of methionine-rich proteins has met with mixed success. In GM rice, an increase of up to 75% in the total seed methionine was reported by using sulphur-rich 2S albumin from sesame (Lee et al. 2003). In contrast, no significant increase in seed methionine was produced in GM rice with the

expression of the sunflower 2S albumin. In the latter case, endogenous seed protein composition changed in a way that resembled the well-characterized responses of seed proteins to plant sulphur nutritional stress (Hagan et al. 2003). While expressing the sunflower protein in the GM rice grain, the endogenous, sulphur-poor proteins were upregulated and the sulphur-rich proteins were down regulated. This reallocation of limited sulphur reserves within the developing rice grain resulted in mature GM grain with different protein composition, with almost same concentration of sulphur-containing amino acids as that of the parental genotype. It is yet not clear why under the control of similar seed-specific promoters, the expression of the two very alike 2S albumins in rice, should produce such contrasting results. However, there are a number of reports of compensatory changes in the endogenous pools of sulphur in GM seeds expressing added, sulphur-rich proteins. The individual kernels of GM maize, overexpressing a methionine-rich 10 kDa zein, showed reduced levels of a separate endogenous sulphur-rich 12 kDa zein (Anthony et al. 1997). Similarly, the endogenous sulphur-rich proteins in GM soya bean seeds were also under-represented that accumulated 2S protein in the Brazil nut (Jung et al. 1997). The GM lupins, expressing the sunflower albumin, had reduced levels of transcripts encoding endogenous sulphur-rich seed storage proteins (Tabe and Droux 2002). The GM lupins also contained a smaller amount of oxidized sulphur than the parental seeds grown in matched conditions. Likewise, the GM narbon beans, expressing the Brazil nut albumin, contained lesser endogenous pools of sulphur in the form of the tri-peptide γ-glutamyl-*S*-ethenyl-cysteine than the parental control seeds (Muntz et al. 1997). In the GM seeds, both non-protein and protein pools of sulphur were apparently arrayed to source methionine for the synthesis of the added sulphur sink protein. In summary, by plant genetic modification, it has assuredly been possible to increase total seed methionine, though the evidence specifies that, in numerous cases, rather than increased delivery of sulphur to the seeds, the reallocation of endogenous pools of sulphur have been involved. In few instances, the data suggest that the methionine enrichment has been achieved through increased rates of methionine biosynthesis in the developing seeds (Tabe and Droux 2002).

1.3.6 Combined Approaches

The manipulation of methionine biosynthesis in plants has greatly expanded the understanding of the regulation of flux through the pathway but, as a means of improving methionine content, this approach suffers from the lack of stable storage of the additional methionine. On the other hand, addition of genes for methionine-rich storage proteins has produced such GM seeds that, in some cases, are predicted to harbour enough S- amino acids to satisfy the growth requirements of both humans as well as animals. Yet, in other cases, the results indicate that methionine biosynthesis in the developing seeds became limiting; for example in lupins, whose starting concentration of methionine was very low (Tabe and Droux 2002). The clear answer of merging the addition of a sulphur sink with modification of the sulphur-containing amino acid biosynthetic pathway is the subject of the present

work. Some success has also been reported; for example, the expression of both the 2S albumin from the Brazil nut and a feedback-insensitive aspartate kinase have given additive increases to the total methionine in the seeds of the GM narbon beans, although most of the effect was apparently due to the Brazil nut protein (Demidov et al. 2003). It has been reported that co-expression of an *Arabidopsis* CGS enzyme with a sulphur-rich zein in the GM alfalfa leaves has increased the accumulation of the zein when compared with its expression alone in the GM alfalfa (Bagga et al. 2005).

1.3.7 High-Methionine Mutants

A number of plant mutants, with increased levels of methionine, have been isolated by the selection on ethionine – a toxic analogue of methionine. Using this method, total three distinct groups of mutated genes have been characterized in *A. thaliana*, and have been found to define three enzymes from the methionine and *S*-adenosylmethionine biosynthetic pathways (Shen et al. 2002). Recently, a soya bean mutant, with increased total methionine in its mature seeds, was isolated using an initial screen for ethionine resistance. The outcome of this work was a soya bean variant, that was predicted to supply enough methionine for ideal animal nutrition without demanding supplementation with synthetic amino acid (Imsande 2001). The mainstay of the many essential nutrients that support human life, health and well being are crops and live stock. The specific role of key nutrients in human nutrition, it is also becoming apparent that the supply of some nutrients is compromised and in some cases may not be sustainable into the future from current resources. The most notable of these potential shortfalls relate to the long chain polyunsaturated fatty acids (LC-PUFA) of the omega-3 (ω3) class, such as eicosapentaenoic acid (EPA, 20: 5Δ5,8,11,14,17) and docosahexaenoic acid (DHA, 22:6Δ4,7,10,13,16,19), that are found predominantly in fish and other seafood in Western-style diets that are low in seafood and have been associated with increased incidence of cardiovascular disease, cancer, stroke, diabetes, inflammatory disease, neuropsychiatric disorders and many other conditions prevalent in Western societies as a result od inadequate levels of EPA and DHA (Simopoulos 2003). Significant increases in consumption of fish and other seafood rich in EPA and DHA are regularly recommend by nutritionists and health authorities. However, it is now widely acknowledged that global fisheries are fully exploited, with many on the verge of collapse (Myers and Worm 2003), and they may be inadequate to sustain even current levels of fish consumption. To overcome the declining catch from wild fisheries, many aquaculture systems rely heavily on wild fisheries for feeds and are actually net consumers, not producers, of ω3 LC-PUFA. This situation means that existing marine-based sources of ω3 LC-PUFA are unlikely to be sufficient to sustain current levels and anticipated future increases in human needs. Genetic engineering technologies is now providing a solution to this dilemma through the development of transgenic plants equipped with the ability to synthesize ω3 LC-PUFA. Genes encoding transfer of EPA and DHA biosynthetic pathways from

marine microalgae and other microorganisms into agricultural crops, in particular oilseed crops is achieving this. The ability to of higher synthesize the main C18-PUFA, linoleic acid (LA, 18:2Δ9,12) and α-linolenic acid (ALA, 18:3Δ9,12,15), and some can also synthesize γ-linolenic acid (GLA, 18:3Δ6,9,12) and stearidonic acid (SDA, 18:4Δ6,9,12,15) is the characteristic feature of higher plants but higher plants are unable to further elongate and desaturate these ω3 C18-PUFA to produce ω3 LC-PUFA that are characteristic of the marine microalgae that are the ultimate source of EPA and DHA found in fishes. introduction of genes encoding all of the biosynthetic enzymes required to convert ALA into EPA and DHA is therefore required to synthesis of ω3 LC-PUFA in higher plants. Substantial parallel gene discovery efforts conducted over the last 10 years in a range of LC-PUFA-synthesizing organisms have resulted in the cloning of genes for all of the fatty acid desaturase and elongase enzymes involved in the aerobic pathway for LC-PUFA synthesis (Sayanova and Napier 2004). It is probable that additional or alternative metabolic manipulations will be required in order to achieve significantly higher levels of DHA synthesis and accumulation in transgenic seed oils. However, it is now clearly apparent that seeds can be engineered to produce the range of ω3 LC-PUFA required in the human diet and potentially in concentrations that should be nutritionally effective. To overcome the inadequate and potentially unsustainable supply from traditional marine sources, Crop plants engineered in this way will ultimately provide the affordable, renewable and sustainable sources of ω3 LC-PUFA.

1.3.8 Sustainable Industrial Raw Materials Supply

To achieve a sustainable increase in the supply of nutritional oils, genetic manipulation of fatty acid metabolic pathways in plants can also open the way for a more sustainable supply of industrial raw materials, by enabling these to be sourced from renewable plant resources rather than from increasingly scarce and non-renewable petroleum. Pessimistic supply forecasts have driven a considerable expansion in the use of plant-based fuels, such as ethanol and bio-diesel, as commodity scale alternatives to conventional fuels as Escalation in the price of petroleum. The recent persistent escalation in the price of petroleum and predominantly pessimistic supply forecasts have driven a considerable expansion in the use of plant-based fuels, such as ethanol and bio-diesel, as commodity scale alternatives to conventional fuels. It is anticipated that in the future other higher-value specialty industrial products currently produced by the petrochemical industry will be produced on a renewable basis from oleochemical sources, predominantly from plants producing specific molecular structures required as starting materials for advanced chemicals and polymers. These products will be generated by metabolic engineering of plant biosynthetic pathways either by redirecting pathways towards the accumulation of current intermediate compounds, such as in the production of lauric acid (C12:0) in rapeseed (Voelker et al. 1996), In this regard, the engineering of fatty acid metabolic pathways in oilseeds is likely to be a particularly fruitful area. The major oilseeds

are very restricted in the range of fatty acids that they contain usually only five (palmitic, stearic, oleic, linoleic and linolenic) have been selected and bred mainly for food purposes. There is an enormous diversity of fatty acid structures (Badami and Patil 1981), including much functionality such as hydroxylation, epoxidation, acetylenation and conjugation that impart properties required for specific industrial uses. The enzymes responsible for these functionalities has been enabled due to gene technology to be cloned from various sources and expressed transgenically in oil accumulating crop species in order to develop novel industrial oils. Most attention has been focused on C18 fatty acids that are modified at the Δ12 position by the addition of epoxy or hydroxy groups, or by the formation of triple bonds (acetylenic) or conjugated double bonds. FAD2-like genes encoding Δ12 epoxygenases, hydroxylases, acetylenases and conjugases have all been cloned (several years ago) and recently reviewed (Jaworski and Cahoon 2003). In Arabidopsis Transgenic expression of these divergent FAD2 genes and other oil-accumulating seeds has generally resulted in synthesis of the Δ12-modifed fatty acid, but in disappointingly low concentrations (less than 10% of oil), even though the modified fatty acids are present at very high concentrations in the source plants (60–90%). However, in each case, the level of vernolic acid synthesis was initially low regardless of whether the Δ12-epoxygenase was a divergent FAD2 type such as from Crepis palaestina or a cytochrome P450 type such as from Euphorbia lagascae. It has subsequently been demonstrated that the level of vernolic acid synthesized in Arabidopsis seeds expressing the Crepis palaestina FAD2-like Δ12-epoxygenase can be enhanced from initial levels of approximately 6% (Singh et al. 2000a, b) to approximately 20% of total fatty acids (Zhou et al. 2006) by increasing the availability of linoleic acid substrate. This was achieved by co-expressing the Δ12-epoxygenase with additional Δ12-desaturase genes in a mutant Arabidopsis genotype lacking the fatty acid elongase (FAE1) and Δ15-desaturase (FAD3) enzymes that would otherwise compete for substrates involved in synthesis of Δ12-epoxy fatty acids.

1.3.9 Discovery and Usage of Genes for Improved Disease Resistance in Crop Plants

The use of disease-resistant crop cultivars provides an effective method of controlling a large number of diseases. However, continuous breeding efforts are required to counter evolution or migration of new pathogen strains. One stumbling block continues to be the lack of agreement regionally between breeders as to the most effective deployment of valuable R genes to prevent their stepwise erosion by pathogen evolution. Plant molecular biology is and will make increasing contributions to resistance breeding by making resistance breeding more effective and more efficient, especially through the use of markers for breeding and providing resistance genotypes for varieties to improve decision making about their deployment.

1.3.9.1 DNA Markers for Breeding

Our efforts have been mainly targeted at rust, nematode diseases of cereals and barley yellow dwarf virus, and molecular markers have been developed for improved breeding efficiency. The Cre1and Cre3 genes currently provide effective genetic resistance in wheat for cereal cyst nematodes. Breeding new resistant varieties has, however, been hindered by the slow and laborious nature of the plant bioassay for nematode resistance. DNA markers have now been identified for both resistance genes based on cloned genes of the nucleotide-binding site–leucine rich repeat disease resistance gene class (de Majnik et al. 2003). These genes co-segregate with the *Cre1* and *Cre3* resistance genes and although there is no direct evidence to indicate that the cloned genes themselves control nematode resistance, they have provided excellent sources for development of simple, rapid and accurate PCR-based markers that are currently being used by wheat breeders. Wheat breeding has relied heavily on genetic resistance to rust disease to control stem, stripe and leaf rust. Breeding efforts have been particularly successful for stem rust using major genes for resistance and DNA markers for resistance are being increasingly used. In areas where stem rust resistance has been a major breeding objective, success has been achieved mainly by using varieties carrying several different stem rust resistance genes, diversity of resistance genotypes and discouragement of the cultivation of susceptible varieties. DNA markers are now being used increasingly for these breeding efforts. DNA markers need to be simple to use and also applicable to as wide a range of breeders germplasm as possible. For example, while some markers can be useful for genetic mapping of resistance genes in particular crosses, they are frequently not useful in all breeder lines where they fail to detect polymorphisms between resistance gene donors and susceptible recurrent parents. Consequently, there can be a long development stage between marker identification and application that involves fine-tuning to produce a robust marker across a range of useful genotypes. Many wheat varieties carry the durable stem rust resistance gene *Sr2* that is effective in providing partial resistance against all strains of stem rust at the adult stage of growth. PCR-based DNA markers have now been developed for marker-assisted breeding using the *Sr2* gene (Spielmeyer et al. 2003), and have provided an entry point to finely map this gene for future molecular cloning (Kota et al. 2006) with the aim of understanding the molecular basis of an adult plant, durable, non-strain-specific resistance gene. Several other stem rust resistance gene markers have been developed and are described below. Good progress is being made in developing a PCR-based marker for the durable adult plant leaf and stripe rust gene pair *Lr34–Yr18*.

1.3.9.2 DNA Markers Useful for Gene Stacking

Pyramids or gene stacks of multiple stem rust resistance genes in a single variety can provide durable resistance. Traditionally, R gene pyramids are achieved using sequential bioassays with rust strains capable of differentiating those different resistance genes. This becomes more difficult for breeders if each of the genes used provide resistance to all available pathogen strains. This is where DNA markers will

make a big contribution to providing simple tests for the presence of specific R genes. For stem rust, markers for *Sr38*, *Sr24*, *Sr26*, *SrR* and *Sr31* have now been developed (Mago et al. 2005a, b). The latter four genes provide resistance to all stem rust strains currently found in Australia and the markers for *Sr24* and *Sr26* that provide resistance to the proliferating strain Ug99 now found in Africa will have global applications.

1.3.9.3 DNA Markers for 'Value Adding' to Alien Resistance Sources

Many of the currently effective stem rust resistance genes are derived from wheat relatives and many have negative dough characteristics that are physically linked to the same chromosome region as the resistance genes. They are consequently not suitable for use in high-quality bread wheats. For several of these R gene sources, the flanking alien chromatin regions have been reduced by recombination in *ph1b* mutant background (Lukaszewski 2000). DNA markers are also being used to detect recombinants carrying the R gene, but with reduced alien flanking DNA (Rogowsky et al. 1991). Retained DNA markers are being used for the deployment of the modified sources of *Sr31*, *SrR* and *Sr26* to produce near-isogenic lines for assessment of yield and quality effects and introduction as pyramids into adapted cultivars.

1.3.9.4 Cloned Rust Resistance Genes

The first rust resistance genes have been cloned from flax (Lawrence et al. 1995). Apart from providing the first insights into how rust resistance genes function, cloned genes will make a positive impact on plant breeding. An interesting and valuable rust resistance gene for stem rust *Rpg1* has been cloned from barley. This gene, which is not from the most common NBS—LRR class of plant disease resistance genes, has provided durable stem rust resistance in barley.

Initial observations with cloned disease resistance transgenes indicated that they might only function in species closely related to the source plant (Tai et al. 1999a, b). More recent data show this is not necessarily the case. When co-expressed in tobacco, the flax rust resistance protein L6 recognizes the corresponding flax rust avirulence protein AvrL567 and induces a hypersensitive response characteristic of a disease resistance reaction. This is likely to be due to direct interaction of the resistance protein and the avirulence protein (Dodds et al. 2004). Whether the gene functions in tobacco to give rust resistance is not possible to determine because tobacco is a non-host for the flax rust. Nevertheless, the transfer from the Linaceae family to the Solanaceae family shows that wide transfers of resistance genes between species can function.

When the current regulatory and political blockages to GM versions of food crops like wheat and barley are removed, a number of possibilities for GM resistance breeding should become available. For example, in barley and wheat, much specificity for powdery mildew occurs at the *Mla* and *PM3* resistance loci, respectively (Shen et al. 2003; Srichumpa et al. 2005). Cloning studies have shown that these are alleles and so cannot be easily recombined to produce gene pyramids for stable resistance—only one allele at a time can be deployed in a homozygous line. This nexus could be broken using transgenic plants and multiple R transgenes

can be transferred to wheat or barley to make otherwise unobtainable resistance gene pyramids (Maghari and Ardekani 2011).

Cloned genes from cereals are providing perfect markers for breeding in stem rust resistance in wheat. Furthermore, we are aiming to clone three or more resistance genes, package them into a single gene construct and introduce them into wheat using Agrobacterium. Two advantages over traditional methods are envisaged. Firstly, using cloned genes, the effect of linked genes with quality and yield defects can be removed. Secondly, by packing them in a single transgene cassette, the three genes will segregate during breeding as a single unit. Using traditional breeding, individual progeny plants homozygous for three unlinked genes are rare in segregating families. So far, cloning R genes from large cereal genomes is still difficult, but technology is advancing rapidly with increasing genome sequence data available. Rust resistance breeding in cereals is set to make a big jump with both marker-assisted and transgenic breeding. Furthermore, biotechnology can deliver surprises and the recent reports that round-up ready wheat shows high levels of rust resistance after spraying with glyphosate provides a challenge to develop agronomic practices for wheat that combine both weed and rust control using round-up (Anderson and Kolmer 2005)

1.3.10 GM Insect Protected Cotton: A Transgenic Plant Improvement

By the mid 1990s, the Australian cotton industry was beginning to stretch at the seams as this relatively young agricultural enterprise began to experience difficulties in containing its main insect pests, two caterpillars of the *Helicoverpa* complex. Failures in pest control were not new to cotton and it was still fresh in the minds of many how the fledgling industry in the Ord Irrigation Scheme (in the far north of Australia) had gone into a catastrophic spiral of insecticide resistance and increasing pesticide application in the 1960s. This resulted in the use of 35 or more insecticide sprays per crop per season, still without reaching any profitable level of production. This unsustainable dependence on pesticides resulted in the closure of cotton production in that region in the 1970s and the transfer of Australia's efforts to Eastern Australia where pest pressures were still high, but not as extreme as in the more tropical North. Despite its sensitivity to drought and the variable availability of irrigation water, which results in periodic reductions in overall output, cotton production in the East has worked well for many years, climbing to Australia's fifth largest agricultural export and earning the country over AUS$1.6 billion in 2001. Production has continued to increase, but by the early 1990s, it was apparent that despite the availability of new and effective pesticides (that had replaced many of the older more toxic chemicals used in the Ord), the same spiral of evolving insecticide resistance and increasing reliance on higher doses or more toxic mixtures of insecticides was being played out again in the East.

Biotechnology offered a new hope in pest control with the development by the Monsanto company of the gene constructs expressing the insecticidal delta

endotoxin protein, the active ingredient of commonly used biological pesticides (e.g. Dipel). The Cry1A insecticidal toxins of the *Bacillus thuringiensis* are highly potent to both *Helicoverpa armigera* and *Helicoverpa punctigera*, the two main insects being controlled by 80% of the pesticides then applied to cotton. CSIRO played a central role in the breeding of the new insecticidal trait (*Cry1Ac*, sold under the Ingard brand name in Australia) into adapted, high-performing germplasm for Australia, its subsequent deployment and the research that underpinned the management strategies and agricultural practices needed to make it a sustainable pest management tool. At the time of its introduction, the industry was already undergoing some critical self-evaluation about its environmental practices and had instituted many reforms that were already having a impact on reducing pesticide usage, including the introduction of best management practice (BMP) into cotton production and appropriate certification of individual and corporate growers (CRDC 2003). By 2002, 60% of the Australian cotton crop was produced under BMP and incorporated the use of the GM insect protected varieties being developed by CSIRO with the Monsanto genes included in this genome. The Ingard genes were introduced into Australia as cotton seed in the variety Coker 312 (an obsolete Texan variety, one of few cotton varieties amenable to genetic transformation and regeneration) that was itself unsuited for growth under Australian environmental and agricultural production conditions. Conventional backcross breeding was used to improve the germplasm base of the GM cotton by repeated backcrossing to elite CSIRO varieties that were among the best in the world for yield, fibre quality and disease tolerance. Multi-site evaluation across the cotton production area ensured that the new GM versions were well adapted and retained the high yield and other qualities of their recurrent parents. By 1996, CSIRO had produced sufficient seed of five Ingard varieties for an initial trial planting of approximately 40,000 ha. In the meantime, researchers were gathering all the necessary data for regulatory approval, crop agronomy and resistance management that were a necessary precursor to any commercial scale use of the new technology.

Regulation of GM products in Australia was handled by a two-component system that included an voluntary advisory panel of scientists (the Genetic Manipulation Advisory Committee) who assessed the safety of GM products and provided advice to a variety of State and Federal Statutory Agencies with responsibilities for particular areas of regulation of human health, food safety, occupational safety and the environment. Subsequent trials increased steadily in size to allow further pollen movement studies, efficacy assessments, breeding selections and seed increase, as well as the ecological impact studies required by regulators. Pollen flow studies indicated that cotton was easily contained within trials (cotton being a predominantly in-breeding plant) and required a relatively modest surrounding buffer crop extending only 20 m beyond the edge of the GM plots to act as a decoy for foraging insects such as bees that were the most likely vectors of pollen dispersal. Efficacy of pest control was not absolute and although it proved to be high during the first part of the growing season, it was noted to decline after flowering (Fitt 2004). This was subsequently shown to translate into commercial production with most of the savings in pesticide applications occurring during the first half of the season, where

H. punctigera was the main pest. Ecological impact studies measured any non-target impacts on the myriad of insects and other invertebrates that frequent cotton crops. In addition, the possibility of movement of the transgene out of cultivated cotton into native *Gossypium* species with a resultant disruption of the fine balance of these species was required to be assessed.

Given the existing knowledge on the host range of the toxicity of the delta-endo toxins, it was expected that the GM cotton plants would not have a negative impact on other invertebrates and this was borne out by extensive surveys of insect abundance in relatively large (10 ha) plots in replicated trials over a couple of years (Fitt and Wilson 2002). Impacts of the Ingard cotton were restricted to reductions in numbers of *Helicoverpa* larvae and other lepidopteran species known to be sensitive to the Cry1Ac protein, with a secondary effect on some lepidopteran-specific wasp parasites that normally feed within *Helicoverpa* caterpillars. Other beneficial insects tended to be more abundant in the Ingard cotton crops and were certainly much more abundant than in cotton crops sprayed with the conventional spectrum of pesticides normally used to control *Helicoverpa*species. Detailed genetic studies concluded that the risks of outcrossing to Australian native G or C genomic species, *Gossypium sturtianum* L., of the transgenes present in the GM cotton (AD genome allotetraploids) were negligible (Brown et al. 1997), although some of the K genome species in the more remote parts of Northern Australia might require further examination, should a cotton industry ever be established there.

The only major remaining concern of both growers and regulators was whether the technology would last beyond a couple of seasons if the target insect species could develop resistance to the insecticidal protein expressed in the plants. Previous research had reported resistance to Cry proteins in the Indian meal moth (*Plodia interpunctella*) and the diamondback moth (*Plutella xylostella*). Akhurst et al. (2003) were able, under laboratory conditions, to select a strain of *H. armigera* that was resistant to the toxicity of Cry1Ac proteins, so it was clear that target pests could possibly develop resistance to the active ingredient of Ingard cotton. The cotton industry had for years grappled with the problem of chemical insecticide resistance and was reluctant to see Ingard technology wasted. They set up a Transgenic and Insect Management Strategy committee to oversee the deployment of this new technology and make recommendations to both growers and regulators on all aspects of resistance management in an effort to preserve the new GM technology. Australian growers voluntarily adopted a strict area restriction on the use of the single gene Ingard cotton that saw every farm plant a maximum of 30% by area of Ingard varieties until such time as a second generation product was available that contained two different insecticidal toxins that would be more robust in countering any resistance development in the crop pests. This restriction was put in place to ensure that any resistance genes selected in the insects in the transgenic crops would not be fixed in the population, but would always find mates emerging from the non-transgenic crop that carry sensitive alleles for susceptibility to the Cry1Ac toxin and hence continually dilute out the resistance, keeping resistance allele gene frequencies very low within the target insect populations (e.g. Roush 1997). These so-called ‘refugia strategies’ require the presence of non-transgenic

crops in close proximity to the GM crop and have been adopted around the world in a variety of crops carrying GM insecticidal traits; they are an important component of management to delay resistance to insecticidal genes. Other management components included specified planting and harvesting windows, obligate crop destruction after harvest to prevent regrowth and cultivation to destroy overwintering pupae. These strategies have been successful and no field resistance selected in GM crops has been reported in any *Helicoverpa* species or other target lepidopteran insects (Tabashnik et al. 2005). By 2002, CSIRO had produced 15 different GM cotton varieties (combinations of Ingard and the herbicide-resistant Roundup Ready cotton) and continually updated their variety suite to keep pace with developments in conventional cotton germplasm. Despite changes from year to year in variety adoption, the 30% cap on Ingard cotton remained for 6–7 years during which time growers maximized the environmental benefits from the reduced pesticide spraying required on Ingard and in general used the new cottons on their more sensitive environmental sites close to towns, rivers or other dwellings where pesticide drift was likely to be a problem.

In 2003, CSIRO released a new suite of GM varieties that contained the *Cry1Ac* and a second insecticidal gene, *Cry2Ab* (also developed by Monsanto), that were sold as Bollgard II cotton. Bollgard II went through the same regulatory assessment as Ingard cotton, under a new regulatory regime that replaced the previous voluntary system. In 2000, the Australian government had put in place legislation to regulate biotechnology through a newly created statutory authority the Office of the Gene Technology Regulator. This represented a somewhat radical departure from previous systems as its primary goal was to put GM regulation on as open and transparent a footing as anywhere in the world. The requirement for accreditation and the issuing of licenses for the conduct of all GM research as well as a capacity for significant legal and monetary penalties have been put in place to ensure a high level of compliance by both research organizations and biotech and seed companies (as well as opponents of GM who might be tempted to interfere with field trials). Australia has not seen the fierce opposition to GM crops characteristic of European countries and GM cotton in particular has had a relatively straightforward introduction into agriculture (primarily because there was a strong desire for the technologies on the part of farmers and very obvious environmental benefits). The same has not been true for GM canola despite its success in Northern America. GM canola foundered at a State political level, even though it was given Federal regulatory approval (Sorek et al. 2014).

Bollgard II cotton has done extremely well in Australia and within 2 years of its introduction constituted over 90% of all the cotton planted in this country, the majority of it as Bollgard II/Roundup Ready varieties that allowed growers better insect and weed control. The greater efficacy in the control of Lepidopteran pests and the presence in the cotton of two different insecticidal toxins offering greater protection against the development of resistance in the target pests have seen the removal of the planting area restrictions and a reduction in the sizes of the required refuges. Initial indications are that Bollgard II has slashed pesticide usage for Lepidopteran control by more than 80%. One of the key developments with this new

insect control technology is that it has fostered a greater adoption of integrated pest management in cotton, which is leading to even further reductions in pesticide usage (Wilson et al. 2004). The success of GM cotton in Australia has highlighted the value of GM solutions to agricultural sustainability and bodes well for future agbiotech products. Success will depend on the right genetics (getting the products into the right genetic backgrounds), the right management (researching the appropriate management scenarios to ensure the delivery of the benefits promised by the technology) and the right communication (making sure that the community, both the agricultural community and the wider community, are aware of the benefits) for the commercialization of those products.

1.4 Conservation Agriculture and Sustainable Development

Conservation agriculture (CA) is well defined, as minimal soil disturbance and permanent soil cover or mulch combined with rotations, is a contemporary agricultural management system most popular in world now a days. The word 'sustainable' as per the Oxford English dictionary defines it as 'capable of being borne or endured, upheld, defended, maintainable'. Somewhat that is sustained is 'kept up without intermission or flagging, continued over a long period'. An important conception in today's agriculture, as we know that the human race will not want to compromise the ability of its future offspring to produce their food needs by detrimental the natural resources used to feed the population nowadays.

1.4.1 Cultivation Techniques or Tillage

The description of tillage dates back many millennia at what time humans changed from hunting and gathering to more sedentary and settled agriculture mostly in the Tigris, Euphrates, Nile, Yangste and Indus river valleys. Lal (2001) enlightened the historical progress of agriculture with tillage being a major constituent of management practices. The mechanical power and tractors became accessible to undertake tillage operations in nineteenth century and today; an assortment of equipment's is accessible for tillage and agricultural production. The reasons for use of tillage are documented as:

(a) Tillage is generally used to loosen the soil and further preparing a bed wherein seed is to be located easily at an appropriate depth into moist soil using other equipments which resulting in good uniform seed germination.
(b) Farmers were able to shift the benefit from the weed to the crop and let the crop to grow deprived of competition early in its growth cycle with subsequent higher yield by tilling.
(c) Tillage speedup mineralization and oxidation afterward exposure of soil organic matter to air.

(d) Availability of nutrients to roots via tilling process
(e) Tillage is considered a critical management practice for controlling soil borne diseases and some insects as well.

As documented that Faulkner's genius was to question the very basis of agriculture itself-the plough. He began to see that the curved moldboard of the modern plough, rather than permitting organic matter to be slogged into the soil by worms and other burrowing animals, in its place buries this nutritious matter under the subsoil (Faulkner 1987).

1.4.2 Conservation Tillage and Conservation Agriculture

Baker et al. (2002) described conservation tillage as the combined umbrella term usually given to no-tillage, direct boring, minimum-tillage or ridge-tillage, to signify that the explicit practice has a conservation goal of some nature. Generally, the 30% retention of surface cover by residues characterizes the lower limit of organization for conservation tillage, however other conservation objectives like conservation of time, fuel, earthworms, soil water, soil structure and nutrients are included for the practice also. Consequently residue levels alone do not sufficiently define all conservation tillage practices (Baker et al. 2002). However the conservation agriculture aims to conserve, improve and make extra effective use of natural resources through combined management of available soil, water and biological resources collective with external inputs. It provides to environmental conservation as well as to enhanced and sustained agricultural production. It is some times also referred to as resource efficient or resource effective agriculture.

Conservation tillage is a amalgam of practices that leave crop residues on the surface, which increases water infiltration and reduces erosion practiced in conventional agriculture to lessen the effects of tillage on soil erosion. Nonetheless, it still depends on tillage as the structure-forming element in the soil. Meanwhile, conservation tillage practices like zero tillage practices can be switch off steps towards conservation agriculture.

Conservation agriculture sustains a permanent or semi-permanent organic soil cover by developing crop or dead mulch. The main function is to protect the soil physically from sun, rain and wind and to feed soil biota. The soil microorganisms and other fauna take over the tillage function and soil nutrient balancing. Usually the mechanical tillage disturbs however no or minimum tillage and direct seeding are important elements of conservation agriculture. A diverse crop rotation is also significant to avoid disease and pest problems. Derpsch (2005) indicated that the extent of no tillage implementation worldwide is just over 95 Mha. However it is considered as proxy for conservation agriculture. Although not all of this land is permanently no tilled or has permanent ground cover. Table 1.1 depicts the extent of no-tillage by country worldwide. Six countries have more than 1 Mha. South America has the highest adoption rates and has extra permanent non-tillage and permanent soil cover. Both Argentina and Brazil had significant lag periods to reach

Table 1.1 Extent of no-tillage adoption worldwide

Country	Area under no-tillage (Mha) 2004/2005
USA	25.30
Brazil	23.60
Argentina	18.27
Canada	12.52
Australia	9.00
Paraguay	1.70
Indo-Gangetic Plains	1.90
Bolivia	0.55
South Africa	0.30
Spain	0.30
Venezuela	0.30
Uruguay	0.26
France	0.15
Chile	0.12
Colombia	0.10
China	0.10
Others (estimate)	1.00
Total	95.48

Derpsch (2005); includes area in India, Pakistan, Bangladesh and Nepal in South Asia

1 Mha in the early 1990s and then prolonged rapidly to 18.3 and 23.6 Mha for these countries respectively. By approving the no-tillage system, Derpsch (2005) estimated that Brazil increased its grain production by 67.2 million tons in 15 years with additional revenue of 10 billion dollars. Derpsch also estimated that at an average rate of 0.51 t haK1 yrK1 Brazil sequestered 12 million tons of carbon on 23.6 Mha of no-tillage land. The three key principles of conservation agriculture are permanent residue soil cover, minimal soil disturbance and crop rotations.

1.4.3 Permanent or Semi-Permanent Organic Soil Cover

Unger et al. (1988) appraised the role of surface residues on water conservation and documented this association between surface residues, enhanced water infiltration and evaporation led to the adoption of conservation tillage after the 1930s dust bowl problem. However, Bissett and O'Leary (1996) showed that infiltration of water under long term conservation tillage was greater compared to conventional tillage on a grey cracking clay and a sandy loam soil in southeastern Australia. Lal (2001) also defined the efficiency of these systems depends on proper construction and regular care otherwise it can be catastrophic. The crop residues of cultivated crops are a substantial factor for crop production through their effects on soil physico-chemical and biological functions besides water and soil quality (Kumar and Goh 2000). The composts and manures as an external mulch can also be applied, though

economically it may restrict its use to higher-value crops like vegetables. Mulch intercepts the suns energy and protects the surface soil from soil aggregate destruction, enhances the infiltration of water and reduces the loss of soil by erosion. Surface mulch helps reduce water losses from the soil by evaporation and also helps judicious soil temperature endorses biological activity and enhances N-mineralization, especially in the surface layers (Hatfield and Pruegar 1996) generally suitable in tropical and subtropical environments (Swanson and Wilhelm 1996). Fabrizzi et al. (2005) showed that no tillage had lower soil temperatures in the spring in Argentina, but conventional tillage had higher maximum temperatures in the summer, and that average temperatures during the season were similar. Roldan et al. (2003) exhibited that no tillage after 5 years for maize in Mexico, soil wet aggregate stability had increased over conventional tillage as had soil enzymes, soil organic carban and microbial biomass inferring that no tillage is a sustainable technology. A cover crop and the subsequent mulch or prior crop residue help reduce weed plague through competition and not allowing weed seeds the light frequently desirable for germination citing the evidence of allelopathic properties of cereal residues in regard to inhibiting surface weed seed germination (Jung et al. 2004). Farming practice that sustains soil microorganisms and microbial activity can also lead to weed conquest by the biological agents (Kennedy 1999). Cover crops contribute to the addition of organic matter in the surface soil horizon (Madari et al. 2005; Riley et al. 2005). Mulch also benefits with recycling of nutrients, especially when legume cover crops are used, through the connotation with belowground biological agents and by providing food for microbial populations (Campbell et al. 1995). Others have shown that this is restricted to the surface horizons, and that the reverse occurs at greater depths in humid soils of eastern Canada (Angers et al. 1997). Soil microbial biomass has usually been used to evaluate belowground microbial activity and for that it is considered as sink and source for plant nutrients. Alterations or any amendments like residues and manures promote while burning and removal of residues decrease soil microbial biomass (Heenan et al. 2004; Alvear et al. 2005). Increased microbial biomass will enhance soil aggregate formation, nutrient cycling through slow release of organically stored nutrients and also assisted in pathogen control (Carpenter-Boggs et al. 2003). Cover crops help endorse biological soil tillage through their rooting, the surface mulch provides food, nutrients and energy for earthworms, and arthropods and microorganisms below ground that also biologically till soils. The usage of deep-rooted cover crops and biological agents will support to relieve compaction under zero-tillage systems. Though from the available data, it looks at the outcomes of burning, incorporation and removal of crop residues on soil properties. It is further reported that Zero-tillage acts in a better balance of microbes and other organisms and a healthier soil. Ground cover endorses an increase in biological diversity both below and above ground (Jaipal et al. 2002). The interactions between root systems and rhizobacteria affect crop health yield and soil quality. Release of exudates by plants activate and sustain specific rhizobacterial communities that improve nutrient cycling, N- fixing, bio control of plant pathogens, plant disease resistance and plant growth stimulation.

As mentioned above the general comparisons between tillage and zero-tillage systems are made to highpoint some other benefits and in this section other important parameters are documented. For example- Tractors consume large amounts of fossil fuels that enhance to costs while also emitting greenhouse gases (mostly CO_2) and contributing to global warming when used for ploughing (Grace et al. 2003). The animal-based tillage systems are also expensive since farmers have to continue and feed a pair of animals for a year for this purposefulness. Animals also emit methane, a greenhouse gas 21 times more potent for global warming than CO_2 (Grace et al. 2003) so zero-tillage reduces these costs and emissions. The zero-till of wheat after rice reduces costs of production by US$60 per hectare mostly due to less fuel (60–80 l haK1) and labour as studies from farmer surveys in Pakistan and India (Hobbs and Gupta 2004). Tillage takes appreciated time that could be used for other useful farming activities or employment. Zero tillage minimizes time for establishing a crop. The time required for tillage can also delay timely planting of crops, with subsequent reductions in yield potential (Hobbs and Gupta 2003). By reducing about-turn time to a minimum, zero-tillage can get crops planted on time, and thus increase yields without greater input cost. Turnaround time in this rice- wheat system from rice- wheat varies from 2 to 45 days, since 2–12 passes of a plough are used by farmers to get a good seedbed (Hobbs and Gupta 2003). With zero-till wheat this time is reduced to just 1 day. Tillage and current agricultural practices result in the decline of soil organic matter due to increased oxidation over time, leading to soil degradation, loss of soil biological fertility and resilience (Lal 1994). Although this SOM mineralization liberates nitrogen and can lead to improved yields over the short term, there is always some mineralization of nutrients and loss by leaching into deeper soil layers. This is particularly significant in the tropics where organic matter reduction is processed more quickly, with low soil carbon levels resulting only after one or two decades of intensive soil tillage. Zero-tillage, on the other hand, combined with permanent soil cover, has been shown to result in a build-up of organic carbon in the surface layers (Lal 2005). No-tillage minimizes SOM losses and is a promising strategy to maintain or even increase soil C and N stocks (Bayer et al. 2000). Although tillage does afford some relief from compaction, it is itself a major cause of compaction, especially when repeated passes of a tractor are made to prepare the seedbed or to maintain a clean fallow. Zero tillage reduces dramatically the number of passes over the land and thus compaction. However, natural compaction mechanisms and the one pass of a tractor-mounted zero-till drill will also result in compaction (Sayre and Hobbs 2004). Some farmers feel that sub-soiling may be needed to resolve belowground compaction layers before embarking on a NT strategy, especially in drier areas. Higher bulk densities and penetration resistance have been reported under zero-tillage compared with tillage (Gantzer and Blake 1978) and are described as natural for zero-tillage. This problem is greater in soils with low-stability soil aggregates (Ehlers et al. 1983). Bautista et al. (1996) working in a semi-arid ecosystem found that zero-tillage plus mulch reduced bulk density. The use of zero-till using a permanent residue cover, even when bulk density was higher, resulted in higher infiltration of water in no tillage systems (Sayre and Hobbs 2004). Scientists assumed that continued use of reduced, shallow and

zero-tillage would require a shift to short-term total tillage to correct soil problems. However, Logsdon and Karlen (2004) showed that bulk density is not a useful indicator and confirm that farmers need not worry about increased compaction when changing from Total to no tillage on deep loess soils in USA. Fabrizzi et al. (2005) also showed higher bulk density and penetration resistance in no tillage experiments in Argentina, but the values were below thresholds that could affect crop growth; wheat yields were the same as in the tilled treatments. Leake (2003) determined that the role of tillage on diseases is unclear and recognizes that a healthy soil with high microbial diversity does play a role by being antagonistic to soil pathogens and suggested that no tillage farmers need to adjust management to control diseases through sowing date, rotation and resistant cultivars to help shift the advantage from the disease to the crop.

Crop rotation is an agricultural management tool with prehistoric origins. Howard (1996) studied the cultural control of plant diseases from an historical view and included examples of disease control across rotation. The rotation of altered crops with different rooting patterns collective with minimal soil disturbance in zero-till systems endorses a more extensive network of root channels and macropores in the soil. Integrated pest management must also be added to the conservative agriculture set of recommendations, since if one of the necessities is to promote soil biological activity, negligible use of toxic pesticides and use of substitute pest control methods that do not affect these critical soil organisms are needed (Leake 2003).

1.4.4 Climate Change and Conservation Agriculture

Climate change is likely to strongly affect rice–wheat, rice–rice and maize-based cropping systems that, today, account for more than 80% of the total cereals grown on more than 100 Mha of agricultural lands in South Asia. Global warming may be beneficial in some regions, but harmful in those regions where optimal temperatures already exist; an example would be the rice–wheat mega-environments in the IGP that account for 15% of global wheat production. Agronomic and crop management practices have to aim at reducing CO_2 and other greenhouse gas emissions by reducing tillage and residue burning and improving nitrogen use efficiency. In the IGP, resource-conserving technologies continue to expand in the rice–wheat cropping systems and save 50–60 l of diesel haK1 plus labour, and significantly reduce release of CO_2 to the environment. Methane emissions that have a warming potential 21 times that of CO_2 are common and significant in puddled anaerobic paddy fields and also when residues are burnt. This GHG emission can be mitigated by shifting to an aerobic, direct seeded or NT rice system. A review of the other benefits of direct seeding and NT in RW areas of South Asia can be found in Grace et al. (2003). Nitrous oxide has 310 times the warming potential of carbon dioxide, and poor nitrogen management affects its emissions. Sensor-based technologies for measuring normalized differential vegetative index and moisture index have been

used in Mexico and South Asia to help improve the efficiency of applied nitrogen and reduce nitrous oxide emissions. Lal (2005) suggested that by adopting improved management practices on agricultural land (use of NTand crop residues), food security would not only be enhanced but also offset fossil fuel emissions at the rate of 0.5 Pg C yrK1.

1.5 Conclusion and Future Perspective

Genetic alteration to the biological software of our major food and fibre production plants will endure to improve the yield and sustainability of agricultural systems. DNA technology is now consistently used in plant improvement programmes with DNA sequence markers enhancing both the speed and the power of selection schemes. The rapidly increasing knowledge of the functioning of crop genomes has already provided enhanced performance in conventional breeding programmes and although transgenic crops have not been welcomed in all parts of the world. These transgenic crops, comprising cotton, fiber crop, food and feed crops like maize, soya bean and canola, have all been considered in the various countries of the world in which they are grown and have entered successfully into markets. This signifies a noteworthy growth incorporation of transgenic modifications into breeding systems. The appreciative molecular bases of plant processes that have been gained from the developments in genomics and our collective knowledge of gene regulation are opening up a new generation of breeding advances, both through transgenic breeding and conventional breeding. One of the rewards in many crops is that once specific breeding objectives have been defined by research that has used all the power of the new technologies, then breeders are able to use new investigative tools to achieve the desired objectives through conventional breeding programmes providing a bridging period of improvement in plant breeding while our societies move towards general acceptance of transgenic tools in plant improvement programmes for our food, feed and fibre crops. The profit from modification of internal architecture, the anatomy of plant tissues; for example, the ratio of palisade and spongy mesophyll leaf cells and the geometry of tissues in the root system are areas in which we can expect telling alterations. A reasonable conclusion is that genetic modification of crops, which has been so powerful and so rewarding in terms of yield and management of many of the major production species over the past few decades, will hold enormous potential in all of the crop species we deal with. We have an increasing knowledge and power to modulate the development and functional operation of crop plants so as to provide optimal performance in our agricultural production system environments. Agricultural performance rests on the interactions of genetics, management and the environment. And a variety of production environments to have the genetic modifications, coupled with appropriate management regimes, to result in an increased efficiency and sustainability of agri-business can be expected in future.

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Plant Metabolomics: Sustainable Approach Towards Crop Productivity

2

Abstract

Metabolomics signifies a rapidly growing and interdisciplinary field of science by combination of biochemistry, analytical chemistry, bioinformatics, medicine etc. Metabolomics allows achieving a sophisticated level of information about biological systems and holding great promise for development of novel diagnostic tests and therapies including personalized medicine. Notwithstanding its powerful analytical and computational systems integration still there remains many challenges pertaining to metabolic and analytical challenges. Metabolomics combined with other technologies permits us to resolve key issues of agronomic performance that remained unsettled hitherto. Metabolomics is also developing into a valuable tool that can be used to monitor and assess gene function, and to characterize post-genomic processes from a broad perspective Many efforts can be focused to crop plants that have detailed info on performance in varied environments These challenges are largely caused by the high degree of chemical diversity among metabolite pools as well as the complexity of spatial and temporal distribution within living tissues. In this chapter role of metabolomics for improving various agricultural crops including GMO varieties are discussed in detail besides various networking approaches as well. The role of plant bioactive substances for stimulating the soil microbial communities is also elaborated in concluding section.

Keywords

Metabolome · Genomic approach · Priming food crops

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2.1 Plant Metabolme Analysis

With availability of transgenic system, the metabolomics is being increasingly used in many crop species and has the potential to facilitate selection of superior traits and improvement of breeding materials (Oikawa et al. 2008; Fernie and Schauer 2009; Daygon and Fitzgerald 2013; Simó et al. 2014a, b and Zivy et al. 2015). In advances with metabolomics, cost-effective genotyping assays opens exciting opportunity to effectively integrate metabolomics in crop breeding programs and the availability of whole genome sequence, genome-wide genetic variants (Hall et al. 2002; Fernie and Schauer 2009).

The metabolomics study has witnessed substantial improvement with advent of mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy. As of now accessible metabolomics stages have the ability to permit huge scale metabolite studies covering both referred to just as obscure metabolites. The downpour of such information, in any case, makes explanation of metabolites an impressive test (Matsuda et al. 2010; Lei et al. 2011). In this specific circumstance, the regularly becoming stronger of bioinformatics apparatuses combined with the foundation of metabolomics databases, for example, for model plant Arabidopsis, and others for different plant species have more noteworthy ramifications for metabolite comment (Tohge and Fernie 2009; Afendi et al. 2012). A lot of information has come about because of metabolic reviews, which may bolster plant enhancement plans concentrating on the characteristics of agrarian significance, for example, yield and stress resilience. In order to devise a holistic way of improving traits of interests, the rapid generation of genome scale data by sequencing of DNA and RNA, and by MS quantification of proteins and metabolites necessitates integration of this information (Pandey et al. 2016). Although, most of the current studies are coming in well-established model organisms, such studies may be of common occurrence in other plant species as well. Scientific community currently faces a herculean challenge of dealing with massive multi-omics data for conducting systems-level analyses (Suravajhala et al. 2016). In such situation, enhanced measurable and bioinformatics apparatuses will be required to break down these informational collections together for better solidification, which can in the long run be interpreted for enhancing plant execution.

2.2 Metabolomic Approach Towards Improvement of Crops Yields Under Environmental Stress

2.2.1 Metabolomics for Improvement of Fruits

Metabolomics have given more prominent bits of knowledge in natural product science uniquely identified with aging and quality. Tomato (Solanum lycopersicum) is a rich wellspring of carotenoids, enemies of oxidants and flavonoids (Tohge and Fernie 2015). Metabolite isolation example of 50 tomato cultivars indicated close concurrence with isolation of organic product size (Moco et al. 2008). Metabolome

is helpful to dissect the aging occasion by plotting a connection with natural product transcriptome (Osorio et al. 2011). Metabolomics can be utilized to illustrate various and differential biochemical pathways exist in the products of tomato ILs and Ecotypes and the tribal species through genome wide metabolic review (Perez-Fons et al. 2014). Apple (*Malus* spp.) contains various supplements in the strip and flesh, including cell reinforcements that lessen the danger of interminable diseases like asthma, malignant growth, cardiovascular sickness, and diabetes. The metabolite substances of the apple organic products are utilized to separate monetarily imperative cultivars (Cuthbertson et al. 2012). For instance, the cultivar 'Brilliant Delicious' contains a high heap of myo-inositol, sugars and succinic corrosive; while, the cultivars 'Red Delicious' and 'Fuji' indicate generally higher plenitude of triterpene/sterols, flavonoids, phenolic acids, stearic corrosive, anthocyanins, and starches. The natural product strip concentrate of 'Fuji' contained elevated amounts of starch including glucose and sorbitol, and was altogether separated from 'Red Delicious' and 'Granny Smith' which contain large amounts of unsaturated fats (oleic and linoleic corrosive). Spatial conveyance of sugars and natural acids between organic product layers has been explained in an ongoing report on metabolic profiling of apple natural product (Cebulj et al. 2017). The roasting of apple natural products amid capacity renders them unmarketable, accordingly applying an unfriendly effect on the apple business. The metabolomics think about on put away apple natural products demonstrated a distinction in the dimension of essential metabolites with various time length. The expanded dimensions of mannose and xylose amid post-reap demonstrated a breakdown of cell divider hemicellulose, which improves organic product senescence. The investigation by Hatoum et al. (2016) set up a connection between metabolic direction amid post-gather stockpiling and cell breath and stress.

In international markets over few past years, the Kiwifruit (*Actinidia Lindl.* spp.) has gained popularity due to its health benefiting nutrients such as vitamin C, fiber and distinct appearance as total of 51 metabolites were detected during kiwifruit development and ripening (Ward and Courtney 2013). During its ripening process, the content of soluble sugars and ascorbate significantly changes, which eventually determines the fruit quality and taste and flavor which can be improved by targeting the metabolites that can render consumer acceptance. In Kiwifruit, application of synthetic cytokinin *N*-(2-chloro-4-pyridyl)-*N′*-phenylurea significantly increases fruit size, and affects the ripening processes by altering the accumulation pattern of metabolites such as amino acids, sugars, organic acids etc., (Ainalidou et al. 2015).

The metabolite content such as organic acids, sugars, vitamins, flavonoids, and carotenoids is used to predict quality and taste of orange fruit (*Citrus* spp.) as one of the metabolomics study on orange bud mutant 'Hong Anliu' (accumulates higher levels of lycopene and sweeter than wild type) led to the identification of 130 metabolites that include acids, sugars, flavonoids, alkaloids, limonoids, coumarins, amino acids, and plant hormones (Pan et al. 2014). The flavor and the taste of 'Hong Anliu' sweet orange was determined by the higher levels of soluble sugars and

lower levels of organic acids along with differential levels of flavonoids at ripe stage.

The contamination of Candidatus Liberibacter asiaticus, causative operator of Citrus Huanglongbing illness, disintegrates juice quality (Slisz et al. 2012). The contamination prompts serious decline in aldohexose, fructose, saccharose and amino acids, for instance, alanine, arginine, isoleucine, leucine, proline, threonine, and valine; whereas, it upgrades the size of turn and essential amino acid. Heat treatment of organic product is generally used as the way to evade natural product sickness amid post-gather reposition that is all around bolstered by metabolomics. With associate degree of investigation, the heat treatment essentially diminished the substance of natural acids and amino corrosive; in any case, it advanced the amassing of metabolites, for instance, 2-keto-D-gluconic corrosive, tetradecanoic corrosive, oleic corrosive, ornithine, natural resin corrosive, myo-inositol, glucose, fructose, sucrose, and turanose, that decreases the danger of post-gather sickness. As of behind schedule, ABA is accounted for to fill in as a controller of citrus shell wax synthesis amid organic product improvement (Wang et al. 2016).

The fruit setting in grape (*Vitis vinifera*), depends upon the metabolic abundance which in turn is regulating by sugar metabolism and hormonal pathway. Sun et al. (2012) documented the effect of geographical distribution on metabolite content. The regions which perceives high sun light-low rainfall show improved content of sugars and amino acids, Na and Ca, along with low levels of organic acids, suggesting the role of extrinsic factors on fruit quality. Cuadros-Inostroza et al. (2016) reported that the abundant metabolic content in grapes berry that is stage specific and cultivar dependent, regulates the ripening processes. The major polyphenols that determine the quality of drinking wine has been reported in Stilbenes as the MS analysis of grapes allowed the detection of several bioactive stilbenes like caraphenol, ampelopsin H, trans-resveratrol, isohopeaphenol,, *Z*- and *E*-astringin, *Z*- and *E*-piceid, piceatanno, B pallidol and pallidol-3-*O*-glucoside and parthenocissin A. Near about 450 compounds has been identified in the studies which focused on the polyphenolics content of the grape including anthocyanin, glycoside aroma precursors, flavanols and procyanidins, flavones and flavanones, phenolic acids and stilbenes. Particularly, this study allowed identification of several 100 compounds, which were used to build a new database of putative compounds (Grape Metabolomics).

One of the member of Rosaceae family is Pear (*Pyrus communis*), which is grown worldwide for its unique 'melting' texture as leading producers of pears is Japan and its metabolomic analysis defined differential accumulation of ~250 metabolites during fruit development and ripening (Oikawa et al. 2015). Ripening of this fruit manifested accumulation of sugars (e.g., sucrose), phytohormone such as ABA, brassinosteroids and also sulphur-containing amino acids. As 15 phytohormones including abscisic acid, auxin, brassinosteroids, gibberellins, jasmonic acid and salicylic acid reported by this type of studies. The blooming stage shows a substantial increase of the metabolites (amino acids and organic acids), which further decreases during fruit development.

Likewise, Aharoni et al. (2004) illustrated strawberry (*Fragaria* × *ananassa*) is rich in secondary metabolites such as flavonoids and also its cultivated species predominantly contain terpenoids such as sesquiterpene nerolidol and the monoterpene linalool while as the wild species were rich in the olefinic monoterpenes and myrtenyl acetate. Surprisingly, these were absent in the cultivated species (Aharoni et al. 2004). At seven different stages of its fruit development, the untargeted (GC-MS) and targeted (HPLC) based studies of strawberry fruits were employed. The metabolic study revealed a shift in the metabolite content during fruit development and ripening. The strawberry ripening involved rise of free amino acid content, with change in sugar content, including substantial changes in other major metabolic pathways such as ester biosynthesis, shikimate, and tricarboxylic acid (Zhang et al. 2011).

The quantitative estimation of primary and secondary metabolites accumulated in the infected and non-infected fruits was evaluated by studying the effect of biotic stress and the fungicide (to avoid biotic stress) on fruit quality. The reduction in the organic acid content and increment in the quantity of sugars has been found in fruit which is infected by *Colletotrichum nymphaeae* and also the infected fruits showed altered content of metabolites such as total phenolics, flanonols, flavan-3-ols, ellagic acid derivatives and oligomeric procyanidins. Nagpala et al. (2016) recently revealed that in an infected strawberry showed increased amount of polyphenol in white-fruited species due to infection from fungal pathogens *viz. Botrytis cinerea* and *Colletotrichum acutatum*.

2.2.2 Metabolomics for Improvement of Legume Crops

Grain legumes satisfy about 33% of required human dietary protein as forage and grain legumes contribute 27% of the world gross primary crop. While cater alone, contributes to food security and environmental sustainability. In spite of the extensively investigated model legumes, metabolomics studies in other legumes remain limited. Concerning model legume, investigation of the effect of rhizobial node factor (Nod) in Medicago revealed a decrease in oxylipins (Zhang et al. 2011). Sanchez et al. (2011) revealed in another study that the metabolic profiling of salt tolerant *Lotus* species uncovered a series of changes involving metabolic adjustments of shoot constituent for survival.

In soybean, stress conditions such as salinity and anoxia results in an accumulation of alanine, and its biosynthesis co-substrates such as glutamate and GABA, and succinate. Differential expression was also obtained for genes involved in nitrogen fixation and fermentation in root. Interestingly, a negative correlation was observed for the amino acid derived from glycolysis and the TCA cycle during water logging, and several TCA cycle enzymes were induced upon exposure to water logging. Similarly, Komatsu et al. (2011) attempted to elucidate the metabolic changes associated with flooding stress in soybean led authors to identify a set of 81 mitochondria associated metabolites, thus suggesting a boost in concentrations of

metabolites involved in glycolysis and respiration such as, amino acids, NAD and NADH coupled with the depletion of free ATP. Muscolo et al. (2015) studied metabolite phenotyping of four Mediterranean accessions of lentil. He found intermediates of the TricarboxyCA cycle and glycolytic pathway decreased in response to drought and salinity stress. Furthermore, this study yielded metabolite markers for specific stresses like asparagine/ornithine, threonate, and alanine/homoserine for drought, NaCl, and salinity, respectively. Pinheiro et al. (2004) studied the effect of water deficiency on *Lupinus albus* and reported that its stem serves as a storage organ for amino acids and sugars. It has been analyzed Stress resistant plants considerably accumulate, glucose, proline, sucrose and asparagine in their stem stelar region (Pinheiro et al. 2004). Moreover the authors have reported reorganization of carbon and nitrogen metabolic pathways in salt resistant plants. Tripathi et al. (2016) in soybean, reported that drought-stressed resulted in increased production of pinitol. Similarly, water stress stimulated accumulation of soluble proteins, sucrose and free amino acids in tolerant soybean (Tripathi et al. 2016).

2.2.3 Metabolomics for Improvement of Cereal Crops

Globally, the primary sources of nutrition are the Cereals, which are rich in vitamins, minerals, carbohydrates and fats. These have been widely studied in order to quantity variation in metabolites and their association with sequence variation (Chen et al. 2016a, b). Various research groups explored the potential of metabolomics in order to harness the metabolites diversity between different varieties and natural variants in the rice (Okazaki and Saito 2016). Likewise in maize, researchers differentiate and subsequently select the superior genotypes for metabolomics with enhanced nutritional composition (Venkatesh et al. 2016). Recently, metabolomic approach has been utilized to survey chemical diversity between different maize and rice variety and its natural variants (Chen et al. 2016a, b). The amino acid metabolism and Photorespiration has been regulated by water scarcity in maize where the amino acids involved in this pathway are glycine and serine, rendered up-regulated. Obata et al. (2015) further reported that under drought conditions, the accumulation of myo-inositol and glycine has to relate with grain size of maize, where metabolites can be used as potential markers for identifying drought tolerant variant. Analogous to the previous studies, drastic increment in certain compounds like as glucose, galactaric acid, gluconic acid, allantoin glucopyranoside and salicylic acid, which could be considered as metabolite markers to address water stress in rice. Ogbaga et al. (2016) illustrated that the ability of a plant to adapt with drought regarding its metabolic status and showed considerable variation within species. Sorghum variety showed greater tolerance to water scarcity (Samsorg 17) as accumulated sugars and alcoholic sugars in comparison with less drought tolerant variety (Samsorg 40) that accumulated free amino acids. Under the salt stress conditions, abundant amount of amino acids and soluble sugars has been observed in the roots of tolerant barley plants (Shelden et al.

2016). However, chilling stress is also known to induce accumulation of amino acids and carbohydrates as like water stress. As the chilling stress is known to cause extensive changes in metabolic profiles of rice varieties which includes the 19–11 (*Indica*) and Nipponbare (*Japonica*) (Zhang et al. 2016). Zhang et al. (2016) reported that one of the rice variant i.e., Nipponbare used to activate antioxidation pathway by modulating key metabolites such as γ-glutamylisoleucine, γ-glutamylglutamine, 5-oxoproline, glycine, glutamate, adenine dinucleotide and putrescine in order to adapt with chilling stress. Due to cold stress, in barley and wheat expedites the amino acid pool and induces the GABA-shunt genes to promote conversion of glutamate to GABA (Mazzucotelli et al. 2006). Cereal grains used to accumulate flavones or flavone-glycosides, which protects plants from various stresses (Caasi-Lit et al. 2007). For example, rice produces plenty of flavone-glycosides to protect it from abiotic stress and herbivores (Matsuda et al. 2012). However, examination of herbivore-induced defense system in maize showed an increase in azealic acid, *N*-hydroxycinnamoyl tyramines, phospholipids, tryptophan, and 1,3-benzoxazin-4-ones (Marti et al. 2013). Accumulation of resistance related metabolites are also reported during plant–pathogen interaction. For instance, a tolerant variety of wheat can accumulate a wide range of metabolites conferring tolerance such as coumaroylputrescine and coumaroylagmatine during fusarium head blight (Kage et al. 2016). Further, evaluation of these hydroxycinnamic acid amide compounds and their placement on metabolic pathways has led to the identification of an important gene *agmatine coumaroyl transferase* (*ACT*).

2.3 Genome Wise Metabolic Modeling

2.3.1 Generating a Draft Reconstruction Based on the Genome Sequence

Current sequencing innovation is delivering a great many sequenced genomes every year, changing the fields of genomics and bioinformatics and expanding interest for new apparatuses that empower high throughput age of working genome-scale metabolic models. With a working genome-scale metabolic model, culture conditions can be anticipated, phenotypes can be anticipated and accommodated with test information, and ineffectively clarified districts of the metabolic system can be recognized. To put it plainly, genome-scale metabolic models are fundamental to the utilization of grouping information to create itemized and quantitative forecasts of living being conduct. The well ordered directions for making a genome-scale remaking have been distributed as of late (Feist et al. 2009; Thiele and Palsson 2010), thus we will forego a point by point depiction of these means for putting accentuation on explicit issues that we have encountered to be basic, troublesome, not entirely obvious, or a blend of these. The means toward a remaking are (Feist et al. 2009; Thiele and Palsson 2010, with numerous representations or a stream graph in Francke et al. 2005; Henry et al. 2010):

(i) Creating a draft recreation dependent on the genome arrangement.
(ii) Recognize and resolve blunders, holes, and irregularities in the system.
(iii) Characterize outer metabolites and the biomass condition.
(iv) Approve the model with extra investigations.

Tools and methods exist that take either a sequence or an annotated genome and produce a first draft of a metabolic reconstruction automatically. One such system is Pathway Tools which underlies the BioCyc collections including the famous EcoCyc database (Keseler et al. 2011). It takes annotated genomes and identifies the metabolic genes based on EC code and name matching. Pathway Tools also includes algorithms for automatic gap filling. In our experience, the information in this resource is wonderful, but the automatically generated output of Pathway Tools results in a rather fragmented set of pathways that need extensive curation (Teusink et al. 2005). Also, the system is not explicitly designed for modeling, but rather, for making an inventory ("encyclopedia") of metabolic pathways, and their regulation. The SEED, a pipeline system for metabolic reconstruction, has been recently made publicly available (Henry et al. 2010). This system uses as input a sequenced genome and produces an SBML (Hucka et al. 2003) model that is able to produce biomass. However, we agree with Feist (Feist et al. 2009), and have stressed ourselves (Francke et al. 2005), that extensive manual curation cannot be fully replaced by automated methods. This is simply because there are too many important organism-specific choices that confer their own idiosyncrasies and set them apart from others. Expert domain knowledge about the organism under study is required to make these choices. With this in mind, we ourselves have developed an automated method that uses orthology prediction between genes of a query genome and genes from already existing genome-scale metabolic models, to copy the gene– protein–reaction associations (Notebaart 2006). The philosophy of this AUTOGRAPH method is that information of the manual curation process of the existing genome-scale metabolic models is being reused as much as possible. Issues resolved during manual curation relate to (i) reaction stoichiometries of specific reactions not found in the databases (or with wrong stoichiometries, still not uncommon); (ii) choices in cofactor usage; and (iii) annotations of genes on the basis of gap filling, extensive bioinformatic analyses, or experimental results.

2.3.2 Identify and Resolve Errors, Gaps, and Inconsistencies in the Network

After the initial draft of the model, manual curation involves going through the draft gene–protein–reaction associations to check for omissions, wrong assignments and gaps, and inconsistencies (Breitling et al. 2008). One insightful inconsistency that we have found (Francke et al. 2005) is the annotation of metA in *L. plantarum* to homoserine succinyl CoA transferase, while this organism lacks a TCA cycle and hence cannot make succinyl CoA. All databases nevertheless agree on this function for metA, presumably because it is a member of a distinct orthologous group that

includes the *Escherichia coli* enzyme, which does take succinyl CoA. However, the protein from B. subtilis, also in this orthologous cluster, has been experimentally shown to take acetyl CoA, but somehow this result did not find its way into BRENDA or any other database. So, sequence similarity, even one-to-one orthology, is not a guarantee for function transfer. Interestingly, the model SEED has kept the succinyl CoA-dependent annotation and hence, the gap, but allows biomass production through methionine transport, even though domain experts will know that L. plantarum grows without methionine (Teusink et al. 2005).

2.3.3 Define External Metabolites and the Biomass Equation

For a reconstruction to become a model, there are a number of additional steps that involve a minimum of experimental data without which the model is of poor predictive value. They come in three flavors:

(a) Biomass composition: biomass composition must be defined, ideally for the different experimental conditions under study. The Supplementary Material in Oliveira et al. (2005), on the biomass of *L. lactis*, gives an extensive and useful account of the data and computation that is required. Protein, RNA, and lipid content change with growth rate and may affect the model simulations.
(b) Data on the bioenergetics: this pertains to information on stoichiometry of pumps and the respiratory chain (P/O ratio), and on maintenance energy and ATP requirement for growth, often referred to as YATP (Tempest and Neijssel 1984). These data can be estimated by carefully monitoring product formation and biomass yield under a sufficient number of substrate/growth rate regimes; for excellent reviews on the techniques involved, see van Gulik and Heijnen (1995), Vanrolleghem et al. (1996), and Vanrolleghem and Heijnen (1998). Surprisingly, only very recently these parameters were carefully estimated for E. coli (Taymaz-Nikerel et al. 2010). But there is an important assumption here: measurement and inclusion of energetic parameters are based on full coupling between ATP production by catabolism and ATP utilization through anabolic processes. Without this assumption, we cannot do the calculations, and the predictions will be imprecise (Teusink et al. 2006).
(c) Essential and possible nutrients: growth requirement data (e.g., on specific auxotrophies for vitamins and amino acids, or possible carbon, nitrogen and sulfur sources) are used to decide on the biological relevance of a gap, for example, if a vitamin is not essential, then a gap in the vitamin biosynthesis pathways may be benign (Teusink et al. 2005). These data can also be used to determine the potential external metabolites, that is, sources for the network, and the necessary transport systems to take them up. Although it is not very often pointed out, sinks are equally important as sources, as they also require transport reactions, even for simple diffusion over the membrane. Easily overlooked sinks include by-products of biosynthesis pathways (e.g., folate biosynthesis produces glycolaldehyde), or diffusible substrates such as water and CO_2. Because genome-

scale models are not exclusively bottom-up but largely based on genome content, they tend to contain more (futile) cycles, parallel routes, and dead-end pathways which a biochemist or biochemical engineer would never put into his or her bottom-up model. These cycles are nevertheless potentially there in the network (Pinchuk et al. 2010; Teusink et al. 2006) and are either thermodynamically infeasible (Beard et al. 2002). The dead-end pathways point to missing biochemical knowledge (Hols et al. 2005) and can therefore be valuable leads to new discoveries, especially in combination with the data on substrate use and product formation. Metabolic reconstructions and their modeling therefore not only rely on gene annotation, but it can also help in functional annotation by identifying functions that have to be there.

2.3.4 Validate the Model with Additional Experiments

Once the model can form biomass by providing all the necessary components that form the biomass, validation of the model requires comparison with additional experimental data. One such set of data are phenotypes of deletion strains; comprehensive sets are unfortunately usually not available but for model organisms such as E. coli and yeast. Their metabolic reconstructions have been extensively tested against the deletion strain phenotypes, with relatively high success rates in the order of 80–90% (Covert et al. 2004; Kuepfer et al. 2005). A more accessible set of data that can be used is physiological data on growth rates, yields, substrate utilization, and product formation rates. Especially chemostat data are useful, as they are the best experimental setup for establishing growth in steady state. The model should be compatible with the measured fluxes, or, at a higher level of validation, should be able to predict some of the fluxes as a function of some input fluxes. Oberhardt (Oberhardt et al. 2009) gives a good overview of the different models that exist and the extent to which they have been validated through experimental data.

2.4 Advanced Metabolic Technologies

Quantitative plant metabolomics provides us with an in-depth understanding of plant metabolism, and helps to improve crop yields (Fernandez et al. 2016a, b). At present, NMR and GC/LC-MS techniques dominate the data acquisition strategies in metabolomics studies, which enable the identification of a myriad of species belonging to the three major classes of nutrient components (i.e., carbohydrate, amino acid, and lipids) in plants (Hagel et al. 2015; Fernandez et al. 2016a, b). Other chromatographic techniques, including capillary electrophoresis (CE) and supercritical fluid chromatography (SFC), can also be coupled to MS for metabolomics studies. However, CE and SFC are being applied to a less extensive extent than conventional GC/LC analyses due to the drawback of poor migration

time reproducibility and lack of reference libraries for CE (Obata and Fernie 2012), and fluid compressibility for SFC (Lesellier and West 2015).

2.4.1 Metabolite Coverage of NMR in Plant Metabolomics

NMR spectroscopy, one of the two leading analytical techniques in the field of metabolome research, is characterized by its reproducibility in quantification, structure identification, and nonbiased detection of metabolites (Ussher et al. 2016). NMR can quantify metabolites in large batches of samples with higher reproducibility and greater accuracy, coupled with a wider time span and dynamic ranges than GC/LC-MS can perform. Particularly in untargeted MS-based metabolomics, the measurements are semi-quantitative. NMR guarantees stable sensitivity as samples and instruments are devoid of contact during detection, eliminating the concerns of gradual contamination by residual metabolites that may compromise sensitivity in MS analyses. In addition, NMR provides the same signal sensitivity for all metabolites regardless of the complexities of the biological matrix, and is independent of the chemical properties of the metabolites (Nagana Gowda and Daniel 2015). NMR is a powerful technique for the analysis of metabolite structures, as it can differentiate compounds with identical masses and two differ only in spatial configuration (Imai et al. 2015). NMR is also preferred in metabolomics studies due to its simple detection requirements using intact bio-specimens without requirements for prior separation. In terms of plant metabolome analysis, NMR mainly covers metabolites, carbohydrate, amino acids, and organic acids (Hagel et al. 2015; Fernandez et al. 2016a, b). A major drawback of NMR technique, however, lies in its sensitivity (Imai et al. 2015; Chen et al. 2016a, b), which restrains its application to the detection of metabolites of low-abundance in plants. In addition, as molecular weight increases, for example, for lipids comprising long fatty chains, its identification capacity is weakened rapidly due to more complex and overlapping signals from the extended hydrocarbon chains in such compounds. The long-carbon-chain lipids, such as fatty acids (FAs) and phospholipids carrying single or multiple long fatty chains, can be further differentiated into hundreds of thousands of subtype species according to the lengths of fatty chains, the number of double bonds, and the functional groups, etc. As NMR can only provide classification based on the characteristic signals located in functional groups, it falls short in terms of conferring specific compound identification due to overlapping signals of methylene in long fatty chains.

2.4.2 Metabolite Coverage of GC/LC-MS in Plant Metabolomics

MS coupled to GC or LC is by far the most frequently applied analytical technique in plant metabolomics studies due to its unparalleled sensitivity and extensive coverage of biological information relevant to the metabolism of the organism (Kueger et al. 2012; Jorge et al. 2016). The plant metabolome reported to date is

composed of approximately 30,000 endogenous metabolites that mainly comprises various lipid classes, the majority of which can be easily characterized and quantified via MS. GC-MS is excellent for the detection of biological samples with very complex matrices, offering highly efficient separation and resolution. Not only can it analyze many of the aforementioned carbohydrates, amino acids, and organic acids detectable by NMR, but it aslo accurately identify a plethora of volatile and thermally stable lipids, or volatile derivatized metabolites, such as FAs (Tsugawa et al. 2011). More importantly, an immense number of well-curated compound reference libraries, including the NIST (Kumari et al. 2011), FiehnLib (Kind et al. 2009) and Golm metabolic databases (GMD (Kopka et al. 2005) are availabe for peak identification and prediction across different models of mass spectrometers, which have been proven very useful in terms of analysing metabolome data. Major limitations of GC-MS, however, lie in its inability to ionize thermolabile metabolites, such as di- and triphosphates, lysophosphatidycholine (LPC), lysophosphatidylethanolamine (LPE), or higher molecular masses phosphatidylcholine (PC) and phosphatidylethanolamine (PE), due to their non-volatile properties even after derivatization, circumscribing its application for global metabolic profiling in plants. This limitation narrows the GC-MS-derived metabolome both in terms of metabolite number and subtypes compared to that obtained using LC-MS. In comparison to GC-MS, LC-MS is useful in handing thermolabile, polar metabolites, and high-molecular weight compounds without derivatization, such as phosphatidylinositol (PI), PE, phosphatidic acid (PA), phosphatidylglycerol (PG), phosphatidylserine (PS), sulfoquinovosyldiacylglycerol (SQDG), PC, monogalactosyldiacylglycerol (MGDG), and digalactosyldiacylglycerol (DGDG) (Burgos et al. 2011). More importantly, with the advancement in ionization techniques, increasing scan speed, and improvement in terms of instrument sensitivity, metabolite coverage of LC-MS can be expanded into greater array of metabolite classes, traditionally dominated by GC-MS Tian et al. 2013). For example, volatile metabolites involved in tricarboxylic acid cycle (TCA), which are generally detected by GC-MS, can now also be analyzed by LC-MS (Shao et al. 2015). Even though GC-MS exhibits higher senstitivity for these volatile compounds aforementioned than LC-MS; the detectability of such compounds in LC-MS per se is enough for the quantitative analysis of targeted metabolites in plant.

2.5 Priming Concept and Salt Tolerance: Seed Priming

Priming allows some of the metabolic processes necessary for germination to occur without germination take place. In priming, seeds are soaked in different solutions with high osmotic potential. This prevents the seeds from absorbing in enough water for radicle protrusion, thus suspending the seeds in the lag phase (Taylor et al. 1998). Seed priming has been commonly used to reduce the time between seed sowing and seedling emergence and to synchronize emergence (Parera and Cantliffe 1994). In seed priming, the osmotic pressure and the period for which the seeds are

maintained in contact with the membrane are sufficient to allow pre-germinative metabolic processes to take place within the seeds up to a level limited to that immediately preceding radicle emergence. Methods for germinating seed and inducing desiccation tolerance in seed are also provided. Preferably the semi- permeable membrane is provided in the form of a tube of circular or polygonal cross-section, which is rotated with the seeds on its inner surface, and the solution retained between its outer surface and a further body to which the membrane is sealed in a watertight manner. Seed priming have important role in increasing the yield of different crops in relation to enhance 37, 40, 70, 22, 31, 56, 50 and 20.6% in wheat, barley, upland rice, maize, sorghum, pearl millet, and chick pea respectively (Harris et al. 2005). Seed priming technique has been practiced in many countries including Pakistan, China and Australia and more than thousand trials had been conducted to evaluate the performance of priming in a variety of crops. Fifty three farmers tested maize seeds priming in the kharif season in 1996 in tribal areas of Rajasthan, Gujarat and Madhya Pradesh; India (Harris et al. 1999). Almost all farmers thought that primed crops grew more vigorously, flowered and matured earlier and produced bigger cobs and higher yield. Independent measurements on a subset of 35 trials showed a mean increase in cob weight of 6% (Harris et al. 2001). Farmers in the project area of priming reported that primed crops grew more vigorously, tolerated dry spells better, flowered earlier (typically 7–10 days) and matured earlier (8–10 days) (Harris et al. 1999). Across different sites in farmer managed trials, priming led to a significant increase in maize grain yields by 105 kg ha^{-1} and 182 kg ha^{-1} higher than those from un-primed maize. An increase of 14% and 18% was recorded during 1999–2000 and 2000–2001, respectively in economic yield (Clark et al. 2001). The plants grown from primed maize seed were consistently larger and also flowered and matured earlier than non-primed seed (Murungu and Madanzi 2004). In six farmer implemented trials, total biomass (10.81 t ha^{-1}), straw yield (7.49 t ha^{-1}), cob yield (3.32 t ha^{-1}), grain yield (2.74 t ha^{-1}) of maize were significantly increased by priming with water compared to not primed treatment (Harris et al. 2007). Forty farmers primed sorghum seed in Zimbabwe during the 1997 and 1998 season and most of the farmers agreed that priming accelerated emergence and plants flowered and matured earlier relative to non-primed crops (Harris et al. 2001). Priming technique is the need of present time to get the enhanced germination and establishment in maize in order to utilize the soil moisture and solar radiation to a maximum extent. In this way plants would be able to complete their growth before the stresses arrive (Subedi and Ma 2005). Osmopriming is commercially used technique for improving seed germination and vigour. It involves controlled imbibition of seeds to start the initial events of germination followed by seed drying up to its original weight. Osmopriming has many advantages including rapid and uniform emergence, improved seedling growth and better stand establishment under any environmental and soil conditions (Chiu and Sung 2002). Polyethylene glycol and KNO3 solutions increased the fresh and dry weight of roots in maize at 2% and 5% concentration primed for 12 h and 18 h. In addition they also increased the vigour index (Abandani and Ramezani 2012). Grain yield was significantly increased in many crops subjected to priming as compared to

non-primed crops. The increase in yield was 13% in case of hydro-priming alone and 26% when primed with ZnSO4 solution. So, it can be concluded that both the treatments have contributed well to the total increase in yield. Final emergence, emergence index, plant height, leaf area, stover dry weight, total dry weight, individual cob weight, cob yield, cob number and number of grains per cob were observed to indicate almost same kind of response to priming treatments in increasing the final yield (Harris et al. 2007). Research on priming has proved that crop seeds primed with water germinated early, root and shoot development started rapidly, grew more vigorously and seedling length was also significantly greater than non- primed seeds. It could also improve the performance of crop by alleviating the effect of salts under saline soil conditions (Mohammadi et al. 2017). Soaking seed in water overnight before sowing can increase the rate of germination and emergence even in soil conditions where moisture content is very low (Clark et al. 2001). Using the leaf checked inhibition of fungal contaminants and bark water extracts of Jatrophacurcas and Moringaoleifera.

2.6 Conclusion and Future Perspectives

With the growing interest in the use of metabolomic technologies for a wide range of biological targets, plant metabolomics have dramatically improved in recent years. The combination of the capabilities of available analytical platforms for the analyses of complex samples, together with the integration of metabolomics with other "omics" and functional genetics, is able to provide novel insights into genetic and biochemical aspects of cellular function and metabolic network regulation. Plant metabolomics, alone or combined with functional genomics, has been applied in many fields. Even though it has some limitations currently, it is no doubt an important tool that is revolutionizing plant biology and crop breeding. The full elucidation of biochemical and genetic mechanisms underlying plant developmental and stress responsive biology depends largely on the comprehensive investigations using systematic omics techniques, which is the foundation for the application of metabolomics in plant science. Among them metabolomics is of particular importance, because the metabolites are more relevant to the plant phenotype (both physiological and pathological phenotypes) as compared with DNAs, RNAs or proteins. Therefore, future studies in this area will focus on both directions: one is the improvement of the metabolomic platform to facilitate the accurate and effective identification and quantification of as many as possible metabolites, the precise interpretation of generated data, and the rapid integration with other omics platforms; the other is the comprehensive investigation into molecular and biochemical mechanisms of metabolic variations in plants using both non-targeted and targeted approaches, to expand and enrich the understanding of plant metabolism in growth and development under both normal and stressed conditions, and the application of metabolomics to plant breeding for better crop yield and quality.

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Rhizosphere Engineering and Agricultural Productivity

3

Abstract

Animal and plant microbiomes encompass diverse microbial communities that colonize every accessible host tissue. These microbiomes enhance host functions, contributing to host health and fitness. A novel approach to improve animal and plant fitness is to artificially select upon microbiomes, thus engineering evolved microbiomes with specific effects on host fitness. We call this engineering approach host-mediated microbiome selection, because this method selects upon microbial communities indirectly through the host and leverages host traits that evolved to influence microbiomes. In essence, host phenotypes are used as probes to gauge and manipulate those microbiome functions that impact host fitness. To facilitate research on host-mediated microbiome engineering, we explain and compare the principal methods to impose artificial selection on microbiomes; discuss advantages and potential challenges of each method; offer a skeptical appraisal of each method in light of these potential challenges; and outline experimental strategies to optimize microbiome engineering. Finally, we develop a predictive framework for microbiome engineering that organizes research around principles of artificial selection, quantitative genetics, and microbial community-ecology.

Keywords

Microbiome · PGPR · Biocontrol · Stress · Tolerance · Plant architect

3.1 Microbes and Biotechnology

Microbes which include bacteria, fungi, protozoa, microalgae, and viruses are too small to be seen by the unaided eye. Microbes exit in all habitats that include soil, water, food, and animal intestines, as well as in more extreme settings such as rocks, glaciers, hot springs, and deep-sea vents. Huge diversity of biochemical and

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metabolic traits that have arisen by genetic variation and natural selection in microbial populations is reflected by microbial habitats. Genomic studies will lead to improved vaccines and better disease diagnostic tools, improved microbial agents for biological control of plant and animal pests, modifications of plant and animal pathogens for reduced virulence, development of new industrial catalysts and fermentation organisms, and development of new microbial agents for bioremediation of soil and water contaminated by agricultural runoff. For critical advances in food safety and food security, human nutrition and functional foods both microbial genomics and microbial biotechnology research is important. Microbes have been classified in different groups such as bacteria, fungi, protozoa, micro-algae and viruses. Men used some of microbial diversity in the production of fermented foods such as bread, yogurt, and cheese. Some soil microbes release nitrogen that plants need for growth and emit gases that maintain the critical composition of the Earth's atmosphere. Other microbes challenge the food supply by causing yield-reducing diseases in food-producing plants and animals. In our bodies, different microbes help to digest food, ward off invasive organisms, and engage in skirmishes and pitched battles with the human immune system in the give-and-take of the natural disease process. .. The habitats these organisms prefer include in soil, water, food, animal intestines and other different environments. Microbial diversity had been used by men from long ago for the production of fermented foods like bread, yogurt, and cheese. Plants need nitrogen for their growth and microbes release that nitrogen and also helps to fix atmospheric nitrogen hence maintaining the critical composition of the Earth's atmosphere. Microbes are not always beneficial some microbes challenge by causing yield-reducing diseases in food-producing plants and animals in human body microbes play an important role for digestion of food ward off invasive organisms, and engage in skirmishes and pitched battles with the human immune system in the give-and-take of the natural disease process.

Different organisms have different size of genome and obtaining the complete genome sequence of a microbe provides crucial information about its biology, but it is only the first step toward understanding a microbe's biological capabilities and modifying them, if needed, for agricultural purposes.

3.1.1 Microbial Biotechnology in Agriculture Natural Fermentation

Naturally occurring microorganisms have been used to develop biofertilizers and bio-pesticides to assist plant growth and control weeds, pests, and diseases to improve agricultural productivity. Microbes help in absorbing the nutrients from the soil in return gets food in the form of waste by- products. Scientists have utilised this property for developing the biofertilizers. Rhizobium is a bacteria used to make biofertilizers. This bacterium lives in nodules which are biological factories that can take nitrogen out of the air and convert it into an organic form that the plant can use. This fertilization method has been designed by nature. Phosphate and nitrogen are important for the growth of plants. These compounds exist naturally in the

environment but plants have a limited ability to extract them. Either coating seeds with the fungus as inoculation, or putting it directly into the ground applies bio fertilizer made from this organism. Phosphate plays an important role in crop stress tolerance, maturity, quality and directly or indirectly, in nitrogen fixation. A fungus, Penicillium bilaii helps to unlock phosphate from the soil. It makes an organic acid, which dissolves the phosphate in the soil so that the roots can use it. Biofertilizers help farmers to reduce the amount of chemical fertilizers by using the naturally all the food available in the soil thus there by preserving the environment for the future generations. All microbes are not beneficial some of the microbes which are pathogens can cause disease or damage plants but scientists have used this property of microbes to control weeds and pests naturally. Weeds not only compete with crops for water, nutrients, sunlight, and space but also harbor insect and disease pests; clog irrigation and drainage systems; undermine crop quality; and deposit weed seeds into crop harvests hence are problematic for farmers. The solution for such problem are bio-herbicides, which not only control weeds but also protect the environment. Microbes have given solution for this problem, microbes possess invasive genes that can attack the defence genes of the weeds, thereby killing it. The benefit of using bioherbicides is that it can survive in the environment long enough for the next growing season where there will be more weeds to infect. It is cheaper than synthetic pesticides thus could essentially reduce farming expenses if managed properly. Further, it is not harmful to the environment compared to conventional herbicides and will not affect non-target organisms.

Fungi can cause diseases in 200 different insects and this property of causing the diseases is being used for production of bioinsecticides. Spores are harvested and packaged so these are applied to insect-ridden fields. When the spores are applied, they use enzymes to break through the outer surface of the insects' bodies. Once inside, they begin to grow and eventually cause death. Some researchers as having the best potential for long-term insect control recommend fungal agents. Since these bioinsecticides attack in different ways it is very difficult to develop resistance against these bioinsecticides. Baculoviruses affect insect pests like corn borers, potato beetles, flea beetles and aphids. One particular strain is being used as a control agent for bertha armyworms, which attack canola, flax, and vegetable crops. Traditional insecticides do not affect the worm until after it has reached this stage and by then much of the damage has been done. Cold environment microbes are of particular importance in global ecology as majority of terrestrial as well aquatic ecosystems of earth are permanently or seasonally submitted to cold temperatures. The microbial communities have developed physiological adaptations to low temperature and chemical stress and have attained the focus of applied research not only in terms of biotechnological prospects but also to understand the use of primitive analogues of biomolecules existed during early Earth environments (Yadav 2015; Saxena et al. 2016). Cold adapted microbes are now been extensively investigated from past few years with a focus on culture dependent and culture-independent techniques. Many novel microbes have been isolated from cold environments which include Halobacterium lacusprofundi (Franzmann et al. 1988), Sphingobacterium antarcticus (Shivaji et al. 1992), Octadecabacter arcticus (Gosink et al. 1997),

Hymenobacter roseosalivarius (Hirsch et al. 1998), Cellulophaga algicola (Bowman 2000), Flavobacterium frigidarium (Humphry et al. 2001), Oleispira antarctica (Yakimov et al. 2003), Flavobacterium psychrolimnae (Van Trappen et al. 2005), Psychromonas ingrahamii (Auman et al. 2006), Exiguobacterium soli (Chaturvedi et al. 2008), Pseudomonas extremaustralis (López et al. 2009), Cryobacterium roopkundense (Reddy et al. 2010), Sphingomonas glacialis (Zhang et al. 2011), Pedobacter arcticus (Zhou et al. 2012), Sphingobacterium psychroaquaticum (Albert et al. 2013), Lacinutrix jangbogonensis (Lee et al. 2014), Massilia eurypsychrophila (Shen et al. 2015), Glaciimonas frigoris (Margesin et al. 2016) and Psychrobacter pocilloporae (Zachariah et al. 2016). For novel and potential psychrotrophic microbes there are several reports on whole genome sequences (Kim et al. 2014). Worldwide isolation of these novel species of psychrotrophic microbes have been reported from different domain archaea, bacteria and fungi which included members of phylum Actinobacteria, Proteobacteria, Bacteroidetes, Basidiomycota, Firmicutes and Euryarchaeota (Shen et al. 2015). From the NW Himalayas some microbial species that have been isolated and reported for the first time include Arthrobacter nicotianae, Brevundimonas terrae, Paenibacillus tylopili and Pseudomonas cedrina and these microbes exhibited multifunctional plant growth promoting (PGP) attributes at low temperatures (Yadav et al. 2015a). study carried out by Yadav et al. (2015b), the microbial species Alishewanella sp., Aurantimonas altamirensis, Bacillus baekryungensis, B. marisflavi, Desemzia incerta, Paenibacillus xylanexedens, Pontibacillus sp., Providencia sp., P. frederiksbergensis, Sinobaca beijingensis and Vibrio metschnikovii have been reported first time from high altitude and low temperature environments of Indian Himalayas while as Wheat associated psychrotrophic bacteria Arthrobacter methylotrophus and Pseudomonas rhodesiae have been reported first time from wheat growing in North hills zone of India (Verma et al. 2015). In a specific search of economically important Bacillus and Bacillus derived genera (BBDG) at low temperature, Various BBDG such as *Bacillus psychrosaccharolyticus, B. amyloliquefaciens, B. altitudinis, B. muralis, Paenibacillus tylopili, P. pabuli, P. terrae and P. lautus* with efficient PGP attributes like biomass degradation, fuel production, mineral recovery and nutrient recycling.

3.1.2 Microbes in Biofuel Production

Biofuel which is actually derived from lignocelluloses component of the plant is derived from the biological origin and is a fine alternative of conventional energy sources like Coal, Petrol, and Diesel etc. The main constituents of lignocellulose are cellulose, hemicellulose and lignin (Reddy and Yang 2005). Cellulose is the main structural component of plant cell walls. It is a long chain of glucose molecules, linked by glycosidic bonds. Hemicellulose, the second most abundant constituent of lignocellulosic biomass, is a family of polysaccharides, composed of

monosaccharide units. The most potent constituent of biomass to serve as a source of bioethanol production is hemicellulose after cellulose. Three dimensional polymer of phenylpropanoid units is Lignin. The carbohydrate fractions of the plant cell wall can be converted into fermentable monomeric sugars through acidic and enzymatic (hemicellulase/cellulase) reactions, which have been exploited to produce biofuels like ethanol, butanol, isobutanol via microbial fermentation processes. Thus simple sugars are subsequently converted into fuels by microorganisms (Bacteria, cyanobacteria, yeast). There are diverse microbial strategies for degrading lignocellulose. The current knowledge about the biomass degradation is limited and research is going on throughout the world to know more about biomass degrading microbes. Currently few species like Trichoderma reesei and the bacterium Clostridium thermocellum (DelRio et al. 2007) are known to be modal organisms to degrade biomass. Thus, enzymes derived from thermophilic and acidophilic organisms known to degrade lignocellulose, hold significant promise for industrial processes (Viikari et al. 2007). Many novel enzymes and enzyme systems that have evolved to make use of cellulosic biomass are present in those microbes, which is difficult-to-culture (Hugenholtz 2002). The availability of a wide range of naturally occurring lignocellulose-degrading enzymes increases the chances of successful enzyme optimization for industrial processes. Optimization of the saccharification process is crucial because the cost of cellulases remains a key barrier to economical production of biofuels (Himmel et al. 2007). A more diverse set of candidate enzymes identified through a combination of conventional cultured microbial studies coupled with environmental prospecting methods will improve the likelihood of obtaining enzymes with activities and stability suited to a variety of industrial processes Whereas the conversion of starch-based biomass results primarily in hexoses, and also the pentose sugars D-xylose and L-arabinose. In contrast to the hexose sugars, the pentose sugars cannot be fermented by wild-type Saccharomyces cerevisiae (Van Maris et al. 2007). Genetically engineered Escherichia coli because of it has capacity for the conversion of all hexose and pentose sugars both (Görke and Stülke 2008). Recently *E. coli* has been engineered for large scale production of isobutanol and other alcohols via a non-fermentative pathway. Genomics studies and pathway-engineered microbes will considerably facilitate the process of biodegradation and biofuel production in the future. Thus biofuels produced by microorganisms from renewable materials are promising substitutes for traditional fuels derived from fossil sources. The demand for sustainable alternative fuels based on renewable resources is already high today but will dramatically increase in the future. Today biofuel industry primarily produces ethanol from corn starch or sugar cane, and biodiesel is generated from vegetable oils and animal fats. However, these first generation biofuels, especially ethanol produced from starch, are in competition with the food and animal feed industry. In contrast, lignocellulosic biomass like crop wastes, forestry residues and municipal solid waste offers a high potential as feedstock for biofuels because it is the most abundant sustainable raw material worldwide. Fuels produced by microbes should help meeting energy- crisis world over.

3.1.3 Microorganism in Metal & Mineral Recovery

Mineral resources are being depleted at fast rate and high-grade ore deposits are easily accessible its necessary to shift our attention towards low grade deposits or to extract valuable metals from industrial wastes. But it's also true that there is no appropriate technology available for recovery of low grade deposits, but certain microbes have shown promising results do it efficiently and efforts are being made to use them for enhanced recovery of minerals. This whole process of using microbes for extraction of minerals from natural deposits is called bioleaching or microbial mining. In other words Microbial mining is the process of bioleaching recovers metals from ores that are not suitable for direct smelting due to their low metal content. Only ores containing sulfur can be bioleached because the bacteria feed on sulfur. Metabolic activities of bacteria are being used for the recovery of metals from the low grade ore and the bacteria having potential to do that are Thiobacilli, particularly T. ferrooxidans, T. thiooxidans, Acidithiobacillus ferrooxidans and Acidithiobacillus thiooxidans (Pradhan et al. 2010). They are thermoacidophilic autotrophic archaebacteria grow in acidic and hot environments. They thrive at extremely low pH (pH 1–2) and fixes both carbon and nitrogen from the atmosphere. It solubilizes copper and other metals from rocks and plays an important role in nutrient and metal biogeochemical cycling in environments. It has been demonstrated that these Thiobacillus spp. can be used for extraction of copper and uranium from insoluble minerals. This implication of microbial activity in leaching and deposition of mineral ores could develop into a recent field of biotechnology known as biohydrometallurgy.

The basic principal for bioleaching is that microbes actually get energy by breaking down minerals into their constituents. The bacteria feeds on nutrients in minerals, thereby separating the metal that leaves the organism's system; then the metal can be collected in a solution. The bacterium uses a chemical reaction called oxidation to turn metal sulphide crystals into sulfates and pure metals Recovery of oil using microbes is termed as Microbially Enhanced Oil Recovery (MEOR) and Xanthomonas campestris is used in this process. Fungi has shown a promising result to be used in the process of bioleaching. Fungi can be grown on many different substrates, such as electronic scrap, catalytic converters, and fly ash from municipal waste incineration. Experiments have shown that two fungal strains (Aspergillus niger, Penicillium simplicissimum) were able to mobilize Cu and Sn by 65%, and Al, Ni, Pb, and Zn by more than 95%. Aspergillus niger can produce some organic acids such as citric acid. This form of leaching does not rely on microbial oxidation of metal, but rather uses microbial metabolism as source of acids which directly dissolve the metal.

The process of bioleaching has certain advantages (i) Bioleaching is generally simpler and therefore cheaper to operate and maintain than traditional processes, since fewer specialists are needed to operate complex chemical plants. (ii) The process is more environmentally friendly than traditional extraction methods. For the company this can translate into profit, since the necessary limiting of sulfur dioxide emissions during smelting is expensive. Less landscape damage occurs, since the

bacteria involved grow naturally, and the mine and surrounding area can be left relatively untouched. As the bacteria breed in the conditions of the mine, they are easily cultivated and recycled. Yet overall, bioleaching creates less air pollution and minimal damage to geological formations, since the bacteria take place there naturally. Microorganisms play a significant role in the recovery of metal and minerals, which is extensively used for our need and survival.

3.1.4 Environmental Nutrients and Microbes

Nutrients are essential for every living organism because they are used in an organism's metabolism, which must be taken in from its environment (Elanor and Rolfes 2005). Plants and animals consume large quantities of nutrients. There are two types of nutrients (i) Organic nutrients: include carbohydrates, fats, proteins and vitamins. (ii) Inorganic chemical compounds such as carbon, hydrogen, oxygen, nitrogen, phosphorus, and sulphur, water, and oxygen may also be considered nutrients (Sizer and Whitney 2007). Carbon (C), Hydrogen (H), Oxygen (O), nitrogen (N), phosphorus (P), and Sulphur (S) are the elements considered basic for cell growth. Microbes (most frequently bacteria) play an important role as mineralizers of organic detritus and recyclers of essential nutrients in environment.

Foundation of ecosystem is constituted by bacteria, being responsible for degradation and recycling of elements. Minerals, or intermediate products of their decomposition, may be directly or indirectly necessary to their metabolism. The dissolution of sulphide minerals under acidic conditions, the precipitation of minerals under anaerobic conditions, the adsorption of metals by bacteria or algae, and the formation and destruction of organometallic complexes are all examples of indirect microorganism participation. There are three categories of oxidation-reduction reactions for minerals with microorganisms: (i) Oxidation by autotrophic or mixotrophic organisms. Energy derived from the oxidation reaction is utilized in cell synthesis. (ii) Electron acceptance by minerals (reduction) for heterotrophic and mixotrophic bacteria. Chemical energy is used to create new cell material from an organic substrate. (iii) Electron donation by minerals (oxidation) for bacterial or algal photosynthesis (reaction is fuelled by photon energy).

Nutrient cycle occurs when nutrients are available in the environment. The nutrient cycle is essential to maintain the percentage of the nutrients and therefore it involves movement of nutrients across various trophic levels in the food chain. Nutrient cycle is a continuous process as nutrients are recycled back from their origin. If microbes do, not break down the dead material, or detritus, those nutrients will never become available to help sustain the life of other organisms. As we know that C, H, O, N, P and S are important for living organism. Recycling of these elements in environment is called as biogeochemical cycle or nutrient cycle. Hence biogeochemical cycle or nutrient cycles ate the path ways in which a element or molecule moves through all components of biosphere. Microbes play an important role for CO2 fixation and recycle carbon in atmosphere. Recycling of H and O is actively involved with the other cycles like the carbon cycle, nitrogen cycle, sulfur

cycle and phosphorous cycle as well. Among all biogeochemical elements recycling, most studies on recycling have focused on N, P, S because these elements often limit primary production (Vanni 2002). Nitrogen is a substance that is essential for all life on the earth. Most nitrogen can be found in air in the gaseous form (78%), but nitrogen can also be found in water and soil in dif¬ferent forms. There, it will be decomposed by bacteria (decomposer) and absorbed by plants and animals. Nitrogen is a part of vital organic compounds because it is chief constituents of amino acids, proteins and DNA. Nitrogen in the gaseous form cannot be absorbed and used as a nutrient by plants and animals; it must first be converted by nitrifying bacteria, so that it can enter food chains as a part of the nitrogen cycle. During the nitrogen fixation process cyanobacteria first convert nitrogen into ammonia and ammonium (ammonium fixation). During the assimilation process, plants absorb ammonium and nitrate, after which they are converted into nitrogen-containing organic molecules, such as amino acids and DNA.

3.2 Root Micrbiome Engineering

The main aim from the early days of agriculture is breeding crops for bigger and more nutritious value, but this cannot be done only by genetic manipulation only. In the Arabidopsis experiments, bacteria from the roots of the largest plants were harvested with a filter and given to other plants growing from seed. Over time, the plants, which were genetically identical and therefore could not evolve by themselves, grew better because of their evolved and improved microbiomes.

3.2.1 Rhizosphere Design and Engineering

For variety of reasons plant ecosystem are valued, e.g., food, feed, and fuel productivity (and hence human livelihood and income), climate regulation, carbon and water cycling, carbon storage, nutrient trapping, provision of wildlife habitats, and recreational activities. Given the wide range of genotypes that can be collected and/or generated per a specific plant species, genetic diversity is a potentially important asset in maintaining or increasing plant ecosystem values, e.g., in controlling stability and stress resilience in native and cultivated ecosystems, productivity in cultivated ecosystems, and ecosystem function. Hence in ecosystem design and breeding programs selection of genotypes and species should be taken in consideration.

Promising ways to accumulate favorable alleles associated with stress tolerance in a plant genome include traditional plant breeding approaches and advanced plant genome editing-based methods. Root exudation is the one of the main ways plant modifies the rhizosphere and there have been few attempts in this context to engineer the rhizosphere by manipulating the efflux of H+ and organic anions from the roots in transgenic plants (Ryan et al. 2009a, b). There are ample examples of genes controlling exudates but its seems easy and feasible to modify expression levels of those genes in plants to redesign rhizosphere for improved features. For example,

transgenic rice and tomato plants transformed with the Arabidopsis vacuolar H + -pyrophosphatase gene AVP1, showed approximately 50% greater citrate and malate efflux than wild-types when treated with AlPO4. This was interpreted as a means to enhance resistance to Al3+ stress and improve the ability to utilize insoluble phosphorus (Yang et al. 2007). However, it is important to note that plant engineering to impact rhizosphere could be a very complex process due to degradation or inactivation of the engineered compound in the soil, small rate of exudation to influence the rhizosphere, limited knowledge about root exudates composition, and changing of exudate releasing time and levels with plant development and external stimuli.

For plant/crop growth promotion, disease resistance, and stress tolerance/regulation bioengineering of synthetic microbial communities presents a unique opportunity. There are hundreds of bacterial strains which have been identified for beneficial effects; engineering a sustainable synthetic microbial community represents a significant challenge. For example, in a simple two strain co-culture, six ecological interaction factors must be taken into account (Grosskopf and Soyer 2014a, b): (1) Commensalism, that is one strain benefits from the other without affecting it, for example products from one strain serves as substrates for the other; (2) Competition, for example substrate competition; (3) Predation, that is the predator benefits while the prey is disadvantaged; (4) No interaction, a net zero effect with no shared substrates, no predator-prey relations, and no competition; (5) Cooperation, both strains benefit from each other, or syntrophy or cross-feeding: and (6) Amensalism, one strain is negatively affected while the other strains remain unaffected. For example, a Bacillus spp. could be engineered to contain a nitrogen-fixation machinery (e.g., NifH from Paenibacillus (Kim and Timmusk 2013), produce high concentrations of plant hormones (Arkhipova et al. 2005), or add pathways from other Bacillus spp. to control pathogens (Köberl et al. 2013, 2015). Pseudomonas, Rhizobium and/or Bradyrhizobium genera could be added for increased nitrogen fixation. A simple three-strain member consortium, including an engineered *Bacillus* spp. with two natural or engineered nitrogen fixers like Pseudomonas, Rhizobium and/or Bradyrhizobium, could provide many of the benefits of a more complex natural rhizosphere community. To add an element of control towards cooperation over competition, genes could be eliminated from each member such that synthesis of an essential co-factor would require the biosynthetic machinery of all strains. A conceivable scenario would be a system where the Bacillus needs a co-factor from the Pseudomonas, the Pseudomonas is dependent of an essential gene from the Rhizobium, and, finally, the Bacillus could remove a waste product produced by the Rhizobium that is subsequently recycled by the Bacillus for mutual use. The potential ecological functional interactions increase with the number of strains added; a three-strain consortium would potentially contain >729 predicted interactions, and a four-member consortium 531,441 predicted interactions (Grosskopf and Soyer 2014a, b). Efforts should be taken to limit the number of strains within a synthetic microbial community to three strains in order to exert control of potential interactions. In designing a microbial community for an engineered rhizosphere, critical elements must be considered in the realm of microbial competence (Bashan et al. 2014).

Microbes that are motile and chemotactically driven to roots by the host's plant root exudates and with strong attachment to the host plant allows for stronger association and possibly better beneficial effects to the plant (Bashan et al. 2014). The density plays an important factor to decide that whether or not the microbe will have a beneficial effect. *Pseudomonas* spp. is the best example, which needs a minimal growth density (i.e., 105–106 CFU g^{-1} of root) in order to protect plants from pathogens. Microbial consortia must be able to tolerate herbicides fertilizers, and pesticides without losing any of the beneficial effects If standard agronomic farming practices are used. Besides these challenges the other challenge is how, when, and where to inoculate? The proper microbes which have been selected. For example, does inoculation work better during the day or night; should inoculation be applied to root, leaves, or both; and does the inoculum need to be alive or can it be lyophilized (O'Callaghan (2016).

3.2.2 Advances in Soil Microbiome Analysis

Soil microbiome contains thousands of ecosystems with high diversity of interacting microorganisms. Rapid sequence and identification of soil DNA has enabled metagenomic analysis technique. For example, inter- rogation of the genetics of whole microbial communities allows us to probe the physiological characteristics and potential of plant-associated microorganisms (Knief 2014). For characterisation of marker genes, typically 16S rRNA in the case of bacteria helps us to chracterise the relative abundance of different species in phyllosphere and rhizosphere communities, while metatranscriptomic approaches may be used to examine the metabolic activities and regulatory mechanisms that function in different environments. It is important to know metabolic, natural product and genomic diversity rather than Understanding microbial function (Seaton and Silby 2014) Metabolic behaviour of the nitrogen fixing species Rhizobium varies profoundly between the rhizo-spheres of different plant species. The relative abundance of individual gene in the population of a single species group is influenced by environmental variation. Culturing and isolation of microbes using newly developed methods will markedly increase our capacity to understand both the overall microbiome, and the individual species within it. Complete assignation of functional genes to particular microbial OTUs in the soil is challenging, although the reconstruction of a draft genome from a novel soil methanogen indicates that this may become more common place in the future (Mackelprang et al. 2011). Microbiomes are not passive players rather, microbes are capable of altering host development physiology, and systemic defenses, enable toxin production and disease resistance, increase host tolerance to stress and drought, modulate niche breadth, and change fitness outcomes in host interactions with competitors, predators, and pathogens (Friesen et al. 2011). The reason being that microbiomes contain hundreds folds gene than host genomes, and because this 'hologenome' _of a host– _microbiome association can vary over space and time, microbiomes can function as a phenotypically plastic buffer between the host-genotype's effects and the environmental effects that interact to shape host

phenotypes. Thus expression of any host phenotype depends on the presence and taxonomic makeup of host associated microbes to some extend. The other option is to employ metatranscriptomics, metaproteomics or metagenomics to infer functional properties of the whole microbial community or of focal microbial taxa within it. Another option available is experimentally manipulation of taxonomical makeup of microbiomes to test hypotheses about microbiome function. For example, gnotobiotic hosts can be maintained with a defined set of microorganisms, and microbiomes can be manipulated with antibiotic treatments or transfer of microbiomes between hosts (Koch and Schmid-Hempel 2011). With any of these approaches, it remains challenging to elucidate specific functional roles of the microbiome in shaping host performance traits (e.g., growth, health, enemy deterrence, mate attraction, fertility, and overall fitness). Above all the central to this challenge is the complexity of microbiome properties which can vary with both the host genotype and the environment (Heath and Stinchcombe 2014).

To improve animal and plant performance by altering their microbiomes is the new research horizon in medicine and agriculture. A novel and underutilized approach employs artificial selection on a host–microbial association to engineer microbiome function, a process that we term host-mediated microbiome selection, or more simply microbiome engineering. The aim of microbiome selection is to improve host performance via artificial selection on the microbiome. Host performance can include any trait that is biologically, medically, or economically important (e.g., growth rate or disease resistance). Artificial selection on a microbiome can be efficient because (i) many important traits in animals and plants are directly influenced by interactions with microbes, and (ii) hosts can mediate microbiome assembly and relative abundance of microbial components (Bakker et al. 2014). Host-mediated microbiome selection therefore leverages host traits that have evolved to manipulate microbiomes in ways to enhance host fitness. Microbiome functions that have been artificially selected can then be analysed by comparing taxonomic makeup and genomic properties among diverged communities that evolved under different selection regimes (e.g., selection for microbiomes promoting early versus late flowering, to quantify types and diversity of microbiome taxa that diverged under different selection treatment, to resolve candidate drivers of altered functions, and to identify microbial taxa for focal experiments. To our knowledge, only a handful of studies have used such an experimental-evolutionary approach to shape microbiomes or to elucidate microbiome function, yet both theoretical modeling and empirical work suggest that host-mediated artificial selection can generate diverged microbial communities with significantly improved effects on hosts (Bernstein and Carlson 2012). The next research frontier is to optimize microbiome engineering for key host traits (e.g., drought tolerance, immune defense, rapid growth, or fecundity) and to accumulate vital information for elucidating the nature of microbial communities that can modify these host phenotypes. Towards these goals, host-mediated microbiome selection is a novel tool that is complementary to prevailing research approaches The traits of animals and the plants evolved to recruit selectively beneficial microbes into symbiosis, reward beneficial genotypes, and exclude or sanction ineffective symbionts for microbiome engineering. t

the initiation of a host–microbe interaction, host control occurs via partner choice or screening, in which the host selectively alters the subset of microbes that are allowed to colonize or persist in association with the host (e.g., via resistance, immunity, and genotypic specificity (Archetti et al. 2011). Finally, some hosts captured a subset of microbes by transmitting them vertically to their offspring, as occurs in bacterial and fungal symbionts that are co-propagated from parent to offspring within animal and plant lineages. The fitness interests of the microbes with their hosts is firmly bound by the combined effect of the host control and vertical transmission (Mitri et al. 2011) and also permit the host to assure that all their progeny receives the specific genotype hence the microbiome with the help of microbiome engineering, advanced and novel functions of microbiome can be chosen without having any information of the microbiome composition and their synergistic interactions. The synergy that for example comes from the relationship between the members of microbiome help to metabolize toxic substances or to reduce stress that would otherwise kill each individual member of the community found in isolation. The synergy is also seen in the host-microbe interaction that exists in root-nodule symbiosis in which the trait responsible for nitrogen fixation is present only in well-matched genotype pairs of plant and bacteria, but neither of the partner expresses the trait for nitrogen fixation if the other partner is absent.

The microbiome functions are usually expressed as an incessantly varying phenotypes of the host. Therefore, the quantitative-genetic outlook of microbiome engineering lies in the fact that the artificial selection acts directly upon the host since the phenotype of the host is premediated diectly while the selection of the microbiome is indirectly through their consequences on the phenotype of the host. However, the indirect selection is considered to be more proficient and cost-effective in comparison to direct selection (Mitri et al. 2011). Indirect selection is highly valuable and mostly useful method because the directly selected trait is generally very complicated and expensive to measure (Bernstein et al. 2012), as is usually the case for microbiome properties as compared to the easiness of measuring the traits of host.

However, the microbiome engineering can modify the microbiome by means of two processes that include: (i) ecological processes that involve the alteration in community evenness and diversity, relative abundance of species and the structure of interactions networks between host-microbe and microbe-microbe and (ii) evolutionary processes which include the modification in allele frequency, mutation, extermination of microbial types in the community and horizontal gene transfer that help in reshuffling of microbial genes. The high-throughput DNA sequencing techniques help in tracking both the evolutionary and ecological processes that surmise the presence and absence of the taxon, active microbial functions which are being expressed and also allow the mechanistic inferences of microbiome functions. The Host-mediated microbiome engineering is therefore regarded as the most powerful method that helps to control, manipulate and understand the functions of the microbiome. A large number of species of both plants and animals contain such traits that allow the continuation of microbiome to other hosts, via the transfer of microbes among the individual members belonging to same generation (horizontal transfer) or via the inheritance of microbiome from parent to progeny between generations of

the host (vertical transfer) (Mitri et al. 2011). The horizontal transmission may occur through infections, contact transmission with the host or exclusion of microbial partners into the environment that thus becomes available to the new hosts. In simplest host-microbe association such as that of bacterial endosymbionts found in insects, the vertical transmission is readily seen. The vertical transmission takes place through a number of different pathways that include transovarial transmission or through behavioral mechanisms like coating an egg covering with bacteria by the mother, which is later attained by the young ones upon hatching. Similarly, the inheritance of microbiome components from mother to offspring is seen in case of humans during or even before birth and likewise the microbiome of honeybee gut from clusters of eight bacterial species is transmitted by newborn bees from sibling workers or from the environment of hive (Moran 2015). Considerably, the vertical transmission of microbiome may arise with various degrees of fidelity that is measured as the likelihood with which an individual microbial genotype or the community as a whole is effectively transmitted from the mother to her offspring.. The fidelity may ranges from nearly 100% in case of transovarial transmission (where all the maternal symbionts are inherited to the progeny and the relative abundance of such transmitted symbionts may change little between generations), to lower values of fidelity as in case of gut or surface microbiomes which are inherited little faithfully from the mother to the offspring. Conversely, the inter-individual maintenance of the microbiome or its components across the generations may produce microbiome-dependent phenotypic variation in host phenotypes on which the natural selection can act upon (Foster and Wenseleers 2006). The chief characteristic feature to optimize the microbiome function is the host's ability to selectively inherit the useful microbiome to the succeeding generations or experimentally imposed high fidelity transmission.

However, the vertical transmission may help to stabilize the microbiome community over time and the effectiveness of host-mediated microbiome engineering could be limited by the swift turnover in the composition of microbiome community. The microbiome properties which are shaped by the artificial selection can be eroded by the stochastic loss of microbial genotypes or by conscription of new genotypes. Though the two principal mechanisms may stabilize the microbiome across host generations and can help in preserving the changes in microbiome composition that exists between generations, thereby increasing the heritability of the microbiome (Peiffer et al. 2013). Firstly, the co-dependency of microbial associates or partners decreases the possibility that one among the partners may be lost from the microbiome and secondly, the choice of symbiont put forth by the host can differentially attain, strengthen and maintain the specific microbial types with beneficial effects upon the fitness of the host thus reducing the turnover again. Microbiome is considered to be inherently stable because they have an ability to quickly return to the original state if disturbed that is what we call "community resilience", thus they are not easy to invade once established i.e., community resistance. Due to the turnover reducing mechanisms one might be more likely to attain such a response to indirect selection on the microbiomes which are having inherent co-dependencies or on the microbiome on which the co-dependency may be experimentally imposed,

or on that microbiomes which is transmitted vertically as in microbiome of honeybee gut, rather than environmentally attained microbiome as in case of rhizosphere microbiome. In order to preserve the useful properties of engineered microbiome, the environmentally restructured microbiomes needs a continuous selection for example rhizosphere microbiomes that are engineered in the greenhouse agriculture, while engineered microbiomes that are transmitted vertically as in case of microbiome of honeybee gut that is more likely to persist across many generations of honeybee even if the continuous selection is absent thus maintaining the functions of microbiome.

The recent approaches like quantitative-genetic and community-ecology approaches have converged to model the evolution and ecology of the microbiome that co-propagates with the host lineages over many generations (Fitzpatrick et al. 2015). In case of quantitative-genetic approach, the host's phenotype is considered to be the most developing property of the genotypes of several interacting partners (such as host and associated symbionts). Such type of interactions may change the evolution of host by contributing to the phenotypic variation of host, probably shifting the response to selection on the host. Indirect selection of microbiomes which is host-mediated can be predicted on such community heritability or microbiome heritability, particularly that a property of microbiome which exists in one generation can be perpetuated to the next generation across the selection cycle as shown by the experiments that are selected artificially on host-associated microbiome. The methods of Host-mediated microbiome engineering may be imposed by either through one-sided host-microbiome selection by selecting a microbiome in a specific host-genotype background as in an inbred host line in a stable environment, or by concurrently selecting both the host and the microbiome i.e., two-sided host-microbiome selection. Furthermore, presently there are no reports available that can compare the efficiency of these selection methods, however one can expect a more efficient selection on the microbiome in a specific host-genotype background, for example in an inbred or clonal host and with stable environment, relative to the host environment which is genetically heterogeneous that introduces genetic changes underlying the host phenotype which is not correlated to the properties of the microbiome. In order to develop the new host-microbiome system for microbiome engineering, it is important to carefully manage all the environmental noise that can obscure any signal and keep the whole experimental setup simple and time-efficient (Garland and Rose 2009). However, the very essential element of protocol for design of microbiome engineering is to carefully choose that host trait or phenotype which could act as direct target of selection. For allowing the indirect selection for the function of microbiome, it is important to choose such a host trait which is significantly affected by the microbiome. Before the transfer of microbiome into the host generations the host trait should ideally be easy to measure so that it could be evaluated readily during the experiment. Lastly, all the traits of the hosts need to be biologically, economically or clinically significant for examples surrounding alternatives of host health, tolerance to stress, resistance to desiccation, metabolism or any other phenotypically plastic trait whose microbiome influences are known. In order to produce the different and functionally variable associations between

host-microbiome the native microbiomes are considered to be well suited. For example, the use of microbiome from the wild hosts as in case of soils that enclose the resident plant roots, rather than those from random microbiomes as in case of bulk soils which does not surround any plant, need to expedite the response to selection, since a randomly selected microbiome needs initially to adjust with the new environment of the host during the primary selection cycles. Also, the host-mediated microbiome engineering is thought to be highly efficient when using wild hosts instead of domesticated hosts. It is because during domestication the genes that allow the host to control interactions with microbes may be lost (Mitri et al. 2011) and the agricultural soil microbiomes vary significantly between the succeeding generations of plant in the dearth of host-microbiome co-propagation. However in comparison to domesticated plant species, both the honeybees and their respective gut associated microbes that are vertically transmitted are being shaped by artificial selection during domestication, and thus both the wild as well as domesticated honeybees may be an appropriate replica for microbiome engineering. Generally, a huge number of design criterion that are available for host-mediated microbiome engineering recommend multiple possibilities for variation in the experimental design. The best possible choice of selection regime depends on the experimental system for example animal or plant, or on experimental control in order to minimize within- treatment variation and all the available resources in order to conduct a long-term engineering experiments. Concluding Remarks and Future Research Directions: The host-mediated microbiome engineering have miscellaneous applications in varied fields predominantly in agricultural research that aim to increase the plant productivity and provide resistance to a number of diseases as well as to drought and salt tolerance. The Microbiome engineering also assist in research system that is relevant to understand and manipulate the human microbiome, besides it has application in improving health, growth and the productivity of domesticated animals (Fitzpatrick 2014).

Since the colonization of terrestrial ecosystem, the land plants have developed the association with microorganisms. The interaction that exists between plants and microbes has proven to be beneficial to plants in number of ways like by increasing the uptake of nutrients, by producing different types of growth hormones and protecting against enemies. Root microbiota reduces the performance of plants as they compete for limited nutrients and by attacking plants like pathogens. As per the recent study it has been shown that the roots of plants gather two distinct microbiomes or microbial compartments from the pool of soil microbial diversity one being the rhizosphere containing microbes that surround the roots and the other is the endosphere containing microbes within roots. The assembly of root microbiome is a multistep process that is being shaped by both soil type and host differences (Edwards et al. 2015). Though, the understanding about how the variation between the host species shapes the endosphere and the rhizosphere assembly still remains incomplete, however it is essential for understanding how the root microbiota add to the ecology and performance of the host. The plants have to compete with a large number of stressors related to environment during their lifetime. The important biotic stressor that shapes the ecological as well as the evolutionary

outcomes is the competition that exists between the plant species for all the resources they share.

The microbes present that are present in the soil are regarded as key components to plant competition. For example, through the recruitment of microbes present in the soil plants can indirectly compete among themselves, and thus the performance of one plant is affected by the microbial recruitment of another plant by feedback mechanism. Therefore, the competitive interactions that exists between plants and are mediated by "plant-soil feedback" (PSF) are known to affect the primary processes of terrestrial ecosystem like community assembly and succession, plant invasions and primary productivity of plants (Lau and Lennon 2012). The biotic drivers of plant-soil feedbacks include the recruitment of assemblages of microbiota of roots across the host plants. In both the natural and man-made systems, drought represents one of the most important abiotic stressors that negatively affects growth and productivity in plants. Due to lack of mobility, plants employ a wide repertoire of phenotypic mechanisms in order to mitigate stress caused by drought, including life history, physiological, molecular as well as morphological changes. It has been suggested as per emerging evidences that the soil microbes play a vital role in plant drought tolerance. For example, the microbes present in the soil can seize hormones in plants thereby leading to dampened response to drought response (Mayak et al. 2004) and also the negative fitness effects of drought can be reduced by the drought-induced shifts in soil microbial communities (Lau and Lennon 2012).

3.3 Harnessing Beneficial Microbes for Biocontrol Agents

Biological control is defined as the decline in the quantity of inoculum or the disease-causing activity of a pathogen by the process that involves the use of one or more non-human organisms (Cook and Baker 1983). Biological control includes numerous approaches like mass introduction of antagonists, plant breeding and cultural practices that are specific and are thus aimed at amending the microbial balance (Alabouvette et al. 1996). Though this approach was initially projected during mid-twentieth century, but it is only since 2005 the researchers have started to focus on substituting the chemical pesticides with the biological control agents. For the control of pathogens that are found in plants (mainly soil-borne fungi), the bacteria and fungi are regarded as the main biological agents. Besides, a number of viruses, nematodes, amoeba as well as arthropods have been considered as possible biocontrol agents (Kulik 1995).

Plants are delimited by various forms of mesofauna as well as different kinds of microbial organisms, a few of which often contribute in controlling the diseases related to plants. There are certain known PGPR that produce volatile compounds which can reduce the signal molecules of pathogens (Ovadis et al. 2004), Quorum sensing can be disrupted in a number of plant pathogens as well as in Agrobacterium, Chromobacterium, Pectobacterium, and Pseudomonas by certain volatile organic compounds that are being produced by strains of Pseudomonas fluorescens and Serratia plymthica. Since, PGPRs are used as agricultural inputs in several crops,

quorum-quenching can serve as a novel strategy for disease management. Individual bacteria secretes a chemical substance into the surrounding environment which helps them to sense the presence of other member in the vicinity. Thus, the ample amount of such substances suggest a high population and thereby triggering few activities that are population-dependent. For example, the production of light in bioluminous bacteria is triggered at high population density. Several strains of PGPR that include B. subtilis GB03, B. amyloliquefaciens IN937a, and E. cloacae JM22 are proved to release a blend of certain volatile components mainly 2,3-butanediol and acetoin (Ryu et al. 2004a, b). The volatile organic compounds that are produced by different strains of rhizobacteria may act as signaling molecules to intervene plant-microbe interactions. In order to trigger the plant responses, the volatile compounds that are produced by PGPR and colonize roots are to be generated in higher concentrations (Ryu et al. 2004a, b). The plant volatile compounds like terpenes, jasmonates and green leaf components are the low molecular weight compounds that have been identified as potent signal molecules for the living organisms (Farmer 2001). The plant volatile compounds like terpenes, jasmonates and green leaf components are the low molecular weight compounds that have been identified as potent signal molecules for the living organisms (Farmer 2001).

3.3.1 Mycorrhizal Activity

Mycorrhizae, which are formed as the result of mutualist symbioses between fungi and plants, occur on most plant species. Because they are formed at an early stage of plant development, they represent nearly ubiquitous root colonists that assist plants with the uptake of nutrients (especially phosphorus and micronutrients) (Morton and Benny 1990). During colonization, mycorrhizal fungi can prevent root infections by reducing access sites and stimulating host defenses (Linderman 1994). Arbuscular mycorrhizal fungi (AMF) protect a host plant against root-infecting pathogenic bacteria. The damage caused by Pseudomonas syringae on tomato may be significantly reduced when the plants are well colonized by mycorrhizae. The mycorrhizal fungi (e.g., Pisolithus and Glomus spp.) can limit subsequent infections of plant pathogens. Because multiple infections can and do take place in field-grown plants, weakly virulent pathogens can contribute to the suppression of more virulent pathogens via the induction of host defenses. Because various epiphytes and endophytes may contribute to biological control, the ubiquity of mycorrhizae deserves special consideration. The mechanisms involved in these interactions include physical protection, chemical interactions, and indirect effects (Fitter and Garbaye 1994). The other mechanisms used by AMF to indirectly suppress plant pathogens include enhancing the availability of nutrition to plants, increasing lignification to cause morphological changes in the root, and changing the chemical composition of plant tissues with antifungal chitinase, isoflavonoids, etc. (Morris and Ward 1992). AMF may also alleviate abiotic stress and change the microbial composition of the mycorrhizosphere (Linderman 1994). Successful mycorrhizal relationships depend upon AMF species, pathogen species, nutrients, and the synergistic and antagonistic

effects of other soil microorganisms to AMFs. Although the specifics of such factors may be unknown, results against fire blight (Erwinia amylovora) were more successful with Glomus intraradices than with an extensively used copper compound. Along with the economical and environmental advantages, gaining a new approach for fire blight control should encourage more detailed studies on AMFs (Bargabus et al. 2002).

3.3.2 Bacteriophages

The use of bacteriophages as an effective phage therapy strategy faces significant challenges for controlling plant diseases in the phyllosphere. A number of factors must be taken into account when considering phage therapy for bacterial plant pathogens. Given that effective mitigation requires high populations of phage be present in close proximity to the pathogen at critical times in the disease cycle, the single biggest impediment to the efficacy of bacteriophages is their inability to persist on plant surfaces over time due to environmental factors. Successful results were obtained by the use of phage therapy in plant pathosystems such as Erwinia carotovora subsp. carotovora (Ravensdale et al. 2007), Xanthomonas citri subsp. citri and Xanthomonas fuscans subsp. citrumelonis (Balogh et al. 2008), Xanthomonas axonopodis pv. vignaeradiatae (Borah et al. 2000), Pseudomonas tolaasii (Munsch and Olivier 1995), Xanthomonas axonopodis pv. allii (Gent and Schwartz 2005), Xanthomonas campestris pv. vesicatoria, Erwinia amylovora (Schnabel and Jones 2001), Xanthomonas arboricola pv. pruni (Zaccardelli et al. 1992), Ralstonia solanacearum, Xanthomonas campestris pv. vesicatoria (Balogh et al. 2003a, b), Rhizobium radiobacter (Boyd et al. 1971), and Xanthomonas campestris pv. juglandis (McNeil et al. 2001).

3.3.3 Quorum Sensing (QS)

Quorum sensing is the population-density-dependent regulation of gene expression in bacteria. Bacterial pathogens and symbionts depend substantially on QS to colonize and infect their hosts, because it enables the individual bacterial cells in a local population to coordinate the expression of certain genes, which helps them to act somewhat like a multicellular organism. QS, which is particularly important for the ability of pathogenic bacteria to successfully infect plant hosts, is also important to the regulation of gene expression and behaviors in bacterial symbionts of plants. Genome-level transcriptional studies are needed to gain a better perspective of the full range of functions that are altered in plants that respond to N-acylhomoserine lactones (AHLs). Chemical identification and synthesis are central to analyzing the biochemical mode of action of mimics, determining their effects on relevant bacteria, identifying the enzymatic pathways of mimic synthesis, and exploring the potential for the use of plant QS mimics to improve the health of plants (Bauer and Mathesius 2004). QS is crucial to aspects of bacterial physiology, including

regulation of rhizospheric competence factors (e.g., antibiotic production), horizontal gene transfer, and control of functions that are directly or indirectly related to plant–microbe interactions (Whitehead et al. 2001).

Abolishment of the production of QS signals, a process known as quorum quenching (QQ), results in significantly defective biofilm formation and thereby reduces the ability of a pathogen to colonize a host (i.e., biocontrol). The mechanisms and functions of QQ have been evaluated in order to reveal its possible applications in the control of plant diseases and promotion of plant health. In this new concept, rhizobacterial volatiles are an important alternative to antibiotics in the biocontrol of various plant pathogens because they are capable of inhibiting the QS network mediated by AHL signal molecules. The potential of QQ to develop novel biocontrol strategies for plant pathogens has been recognized (Dong et al. 2007). These studies clearly indicate that QQ can be used for the biocontrol of pathogenic microorganisms by targeting the QS pathway.

QS are critical for the infection and invasion processes of many phytopathogens. QS via AHLs are used by many proteobacteria to regulate the expression of virulence-associated factors that orchestrate their temporal and spatial production within a plant. Many phytopathogenic bacteria use AHL-QS in order to spatially and temporally express virulence-associated factors in planta (Venturi and Fuqua 2013). Many AHL-producing bacteria colonize the rhizospheres of plant roots (Elasri et al. 2001). Most of these bacteria (e.g., PGPR) are beneficial to the plant, which means they can promote its growth and/or protect it from microbial pathogens (Lugtenberg and Kamilova 2009a, b). Mechanisms of disease suppression of microbial pathogens by PGPR strains include niche exclusion by competition for nutrients, production of antimicrobial compounds, and induction of ISR during which the inducing PGPR and pathogen do not undergo any type of direct interaction. ISR differs from pathogen-induced and salicylate-mediated SAR (Venturi and Fuqua 2013). ISR that follows colonization of PGPR strains leads to the enhanced expression of various signaling pathways, including jasmonate and ethylene.

The AHLs affect gene expression by altering the levels of many proteins, including those involved in hormonal and defense functions. Many phytopathogenic bacteria use AHL-QS to temporally express virulence-associated factors in the plant after successful colonization. Experiments have been performed with transgenic plants that produce AHLs by expressing a bacterial luxI-type gene by studying plant susceptibility after challenge with a bacterial pathogen. Plant mimics, that act as antagonists of AHL-QS might lead to pathogen confusion and decreased pathogenicity. More progress with respect to AHL mimics has been achieved using algae, especially for compounds that inhibit AHL-QS systems (Manefield et al. 2002). A compound that inhibits AHL-QS has also been identified from leguminous plants; it has been identified as L-canavanine (an arginine analog) (Keshavan et al. 2005). All of these inhibiting compounds have been implicated in the enhancement of the proteolytic degradation of QS LuxR receptors, and therefore to affect the QS response (Koch et al. 2005). Research continues to focus on identifying QS inhibitors, because these are believed to have considerable applications in agriculture for controlling bacterial populations.

A new approach to protect plants against bacterial diseases is based on interference with the communication system of QS, used by several phytopathogenic bacteria to regulate expression of virulence genes according to population density (Cui and Harling 2005). The enzyme, AiiA, isolated from bacterial strain Bacillus sp. 240B1, was found to degrade the QS-signaling molecule of the soft rot pathogen Pectobacterium carotovorum (formerly Erwinia carotovora), and thereby to render the bacteria incapable of infecting the host. Transgenic expression of AiiA in planta was subsequently demonstrated to provide significant enhancement of resistance against soft rot in potato (Dong et al. 2001). The strategy looks technically very promising because the microbial target is likely to be strongly conserved.

3.3.4 Cyanobacteria

Cyanobacteria are Gram-negative, oxygenic photosynthetic prokaryotes. They convert light into chemical energy using water as an electron donor, and release oxygen (O2) as a byproduct during photosynthesis. Early unicellular and filamentous cyanobacteria formed 3.5 billion years ago; the endolithic forms appeared about 1.5 billion years ago (Whilmotte 1994).

It is widely accepted that cyanobacteria are responsible for the formation of atmospheric O2 and that they are the antecedents of the present-day chloroplasts, algae and green plants (Mulkidjanian et al. 2006). They are found in almost all kinds of environments: aquatic, including freshwater and marine; terrestrial, including deserts, rocks, and mountain soils; and extreme conditions, including hot springs and polar regions; as well as in alkaline and acidic conditions, and symbiotic environments (Abed et al. 2009). They are a morphologically diverse group of organisms that exist in unicellular (e.g., Synechocystis), colonial (e.g., Gloeothece), trichomatous (e.g., Oscillatoria and Spirulina), filamentous (e.g., Lyngbya and Phormidium), heterocystous (Anabaena, Nostoc, and Calothrix), false filamentous (Scytonema), and heterotrichous (e.g., Stigonema) and have been grouped into three orders (Chroococcales, Chaemosiphonales, and Hormogonales) by Geitler (1932); five orders (Chroococcales, Chaemosiphonales, Pleurocapsales, Nostocales, and Stigonemetales) by Desikachary (1959); and four orders (Chroococcales, Oscillatoriales, Nostocales, and Stigonematales) by Anagnostidis and Komárek (1990). Several cyanobacteria have the ability to fix atmospheric N2, which leads to the formation of ammonia and a byproduct, hydrogen (H2) (Burris 1991), using specialized cells called heterocysts (Thiel and Pratte 2001). Cyanobacteria possess chlorophyll a, phycocyanins, and phycoerythrin as photosynthetic pigments. These pigments give the characteristic blue-green color to cyanobacteria, hence known as blue-green algae.

An immense body of knowledge on the diversity and physiology of cyanobacteria serves as an appropriate base for exploring their biotechnological applications (Abed et al. 2009). Since 2000, cyanobacteria have been considered to be a promising and rich source of bioactive compounds with antibacterial, antifungal, antiviral,

anticancer and immunosuppressive properties (Koehn et al. 1992). However, certain toxic cyanobacteria or harmful algal blooms excrete bioactive compounds, called cyanotoxins, into surrounding waters. These compounds showed inhibitory effects on the growth, photosynthesis, respiration of other algae (Shunmugam et al. 2014) and higher plants (Shunmugam et al. 2013). Cyanotoxins have been classified according to the symptoms they cause in humans and vertebrates: hepatotoxin (such as microcystin, nodularin, and cylindrospermopsin), neurotoxins (e.g., anatoxin-a, anatoxin-a(s), and saxitoxin) and irritant-dermal toxins (Carmichael 2001). In addition to bioactive compounds, cyanobacterial extracts are known to contain plant growth regulators such as gibberellin, indole 3-acetic acid, and indole 3-butyric acid, which promote seed germination and somatic embryogenesis in plant species such as Daphne spp. (Wiszniewska et al. 2013), Daucua carota L. (Hellebust 1974), Gossypium hirsutum L. (Gurusaravanan et al. 2013), Arachis hypogaea L., and Moringa oleifera Lam. (Gayathri et al. 2015).

The inherent properties of cyanobacteria that make them attractive candidates for use in biotechnological applications include photosynthetic efficiency, ease of genetic modification, and inexpensive growth requirements (i.e., sunlight, CO2, and water along with a few essential mineral nutrients). More than 35 genomes of cyanobacteria have been sequenced and metabolomes of them have been characterized, all of which makes these organisms recommended for highly innovative biotechnological approaches (Lu 2010). Cyanobacteria such as Spirulina platensis and Arthrospira sp. have been found to contain medically important gamma-linolenic acid, which is converted inside the human body into arachidonic acid and further into prostaglandin E2. This (gamma-linolenic acid) compound lowers the blood pressure and plays an important role in lipid metabolism (Abed et al. 2009). Cyanobacteria also produce commercially important UV-absorbing compounds such as mycosporine-like amino acids and scytonemin (Fleming and Castenholz 2007). In addition, cyanobacterial pigments such as carotenoids and phycobiliproteins have been extensively utilized in nutraceutical and pharmaceutical industries (Nayak et al. 2007).

3.3.5 PGPR and Their Mechanisms of Biocontrol

Plant growth-promoting rhizobacteria exhibit several mechanisms of biocontrol, most of which involve competition and production of secondary metabolites that directly affect a pathogen. Examples of such metabolites include antibiotics, cell-wall-degrading enzymes, siderophores, and hydrogen cyanide (HCN) (Weller 1988). PGPR suppress plant pathogenic bacteria by different modes of action of these, the most common mechanisms include the secretion of antibiotics, bacteriocins, and siderophores, and the induction of systemic resistance. Combining all of these PGPR-mediated mechanisms may result in strong plant protection against various diseases. For example, bacterial wilt caused by R. solanacearum was controlled by rhizobacteria to an extent of 16.66%–83.33% in tomato (Jagadeesh 2000),

whereas inoculation of three strains of fluorescent Pseudomonas (RJA112, RBG114, and Arthrobacter RBE201) resulted in 83.33% suppression. These results may be due to the combined or synergistic effects of different bacteria. These results also indicate that antibiotics, siderophores, bacteriocin, organic acid production, and induced systemic resistance (ISR) mediated by different antagonists play important roles in the suppression of R. solanacearum.

3.3.6 Antagonistic Activity

Plant growth-promoting rhizobacteria antagonize plant pathogenic bacteria by the secretion of metabolites and antibiotics. Huang et al. (2013) reported that PGPRs isolated from a pathogen-prevalent environment possessed better antagonistic activity. The different types of antimicrobial compounds produced by bacteria include volatiles (HCN, aldehydes, alcohols, ketones, and sulfides), nonvolatile polyketides (diacetyl phloroglucinol (DAPG) and mupirocin), heterocyclic nitrogenous compounds (phenazine derivatives: pyocyanin, phenazine-1-carboxylic acid; PCA, PCN, and hydroxy phenazines) (Balsanelli et al. 2014), phenylpyrrole antibiotic (pyrrolnitrin) (Ahmad et al. 2008), and lipopeptide antibiotics (iturins, bacillomycin, surfactin, and Zwittermicin A) (Bouizgarne 2013). Fluorescent pseudomonads and Bacillus species are also active in the suppression of plant pathogenic microorganisms. These bacterial antagonists enforce suppression of plant pathogens by the secretion of the abovementioned extracellular inhibitory metabolites at low concentrations. For example, black rot caused by X. campestris pv. campestris (Xcc) causes severe economic losses in all developmental stages of crucifers, but the lipopeptide-producing Bacillus strains actively suppress Xcc during the late growth phase (Mariano et al. 2001). It has been demonstrated that lipopeptide can stimulate ISR in plants, probably by interacting with plant cell membranes and inducing temporary alterations in the plasma membrane (Ongena et al. 2009). Niu et al. (2013) found that the antibiotic polymixin P was suppressive against *E. amylovora* Ea273 and Erwinia caratovora, which respectively cause fire blight and soft rot diseases. Sometimes, bacterially produced antimicrobial compounds may not be efficient for all groups of pathogenic bacteria. Similarly, some studies showed that in vitro activities could not be related to in situ activity. Numerous investigations have demonstrated that actinobacteria, particularly Streptomyces, produce numerous secondary metabolites with antibiotic properties. Currently, 42% of the 23,000 known microbial secondary metabolites are produced by actinobacteria (Bouizgarne 2013). Actinobacteria, particularly the genus Streptomyces, produce 70%–80% of known bioactive natural products (Berdy 2005). In agricultural production systems, antibiotic sprays are extensively managing bacterial plant diseases. Because this method is often not advisable, the possibility of using actinobacteria as bio-inoculants for the management of bacterial diseases should be explored.

3.3.7 Siderophore Production

Siderophores extracellular compounds with a high affinity for ferric iron, are secreted by microorganisms to take up iron from the environment (Hofte 1993). Their mode of action in the suppression of disease was thought to be solely based on competition with the pathogen for iron (Duijff et al. 1997). Under iron-limiting conditions, PGPR produce low-molecular-weight compounds called siderophores to competitively acquire ferric ion (Whipps 2001). Several fluorescent pseudomonads are known to be highly competitive for iron. Because many pathogens need iron, which must be taken up from the environment, a lack of iron may reduce their pathogenicity. It has been reported that certain endophytic bacteria isolated from field-grown potato plants can reduce the in vitro growth of Streptomyces scabies and X. campestris through the production of siderophore and antibiotic compounds (Sessitsch et al. 2004). Environmental factors such as pH, iron levels, the presence of other trace elements, and the supplies of carbon, nitrogen, and phosphorus, can also modulate siderophore synthesis (Duffy and Défago 1999).

3.3.8 Applications of Siderophore

Siderophores have wide applications in various fields such as environmental sciences and medicine. In agriculture, they are used to improve soil fertility and biocontrol. In medicine, iron excesses as well as the primary iron-overload diseases (i.e., hemochromatosis, hemosiderosis, and accidental iron poisoning) require the removal of iron from the body and especially from the liver. Such diseases can be efficiently treated with siderophore-based drugs, for which siderophore acts as the principal model (Pietrangelo 2002). Siderophore can be used for antibiotic delivery in antibiotic-resistant bacteria. This application uses the iron-transport abilities of siderophores to carry drugs into cells by preparation of conjugates between siderophores and antimicrobial agents, which is called the Trojan Horse strategy. Examples of siderophore-based antibiotics in nature include albomycins (Benz et al. 1982), ferrimycins and salimycins (Vértesy et al. 1995). For Fe^{3+} chelation, the albomycins use a part of the ferrichrome structure that is attached via a serine spacer to a toxic molecule.

Several microorganisms introduce albomycin through the ferrichrome-uptake system into the cell, where the toxic part is released enzymatically with detrimental effects to the cell. Siderophore has been used as an iron chelator in cancer drugs such as dexrazoxane, O-trensox, the desferriexochelins, desferrithiocin, and tachpyridine (Miethke and Marahiel 2007). Siderophore is also used for the clearance of non-transferrin-bound iron in serum that occurs as a result of some chemotherapy (Chua et al. 2003). Siderophore produced by Klebsiella pneumoniae acts as an antimalarial agent (Gysin et al. 1991). Desferrioxamine B produced by Streptomyces pilosus is active against P. falciparum both in vitro and in vivo.

Siderophore enters the P. falciparum cell and causes intracellular iron depletion. Therefore, siderophores used as environmental applications by naturally occurring ligands may affect actinide mobility in waste repositories and in the environment (Ruggiero et al. 2000). Excessive accumulation of heavy metals is toxic to most plants and contaminates the soil, which results in decreased soil microbial activity as well as losses in soil fertility and decreased yields (McGrath et al. 1995). In this context, hydroxamate-type siderophore present in soil plays an important role in the immobilization of metals. Siderophores also have wide applications as biocontrol agents by suppressing the growth of many bacteria and pathogens and thereby decreasing yield losses of economically important crops.

In soil, siderophore production activity plays a central role in determining the ability of different microorganisms to improve plant development. During this process, fluorescent siderophores (which have a very high affinity for ferric iron) are secreted during growth under low-iron conditions. The resulting ferric-siderophore complex is unavailable to other organisms, but the producing strain can utilize this complex via a very specific receptor in its outer-cell membrane (Buyer and Leong 1986). In this way, fluorescent Pseudomonas strains may restrict the growth of deleterious bacteria and fungi at the plant root (Loper and Buyer 1991). This efficient iron-uptake mechanism may also be a significant contributing factor to the ability of these strains to aggressively colonize plant roots, thus aiding the physical displacement of deleterious organisms.

Although iron competition among the rhizosphere population has attracted some research attention, it is not yet clear how this affects the iron requirements of plants. Iron deprivation in plants leads to a form of chlorosis (Julian et al. 1983). The influence of fluorescent pseudomonads upon siderophore production spans a wide variety of factors, including iron concentration (Kloepper et al. 1980), nature and concentration of carbon and nitrogen sources (Park et al. 1988), phosphate levels (Barbhaiya and Rao 1985), pH and light (Greppin and Gouda 1965), degree of aeration (Lenhoff 1963), temperature (Weisbeek et al. 1986), and the presence of trace elements such as magnesium (Georgia and Poe 1931), zinc (Chakrabarty and Roy 1964).

Siderophores have been isolated from different soils (Powell et al. 1980). Microbial siderophores enhance iron uptake by plants that are able to recognize the bacterial ferric-siderophore complex (Dimkpa et al. 2009a) and are also important in the iron uptake by plants in the presence of metals such as nickel and cadmium (Dimkpa et al. 2008). However, whether bacterial siderophore complexes can significantly contribute to the iron requirements of the plant remains unclear. Siderophore production confers competitive advantages to plant growth by promoting rhizobacteria (PGPR) that can colonize roots and exclude other microorganisms from this ecological niche (Haas and Défago 2005). Under highly competitive conditions, the ability to acquire iron via siderophores may determine the outcome of competition for carbon sources that have been made available by root exudation or rhizodeposition (Crowley 2006). Among the bacterial siderophores that have been studied, those produced by pseudomonads are known for their high affinity to ferric ion. For example, the potent siderophore pyoverdin can inhibit the growth of bacteria and fungi that produce less potent siderophores in in vitro condition suppressing the growth of other pathogens (Kloepper et al. 1980, 2007).

3.3.9 Induced Systemic Resistance

Another possible mechanism for biological control of plant pathogens is the use of bacterial metabolites that increase a plant's resistance to pathogens by induced systemic resistance (ISR). Resistance that is elicited in plants by the application of chemicals or necrosis-producing pathogens is called systemic acquired resistance (SAR). Pieterse et al. (1998) proposed that ISR and SAR can be differentiated not only by their elicitor molecules but also by the signal transduction pathways that they elicit within the plant. Accordingly, ISR is elicited by rhizobacteria or other nonpathogenic microorganisms, and SAR is elicited by pathogens or chemical compounds. Several PGPR strains can act as inducers of ISR (Kloepper and Beauchamp 1992); in fact, PGPR-mediated ISR may be an alternative to chemical inducers. Plants treated with PGPR provided systemic resistance against a broad spectrum of plant pathogens to reduce the incidence of bacterial disease (Ryu et al. 2004a, b). The expression of ISR is dependent upon the combination of host plant and bacterial strain. Most reports of PGPR-mediated ISR involve free-living rhizobacterial strains, but ISR activity has also been observed in endophytic bacteria. Volatile organic compounds may be key in this process. For example, volatiles released by Bacillus subtilis GBO3 and Bacillus amyloliquefaciens IN937a were able to activate an ISR pathway in Arabidopsis seedlings inoculated with the soft rot pathogen of Erwinia carotovora ssp. carotovora (Ryan et al. 2001). Jones et al. (2005) reported that the combined effect of PGPRs and SAR compounds was effective in controlling bacterial leaf spot disease in tomato caused by X. campestris pv. vesicatoria.

A large number of defense enzymes have been reported to be associated with ISR. These include ascorbate peroxidase (APX), β1,3-glucanase, catalase (CAT), chitinase, lipoxygenase (LOX), peroxidase (PO), phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO), proteinase inhibitors, and superoxide dismutase (SOD) (Van Loon 1997). These enzymes also bring about liberation of the molecules that elicit the initial steps in the induction of resistance (Van Loon et al. 1998). ISR by PGPR has been noticed in a number of crops, including Arabidopsis (Pieterse et al. 1996), brinjal, chilli (Bharathi et al. 2004), carnation (Van Peer et al. 1991), cucumber (Wei et al. 1996), mango (Vivekananthana et al. 2004), potato (Doke et al. 1987), radish (Leeman et al. 1996), rice (Nandakumar et al. 2001), sugarcane (Viswanathan and Samiyappan 1999), and tomato (Duijff et al. 1997) against a broad spectrum of pathogens, including fungi and bacteria.

3.3.10 Accumulation of Cell Wall Components

Inoculation of plants with PGPR, which leads to the strengthening of cell walls, also alters host physiology and metabolic responses, which lead to an enhanced synthesis of plant defense chemicals upon challenge by pathogens (Nowak and Shulaev 2003). Application of endophytic Pseudomonas fluorescens WCS417r on tomato increased the thickening of the outer peripheral and outermost part of the radial side of the first layer of cortical cell walls (Duijff et al. 1997). Grapevine treated with Burkholderia phytofirmans PsJN enhanced the phenolic compound accumulation;

strengthening of cell walls in the exodermis and several cortical cell layers was also observed during endophytic colonization of the bacterium (Compant et al. 2005). When challenged by a pathogen, PGPR-inoculated plants formed structural barriers (e.g., thickened cell wall papillae) due to the deposition of callose and the accumulation of phenolic compounds at the site of attack (Benhamou et al. 1996). This result indicates that structural changes in plant anatomy due to PGPR inoculation are protective against various phytopathogens.

3.3.11 Accumulation of PR Proteins and Defense Related Enzyme

The inoculation of PGPR causes a number of biochemical and physiological changes in plants (Ramamoorthy and Samiyappan 2001), including induced accumulations of pathogenesis-related proteins (PR proteins), chitinases, and some peroxidases (Viswanathan and Samiyappan 1999). However, certain PGPR do not induce PR proteins (Pieterse et al. 1996), but instead increase accumulation of other defense enzymes (Chen et al. 2007). Seed treatment with PGPR strains could increase the level of PR proteins in beans. Similarly, PR proteins were induced in intercellular fluid in the leaves of tobacco plants grown in the presence of P. fluorescens CHA0 (Maurhofer et al. 1994). Rice leaves pretreated with P. fluorescens and challenge-inoculated with X. oryzae pv. oryzae showed increases in lignin content, peroxidase activity, and 4-coumarate CoA ligase activity (Vidhyasekaran et al. 2001). Amrita et al. (2013) reported that the isolate Bacillus pumilus (BRHS/C1) was most effective in improving the growth of Listea monopetala (Roxb.) Pers. by the production of defense-related enzymes such as peroxidase, phenylalanine ammonia lyase, chitinase, and β-1,3-glucanases.

3.3.12 Production of Signaling Compounds

Plant–PGPR interaction regulates the salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) pathways in plant systems, which initiate basal resistance against pathogens. These three signals are important in ISR as well as SAR. Both types of induced resistance are effective against a broad spectrum of pathogens. Serratia marcescens 90–166 mediates ISR to bacterial pathogens by producing SA, using the salicylate responsive reporter plasmid pUTK21. The strain 90–166 induced disease resistance to P. syringae pv. tabaci in wild-type Xanthi-nc and transgenic NahG-10 tobacco that expressed salicylate hydroxylase (Press et al. 1997). Several genera of bacteria, including pseudomonads, are known to synthesize SA. Root colonization of A. Arabidopsis thaliana by the nonpathogenic, rhizosphere-colonizing bacterium P. fluorescens WCS417r was found to elicit ISR against P. syringae pv. tomato (Pst) (Knoester et al. 1999).

3.3.13 Plant Nutrient Management and Its Role in Suppression of Phytopathogens

Plant nutrient management is central to plant disease management. Some nutrients (e.g., N, P, K, Mn, Zn, B, Cl, and Si) are very important for imparting resistance/tolerance to plants against many diseases. Excess nitrogen application causes plants to become vulnerable to various diseases, whereas sufficient potassium content decreases the susceptibility of plants against many diseases (Dordas 2008). Balanced potassium content in plants reduces the incidence of diseases such as.

3.3.14 Modern Use of Phages in Biocontrol

Decades of research on the use of bacteriophages have contributed several important discoveries to the reevaluation of phage therapy. In ancient times, such exploitation was successfully used to control bacterial plant diseases (Thomas 1965). In modern times, phages have been found to have several potential advantages in disease control. First, as natural components of the biosphere, bacteriophages can be readily isolated from bacteria that occur in a range of locations, including soil (Ashelford et al. 2003), water (Miernik 2003), plants (Gill and Abedon 2003), animals (Sulakvelidze and Barrow 2005), as well as hydrothermal vents (Liu et al. 2006). Second, phages are self-replicating and self-limiting; they reproduce only as long as the host bacterium is present in the environment and quickly degrade in its absence (Lang et al. 2007). Third, phages target only the bacterial receptors that are essential for pathogenesis; therefore, the resistant mutants of bacterial strains are attenuated in virulence (Kutter 1997). Fourth, bacteriophages are not only nontoxic to eukaryotic cells (Greer 2005) but also are specific or highly discriminatory; the latter means that they eliminate only target bacteria without damaging other, possibly beneficial indigenous flora (Summers 2001). Fifth, phage preparations are fairly easy and inexpensive to produce and can be stored at 4 °C in complete darkness for months without a significant reduction in titer (Greer 2005). Sixth, phage application can be carried out with standard farm equipment; moreover, because the majority of agrochemicals do not inhibit phages, they can be tank-mixed with them without significant loss in titer (Iriarte et al. 2007). Although copper-containing bactericides have been shown to inactivate phages, this inhibition was eliminated when the phages were applied at least 3 days later.

Borah et al. (2000) treated mung bean bacterial leaf spot caused by Xanthomonas axonopodis with a co-application of antagonistic phylloplane bacteria and a bacteriophage that was known to be active against the pathogen. No protection was obtained with antagonistic bacteria alone, but application of the phage confined the pathogen population in plants to leaves; disease control efficacy was recorded as 68% in comparison with untreated plants. Flaherty et al. (2001) isolated 16 phages against a pathogen, screened for host range and lytic ability, selected lytic phages

with broad host ranges, and developed the H-mutant phage from some of them. Daily application of phage mixtures in foliar spray can reduce disease severity by 75% (relative to control plants) after 6 weeks. Extensive research has been done on suppressing bacterial blight caused by Xanthomonas campestris with phages. However, Neil et al. (2001) revealed that the lytic bacteriophages of X. campestris are widespread in New Zealand soils and could be very easily isolated from the top 2.5 cm. However, for the eight soil samples (out of 11) that yielded these phages, control of bacterial blight was not significantly different than on untreated branches of walnut. Flaherty et al. (2001) effectively controlled bacterial blight in greenhouse and field experiments with a mixture of four phages that were known to be active against two predominant strains of X. campestris.

Streptomyces scabiei, the common scab pathogen of potato, was controlled by the phages Stsc1 and Stsc3 by Goyer (2005). The disease control abilities of phages Stsc1 and Stsc3 were tested in radish seedlings; those that were grown in the presence of S. scabiei weighed significantly less (mass decrease 30%) than the negative controls. When the radish seedlings inoculated with Stsc1 and Stsc3 phages were observed to grow with no disease symptoms, effective disease control of phages in a plant system in vitro was confirmed. Lang et al. (2007) reported that Xanthomonas leaf blight of onion (Allium cepa), caused by Xanthomonas axonopodis pv. alli, was controlled by mixtures of bacteriophages.

The persistence of phages has been recorded as 72–96 h in field and greenhouse conditions. During biweekly or weekly application of phages, 26%–50% of disease severity was observed; this result was better than application with copper hydroxide plus mancozeb. Integration of bacteriophage mixtures with acibenzolar-S-methyl (ASM) appears to be a promising strategy for managing Xanthomonas leaf blight of onion and could reduce grower reliance on conventional copper bactericides. Bacteriophage stability in soil and at different temperatures was studied in Ralstonia solanacearum phages φRSA1, φRSB1and φRSL1 by Fujiwara et al. (2011). The R. solanacearum phages were found to be stable in soil, especially at higher temperatures of 37–50 °C, and the stable nature of phages in soil was confirmed by recovery of φRSL1 phages from roots of treated plants and from soil 4 months post-infection. Based on these findings, the researchers proposed that these phages are effective for the control of wilting in tomato.

In most greenhouse and field studies, bacteriophages reduced the severity of bacterial spot of tomato to levels equal to or lower than those obtained with copper bactericides. However, the effectiveness of phages for biological control depends not only upon the susceptibility of the target bacterium but also on environmental factors that affect phage survival (Flaherty et al. 2000). In general, the survival of microorganisms in the phyllosphere depends upon the limited resources that are available in this habitat and the ability of organisms to cope with varied environmental stress conditions such as fluctuating water availability, heat, and osmotic pressure, as well as desiccation. However, phage persistence in plant phyllospheres for extended periods is limited by many factors, including sunlight irradiation, temperature, and desiccation, especially in the UV zone and when copper bactericides are used (Iriarte et al. 2007). Iriarte et al. (2007) developed formulations that mixed

phages and skim milk to extend phage persistence in plant phyllospheres. This protective formulation eliminated the reduction caused by both of these factors. Although phage persistence was dramatically affected by UV light, the other factors were less effective. In particular, formulated phages reduced the deleterious effects of the environmental factors. Integrated management strategy, which is central to plant disease control in modern agriculture, includes combinations of chemical control agents and has achieved biocontrol. As a part of an integrated disease management approach, Obradovic et al. (2004) used combinations of phages with other biological control agents and plant inducers. Phage-based integrated management of tomato bacterial spot is now officially recommended to tomato growers in Florida, and bacteriophage mixtures (e.g., Agriphage) that reduce this pathogen are commercially available (Obradovic et al. 2008).

3.3.15 Biocontrol Pseudomonads in the Soil Microbiome

As the harmful environmental impacts of chemical pesticides become more apparent, manipulation of the soil and plant-associated microbiota is gaining increasing recognition as a potential alternative treatment for a range of crop diseases and pests. This may occur on a whole-microbiome level, for example through the development of suppressive soils or the control of potato scab by irrigation, or alternatively through the stimulation/introduction of key biocontrol microorganisms, such as Bacillus or Pseudomonas spp. Many important fungal and bacterial diseases including fire blight (Erwinia amylovora, (Stockwell et al. 2010)), potato scab (Streptomyces scabies, (Arseneault et al. 2013)) and take-all (Gaeumannomyces graminis var. tritici, (Yang et al. 2011)) are effectively sup-pressed by members of the Pseudomonas fluorescens species group. These important, widespread soil-dwelling microbes have an established role in the development of take-all suppressive soils Yang et al. 2011), where the fungal pathogen is maintained at a low level in the soil but is unable to cause disease. Take-all is a destructive fungal crop disease that causes substantial losses in cereal crops and is therefore an attractive target for the development of Pseudomonas biocontrol agents. However, to date efforts in this direction have been plagued by inconsistency in large part due to the huge complexity of the plant/pathogen/soil ecosystem. Pseudomonas fluorescens P. fluorescens are a diverse clade of Gram negative, g-proteobacteria that non-specifically colonise a number of different plant species. They represent a major constituent of the rhizosphere microbiome, and exploit root exudates as source of nutrients and energy. P. fluorescens spp. are flexible, generalist bacteria that are able to colonise many different environmental niches and carbon sources. Their genomes are correspondingly complex, encoding around 6000 genes, and with a high degree of intraspecies diversity—the Pseudomonas core genome represents as little as 20% of an individual bacterial genome with much of the accessory genome given over to signal transduction, phenotypic output loci and secondary metabolism (Garrido-Sanz et al. 2016). The high degree of genomic and metabolic plasticity among the soil pseudomonads allows both individual bacteria, and the microbial population as a whole, to

effectively adapt to different plant–soil–microbiome environments. Pseudomonas plant colonisation is a complex, tightly con-trolled process that begins with chemotaxis into the rhizosphere along a gradient of root exudates, followed by surface association and migration on the rhizoplane and ultimately the formation of a bacterial biofilm. The early stages of colonisation are facilitated by flagella and type IV pili, and the production of biosurfactants, which together enable coordinated swarming motility (Lugtenberg et al. 2001). The later stages are characterised by the formation of micro-colonies on the plant surface, then establishment of a mature biofilm. In addition to bacterial cells this protective matrix is composed of proteinaceous adhesins, lipopolysaccharide and various exopolysaccharide molecules (Gal et al. 2003). To successfully colonise the plant rhizosphere, many Pseudomonas spp. produce enzymes that enable them to manipulate plants, encouraging growth and disrupting stress responses. For example, enzymes that synthesize and catabolise auxins and plant growth-promoting volatiles such as 2–3-butanediol and acetoin have been identified in several Pseudomonas genomes (Lugtenberg et al. 2001). In addition, many Pseudomonas spp. produce ACC deaminase, which protects plants from environmental stresses by short-circuiting ethylene production. P. fluorescens in the rhizosphere is under continuous attack from other members of the soil microbiome. This takes the form of competition and antagonism from other microorganisms, as well as predation by nematodes and insects. To counter this second threat, and to prevent insect predation of their host plants, many Pseudomonas spp. produce insecticidal molecules such as the Mcf, IPD072Aa and Fit toxins (Lugtenberg et al. 2001). Meanwhile, to fight against hostile bacteria, oomycetes and fungi, soil Pseudomonas spp. secrete bacteriocins, alongside toxins and other natural products using specialised protein secretion pathways. Type III and Type VI complexes inject toxins and effector proteins into eukaryotic and bacterial cells, and contribute to various cytotoxicity and virulence-associated phenotypes. Type II secretion systems are diverse protein exporters, and facilitate the secretion of bacteriocins, surface adhesins and extracellular enzymes (Hinsa et al. 2003). As well as affecting plant behaviour, some Pseudomonas spp. also disrupt signal transduction by other rhizosphere micro-organisms, for example by producing AHL lactonase to suppress quorum sensing (Jafra et al. 2006). Pseudomonas spp. also produce a diverse array of secreted natural products. These have varied functions, although many serve to kill or suppress plant predators and competing microorganisms. Even those molecules with a well-defined alternative function often function as antimicrobials. These include the metal ion-chelating siderophores, which also inhibit pathogenic fungi by inducing metal ion starvation in model rhizospheres. Phenazines; flavin coenzyme analogues that function as electron shuttles in microoxic environments also inhibit electron transport in plant pathogens and are linked to ecological fitness in take-all infected wheat rhizospheres (Mazzola et al. 1992). Likewise, viscosin and other cyclic lipopeptides act both as surfactants to enable swarming motility and antibiotics that solubilise cell membranes. Soil Pseudomonas spp. also produces a host of dedicated antimicrobials, such as the antifungal compounds pyoluteorin and pyrrolnitrin, phloroglucinols like 2–4-DAPG and hydrogen cyanide (Blumer and Haas 2000). A recently conducted metabolic profiling analysis based on soil isolates

from Rothamsted Research (Harpenden, U.K.) demonstrated a remarkable level of natural product diversity within the rhizosphere Pseudomonas population, with isolates from a single wheat field producing a com-parable natural product complement to an extensive library of global isolates from diverse environmental sources (Nguyen et al. 2016). Depending on the exact conditions in their environment, P. fluorescens populations select from the huge potential within the accessory genome to produce an optimal genetic and metabolic response. Clearly, if we can define the genetic loci and phenotypic characteristics that con-tribute to rhizosphere colonisation and biocontrol, and determine how these change with different plant/soil environments, we will be much better placed to exploit the soil Pseudomonas population to develop better crop management strategies and novel biocontrol agents. Analyzing genomic diversity in plant associated Pseudomonas populations A recent 2-year experiment at Rothamsted (McMillan et al. 2011) presented us with an opportunity to examine the relationship between the Pseudomonas genome and the environment, in the context of infection with take-all. This experiment compared high (Hereward) and low (Cadenza) take-all inoculum building (TAB) wheat varieties, and the impact on crop yield in the second wheat (McMillan et al. 2011). We isolated hundreds of Pseudomonas CFUs from the rhizospheres of second year wheat plants, and subjected them to extensive phenotypic, genotypic and genomic analysis, including whole genome sequencing of 19 isolates. A phylogenetic tree of all Pseudomonas isolates based on ERIC PCR profiles and housekeeping gene sequences showed that the wheat variety grown in year one exerted considerable selective pressure on both the extent and nature of Pseudomonas genomic diversity. Hereward plots showed increased take-all build-up and Pseudomonas genomic richness, alongside yield losses of 3 t/ha. However, while distinct clusters of genotypes were observed when year one wheat variety was considered, no pattern was observed with cultivars from year two. These findings agree with a 16S rRNA gene amplicon sequence analysis of the rhizosphere soil in each plot, which showed that year one Hereward plots contained significantly larger Pseudomonas populations, alongside several different genera of saprophytes. We then took a statistical approach to combine our various datasets, conducting correlation coefficient analyses to identify the phenotypes and genes that were selected by different cultivar combinations over the course of the field trial. This analysis identified several interesting correlations between phenotypes, genotypes, and the wheat varieties from which strains were isolated (Mauchline et al. 2015). At least two distinct, mutually exclusive phenotypic/genotypic groups emerged from our analysis. The first of these showed increased levels of antimicrobial activity towards Streptomyces spp., and contained operons for cyclic-lipopeptide and LPS biosynthesis, type VI secretion and toxin production. The second group produced Pseudomonas and the rhizosphere microbiome. The operons associated with year one Hereward cultivation (high TAB) also positively correlated with Streptomyces suppression, while loci that positively correlated with year one Cadenza (low TAB) strongly associated with increased pyoverdin production (Mauchline et al. 2015). In addition, Pseudomonas isolates from this field experiment were used to construct synthetic community fungal antagonism assays (Stevenson et al. 2013). Increased Pseudomonas spp. richness

positively correlated with in vitro pathogen growth. This supported the field observation that first year Hereward plots, with a higher rhizosphere genotypic richness than first year Cadenza, developed more severe take-all disease, demonstrating a negative biodiversity effect. We propose that the increased levels of senescent root tissue and saprophytic microorganisms that accompany Hereward growth in year one may lead to an increased abundance of pseudomonads that are adapted to niche competition with other microbes, whereas the comparatively benign environment associated with Cadenza rhizospheres favours Pseudomonas genotypes that are better adapted to plant–host communication and increased production of metal scavenging siderophores. Clearly, it remains to be established whether the model we propose for the interplay between wheat, take-all and Pseudomonas is correct, or whether there is a different reason for the population shifts we see. Nonetheless the impact of the first year wheat cultivar was still detectable 2 years after the beginning of the experiment, consistent with substantial selective pressure on the first-year rhizosphere population. A second long-term wheat experiment that will capture the full disease epidemic is underway, as are several laboratory experiments including root exudate metabolomic analysis, to strengthen and refine our initial conclusions. Our experiments indicate that first year wheat genotype affects both the overall Pseudomonas population, and also the distribution of individual genotypes in the second year rhizosphere (Mehrabi et al. 2016). In turn, these experiments support our contention that a better understanding of the soil microbiota, combined with smart manipulation of plant cropping systems may present a reliable future route to sustainable yield improvement and biocontrol.

3.4 PGPR and Stress Tolerance Mechanisms

In the narrow zone of contact between soil particles and roots, the rhizosphere constitutes the first plant-influenced habitat encountered by soil microorganisms (Dessaux et al. 2016). Within this zone of plant-soil interaction, the rhizosphere is a dynamic and densely populated soil area with a complex set of inter- and intraspecies communications and food web interactions that significantly impacts carbon flow and transformation. The rhizosphere has been described to include three zones: the endorhizosphere, as portions of the root cortex and endodermis where microbes and mineral ions reside in the apoplastic space between cells; the rhizoplane, as the middle zone next to the root epidermal cells and mucilage; and the ectorhizosphere, as the outermost zone which extends from the rhizoplane out into the bulk soil (McNear Jr. 2013). It is important not to consider the rhizosphere as a region of definable size or shape, but rather as a gradient of chemical, biological and physical properties along the root (McNear Jr. 2013). The rhizosphere is strongly influenced by plant metabolism through the release of carbon dioxide (CO2) and secretion of photosynthate as an array of root exudates (mainly from the rhizoplane and ectorhizosphere). Root exudates, including phytohormones, facilitate rhizosphere interactions by serving as energy sources for microorganisms and acting as chemical attractants and repellents (Bais et al. 2001). They serve as communicating

molecules to initiate biological and physiological interactions between the soil microbiome and the plant roots by influencing the chemical and physical properties of the soil and the soil microbial community, inhibiting growth of competing plant species, facilitating beneficial symbioses, e.g. with nitrogen-fixing bacteria, mycorrhizal fungi and epiphytes, and by preventing pathogenic bacterial, fungal and insect attacks (Nardi et al. 2000). The rhizosphere is of paramount importance for ecosystem services, such as carbon and water cycling, nutrient trapping, crop production, and carbon uptake and storage (Adl 2016). Global climate change, including rising temperatures and disruptive weather patterns due to increasing levels of atmospheric CO_2, will affect rhizosphere ecology, and hence ecosystem function, through a variety of direct and indirect ways. For example, it has been estimated that increasing global temperatures between 1981 and 2002 reduced the yields of major cereals by almost $5 billion per year. Abiotic stresses such as drought, high temperatures and salinity are major causes for loss of natural vegetation, crop productivity depreciation and, therefore, reduction of capacity for CO2 uptake. Drought stress severely inhibits photosynthesis and root growth (Verslues 2017). Salinity stress leads to ion toxicity due to excessive amounts of Na + and Cl − that causes detrimental effects on plant growth and development (Negrao et al. 2017).

Drought and salinity stresses both cause elevated levels of ethylene, which is inhibitory to root growth and therefore affect a number of plant physiological pathways. Additional environmental stresses negatively impact plant growth and development in a number of ways like disturbing hormone balance and increasing susceptibility to diseases (Saleem et al. 2007). Plant survival under abiotic stress conditions requires extensive physiological adaptations. In this regard, plant hormones play crucial roles in the process of root formation and growth and in regulation of root morphological responses to abiotic stress. Hormone perception and crosstalk create an intricate network in which abiotic stresses can interfere, resulting in root growth alterations in the rhizosphere. The phytohormone auxin is the key regulator for almost all aspects of growth and development in plants. In roots, the most important auxin-associated phenotypes are the increase in the length of root hairs, the bimodal effect of auxin concentration on primary root length, the dose-dependent increase in number of lateral root primordia, and the response to gravity (Peret et al. 2009). Other types of plant hormones like cytokinins and gibberellic acids (GAs) act as negative regulators of root development and positive stimulators of root elongation, respectively. It was shown that exogenous cytokinin treatment inhibits root elongation while reduction of endogenous cytokinin levels increases primary root elongation (Miyawaki et al. 2006). The involvement of abscisic acid (ABA) in responses to abiotic stresses and in particular to drought is well characterized. When the roots sense a decrease in soil water in the rhizosphere region, first ABA biosynthesis in the root tips is increased and then it is transported to the leaves leading to induction of stomatal closure. It was reported that primary root elongation is inhibited by ABA during drought stress (Xiong et al. 2006). Ethylene is a gaseous plant hormone that has significant influences on many aspects of plant growth and development. Ethylene is a strong inhibitor of root elongation and stimulates root hair formation (Negi et al. 2008). Strigolactones and their derivatives

were recently defined as novel phytohormones that play important roles in shoot and root growth. In particular, Strigolactones and their derivatives promote the elongation of primary roots and adventitious roots, while repressing lateral root formation (Sun et al. 2016). Also, it should be noted that hormonal crosstalk is essential for plant growth and development. For example, ethylene controls root formation by regulating auxin transport within the root tip (Swarup et al. 2007), and stimulates the expression of auxin biosynthesis genes, and of AUX1 (auxin transporter protein 1) and PIN2 (PIN FORMED2 auxin transporter), resulting in an increased basipetal auxin transport. Plant phytohormones are also important in the context of beneficial plant-microbe interactions (see below). Therefore, plant phytohormones should be considered as one of the key factors in all efforts dealing with rhizosphere engineering. While plants may acclimate to abiotic stress tolerance through phenotypic plasticity, associations with naturally occurring microorganisms provide another means for enhanced resistance to, or protection from, various abiotic stresses (Farrar et al. 2014).

The microbiota or microbiomes of plants, like those of humans and many other eukaryotes, comprise a staggering diversity of microorganisms inside and outside their tissues, in the endosphere and ectosphere, respectively (Vandenkoornhuyse et al. 2015). Due to their intimate association with microbes, plants could be considered meta-organisms, or holobionts, between the plant per se and its interacting microbiota (Vandenkoornhuyse et al. 2015), and the genome of the plant microbiome is sometimes referred to as the second genome of the plant. The plant-microbe interactome covers a spectrum of associations ranging from mutualistic, commensalistic and parasitic relationships and represents an ancient co-evolution (Heckman et al. 2001). A classic example of ancient symbiosis in plants is with arbuscular mycorrhizal (AM) being the oldest, over 400 million years (Heckman et al. 2001). Of paramount interest are the interactions between plants and the rhizospheric microbial communities (Philippot et al. 2013). This complex plant-associated microbial community is critical for plant health and productivity (Berendsen et al. 2012). As is further detailed below, many isolated bacterial strains have been identified as plant growth-promoting bacteria (PGPB), which can stimulate plant growth through a number of direct and indirect mechanisms (Kloepper and Schroth 1981a, b). Such mechanisms include nutrient solubilization, biological nitrogen fixation, and induction of systemic resistance, production of plant growth regulators, organic acids, and volatile organic.

3.4.1 Interactions Between the Rhizosphere and Other Components of the Plant Ecosystem

Carbon (C) enters the soil as root exudates or via decomposition of root or aboveground biomass. In the soil, C exists in root or microbial biomass, as bioavailable labile organic C, or as more recalcitrant C. Carbon exits the soil as direct emissions, or via root or microbial respiration, with microbial-mediated soil respiration being the major source of CO2 from terrestrial ecosystems. Carbon is also lost from the

ecosystem as volatile organic compounds (VOCs) and methane (CH4), compounds (VOCs) as well as protection by enzymes like 1-aminocyclopropane-1-carboxylate (ACC)-deaminase, chitinase and glucanase. Plant Growth-Promoting Rhizobacteria (PGPR) are a group of PGPBs inhabiting the proximity or surface of the roots and are involved in promoting plant growth and development by direct release of microbial exudates (e.g., metabolites and small peptides/lipids) in the vicinity of rhizosphere (Kloepper and Schroth 1981a, b). The first use of the word "PGPR," was by Kloepper and Schroth (1978), but soil microbes influencing plant growth has been known for over 100 years (Hartmann et al. 2008). PGPR have been shown to increase productivity in a variety of different crops (e.g., tomato, lettuce, wheat, soybean, rice and apples) under normal and stressful conditions (Deshmukh et al. 2016). Their mode of action includes broad-spectrum antagonism in biocontrol of soil-borne pathogens (Nakkeeran and Fernando 2005), immobilization of nutrients (e.g. phosphorus) (Bhattacharyya and Jha 2012), release of plant hormones (Vessey 2003), and direct fixation of nitrogen (e.g. bradyrhizobium and rhizobium) (Zahran 2001). Axenic cultures exhibiting PGPR effects have been obtained from hundreds of rhizospheric isolates (Bhattacharyya and Jha 2012). However, a vast majority of these PGPRs are not amenable to cultivation, or exists as co-culture dependents. The physical and chemical context of the rhizosphere is the result of many competing and interacting processes that depend on soil type and water content, the composition of microbial communities, and the physiology of the plant itself (Ryan et al. 2009a, b). For better plant productivity, all three components of the rhizosphere, plant, soil and microbes can be engineered. The soil can be amended or managed to improve its overall quality by changing its physical and chemical properties, microbiomes can be selected for beneficial traits such as promoting plant growth and root characteristics, and the plants can be engineered to harbor beneficial and novel traits of interest. Natural and synthetic plant-microbe interactions can be utilized to improve nutrient bioavailability. The key chemical compounds used in the plant root-microbe communications can be used as targets for genetic engineering to further enhance these interactions. The present review mainly focuses on plant and microbe (PGPRs) components in rhizosphere engineering.

3.4.2 Harnessing Natural and Beneficial Plant-Microbe Interaction in the Rhizosphere Region

Various strategies have been described to alleviate stress-induced adverse effects on plant growth. Transcriptome engineering is a promising approach for generating abiotic stress-tolerant crops, and to date, constitutive overexpression of single genes encoding enzymes related to the accumulation of osmolytes and proteins that function as reactive oxygen species (ROS) scavengers and ion transporters has been the most common strategy for improving abiotic stress tolerance in plants (Reguera et al. 2012). Nevertheless, due to involvement of multiple pathways in plant acclimation to stress and possible pleiotropic effects on plant growth (Rivero et al. 2007), this approach has met with limited success. Application of agrochemicals (i.e.

pesticides) is another approach in boosting crop productivity but is contentious due to their cost and environmental concerns about their long-term utilization in soil. An alternative strategy to mitigate climate change impacts on plant fitness is the utilization of beneficial mutualistic plant-microorganism interactions in the rhizosphere. Such mutualism can offer enhancement of root nutrient uptake and biomass productivity, and potentially improved plant acclimation to abiotic stresses (Mirshad and Puthur 2017). Identification of rhizospheric microbes capable of conferring stress tolerance to their plant hosts, and employing symbiont-based approaches to understanding and improving root biomass production, whole plant productivity and/or soil carbon storage, could significantly contribute to reducing the negative impact of abiotic stresses on plant ecosystem function. There are several advantages to this approach, including the capability of PGPRs in conferring more than one type of abiotic stress tolerance (e.g. through affecting plant hormone pathways), and the applicability to a wide variety of diverse plant hosts (Coleman-Derr and Tringe 2014). Recently, PGPRs have received substantial attention for their ability to confer benefits to crop productivity and stress tolerance, similar to what have been achieved through time-consuming plant breeding programs (Tank and Saraf 2010). For example, the growth-promoting bacterial, Burkholderia phytofirmans strain PsJN, has been shown to colonize and promote growth of switchgrass under different conditions, especially in the early growth stages (Kim et al. 2012a, b), suggesting it may be a promising candidate for improving bioenergy crop production. Strain PsJN can significantly stimulated growth of specific genotypes of switchgrass under suboptimal conditions, pointing the way to the development of a sustainable feedstock production system (Kim et al. 2012a, b).

3.4.3 Drought

Drought is the single most critical threat to plant and crop productivity; it impairs plant growth and development more than any other environmental factor (Shao et al. 2009). Drought stress is affected by climatic, edaphic and agronomic factors, and in view of climate change and limiting global freshwater supply it has been predicted that the impacts of drought will be further aggravated in the future (Somerville and Briscoe 2001). With forecasted global changes in temperature and precipitation, drought will increasingly impose a challenge not only to food and feed but also to biomass production (Yin et al. 2014). Most of the bioenergy crops have some degree of drought susceptibility as revealed for example through measures of low water-use efficiency (WUE) in Miscanthus (Yin et al. 2014). Thus, in addition to the urgent need of developing drought-tolerant crops for food security, it becomes imperative to improve drought tolerance and WUE in bioenergy crop plantations for sustainable biomass production in arid and semi-arid regions (Ings et al. 2013).

Despite an extensive volume of scientific reports on enhancing plant drought tolerance using a variety of genetic engineering approaches, progress has been slow, demonstrating the complexity of the trait and the large number of genes involved.

The rhizosphere and associated microbiota play critical roles in controlling the ability of plants and plant ecosystems to cope with drought. PGPRs colonize the rhizosphere/endo-rhizosphere of plants and confer drought tolerance by: (i) producing exopolysaccharides (EPS), 1-aminocyclopropane-1-carboxylate (ACC) deaminase, VOCs, phytohormones like abscisic acid (ABA), gibberellic acid, cytokinins, and indole-3-acetic acid (IAA); (ii) inducing accumulation of osmolytes and antioxidants; and (iii) regulation of stress-responsive genes and alteration in root morphology (Vurukonda et al. 2016). For example, IAA-producing Azospirillum spp. improved tolerance to drought stress in wheat by enhancing root growth and formation of lateral roots (Arzanesh et al. 2011). Similarly, PGPR Bacillus thuringiensis stimulated the growth of a lavender species (Lavandula dentate) under drought conditions due to the production of IAA, which improved nutrition, physiology, and metabolic activities of the plant (Armada et al. 2014a, b). In the same vein, Rolli et al. (2015) recently demonstrated the contribution of GFP-labelled Acinetobacter spp. and Pseudomonas spp. isolates in improving acclimation to drought in Arabidopsis and grapevine through a water stress-induced mechanism. Leaves of oriental thuja (Platycladus orientalis) inoculated with Bacillus subtilis increased stomatal conductance and ABA levels in shoots, conferring drought resistance to container-grown plants. Inoculated plants had increased leaf-water content and water potential and an increase in cytokinin concentration, which was linked to the increase in ABA levels (Liu et al. 2013). In another study, Phyllobacterium brassicacearum strain STM196, isolated from the rhizosphere of oilseed rape (Brassica napus), was shown to help alleviate drought stress in inoculated Arabidopsis plants by increasing ABA levels and decreasing leaf transpiration, thereby enhancing osmotic stress tolerance (Bresson et al. 2013). Also, soybean plants inoculated with gibberellin-producing rhizobacterium *Pseudomonas putida* strain H-2–3, were found to show enhanced shoot length and fresh weight under drought stress. These soybean plants also accumulated higher levels of chlorophylls, salicylic acid and abscisic acid in response to stress, compared to control plants (Kang et al. 2014a, b, c). In a promising approach by Timmusk et al. (2014), soil microbial communities were isolated from harsh environments and used to prime wheat plants. Out of a dozen or more isolates, Bacillus thuringiensis strain AZP2 and Paenibacillus polymyxa strain B were able to confer improved drought tolerance to wheat seedlings (Timmusk et al. 2014).

3.4.4 Mechanisms of PGPR Mediated Drought Stress Tolerance

Several mechanisms have been proposed for PGPR mediated drought stress tolerance in plants. It includes phytohormonal activity, volatile compounds, alteration in root morphology, ACC deaminase activity, and accumulation of osmolytes, EPS production, antioxidant defense and co-inoculations. The term Induced Systemic Tolerance (IST) has been coined to accommodate the microbial induced physical and chemical changes in plants, which result in enhanced tolerance to abiotic stresses (Yang et al. 2009a, b).

3.4.5 Alteration of Phytohormonal Activity

Phytohormones such as IAA, gibberellins, ethylene, abscisic acid and cytokinins are produced by plants, which are important for their growth and development (Teale et al. 2006). Phytohormones play a role in plants to abiotic stress to escape or survive stressful conditions (Fahad and Bano 2012). Furthermore, PGPRs are able to synthesize phytohormones that stimulate plant cell growth and division to become tolerant against environmental stresses (Glick and Pasternak 2003).

Physiologically most active auxin in plant growth and development is IAA. Various plant species inoculated with IAA producing bacteria increased root growth and/or enhanced formation of lateral roots and roots hairs (Dimkpa et al. 2009a) thus increasing water and nutrient uptake (Mantelin and Touraine 2004), helping plants to cope with water deficit (Egamberdieva and Kucharova 2009). IAA producing *Azospirillum* enhanced plant's tolerance to drought stress (Dimkpa et al. 2009a). *A. brasilense* producing nitric oxide, a small diffusible gas act as a signaling molecule in IAA inducing pathway and helps in adventitious root development in tomato plants (Creus et al. 2005). Similarly, inoculation with *A. brasilense* Cd increased root projection area, specific root length and specific root area, as compared with non-inoculated controls of common bean (*Phaseolus vulgaris* L.) under drought stress. The effects of *A. brasilense* Cd on root morphology are related to its ability to produce phytohormones, mainly auxins (Dimkpa et al. 2009b).

In case of maize seedlings, inoculation with *A. brasilense* improved relative and absolute water contents compared to non-inoculated plants under drought stress. Bacterial treatment dropped the water potential but increased the root growth, biomass, foliar area, and proline accumulation in leaves and roots. These effects were more significant at 75% reduction in water supply, compared to 50% reduction (Casanovas et al. 2002). Inoculation of *A. brasilense* Sp245 in wheat (*Triticum aestivum*) under drought stress resulted in better grain yield and higher mineral quality (Mg, K and Ca), with improved relative and absolute water content, water potential, apoplastic water fraction and lower volumetric cell wall elasticity suggesting that, in addition to better water status, an 'elastic adjustment' is crucial in increased drought tolerance in plants (Creus et al. 2004). Similarly, *Azospirillum*-wheat association induced decrease in leaf water potential and increase in leaf water content, which was attributed to the production of plant hormones such as IAA by the bacteria that enhanced root growth and formation of lateral roots their by increasing uptake of water and nutrients under drought stress (Arzanesh et al. 2011).

PGPR *B. thuringiensis* helped *Lavandula dentate* plants to grow under drought conditions due to the production of IAA by the bacterium, which improved nutrition, physiology, and metabolic activities of plant (Armada et al. 2014a, b). Soybean plants inoculated with gibberellins secreting rhizobacterium *P. putida* H-2–3 improved plant growth under drought conditions (Sang-Mo et al. 2014). Production of ABA and gibberellins by *Azospirillum lipoferum* alleviated drought stress in maize plants (Cohen et al. 2009). Cellular dehydration induced biosynthesis of ABA, a stress hormone during water deficit condition (Kaushal and Wani 2015). Arabidopsis plants inoculated with *A. brasilense* Sp245 had elevated levels of ABA

compared to non-inoculated plants (Cohen et al. 2008). PGPR *Phyllobacterium brassicacearum* strain STM196, isolated from the rhizosphere of Brassica napus enhanced osmotic stress tolerance in inoculated *Arabidopsis* plants by elevating ABA content, leading to decreased leaf transpiration (Bresson et al. 2013). Inoculation of *Platycladus orientalis* seedlings with cytokinin producing PGPR (Bacillus subtilis) elevated the levels of ABA in shoots and increased the stomatal conductance conferring drought stress resistance (Liu et al. 2013).

3.4.6 Volatiles in Inducing Drought Tolerance

Induction of volatiles takes place when plants are exposed to a multitude of stresses. These stress-induced volatiles serve as signals for developing priming and systemic responses within the same and in neighboring plants. Bacterial priming with Bacillus thuringiensis AZP2 in wheat seedlings under drought stress resulted in enhanced plant biomass and fivefold higher survival under severe drought due to significant reduction of volatile emissions and higher photosynthesis (Timmusk et al. 2014). This suggests that bacterial inoculation improved plant stress tolerance. Volatiles are promising candidates for rapid non-invasive technique to assess crop drought stress and its mitigation during stress development (Timmusk et al. 2014). Root colonization with Pseudomonas chlororaphis O6 prevents water loss by stomatal closure mediated by 2R, 3R-butanediol, a volatile metabolite produced by *P. chlororaphis* O6, whereas, bacteria deficient in 2R, 3R-butanediol production showed no induction of drought tolerance. Microbial volatile 2R, 3R-butanediol helps in inducing resistance to drought stress in *Arabidopsis* (Cho et al. 2008). Further, *Arabidopsis* mutant lines indicated that induced drought tolerance required the salicylic acid (SA), ethylene and jasmonic acid-signaling pathways. Both induced drought tolerance and stomatal closure were dependent on Aba-1 and OST-1 kinase (Cho et al. 2008). Increase in free SA in *P. chlororaphis* O6-colonized plants under drought stress and after 2R, 3R-butanediol treatment suggests the primary role of SA signaling in inducing drought tolerance. Bacterial volatile 2R, 3R-butanediol was a major determinant in inducing resistance to drought in *Arabidopsis* through an SA-dependent mechanism (Cho et al. 2008).

3.4.7 PGPR Alter Root Morphology Under Drought Stress

Plant's physiological status is regulated by cell membranes and rhizobacteria can influence processes taking place at these sites. *A. brasilense* reduced membrane potentials in wheat seedlings and phospholipid content in the cell membranes of cowpea due to the changes in proton efflux activities. Water deficit changes the phospholipid composition in the root, increases phosphatidylcholine and reduces phosphatidylethanolamine (Sueldo et al. 1996), but inoculation with *Azospirillum* prevented these changes in wheat seedlings, though higher phosphatidylcholine and lower phosphatidylethanolamine unsaturation occurred (Pereyra et al. 2006).

Bacteria mediated changes in the elasticity of the root cell membranes is one of the first steps towards enhanced tolerance to water deficiency (Dimkpa et al. 2009a). PGPR improves the stability of plant cell membranes by activating the antioxidant defense system, enhancing drought tolerance in plants (Gusain et al. 2015).

3.4.8 ACC Deaminase Producing Rhizobacteria in Drought Stress Tolerance

Ethylene levels regulate plant activities and the biosynthesis of ethylene is regulated by biotic and abiotic stresses (Hardoim et al. 2008). In the biosynthetic pathway of ethylene, S-adenosylmethionine (S-AdoMet) is converted by 1-aminocyclopropane -1-carboxylate synthase (ACS) to 1-aminocyclopropane-1-carboxylate (ACC), the immediate precursor of ethylene. Under stress conditions, the plant hormone ethylene endogenously regulates plant homoeostasis resulting in reduced root and shoot growth. Plant ACC is sequestered and degraded by ACC deaminase producing bacteria to supply nitrogen and energy. Furthermore, by removing ACC the bacteria reduce the deleterious effect of ethylene, ameliorating plant stress and promoting plant growth (Glick 2005). ACC deaminase producing PGPR *Achromobacter piechaudii* ARV8 significantly increased the fresh and dry weights of both tomato and pepper seedlings and reduced the ethylene production under drought stress (Mayak et al. 2004). Rhizobacteria populating the sites where water is limited with repeated dry periods are likely to be more stress adapting and promote plant growth than those bacteria isolated from the sites where water sources are abundant (Mayak et al. 2004). The seedlings treated with *A. piechaudii* ARV8, a bacterial strain isolated from an arid site, were significantly better in growth than the seedlings treated with a bacterium, *P. putida* GR12–2 that was originally isolated from the rhizosphere of grasses in the high Canadian Arctic areas where water is abundant (Lifshitz et al. 1986).

Inoculation of pea plants with the ACC deaminase producing *Variovorax paradoxus* 5C-2 was more pronounced and consistent under soil drying condition (Dodd et al. 2005). Plants inoculated with ACC deaminase producing bacterium gave more seed yield, seed number and seed nitrogen accumulation and restoring nodulation, which was, depressed in drought stress conditions (Dodd et al. 2005). ACC deaminase producing bacteria eliminated the effects of drought stress on growth, yield, and ripening of pea in both pot and field trials (Arshad et al. 2008). Inoculation of *Pisum sativum* with ACC deaminase producing *Pseudomonas fluorescens* biotype G (ACC-5) induced longer roots, which led to an increased uptake of water from soil under drought stress (Zahir et al. 2008). Inoculation with *V. paradoxus* 5C-2 improved growth, yield and water-use efficiency of droughted peas (Belimov et al. 2009). Systemic effects of *V. paradoxus* 5C-2 showed soil drying-induced increase of xylem abscisic acid (ABA) and an attenuated soil drying-induced increase of xylem ACC (Dodd et al. 2005). Increased nodulation by symbiotic nitrogen-fixing bacteria prevented drought-induced decrease in nodulation and seed nitrogen content. Successful deployment of bacteria in the rhizosphere increased yield and

nutritive value of plants in dry soil, via both local and systemic hormone signaling. Such bacteria provide an easily realized, economic means of sustaining crop yields in dryland agriculture (Belimov et al. 2009). Axenic studies showed that inoculation with ACC deaminase producing rhizobacteria-increased root–shoot length, root-shoot mass and lateral root number of wheat plants compared to control. Better development of roots helped plants to acquire water and nutrients resulting in improved growth and yield under drought stress (Shakir et al. 2012). Co-inoculation of ACC deaminase producing *Bacillus* isolate 23-B and *Pseudomonas* 6-P with *Mesorhizobium ciceris* for mitigation of drought stress and plant growth promotion under axenic conditions on *Cicer arietinum* varieties (*Kabuli* L-552 and *Desi* GPF-2) significantly improved germination, root and shoot length and fresh weight of chickpea seedlings under osmotic potential up to 0.4 MPa over uninoculated control. Proline content was higher in PGPR treated varieties under water stress. Similarly, pepper inoculated with ACC deaminase producing Bacillus licheniformis K11 alleviated drought stress and six differentially expressed stress proteins were identified by 2D-PAGE (Hui and Kim 2013).

3.4.9 Osmolytes in Imparting Drought Tolerance in Plants

Plants adaptation to drought stress is associated with metabolic adjustments that lead to the accumulation of several compatible solute/osmolytes like proline, sugars, polyamines, betaines, quaternary ammonium compounds, polyhydric alcohols and other amino acids and water stress proteins like dehydrins (Close 1996). PGPR secrete osmolytes in response to drought stress, which act synergistically with plant-produced osmolytes and stimulate plant growth (Paul et al. 2008). PGPR *Pseudomonas putida* GAP-P45 inoculation improved plant biomass, relative water content and leaf water potential by accumulation of proline in maize plants exposed to drought stress (Sandhya et al. 2010). Inoculating plants with PGPR adds up to the existing concentrations of proline, a sizeable quantity of proline increased when maize plants were inoculated with *P. fluorescens* under drought stress (Ansary et al. 2012). Drought tolerance of *L. dentate* showed that PGPR *B. thuringiensis* (Bt) inoculation enhanced shoot proline accumulation when compared to control plants under drought stress (Armada et al. 2014a, b). Increased proline content due to up regulation of proline biosynthesis pathway keeps proline in high levels to help in maintaining cell water status, protects membranes and proteins from stress (Sandhya et al. 2010). Similarly, tomato (*Lycopersicon esculentum* Mill) cv. Anakha treated with phosphate solubilizing bacteria (PSB) (*Bacillus polymyxa*) secreted excess proline to cope up with the drought condition (Shintu and Jayaram 2015). PGPR consortia containing *Pseudomonas jessenii* R62, *Pseudomonas synxantha* R81 and Arthrobacter nitroguajacolicus strainYB3, strain YB5 enhanced plant growth in Sahbhagi (drought tolerance) and IR-64 (drought sensitive) cultivars of rice (*Oryza sativa* L.). Higher proline accumulation in inoculated plants indicates higher plant tolerance to water stress (Gusain et al. 2015). In severe drought stress both the consortia treated varieties showed higher proline in almost similar way, the attribute

might differentiate the tolerant and sensitive varieties of rice suggesting the vital role of proline as an osmoregulatory solute in plants (Gusain et al. 2015). Inoculation of maize plant with PGPR *P. putida* GAP-P45 (Sandhya et al. 2010) and *A. lipoferum* (Bano et al. 2013) improved plant growth through accumulation of free amino acids and soluble sugars compared to non-treated plants under drought stress.

Trehalose, a non-reducing disaccharide acts as osmoprotectant by stabilizing dehydrated enzymes and membranes; its biosynthesis imparts osmoprotection (Yang et al. 2010). Enhanced drought tolerance was observed in *P. vulgaris* plants inoculated with *Rhizobium etli* overexpressing trehalose-6-phosphate synthase gene compared with plants inoculated with the wild strain (Suarez et al. 2008). Macro array analysis of 7200 expressed sequence tags from nodules of plants inoculated with the strain overexpressing trehalose-6-phosphate synthase gene revealed up-regulation of genes involved in stress tolerance, carbon and nitrogen metabolism, suggesting a signaling mechanism for trehalose (Suarez et al. 2008). It is well established that trehalose plays an important role as a signaling molecule in plants (Paul et al. 2008). Similar effects were observed in maize plants inoculated with *A. brasilense* overexpressing trehalose biosynthesis gene (Rodriguez et al. 2009). Inoculation imparted drought tolerance in maize and significantly increased the plant biomass. It is hypothesized from the study that very small amounts of trehalose translocated to the maize roots and signal stress tolerance pathways in the plant. Thus, trehalose metabolism in PGPR is key for signaling plant growth, yield, and adaptation to abiotic stress, and its manipulation has a major agronomical impact on plants (Rodriguez et al. 2009).

Choline plays a critical role in plant stress resistance, mainly for enhancing glycine betaine(GB) synthesis and accumulation (Zhang et al. 2010a, b). Evident reports on induced role of B. subtilis GB03 in *Arabidopsis* (Zhang et al. 2010a, b) and *Klebsiella variicola*F2*, P. fluorescens* YX2 and *Raoultella planticola* YL2 in maize showed enhancements in biosynthesis and accumulation of choline as a precursor in GB metabolism, resulting in the accumulation of GB thereby improving leaf relative water content (RWC) and dry mater weight (DMW) (Gou et al. 2015). The increased content of choline in drought stressed maize plants supplied more nutrition as a food additive (Zhang et al. 2010a, b). The relative expression of *PEAMT* gene induced in *Arabidopsis* by *B. subtilis* GB03 were almost threefold as compared with uninoculated plants under osmotic stress, resulting in elevated metabolic level of choline together with GB in osmotically stressed plants (Zhang et al. 2010a, b). Enhanced accumulation of solutes such as GB was induced by PGPR stains under stress conditions that regulated plant stress responses by preventing water loss caused by osmotic stress (Nadeem et al. 2009; Bashan et al. 2014). Similarly, osmotic stressed plants inoculated with PGPR stains such as *B. subtilis* GB03 and *Pseudomonas* spp. accumulated significantly higher GB than those in plants without inoculation (Sandhya et al. 2010), might be attributed to up-regulation of GB biosynthesis pathway by enhancing some key enzymes gene expression such as *PEAMT* (Zhang et al. 2010a, b). Similarly, polyamines are considered as plant growth regulating compounds among them, cadaverine was correlated with root growth promotion or osmotic stress mitigation in plants. Role of cadaverine

producing *A. brasilense* Az39 promoted root growth and helped to mitigate osmotic stress in rice seedlings (Cassan et al. 2009).

3.4.10 Exopolysaccharide (EPS) Production by Rhizobacteria and Alleviation of Drought Stress

Drought stress can make physico–chemical and biological properties of soil unsuitable for soil microbial activity and crop yield. Water availability controls the production and consumption of protein and polysaccharides by the bacteria (Roberson and Firestone 1992) and thus indirectly influences soil structure. Exopolysaccharide (EPS) production by microbes protects them from inhospitable conditions and enables their survival. Capsular material of *A. brasilense* Sp245 contains high molecular weight carbohydrate complexes (lipopolysaccharide–protein (LP) complex and polysaccharide–lipid (PL) complex) responsible for protection under extreme conditions like desiccation. Addition of these complexes to a suspension of decapsulated cells of *A. brasilense* Sp245 significantly enhanced survival under drought stress (Konnova et al. 2001). The EPS released into soil as capsular and slime materials by soil microbes can be adsorbed by clay surfaces due to cation bridges, hydrogen bonding, Van der Waals forces, and anion adsorption mechanisms thus forming a protective capsule around soil aggregates (Sandhya et al. 2009). EPS provides a microenvironment that holds water and dries up more slowly than the surrounding environment thus protecting the bacteria and plant roots against desiccation. Production of EPS by bacteria has been shown to improve permeability by increasing soil aggregation and maintaining higher water potential around the roots, thereby increasing in the uptake of nutrients by plant with an increase in plant growth and protection from drought stress (Selvakumar et al. 2012). Plants treated with EPS-producing bacteria display increased resistance to water stress (Bensalim et al. 1998). Significant increase in root adhering soil per root tissue (RAS/RT) ratio was observed in sunflower rhizosphere inoculated with the EPS-producing rhizobial strain YAS34 under drought conditions (Alami et al. 2000). Inoculation of *Pseudomonas* sp. strain GAP-P45 increased the survival, plant biomass and RAS/RT of sunflower seedlings subjected to drought stress. The inoculated rhizobacteria could efficiently colonize the root adhering soil, rhizoplane and increase the percentage of stable soil aggregates. Better aggregation of RAS leads to increased uptake of water and nutrients from rhizosphere soil, thus ensuring plant growth and survival under drought stress (Sandhya et al. 2009). Seed bacterization of maize with EPS-producing bacterial strains *Proteus penneri* (Pp1), *Pseudomonas aeruginosa* (Pa2) and *Alcaligenes faecalis* (AF3) along with their respective EPS improved soil moisture contents, plant biomass, root and shoot length and leaf area. Under drought stress, the inoculated plants showed increase in relative water content, protein, sugar and proline content and the activities of antioxidant enzymes were decreased (Naseem and Bano 2014). Inoculation of catalase and exopolysaccharides producing *Rhizobium leguminosarum* (LR-30), *Mesorhizobium ciceri* (CR-30 and CR-39) and *Rhizobium phaseoli* (MR-2) showed

beneficial interaction to wheat (non-legume) under drought stress. Inoculation improved the growth, biomass and drought tolerance index of the wheat seedlings under poly ethylene glycol (PEG) 6000 simulated drought. Isolates produced indole acetic acid which enhanced the root length of the seedlings to dilute the drought stress (Hussain et al. 2014).

3.4.11 Altering Antioxidant Defense System

Reactive oxygen species (ROS) generation are results of drought exposure to plants which include superoxide anion radicals (O2−), hydrogen peroxide (H2O2), hydroxyl radicals (OH), singlet oxygen (O12) and alkoxy radicals (RO). Reaction of ROS with proteins, lipids and deoxyribonucleic acid causing oxidative damage and hinders the normal functions of plant cell. Enzymatic components include superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR). Non-enzymatic components contain cysteine, glutathione and ascorbic acid (Kaushal and Wani 2015). several experiments on maize plants inoculated with five drought tolerant plant growth promoting Pseudomonas spp. strains namely P. entomophila, P. stutzeri, P. putida, P. syringae, and P. montelli subjected to drought stress showed significantly lower activity of antioxidant enzymes as compared to uninoculated plants (Sandhya et al. 2010). similar experiments were done with Bacillus species that developed protection against drought stress by reducing activity of the antioxidant enzymes APX and Glutathione peroxidase (GPX) (Vardharajula et al. 2011). In field studies basil plants (Ocimum basilicum L.) treated with Pseudomonas sp. under drought stress, significantly increased the CAT enzyme activity, similarly when treated with microbial consortia containing Pseudomonades sp., Bacillus lentus and A. brasilense highest activity of GPX and APX was observed (Heidari and Golpayegani 2011). several other bacterial species have shown promising results for PGPR activity which includes *B. thuringiensis* (Bt). Hence such studies provide full evidence that PGPRs application in enhancing drought tolerance by plants by altering the antioxidants activity under water deficit conditions (Gusain et al. 2015).

3.4.12 Molecular Studies in Alleviation of Drought Stress by PGPR

To understand and compare the holistic responses of an organism to their environment gene expression studies are powerful tools (Karen et al. 2010). The entire set of transcripts that are expressed with in a cell or organism at a particular developmental stage or under various environmental conditions are in transcriptome. There are various technologies for assaying the transcriptome including hybridization-based microarrays to RNA sequencing (Wang et al. 2009a, b). Gene expression by drought stress was recently characterized using molecular approaches (Yuwono et al. 2005), their physiological roles with respect to tolerance induced by

PGPR. Using Real-Time PCR (RT-PCR), upregulation of stress related genes APX1, SAMS1, and HSP17.8 in the leaves of wheat were identified and increased activity of enzymes involved in the plant ascorbate–glutathione redox cycle conferring drought tolerance in wheat when priming with Bacillus amyloliquefaciens 5113 and A. brasilense NO40 alleviating the deleterious effect of drought stress (Kasim et al. 2013). PGPR enhanced plant tolerance to drought was observed with inoculation of PGPR Paenibacillus polymyxa B2, which enhanced drought tolerance of Arabidopsis thaliana the transcriptional level Using 2-D polyacrylamide gel electrophoresis (2D-PAGE) and differential display polymerase chain reaction (DD-PCR),RNA display showed that an mRNA transcription of a drought-response gene, EARLY RESPONSE TO DEHYDRATION 15 (ERD15) was augmented in inoculated plants compared to uninoculated controls (Timmusk and Wagner 1999). Six differentially expressed stress proteins were identified in pepper plants inoculated with B. licheniformis K11 under drought stress. Among the stress proteins, specific genes of Cadhn, VA, sHSP and CaPR-10 showed more than a 1.5-fold increase in treated plants compared to control (Lim and Kim 2013). Using microarray analysis, a set of drought signaling response genes were down-regulated in the P. chlororaphis O6-colonized A. thaliana compared to those without bacterial treatment under drought stress. Transcripts of the jasmonic acid-marker genes, VSP1 and pdf-1.2, salicylic acid regulated gene, PR-1 and the ethylene-response gene, HEL, were up-regulated in colonized plants, but differed in their responsiveness to drought stress (Cho et al. 2013). Using Illumina sequencing (HiSeq 2000 system) the effects of the association between the diazotroph Gluconacetobacter diazotrophicus PAL5 and sugarcane cv. SP70-1143 under drought stress concluded that bacterial inoculation activated the ABA-dependent signaling genes conferring drought resistance in sugar cane cv. SP70-1143 (Vargas et al. 2014).

3.4.13 Co-Iinoculation of PGPR for Alleviation of Drought Stress

Combination of single strains of PGPR with either mycorrhizal fungi elicits plant drought tolerance (Wang et al. 2012). Several studies carried out has shown greater growth compared to inoculation with Rhizobium alone e.g. Co-inoculation of common bean (P. vulgaris L.) with Rhizobium tropici-CIAT 899, P. polymyxa-DSM36 and P. polymyxa-Loutit Furthermore, co-inoculation exhibited greater nodulation (number and biomass) and nitrogen content compared to drought-stressed plants inoculated with only Rhizobium (Figueiredo et al. 2008). Microbial consortium containing PGPR Bacillus cereus AR156, B. subtilis SM21 and Serratia sp. XY21 termed as BBS improved drought tolerance in cucumber plants. After withholding watering for 13 days, BBS treated plants exhibited darker green leaves with lighter wilt symptoms, decreased the leaf mono dehydroascorbate content and relative electrical conductivity, increased the leaf proline and chlorophyll content and root recovery intension in cucumber plants under drought stress. In comparison with control, the BBS treatment enhanced the SOD activity and mitigated the drought-triggered down-regulation of the genes cAPX, rbcL and rbcS encoding cytosolic

ascorbate peroxidase and ribulose-1,5-bisphosphatecarboxy/oxygenase (Rubisco) large and small subunits, respectively, in cucumber leaves (Wang et al. 2012). Microbial consortia consisting of EPS producing bacterial strains P. penneri (Pp1), P. aeruginosa (Pa2) and A. faecalis (AF3) showed greater potential to drought tolerance in maize compared to single PGPR strains (Naseem and Bano 2014). PGPRs consortia alleviated drought stress in rice plants by reducing oxidative damage and accumulation of proline in rice plants grown under drought there by improved the plant growth (Gusain et al. 2015). GPR strain Pseudomonas mendocina Palleroni and an arbuscular mycorrhizal (AM) fungus (either Glomus intraradices or Glomus mosseae) significantly enhanced the root phosphatase activity; and the proline accumulation and the activities of NR, POD and CAT in the lettuce leaves under moderate and severe drought stress (Kohler et al. 2008). Further co-inoculation of wheat plants with Azotobacter chrocoocum (E1) and Pseudomonas sp. (E2) alleviated drought stress through increased anatomical changes such as thickness of epidermis, ground, mesophyll and phloem tissues, diameter of xylem vessel and dimensions of vascular bundles of the root system, whereas water deficit levels (75, 50 and 25% field capacity) decreased the anatomical values of the un-inoculated wheat cultivars (El-Afry et al. 2012).

3.4.14 Salinity

Environmental stress that severely affects plant productivity throughout the world is salinity. Ion toxicity and ion imbalances in plants is caused by excess salts thus inducing metabolic imbalances and hyperosmotic stress-induced water deficit. Plant synthesizing osmolytes and polyamines, by activating defense mechanisms, reducing ROS accumulation, ion transport, and compartmentalization, in order to cope with salinity stress. Study carried by Upadhyay et al. (2011a, b), wheat seedlings were inoculated with EPS-producing PGPRs (including Bacillus spp. Enterobacter spp. Paenibacillus spp.) showed significantly decreased Na + uptake and increased biomass production under high-saline conditions. Another study carried on tomato inoculated with certain PGPRs were shown to be able to maintain their growth under high-salinity and water-limited conditions by reducing the negative impact of stress-induced ethylene release on root growth through the activity of bacterial ACC-deaminase (Mayak et al. 2004). Bharti et al. (2016) demonstrated the use of a carotenoid-producing halotolerant PGPR Dietzia natronolimnaea strain STR1 in conferring salinity tolerance in wheat. Inoculated plants had higher expression of proline and various antioxidants, enabling these plants to withstand salinity stress (Bharti et al. 2016). Another study by Mahmood et al. (2016) demonstrated that Enterobacter cloacae and Bacillus drentensis acted synergistically with foliar application of silicon to confer salinity tolerance in field-grown Mung beans. Moreover, Brachybacterium saurashtrense strain JG-06, Brevibacterium casei strain JG-08, and Haererohalobacter spp. strain JG-11 conferred improved plant growth in peanuts when seedlings were subjected to salinity stress by adding 100 M NaCl. Plant height, shoot length, root length, shoot dry weight, root dry weight, and total

biomass were significantly higher in inoculated plants compared to uninoculated plants (Shukla et al. 2012) in order to cope with salinity.

3.4.15 Salt Tolerance Mediated by Plant Growth Promoting Rhizobacteria

Research has time and again continuously demonstrated numerous beneficial associations between plants and microbes, beginning with the classic legume–rhizobia symbiosis. The higher abundance of microbial populations is because of the rhizosphere that enriched with nutrient sources.(Lugtenberg and Kamilova 2009a, b). Beneficial activities are being exerted by Free-living beneficial bacteria dwelling in the rhizosphere and these bacteria are being termed as plant growth promoting rhizobacteria (PGPR) which can be facultative endophytes that further invade intercellular spaces of host tissues and thrive as endophytes to establish a mutually beneficial association. The root surfaces are the areas where these PGPR colonizes thrives in spaces between root hairs and rhizodermal layers whereas, some are not physically in contact with the roots (Gray and Smith 2005).

Rhizosphere signaling events and regulation of communication in beneficial plant–microbe interactions is done by root exudates. Chemical signals for bacterial chemotaxis, secretion of exopolysaccharides, quorum sensing and biofilm formation during rhizosphere colonization (Narula et al. 2009) are secreted by roots which include Phenols, flavonoids, and organic acids. PGPR are screened in vitro for plants and then tested in greenhouse and field trials prior to commercialization. GPR promote plant growth and development through diverse mechanisms such as enhanced nutrient assimilation (biofertilizers) by biological nitrogen fixation, phosphorous solubilisation or iron acquisition (Kuan et al. 2016), control pathogens by antagonism and competition (biocontrol agents) (Chowdhury et al. 2015), degrade organic pollutants and reduce metal toxicity of contaminated soils (bioremediation), and facilitate phytoremediation (Weyens et al. 2015). Abiotic stress has been modulated by Inoculation with PGPR via direct and indirect mechanisms that induce systemic tolerance (Yang et al. 2009a, b). Now research is being done to investigate PGRP for their role in improving plant-water relations,

Rhizobacteria- A plant growth promotor- has the ability to regulate stomatal opening and water potential by influencing the transpiration rate and hydraulic conductivity. For example, when Bacillus megaterium is inoculated with Maize plants, the root hydraulic conductivity increased compared to plants without inoculum at 2.59 dsm-1 salinity level. This was due to the increase in expression of two plasma membrane aquaporin protein isoforms (ZmPIP) (Marulanda et al. 2010). Besides PGPR is responsible for inducing phytohormone signaling and osmolyte accumulation in plants in order to overcome the initial osmotic shock upon salinization. Arabidopsis thaliana containing transgenes (proBA) from Bacillus subtilis lead to salt tolerance in plants by enhancing proline synthesis (Chen et al. 2007). Rice inoculated with Bacillus amyloliquefaciens SN13 resulted in increase in salt tolerance of the plant on exposure to 200 mM NaCl in soil as well as hydroponic

conditions and affected the expression of 14 genes by upregulation of four viz.; SOS1, EREBP (ethylene responsive element binding proteins), SERK1 (somatic embryogenesis receptor-like kinase) and NADP-Me2 (NADP-malic enzyme) and downregulating two namely; GIG (glucose insensitive growth) and SAPK4 (serine-threonine protein kinase) in hydroponic conditions. MAPK5 (Mitogen activated protein kinase 5) showed upregulation only under greenhouse conditions. Modulation of genes responsible for ionic and osmotic stress response mechanisms were carried by using SN13 inoculation (Nautiyal et al. 2013).

Beneficial microorganisms possess the ability to enhance the carbohydrate metabolism and transport which in turn implicate source-sink relations, photosynthesis, growth rate and biomass reallocation. Wheat seeds with inoculation of B. aquimaris strains under saline field conditions (ECe = 5.2 dS m^{-1}) increased total soluble sugars as well as reducing sugars resulting in higher shoot biomass. Accumulation of NPK and reduction of Na in leaves (Upadhyay et al. 2011b) inoculation of pepper (*Capsicum annuum*) with *Pantoea dispersa* and Azospirillum brasilense enhanced photosynthesis and stomatal conductance under salinity without affecting photochemical efficiency and concentration of chlorophyll of photosystem II (del Amor and Cuadra-Crespo 2012). Accumulation of large quantities of osmoprotectants in cytosol occur in microbes upon exposure to osmolality fluctuations in their environments (Kempf and Bremer 1998). These circumstances result in osmolyte biosynthesis including betaines proline, glycine and trehalose by PGPR much quicker than host plant associated with it. co inoculation of Paenibacillus polymyxa Rhizobium tropici strain into beans modified to overexpress trehalose 6-phosphate gene resulted in increased N content, nodulation and plant growth. on carrying the microarray analysis of nodules suggested that extracellular trehalose induce salinity tolerance by upregulation of genes tolerant to stress (Figueiredo et al. 2008).

Bacteria have the potential to limit the salt uptake by plants. They trap the cations in the exopolysaccharide matrix and regulate the expression of ion affinity transporters. in addition, alter the structure of roots containing extensive rhizosheaths. PGPR however is known for its capability of enhancing the exchange of nutrients, both micro and macronutrients. it is also known to cause alleviation in nutrient imbalance that is caused by higher influx in Na^+ and Cl^- ions. Microbial miniralisation, organic acids and siderophores cause increased nutrient availability for plants (Lugtenberg et al. 2013). PGPR improves maintenance of ion homeostasis by enhancing K+/Na+ ratios in shoots and reduces the accumulation of Na+ and Cl- in leaves, excludes Na+ through roots and boost the K+ transporter activity having high-affinity. Inoculation of auxin producing Azotobacter strains C5 and C9 in maize plants improve K+ uptake and exclude Na+ under salt stress. PGPR inoculation not only enhance stress response but also increase polyphenol, chlorophyll and chlorophyll content in leaves (Rojas-Tapias et al. 2012). While studying Burkholderia phytofirmans and Arabidopsis thaliana PsJN in order to understand the spatiotemporal regulation of long and -term salt stress, colonized plants exhibited higher tolerance to stress/on altering the patterns of expression of genes responsible for ion homeostasis (SOS1, KT1, NHX2, and HKT1) after salt stress induce rapid molecular change which may be due to the salt tolerance (Pinedo et al. 2015). upregulation

of PtHKT1 and down regulation of PtHKT2 in the roots under high salt gradient were validated by decreased Na + accumulation in Puccinellia tenuiflora which is a halophyte grass (Niu et al. 2016).

Soil bacteria release metabolites, enzymes and exogenous hormones modulating hormone status in plants due to increase in salt tolerance. During stress, fresh synthesis of metabolites and phytohormones in plants take place as a response to events of signaling due to Plant-microbe interactions (Dodd et al. 2010). The biosynthesis of auxins in rhizobacteria occur through a number of pathways. One of them is the tryptophan utilization in root exudates followed by its conversion into IAA (indole-3-acetic acid), that is absorbed by the roots of the plant. In addition, the endogenous IAA pool of the plant triggers the auxin- pathway which in turn stimulates cell proliferation and growth. IAA production of PGPR represents the most commonly studied molecules of bacterial signalling in plant-microbe interactions. Exogenous IAA functioning depends on the endogenous IAA levels in plants. Optimal concentration of Exogenous IAA along with bacterial IAA acquisition may cause inhibition or promotion or even neutral plant growth (Spaepen and Vanderleyden 2011a, b). Bacillus amyloliquefaciens SQR9 when inoculated in maize seedlings in vitro enhance tolerance to salt stress (100 mM NaCl). This inoculation resulted in increase in total and soluble sugar, glutathione and chlorophyll contents, improved the activity of catalase and peroxidase activity and enhanced K+/Na+ ratio. Conformation to these physiological changes to unveil salt stress which lead to upregulation of H (+)-Ppase (encoding H+ pumping pyrophosphatase), RBCS, RBCL (encoding RuBis Co subunits), NHX1, NHX2 and NHX3 and HKT1 genes and downregulation of NCED expression in the inoculated seedlings (Chen et al. 2007). Cytokinin role in salt tolerance is yet to be widely studied. *Pseudomonas extremorientalis* TSAU20, P. extremorientalis TSAU6 and *P. aurantiaca* TSAU22, strains of pseudomonas increase growth up to 52% as compared to control plants. Seed dormancy was also induced in wheat seeds when salinity alleviated (100 mM NaCl) (Egamberdieva and Kucharova 2009). Lettuce seedlings inoculated with *B. subtilis* under water deficit conditions shortened root and increase in accumulation of biomass in shoot with only small effect on root biomass. In addition shoot ABA hindered the role of root -to-shoot signalling in spite of cytokinin increase in shoot (Arkhipova et al. 2007).

3.4.16 Abscisic Acid

Determining the role of exogenous ABA in plant–microbe interactions and whether bacterial ABA influences ABA status of plants under salt stress there are relatively few studies. But the to enhance the growth of salt stressed plants signaling pathways like by ABA and ABA biosynthesis are mediated through PGPR. Halotolerant Dietzia natronolimnaea STR1 induced salinity (150 mM NaCl) tolerance mechanisms in wheat plants via modulation of an ABA-signaling cascade, validated by the upregulation of TaABARE (ABA-responsive gene) and TaOPR1 (12-oxophytodienoate reductase 1) leading to TaMYB and TaWRKY stimulation,

followed by expression of stress response genes including upregulation of TaST (a salt stress-induced gene). Expression of tissue-specific responses of ion transporters and SOS pathway related genes were modulated. Increase in Gene expression of various antioxidant enzymes and proline content was seen. Contributing to enhanced protection against salt stress in PGPR inoculated plants (Bharti et al. 2016). PGPR increased water potential and decreased electrolyte leakage. The inoculated plants showed down-regulation of ABA compared with control plants, while salicylic acid and gibberellin GA4 contents were increased (Kang et al. 2014a, b, c). Seed inoculation of cotton with Pseudomonas putida Rs-198 reduced ABA accumulation and increased plant biomass in salinized soil but the induced salt tolerance can also be attributed to regulated ionic balance and improved endogenous IAA content (Yao et al. 2010). The inoculation of PGPR Strains Arthrobacter protophormiae SA3 and B. subtilis LDR2 in wheat plants built up IAA while conflicted the increase of ABA and ACC content under salt stress conditions (100 mM NaCl).

3.4.17 Extracellular Molecules

Proteins, hormones, volatiles, polyamines, and other compounds are the extracellular secretions of PGPR that have been determined to manipulate signaling pathways and regulatory functions that positively impact plant defense and development by stimulating growth, inducing disease resistance and eliciting stress tolerance (Zhou et al. 2016).

3.4.17.1 Exopolysaccharides

Exopolysaccharides (EPS) secreted by bacteria are responsible for attachment, often along with other bacteria, to soil particles and root surfaces. The EPS increasing water holding capacity and cation exchange capacity and bind soil particles to aggregates, stabilizing soil structures (Upadhyay et al. 2011a, b). The enclosed matrix of microcolonies is usually formed by EPS which confer protection against environmental fluctuations, water and nutrient retention, and epiphytic colonization (Balsanelli et al. 2014). They are also indispensable for mature biofilm formation and functional nodules in legume–rhizobia symbiosis. Stabilization of soil aggregates under field conditions was seen as a result of Inoculation of EPS producing Pseudomonas mendocina with an arbuscular mycorrhizal fungus, Glomus intraradices onto lettuce (Lactuca sativa) (Kohler et al. 2006). Increased growth of chickpea (Cicer arietinum var. CM-98) and soil aggregation with roots under high salt concentrations (up to 200 mM NaCl) (Qurashi and Sabri 2012) was also seen as a result of Inoculation with salt-tolerant Halomonas variabilis HT1 and Planococcus rifietoensis RT4. Further scrutinyis required in determining the role of EPS in plant salinity tolerance no doubt EPS production and composition improves bacterial resistance to abiotic stress.

3.4.17.2 Lipo-Chitooligosaccharides

Salt stress affects Legume–rhizobia symbiosis and high levels of salinity inhibit nodule formation and nitrogen fixation (Zahran 1999). Rhizobia in response to flavonoids present in root exudates and initiate nodule formation secrets Lipochitooligosaccharides (LCOs) as Nod-factors (NFs). LCOs are conserved at the core but diverge in the N-Acetyl chain length, degree of saturation, and substitutions (glycosylation or sulfation), which are crucial in host specificity (Oldroyd 2013). Nod-factors also act as stress response signals in legumes and NF synthesis is modulated by other PGPR and abiotic stresses. High salinity (100–200 mM NaCl) inhibited root hair deformation responses to increase in NF concentrations in Soybean (Glycine max) – Bradyrhizobium japonicum symbiosis (Duzan et al. 2004). Increase in growth was seen as a result of Inoculation of IAA producing Azospirillum brasilense Cd into the Rhizobium-Bean (Phaseolus vulgaris cv. Negro Jamapa) The co-inoculation also promoted Nod-genes expression in R. tropiciCIAT899 and R. etli ISP42 grown in the presence of root exudates (Dardanelli et al. 2008). More resistant to salt stress are Free-living rhizobia than inside their legume hosts. Enhanced nodulation and growth of soybean under salinity levels (36 and 61 mM NaCl) was seen as a result inoculation of *B. japonicum* 532C grown in genistein (a flavonoid), such positive effects become more evident with time (Miransari and Smith 2009) and increased yield up to 21% under salinized field conditions.

3.4.17.3 Bacteriocins

Small peptides secreted by rhizobacteria that are bactericidal or bacteriostatic against relative bacteria, thus providing a competitive advantage to the producer strain but might also promote microbial diversity in an ecologic niche are called Bacteriocins. Expression of proteins involved in carbon and energy metabolism pathways were modulated by the bacterial signals. Proteins involved in photosynthesis including PEP carboxylase, RuBisCo-oxygenase large subunit, pyruvate kinase and proteins of photosystems I and II were upregulated along with other stress related proteins (Subramanian et al. 2016b). Application of thuricin 17, isolated from a soybean endosymbiont Bacillus thuringiensis NEB 17 differentially altered the proteome of salt-stressed (250 mM NaCl) Arabidopsis plants. Thuricin 17 positively manipulate plant proteome profile and enhance physiological tolerance to salinity (Subramanian et al. 2016a).

3.4.17.4 Polyamines

Polyamines (PAs) are low molecular weight aliphatic amines with pronounced antioxidant activity that are ubiquitous in all living organisms and modulate ROS homeostasis by scavenging free radicals and stimulating antioxidant enzymes. Spermidine, spermine, and putrescine are the most abundant polyamines that are implicated in various developmental processes and stress responses in plants (Gupta et al. 2013). Increase abiotic stress tolerance can be attributed to application of exogenous polyamines. But PGPR secretion of polyamines is largely unexplored.

Increased cellular polyamine accumulation in Arabidopsis by Spermidine from Bacillus megaterium BOFC15 thereby activating PA-mediated signaling pathways contributing to the osmotic stress tolerance of plants. Greater biomass, elevated photosynthetic capacity and higher antioxidant enzyme activity was seen as a result bacterial inoculation. Maintaining water balance and stomatal conductance by the ABA dependent stress responses and robust root system architecture were other mechanisms involved (Zhou et al. 2016).

3.4.17.5 Volatile Compounds

PGPR that release Volatile organic compounds (VOC) are known to stimulate plant growth, resulting in increased shoot biomass, and modulated stress responses. Perception of volatiles by plants and subsequently induced mechanisms require further research (Bailly and Weisskopf 2012). B. subtilis GB03 VOCs mediated tissue specific regulations of Na+ homeostasis in salt-stressed plants. Arabidopsis under 100 mM NaCl treated with VOCs decreased Na+ accumulation by concurrently downregulating expression of HKT1 in roots but upregulating it in shoots. Presumably, the induction of HKT1 dependent shoot-to-root recirculation resulted in reduced Na+ accumulation up to ~50% throughout the plant. Treatment with VOCs increased leaf surface area, root mass, and total K+ content when compared with controls whereas, inoculated athkt1 mutants showed stunted growth. Exposure to VOCs reduced the total Na+ level by 18% and enhanced shoot and root growth of sos3 mutants in 30 mM NaCl (Zhang et al. 2008). A putative VOCs blend released from Pseudomonas simiae AU induced salt-tolerance in soybean (*Glycine max*) under 100 mM NaCl by decreasing root Na+ accumulation and increasing proline and chlorophyll content. Protein expression analysis confirmed upregulation of vegetative storage proteins (Na+ homeostasis), RuBisCO large chain proteins (photosynthesis) in exposed soybean seedlings (Vaishnav et al. 2015a, b). Paraburkholderia phytofirmans PsJN VOCs stimulate plant growth and induce salinity tolerance that have been demonstrated both in vitro (150 mM NaCl/15 mM CaCl2) and in soil (200 mM NaCl/20 mM CaCl2). Growth parameters of Arabidopsis plants measured as rosette area, fresh weight, and primary root length were higher than the control plants and exposure to VOCs showed parallel growth promoting effects of direct bacterial inoculation. Genome wide mapping association of Arabidopsis accession lines revealed 10 genetic loci associated with growth stimulation in response to the presence of *P. simiae* WCS417r in vitro, which is partly caused by VOC produced by the bacterium. Even though the study was conducted to select lines for breeding strategies, it is interesting to note that the genotype variation of host plants has different interactions with the associated root microbiome (Wintermans et al. 2016).

3.4.18 Heavy Metals

Presence of heavy metals represent a significant challenge to plant and microbial growth if elevated above tolerance levels and/or above essential needs (Cu, Ni, Cr, Zn are essential at lower levels) phytoremediation potentials are usually influenced

by the toxic effects of metals in soil; however, application of soil bacteria can enhance phytoremediation. This association has been give the term microbe-assisted phytoremediation (Glick 2003). Once microbes inoculated many PGPRs have demonstrated the ability to protect their host plants from heavy-metal toxicity (Shinwari et al. 2015). Genera of PGPRs shown to have this ability cover a wide diversity from the Alpha -proteobacteria (Mesorhizobium, rhizobium, Sinorhizobium, Bradyrhizobium), Betaproteobacteria (Achromobacter), γ-proteobacteria (Azotobacter, Pseudomonas) to Firmicutes (*Bacillus* spp.) (Shinwari et al. 2015). These scenarios involve five main mechanisms:

(a) Extrusion by transport via efflux pumps
(b) Exclusion by removing metals from target sites
(c) Inactivation by complexation
(d) Biotransformation to a less toxic redox state
(e) Methylation and demethylation (Tak et al. 2013).

3.5 Microbial Wealth and Impact on Agriculture

Development of sustainable agriculture in which the high productivities of plants and animals are ensured using their natural adaptive potentials, with a minimal disturbance of the environment (Noble et al. 2004) has been given increased attention. It is our view that the most promising strategy to reach this goal is to substitute hazardous agrochemicals (mineral fertilizers, pesticides) with environment-friendly preparations of symbiotic microbes, which could improve the nutrition of crops and livestock, as well as their protection from biotic (pathogens, pests) and abiotic (including pollution and climatic change) stresses (Yang et al. 2009a, b). Agricultural microbiology is the present paramount research field responsible for the transfer of knowledge from general microbiology and microbial ecology to the agricultural biotechnologies. The broad application of microbes in sustainable agriculture is due to the genetic dependency of plants on the beneficial functions provided by symbiotic cohabitants (Noble et al. 2004). The agronomic potential of plant–microbial symbioses proceeds from the analysis of their ecological impacts, which have been best studied for N2 fixing (Franche et al. 2009). This analysis has been based on 'applied co-evolutionary research' (Arnold et al. 2010), addressing the ecological and molecular mechanisms for mutual adaptation and parallel speciation of plant and microbial partners. For plant–fungal interactions, it has been demonstrated that the host genotype represents the leading factor in the biogeographic distribution of mycobionts and for their evolution within the mutualist↔antagonist and specialist↔generalist continua (Peay et al. 2010). impact of agricultural microbiology on sustainable agriculture would be to substitute agrochemicals (mineral fertilizers, pesticides) with microbial preparations. However, this substitution is usually partial and only sometimes may be complete, e.g. in recently domesticated leguminous crops, which retain a high potential for symbiotrophic N nutrition, typical for many wild legumes (Provorov and Tikhonovich 2003). Plant mixotrophy, e.g. on a

simultaneous symbiotrophic and combined N nutrition can be by application of nutritional symbionts ad is the reason for maximal productivity of the majority of crops is reached using an optimal (species- and genotype-specific) combination of both nutritional types because of which a high sustainability of legume production may be achieved (Provorov et al. 1998).

Removal of N-compounds from the actively N2-fixing symbioses, can improve the balance between symbiotrophic and combined N nutrition as has been suggested for tropical forest ecosystems (Hedin et al. 2009). However, different approaches for improving the nutritional and defensive types of microbial mutualists need to be developed. Remodelling plant developmental or defensive functions may represent a promising field for agricultural biotechnology by application of microbial symbiotic signals or their derivatives. Future development of agricultural microbiology may involve the construction of novel multipartite endo- and ecto-symbiotic communities based on extended genetic and molecular (metagenomic) analyses which needs to create composite inoculants, which simulate the natural plant-associated microbial communities. A combination of N- and P-providing symbionts would appear promising, including the endosymbiotic rhizobia + VAM-fungi (Shtark et al. 2010), for balancing the host plant metabolism. The further development of agricultural microbiology faces several important ecological and genetic challenges imposed by the broad application of symbiotic microbes which include regular human pathogens, which are frequently found in endophytic communities, including *Bacillus, Burkholderia, Enterobacter, Escherichia, Klebsiella, Salmonella and Staphylococcus* species (Ryan et al. 2008). An increased knowledge of microbe-based symbioses in plants could provide effective ways of developing sustainable agriculture in order to ensure human and animal food production with a minimal disturbance of the environment. The effective management of symbiotic microbial communities is possible using molecular approaches which will addressing the ecological and genetic consequences of the broad application of microbes in agricultural practice.

Plant growth mediated by PGPR occurs by the alteration of the whole microbial community in rhizosphere niche through the production of various substances (Kloepper and Schroth 1981a, b). Plant growth is promoted by PGPR directly by either facilitating resource acquisition like nitrogen, phosphorus and essential minerals via biological nitrogen fixation, phosphate solubilization and iron sequestration by siderophore respectively or modulating plant hormone levels such as auxins, gibberellins (GAs), cytokinins (CK), and nitric oxide (NO), or indirectly plant growth promotion such as rhizosphere competition, induced systemic resistance (ISR), biosynthesis of stress-related phytohormones like jasmonic acid (JA), cadaverine (Cad), or the ethylene catabolism-related enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase. Production of exopolysaccharides reduce Na+ uptake in the plant by binding it is the possible reasons production of exopolysaccharides.

3.5.1 Direct Mechanisms

Plant growth is enhanced by plant-growth-promoting rhizobacteria in the absence of pathogens. Plethora of mechanisms are responsible to stimulate plant growth with the help of various soil bacterial species burgeoning in plant rhizosphere. The rooting patterns are affected by the nutrient uptake of microbial activity and the supply of available nutrients to plants.

3.5.2 Nutrient Acquisition

Microorganisms metabolize carbon and nitrogen,microbe oriented molecules are taken up by plants for growth and development (Kang et al. 2015).

3.5.2.1 Nitrogen Fixation

Conversion of nitrogen to ammonia by nitrogen fixing microorganisms using a complex enzyme system known as nitrogenase is called Biological N2 fixation (BNF). BNF accounts for two-thirds of the nitrogen fixed globally. Nitrogen fixing organisms are generally categorized as (a) symbiotic N2 fixing bacteria including members of the family rhizobiaceae which forms symbiosis with leguminous plants (e.g. rhizobia) (Zahran 2001) and nonleguminous trees (Frankia) and (b) non-symbiotic (free living, associative and endophytes) nitrogen fixing forms. Uch as cyanobacteria (Anabaena, Nostoc), Azospirillum, Azotobacter, Gluconoacetobacter diazotrophicus and Azocarus etc. (Bhattacharyya and Jha 2012). However, non-symbiotic nitrogen fixing bacteria provide only a small amount of the fixed nitrogen that the bacterially associated host plant requires (Glick 2012a, b). Fixing of N2 by Plant growth-promoting rhizobacteria in non-leguminous plants are also called as diazotrophs capable of forming a nonobligate interaction with the host plants (Glick et al. 1999, 2007). The activity of the molybdenum nitrogenase is the basic reason for biological fixation and this activity is found in all diazotrophs (Bishop and Jorerger 1990). A number of free-living bacteria, for example Azospirillum spp., are also able to fix nitrogen and provide it to plants. Structural genes, genes involved in activation of the Fe protein, iron molybdenum cofactor biosynthesis, electron donation, and regulatory genes required for the synthesis and function of the enzyme are necessary nitrogen fixation. A cluster of around 20–24 kb with seven operons encoding 20 different proteins are typically found in nif genes which are found in diazotrophic (nitrogen fixing) bacteria.

3.5.2.2 Phosphate Solubilization

The second important plant growth-limiting nutrient after nitrogen is Phosphorus (P) and is abundantly available in soils in both organic and inorganic forms. As the majority of soil P is found in insoluble forms while the plants absorb it only in two soluble forms, the monobasic (H2PO4) and the dibasic (H2PO4) ions (Bhattacharyya

and Jha 2012 that is the reason of low availability of phosphorous to plants. But regular application of phosphate fertilizers is not only costly but is also environmentally undesirable (Kaur and Reddy 2014). This has led to search for an ecologically safe and economically reasonable option for improving crop production in low P soils. Organisms coupled with phosphate solubilizing activity, often termed as phosphate solubilizing microorganisms (PSM), may provide the available forms of P to the plants and hence a viable substitute to chemical phosphatic fertilizers (Khan et al. 2006). Bacterial genera like Azotobacter, Bacillus, Beijerinckia, Burkholderia, Enterobacter, Erwinia, Flavobacterium, Microbacterium, Pseudomonas, Rhizobium and Serratia are reported as the most significant phosphate solubilizing bacteria (Bhattacharyya and Jha 2012).

The growth and yield of crop plants can be enhanced by Rhizobacteria which can solubilise inorganic P sources. Besides, examples of some widely reported P solubilising microbial species intimately associated with a large number of agricultural crops like potato, tomato, wheat, radish, pulses etc., are Azotobacter chroococcum (Kumar et al. 2001), Bacillus circulans and Cladosporium herbarum (Singh and Kapoor 1999), Bradyrhizobium japonicum (Antoun et al. 1998), Enterobacter agglomerans (Kim et al. 1998), Pseudomonas chlororaphis and P. putida (Cattelan et al. 1999) and Rhizobium leguminosarum (Chabot et al. 1998). The ability of PGPRs to solubilize mineral phosphate, therefore, has been of immense interest to agricultural microbiologists since it can enhance the availability of phosphorus for effective plant growth. PGPRs have been recorded to solubilize precipitated phosphates to plants, representing a possible mechanism of plant growth promotion under field conditions. Synthesis of organic acids by rhizosphere microorganisms could be the possible reason for solubilization of inorganic P sources. The commercial application of phosphate-solubilizing PGPB has been quite limited because of variable results. Positive effects of applying phosphate-solubilizing bacteria are seen when these bacteria are co-inoculated with bacteria with other physiological capabilities.

3.5.2.3 Siderophore Production

Siderophores are low molecular weight secondary metabolites produced by microbes under iron deficiency, to supply iron to the organism. In the rhizosphere crops associated with siderophore-producing microbes may obtain iron through microbially-produced siderophores. There are different classes of siderophores such as hydroxamate, catecholate and mixed ligand siderophores. Under laboratory conditions, these siderophores can be produced in liquid and/or solid media. Subsequently, they can be detected and identified by different methods. Iron (Fe) is the fourth most abundant element in the earth's crust. In biology, Fe is involved in several important processes that may be enzymatic (as a cofactor to several enzymes) or non-enzymatic in nature. Examples of nonenzymatic interactions of Fe and soil-plant biosystems include those involving leghemoglobin, ferritin, and siderophore. In response, soil microbes and certain groups of plants - so-called strategy II plants - secrete into the rhizosphere environment a variety of siderophores that perform an Fe scavenging role. Upon secretion, siderophores interact with the ferric Fe; the Fe

is then transported into the cell, where it is reduced to the biologically relevant ferrous (Fe2+) form. There are over 500 biomolecules that are classified as siderophores; hence, different multiple genes and regulators are involved in their biosynthesis, transport, and re-import into the cell. Considering this wide diversity of siderophores, in-depth discussions of the genetics of their production and the biochemistry of their different structures are beyond the scope of this study. An NRPS-dependent mechanism implies that mRNAs are not involved in the biosynthesis process. Still, other siderophores, including many of the hydroxamate-type ones, are synthesized based on mechanisms that are NRPS-independent. Structurally, siderophores can be classified as hydroxamate, catecholate, or mixed hydroxycarboxylic ligand groups. Other siderophores, such as the mixed ligand pyoverdines are fluorescent compounds with variable molecular mass produced mainly by the ubiquitous soil bacteria of the genus Pseudomonas. In addition to the typical repeating dicarboxy-diamide peptide backbone, pyoverdines contain dihydroxyquinoline choromophores that differentiate them from other siderophores and permit fluorescence-based detection and measurement (Dimkpa et al. 2009a). Whereas the need for siderophore production is based primarily on the Fe status of the environment, the process is also driven by complex ecological interactions that involve pH, other environmental factors such as metal contaminants, and their interplay with Fe. Although the bioavailability of Fe is strongly tied to pH, siderophore production by different microbes appears to controvert the pH-dependency of Fe deficiency. For example, acid soils are rich in hydroxamate siderophores produced mainly by fungi and Streptomyces, and reflects in the optimal stability of their ferric complexes at low pH. In contrast, neutral to alkaline soils support the production of both hydroxamate and catecholate siderophores. In *Bacillus*, siderophore-indicative halo production on agar plates was greater between pHs 7.0 to 9.0 than at lower (5.0) and higher (11.0) pH).

3.5.2.4 Phytohormone Production

Production of different phytohormones like IAA, gibberellic acid and cytokinins by PGPR can alter root architecture and promote plant development. Reports of producing IAA by Several PGPRs as well as some pathogenic, symbiotic and free living rhizobacterial in the rhizospheric soil and thereby plays a significant role in increasing the root surface area and number of root tips in many plants (Han et al. 2005). Similarly significant shoot growths in maize and rice dwarf mutants were promoted by gibberellins-like substances excreted by Azospirillum spp. (Boiero et al. 2007). Microbial synthesis of the phytohormone auxin (indole-3-acetic acid/indole acetic acid/IAA) has been known for a long time. It is reported that 80% of microorganisms isolated from the rhizosphere of various crops possess the ability to synthesize and release auxins as secondary metabolites. Generally, IAA secreted by rhizobacteria interferes with the many plant developmental processes because the endogenous pool of plant IAA may be altered by the acquisition of IAA that has been secreted by soil bacteria (Spaepen et al. 2007). Evidently, IAA also acts as a reciprocal signaling molecule affecting gene expression in several microorganisms. Consequently, IAA affects plant cell division, extension, and differentiation;

stimulates seed and tuber germination; increases the rate of xylem and root development; controls processes of vegetative growth; initiates lateral and adventitious root formation; mediates responses to light, gravity and florescence; affects photosynthesis, pigment formation, biosynthesis of various metabolites, and resistance to stressful conditions. Bacterial IAA provides the plant greater access to soil nutrients by increasing root surface area and length. These also result in facilitates an increasing amount of root exudation that provides additional nutrients to support the growth of rhizosphere bacteria (Glick 2012a, b) by loosening the plant cell walls. Thus rhizobacterial IAA are called as an effector molecule in plant– microbe interactions, both in pathogenesis and phytostimulation (Spaepen and Vanderleyden 2011a, b). The main precursor for the IAA is an important molecule that alters the level of IAA synthesis is the amino acid tryptophan but for tryptophan anthranilate, a precursor for tryptophan reduces IAA synthesis.

By this mechanism, IAA biosynthesis is fine-tuned because tryptophan inhibits anthranilate formation by a negative feedback regulation on the anthranilate synthase, resulting in an indirect induction of IAA production (Spaepen et al. 2007). However, supplementation of culture media with tryptophan increases the IAA production by most of the rhizobacteria (Spaepen and Vanderleyden 2011a, b). Biosynthesis of tryptophan starts from the metabolic node chorismate in a five-step reaction encoded by the trp genes. Starting with tryptophan, at least five different pathways have been described for the synthesis of IAA, and most pathways show similarity to those described in plants, although some intermediates can differ (Spaepen and Vanderleyden 2011a, b): (1) IAA formation via indole-3-pyruvic acid and indole-3-acetic aldehyde is found in a majority of bacteria like, Erwinia herbicola; saprophytic species of the genera Agrobacterium and Pseudomonas; certain representatives of Bradyrhizobium, Rhizobium, Azospirillum, Klebsiella, and Enterobacter (2) The conversion of tryptophan into indole- 3-acetic aldehyde may involve an alternative pathway in which tryptamine is formed as in pseudomonads and azospirilla and (3) IAA biosynthesis via indole-3-acetamide formation is reported for phytopathogenic bacteria *Agrobacterium tumefaciens, Pseudomonas syringae, and E. herbicola; saprophytic pseudomonads* like (*Pseudomonas putida and P. fluorescens*). (4) IAA biosynthesis that involves tryptophan conversion into indole-3-acetonitrile is found in the cyanobacterium (Synechocystis sp.) and (5) the tryptophan-independent pathway, more common in plants, is also found in azospirilla and cyanobacteria. Most Rhizobium species have been shown to produce IAA (Ahemad and Khan 2012; Ahemad and Khan 2011). Multiple processes including cell division, differentiation and vascular bundle formation, IAA is involved and these three processes are also essential for nodule formation. Hence, it seems likely that auxin levels in the host legume plants are necessary for nodule formation (Spaepen et al. 2007). Producing potential nitrogen fixing root nodules containing up to 60-fold more IAA than nodules formed by the wild-type counterpart in *Vicia hirsute* (Camerini et al. 2008) has been resulted because of introducing inoculation with Rhizobium leguminosarum bv. viciae. Environmental stress factors include acidic pH, osmotic and matrix stress, and carbon limitation (Spaepen et al. 2007) which modulate the IAA biosynthesis in different bacteria, Among genetic factors,

both the location of auxin biosynthesis genes in the bacterial genome (either plasmid or chromosomal) and the mode of expression (constitutive vs. induced) have been shown to affect the level of IAA production. The location of auxin biosynthesis genes can affect the IAA level, as plasmids are mostly present in multiple copies. This can be illustrated by the difference in the IAA level between the rhizobacterial strains, Pseudomonas savastanoi pv. savastanoi and P. syringae pv. syringae. In the former strain, the genes for auxin biosynthesis genes are present on a plasmid, while in the latter one the corresponding genes are located on the chromosomal DNA, resulting in a lower IAA production. Involvement of PGPR formulated cytokinins was also observed in root initiation, cell division, cell enlargement and increase in root surface area of crop plants through enhanced formation of lateral and adventitious roots (Werner 2005). It has been established that the working pathways of these phytostimulators leading to overall development in crop plants are differently regulated by catabolite repression (Zaidi et al. 2009) as physiological regulator of biofilm formation. Generally, ethylene is an essential metabolite for the normal growth and development of plants (Khalid et al. 2006).

3.5.2.5 ACC Deaminase Activity

Enzyme, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, facilitate plant growth and development by decreasing ethylene levels, inducing salt tolerance and reducing drought stress in plants (Zahir et al. 2008) are possessed by Plant growth promoting rhizobacteria. ACC deaminase activity have been identified in a wide range of genera such as Acinetobacter, Achromobacter, Agrobacterium, Alcaligenes, Azospirillum, Bacillus, Burkholderia, Enterobacter, Pseudomonas, Ralstonia, Serratia and Rhizobium etc. (Kang et al. 2015). These bacteria take up the ethylene precursor ACC and convert it into 2-oxobutanoate and NH3 (Arshad et al. 2008). The stress relieved by ACC deaminase producers include effects of phytopathogenic microorganisms (viruses, bacteria, and fungi etc.), and resistance to stress from polyaromatic hydrocarbons, heavy metals, radiation, wounding, insect predation, high salt concentration, draft, extremes of temperature, high light intensity, and flooding (Glick 2012a, b; Lugtenberg and Kamilova 2009a, b). The plant root elongation, promotion of shoot growth, and enhancement in rhizobial nodulation and N, P and K uptake as well as mycorrhizal colonization in various crops are the major noticeable effects of seed/root inoculation with ACC deaminase-producing rhizobacteria.

3.5.3 In-Direct Mechanisms

The application of microorganisms to control diseases, which is a form of biological control, is an environment-friendly approach (Lugtenberg and Kamilova 2009a, b). The major indirect mechanism of plant growth promotion in rhizobacteria is through acting as biocontrol agents (Glick 2012a, b). In general, competition for nutrients, niche exclusion, induced systemic resistance and antifungal metabolites production is the chief modes of biocontrol activity in PGPR (Lugtenberg and Kamilova 2009a, b).

Many rhizobacteria have been reported to produce antifungal metabolites like, HCN, phenazines, pyrrolnitrin, 2,4-diacetylphloroglucinol, pyoluteorin, viscosinamide and tensin (Bhattacharyya and Jha 2012).

3.5.3.1 Competition for Nutrient and Niches

Offensive PGPB colonization and defensive retention of rhizosphere niches are enabled by production of bacterial allelochemicals, including iron-chelating siderophores, antibiotics, biocidal volatiles, lytic enzymes, and detoxification enzymes. Iron is an essential growth element for all living organisms. The scarcity of bioavailable iron in soil habitats and on plant surfaces foments a furious competition (Loper et al. 2007). Under iron-limiting conditions, PGPB produce low molecular-weight compounds called siderophores to competitively acquire ferric ion (Whipps 2001). Although various bacterial siderophores differ in their abilities to sequester iron, in general, they deprive pathogenic fungi of this essential element since the fungal siderophores have lower affinity. Some PGPB strains go one step further and draw iron from heterologous siderophores produced by cohabiting microorganisms (Loper and Henkels 1999). Siderophore biosynthesis is generally tightly regulated by iron-sensitive Fur proteins, the global regulators GacS and GacA, the sigma factors RpoS, PvdS, and FpvI, quorum-sensing autoinducers such as N-acyl homoserine lactone, and site-specific recombinases (Ravel and Cornelis 2003). However, some data demonstrate that none of these global regulators is involved in siderophore production. Neither GacS nor RpoS significantly affected the level of siderophores synthesized by Enterobacter cloacae CAL2 and UW4 (Saleh and Glick 2001). RpoS is not involved in the regulation of siderophore production by Pseudomonas putida strain WCS358. In addition, GrrA/GrrS, but not GacS/GacA, are involved in siderophore synthesis regulation in Serratia plymuthica strain IC1270, suggesting that gene evolution occurred in the siderophore-producing bacteria (Ovadis et al. 2004.). A myriad of environmental factors can also modulate siderophores synthesis, including pH, the level of iron and the form of iron ions, the presence of other trace elements, and an adequate supply of carbon, nitrogen, and phosphorus (Compant et al. 2005).

3.5.3.2 Induced Systemic Resistance (ISR)

The use of induced systemic resistance and systemic acquired resistance as a strategy for pest management is suitable and marketable to the producers. The coevolution flanked by plants and potential microbial pathogens has been depicted as a zigzag model (Jones and Dang 2006). According to the zigzag model, the primary inducible responses are a result of the perception of chemical elicitors, microbe-associated molecular patterns (MAMPs), pathogen-associated molecular patterns (PAMPs) and/or damage-associated molecular patterns (DAMPs). MAMPs explain broad microbe-derived molecules in conjunction with those instigating from PGPRs while as PAMPs exclusively illustrate molecules from pathogenic microbes for example fungi, oomycetes, and bacteria (Newman et al. 2013). As a consequence,

PAMPs are a subgroup of MAMPs (Maffei et al. 2012). These molecules could collectively be described as "patterns that elicit immunity" (PEIs), are frequently prominent by transmembrane pattern recognition receptors (PRRs) in plant cells (Maffei et al. 2012; Newman et al. 2013). Upon identification of MAMP- or DAMP-derived patterns, PTI (PAMP- or pattern-triggered immunity) is activated in the plant and the alleged molecules could be illustrated as immune elicitors. This defense reaction intends to confine the growth of the intruder and can advance towards systemic induced resistance that renders plant relatively tolerant to subsequent pathogen attack (Henry et al. 2012).

Systemic induced resistance is further divided into systemic acquired resistance (SAR) or induced systemic resistance (ISR). Systemic acquired resistance is generally differentiated by localized necrosis, expression of pathogenesis related (PR) protein genes, and entails the salicylic acid (SA) pathway while as ISR is normally activated by plant growth-promoting rhizobacteria (PGPR) (Walters et al. 2013), is not connected with necrosis and encompasses the jasmonic acid (JA) and ethylene (ET) pathways (Henry et al. 2012). Typical responses of PTI comprise cell wall modifications and the assembly of reactive oxygen species (ROS) which can be persistently cytotoxic and are imperative in signaling. Further, responses comprise the generation of phytoalexins, expression of PR proteins, activation of mitogen activated protein kinase (MAPK) pathways, and defense signaling linking calcium (Ca^{2+}) influx from extracellular spaces and variations in free cytosolic Ca^{2+} concentrations (Garcion et al. 2007). To counteract the preliminary plant defense reaction, thriving microbes have developed specific effectors that stimulate identification of defense elicitors or subsequent plant defense mechanisms to promote effector-triggered susceptibility (ETS). Conversely, if these pathogen effectors are in turn identified by cognate plant resistance (R) proteins, the subsequent line of inducible response, effector-triggered immunity (ETI), is instigated that frequently capitulates a hypersensitive resistance response (HR) (Deslandes and Rivas 2012). The product of plant/microbe associations can result in symbiosis, disease resistance and is directed by extra levels of complicated co-evolution. Certainly, it must be identified that pathogen colonization of plants can produce dynamic pathogenic, mutualistic or parasitic interactions of anecdotal intensity and specificity. It is thus indispensable for the plant to assess the range of risk and to escalate suitable and impartial responses. These may vary from priming, being prepared to counteract sooner to definite attack, or expression of PTI-based defense mechanisms to yield incompatibility if the microbe/pathogen is incapable to repress these responses. Numerous research reports have confirmed that few of the inconsistency adept with ISR defense via inoculation with Plant Growth Promoting Rhizobacteria (PGPR) can be overcome by using multiple strains (Pii et al. 2015). Consequently, it is suggested that multiple strains be assorted in concert if at all feasible when endeavoring to stimulate ISR via PGPR inoculation.

3.6 Conclusion and Future Perspective

Agriculture and soil have been carrying the burden of sustaining mankind on planet earth. Indiscriminate exploitation of resources has limited the productive and humans are looking for alternative sources for fulfillment of their livelihood needs. Sustainable agriculture has become a norm in modern agriculture. As environmental and ecological issues continue to impact agriculture, all technologies developed for crop production must be economically feasible, ecologically sound, environmentally safe, and socially acceptable. Numerous non-chemical methods for the control of crop diseases, such as pathogen-free seeds, disease-resistant varieties, crop rotation, application of plant extracts, organic amendments, and biological control are considered less harmful than synthetic chemical pesticides and, therefore, offer great potential for application in conventional agriculture, organic farming, and/or soilless culture. No single method can provide satisfactory control of crop diseases. Integration of all effective and eco-friendly measures in accordance with the dynamics of agro-ecosystem management would be the best strategy for the efficient control of diseases in crops. In this era of energy conservation and environmental protection, research on energy-saving and environmentally sound methods for the sustainable management of crop diseases is both a priority and a challenge. Application of modern tools and techniques for enhancement of PGPR can serve as key in sustainable agriculture by improving soil fertility, plant tolerance, crop productivity, and maintaining a balanced nutrient cycling. Further studies on selecting suitable rhizosphere microbes and producing microbial communities along with exploring multidisciplinary research that combines applications in biotechnology, nanotechnology, agro biotechnology, chemical engineering and material science and bringing together different ecological and functional biological approaches can provide new formulations and opportunities with immense potential.

The expansion of new nanodevices (biosensors, enzyme encapsulation) and nanomaterials (nanotubes, nanowires, fullerene derivatives and quantum dots) with the surfacing of nanotechnology announces probable narrative application in the field of agriculture and life sciences (Dixit et al. 2015). Nanoparticles in plant pathology targets specific agricultural problems in plant-pathogen interactions and provide new ways for crop protection. This includes early detection of biotic stresses and their management, enhancing input use efficiencies and self-life of perishables (fruits, flowers and vegetables etc.). Development of superior or novel PGPR strains by improving above traits can be possible using genetic manipulations. These PGPR-biotechnologies can be exploited as a low-input, sustainable and environment- friendly technology for the management of plant stresses.

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Plant Genetic Engineering and GM Crops: Merits and Demerits

4

Abstract

Genetic transformation in plants agreements a great potential to modify crops for improved agronomic traits including resistance to diseases, pests and good nutritional quality along with enhanced productivity. The transgene could be derived from unrelated plant species and even from non-plant sources leading to a revolution in molecular agriculture. In this chapter, the main approach lies on concept of genetic engineering techniques to improve the plant architect. The concept of GM crops and environmental implications besides their safety assessment is documented in detail and also in the end future perspective for adopting the next generation quantitative genetics is also elaborated.

Keywords

GM crops · Genetic Engineering · Bt crop · Safety assessment · Transformation

4.1 Concept of Plant Genetic Engineering

It may seem like a trivial task today to introduce genes into plants to create new commercially useful varieties. However, in the early 1980s, this was one of the major bottlenecks preventing the completion of an agricultural revolution that began after the discovery and use of restrictive enzymes, followed quickly by the genetic engineering of bacteria for medical and industrial purposes. Since its inception, plant biotechnology has been technologically driven, and the successful establishment of gene transfer technologies for major crops (McCabe et al. 1988; Christou et al. 1991) was a major breakthrough for small biotechnology companies, which led the field in the early 1980s. When it was shown that the soil bacterium Agrobacterium tumefaciens transfers part of the DNA from a resident plasmid to the plant genome, the first model of transgenic plants did not take long (Barton et al. 1983). The first key plant transformation patents on A. tumefaciens and biolistics

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defined the industry and precipitated its transformation and consolidation. While early activities in the field were dominated by start-ups in the US, such as Cetus Madison (Agracetus), Agrigenetics, Calgenetics, Advanced Genetic Systems, Molecular Genetics, and others, as well as Plant Genetic Systems in Belgium and a number of larger, more established agrochemical companies such as Monsanto, DuPont, Lilly, Zeneca, Sandoz, Pioneer, Bayer, and others, the field is now dominated. Insect resistance based on Bacillus thuringiensis (Bt) genes and herbicide tolerance were the first two features to be successfully commercialized. A good example of the broader landscape is the consolidation and turmoil in the Bt industry (Sanahuja et al. 2011).

4.1.1 Evolution of the Commercial Landscape for *Bacillus thuringiensis* (Bt) Crops

The five major companies currently selling Bt seeds have emerged through a series of mergers, acquisitions and spin-offs/demergers as larger companies segregate their agribusiness interests. In its current incarnation, Monsanto Co. was an agribusiness spin-off from Pharmacia in 2002 following the merger with Pharmacia and Upjohn in 2000 of the original Monsanto Co. (established in 1901). In late 2000, Pharmacia established the new Monsanto as an agribusiness subsidiary and in 2002 it became an independent company. Bayer CropScience is an agribusiness subsidiary of Bayer AG, which was formed after Aventis CropScience acquired in 2000. Syngenta was formed in 2000 from the merger of Novartis and AstraZeneca, both of which were spin-offs from agribusiness in previous mergers. Dow AgroSciences is a wholly-owned subsidiary of Dow Chemical Co., which was formed when Dow Chemical Co. purchased Eli Lilly's stake in Dow Elanco (an agribusiness spin-off formed in 1989 by Dow Chemical Co. and Ely Lilly & Co.). Pioneer Hi-Bred International is now DuPont's agribusiness subsidiary, which acquired 20% of the company in 1997 and the remaining 80% in 1999. (Sanahuja et al. 2011) It is interesting that commercial products were first developed and the science behind them came later. It is therefore not surprising that the two original features remain today's most dominant commercial features. Efficiency has improved and the characteristics have been stacked in individual varieties, but the technology remains the same in principle. The academic community's decision to focus on the Arabidopsis thaliana model plant paid beautiful dividends in basic science. In conjunction with advances in DNA sequencing, the genomics field has grown old and it is now considered routine to undertake major sequencing projects for various plant species. Access to major crop gene sequences can now be combined with high-performance transcriptome and proteome analysis, leading to unprecedented advances in gene discovery and functional annotation. Metabolomics and biology of systems now take center stage and generate large amounts of data to create models for the entire plant system. Advances in bioinformatics allow these large data sets to be stored, handled, mined and manipulated, leading to further progress in our understanding of fundamental and more complex plant processes. The impact of this rich stream of

previously untapped data is that targets, such as modulation, were previously considered unattractable such as the modulation of photosynthesis and the ability of plants to fix nitrogen, are now within our reach as shown by the recent substantial investments of time and resources into these areas. Multigene engineering has also helped to develop more complex crops, including extended metabolic pathways that produce valuable compounds such as b-carotene for golden rice (Ye et al. 2000) and three different vitamins for multivitamin corn (Naqvi et al. 2009). The increasingly antagonistic effect of over-zealous regulation was one surprising development that was not planned in the early days of plant biotechnology. A robust regulatory system is required for new technologies, but it should be based on rational principles and evidence rather than political expediency (Farre et al. 2011). The current regulatory environment for genetically modified crops, especially in Europe, is hostile, irrational and inconsistent with the overall effect of seriously impeding scientific progress. The early pioneers of plant genetic engineering foresaw the technology's potential and its ability to increase yields and address our most challenging social problems, such as poverty and food insecurity. While the technology has progressed steadily, the positive impact it could have throughout the world is unnecessarily wasted. My fervent hope is that the change in this situation will take another 30 years (Ramessar et al. 2010).

4.2 Plant Genetic Transformation

Genetic transformation in plants offers a great potential to modify crops for better agronomic characteristics, including disease resistance, pests and good nutritional quality, as well as increased productivity (Vain 2007). Transgenes could be derived from unrelated plant species and even from non-plant sources leading to molecular agriculture revolution. The identification of a wider range of potentially important genes for crop improvement, which are also tailored or redesigned to further improve their properties in specific crops, has subsequently intensified the development of efficient technologies for plant transformation. The combined effort of genetic engineering and conventional breeding programs has enabled the introduction of useful features into commercial crops within an economically viable time frame. In the non-agricultural sector, which includes an alternative source of medicinally important recombinant proteins and vaccines, transgenic plants are more widely used (Fischer et al. 2004). Experiments on genetic transformation of plants began shortly after the discovery of DNA as a transforming genetic material in bacteria (Avery Oswald et al. 1944). However, the development of Agrobacterium-mediated genetic transformation in plants has achieved a successful genetic transformation with reproducibility (Chilton et al. 1977). The limited success of the transformation achieved by Agrobacterium in monocotyledons and other recalcitrant plant species resulted in the discovery of direct DNA delivery methods, including the most commonly used Sanford (1990) method of particle bombardment. Genetic transformation methods can be categorized as indirect and direct DNA delivery systems in general. The indirect method involves the introduction of genes

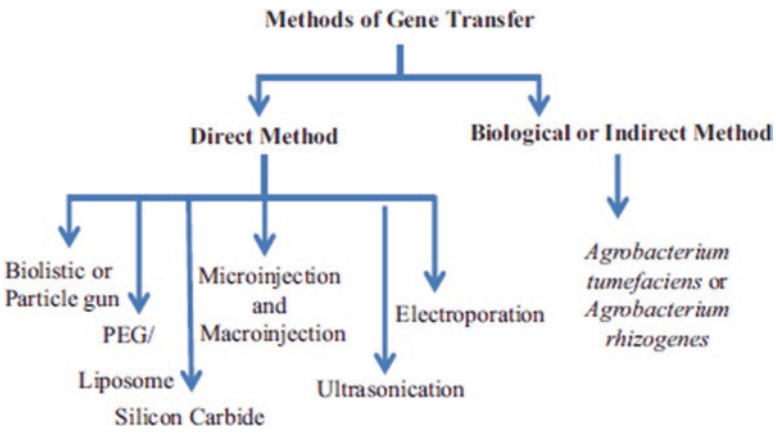

Fig. 4.1 Different methods of genetic transformation

of interest into the target cell by Agrobacterium tumefaciens or Agrobacterium rhizogenes, while no such bacterial cells are used to transfer DNA directly to the target cells (Fig. 4.1).

4.2.1 Methods of Genetic Transformation

4.2.1.1 Indirect/Biological/Agrobacterium- Mediated Genetic Transformation

This method uses the genus Agrobacterium's natural ability to transform plant cells. A. Tumefaciens is a gram-negative bacterium transmitted by oil that causes "crown gall disease," whereas it is called A. The causative agent of hairy root disease is rhizogenes. Other species are A. Rubi causing cane gall disease, A.vitis causing grape galls and avirulent species A. Radio (Otten et al. 1984). The plasmids of A inducing tumor (Ti). Tumefaciens or A plasmids that induce root (Ri). Rhizogenes are pathogenic megaplasmids. These megaplasmids have "T-DNA," also known as "transferred DNA," a region consisting of an oncogenic region, the causative agent. Oncogene is responsible for the production of enzymes involved in the synthesis of auxins and cytokinin, which leads to the development of tumours. The host range of different Agrobacterium strains is the most important factor in the Agrobacterium-mediated transformation. It has been reported that agrobacterium transfers DNA to a relatively large group of organisms, including different dicot and monocot plants (Anderson and Moore 1979) and gymnosperms (McAfee et al. 1993). The transfer of DNA to fungi mediated by Agrobacterium, including yeasts (Bundock and Hooykaas 1996), ascomycetes (Abuodeh et al. 2000) are reported and in recent times, Agrobacterium was reported to transform human cells (Kunik et al. 2001).

4.2.2 Structure of Ti Plasmid and Ri Plasmid

The size of Ti plasmids is between 200 and 800 kbp (Wood et al. 2001). Most plasmids in Ti have four common regions, in other words. (B) gene cluster required for DNA replication, (c) gene cluster required for conjugation and (d) "vir" region also known as "virulence" r (a) T-DNA region, which is so-called due to the transfer and integration of its homologous counterpart into the nuclear genome of host plant cells (this region has the potential to alter the morphology of the host plant by inducing galls (shooty or rooty mutant galls) (B) the gene cluster required for DNA replication, (c) the genecluster required for conjugation and (d) the "vir" region, also known as the "virulence" region, which consists of a gene cluster solely responsible for the encoding of a set of proteins involved in the excision, transfer and finally integration of T-DNA into the nuclear genome of host plant cells. Any mutation in this area leads to a loss of virulence. The components of these natural plasmids were therefore used as a basis for the development of vectors with a high efficiency of plant transformation. The T-region varies from 10 to 30 kbp and generally accounts for less than 10% of the Ti plasmid (Zambryski et al. 1980). Some Ti plasmids consist of only one T-region, while other plasmids have had several T-regions (Suzuki et al. 2000). The T-DNA is divided into "oncogenic" or "onc" and "os" regions and is bordered by a repeat of 25 bp on both sides. The oncogenic region consists of three genes, in other words. Tms1, tms2 and tmr represent' shooty loci with tms1 and tms2 representing' rooty locus.' These oncogenes are primarily responsible for the encoding of two phytohormone biosynthesis enzymes, i.e. Auxin (acetic acid indole) and a cytokinin (isopentyladenosine 5′-monophosphate). The inclusion of oncogenes in the host plant's nuclear genome stimulates the synthesis of phytohormones in the host plant. Phytohormones induced uncontrollable growth of host plant cells, leading to the development of tumors of the crown gall. The 'os' region consists of genes that encode enzymes necessary for the synthesis of specialized chemicals called opines metabolized by the bacteria. Opines are amino acid and sugar derivatives and provide the bacteria with carbon and energy. Ti plasmids are named after the type of opines encoded by their genes, such as oc topine, nopaline, succinamopine and leucinopine. The two most frequently produced opines are octopine and nopaline. The T-DNA contains genes for enzymes octopine synthase and nopaline synthase, which are required for the production of corresponding opines, octopine and nopaline. In addition, the T-DNA region is bordered by 25 base pairs of left (LB) and right border (RB), arranged in a directly repeated orientation (Veluthambi et al. 1988). These bordered sequences serve as a signal for the successful transmission of T-DNA to host plants (Zupan et al. 2000). The presence of polarity between the borders of T-DNA has been observed, as right borders are more important than left borders (Sen et al. 1989). Many right borders of T-DNA have shown the presence of sequences of T-DNA 'overdrive' near them, while such sequences are absent from left borders. The function of enhanced T-strand transmission to plants was attributed to overdrive sequences, but the molecular mechanism of this process is not clear (Hansen et al. 1992). However, it has been suggested that

the protein of Vir C1 binds to the overdrive sequence and may improve the cleavage of T-DNA by endonuclease VirD1/D2 (Toro et al. 1989). The virulence region of pTi is external to the T-DNA region, and genes (called "vir" genes) are grouped into ABCDEFGH operons. These operons are responsible for encoding enzymes responsible for carrying out conjugative transfer of T-DNA to host plant cellular genome. Other operons that facilitate the transfer of T-DNA are the chromosomes containing chv genes (chvA, chvB, chvF). A's smid Ri pla (pRi). The pTi is functionally homologous with rhizogenes. The pRi-like pTi consists of the T-DNA region, the vir region that is primarily responsible for transformat ion (White et al. 1982). The structural analysis of Ri plasmids of the agropine type revealed the presence of two T-DNA regions separated from each other by non-transferred 15 Kb DNA. The RB sequences fl anchored to the T-DNA (T R) contain genes homologous to the T-D NA (Tms1 and Tms2) of pTi (Willmitzer et al. 1982). The loss of virulence in the T-R region of pRi results in y mutation (White et al. 1985). T R -DNA region has been reported to contain genes involved in agropine biosynthesis (ags), but the precise number of genes required for agropine production is not yet recognized (Huffman et al. 1984). The transcripts homologous to the Ri tms loci in A. rhizogenes mediated transformed tissues of *Nicotiana glauca* were of same size as that of tms region derived transcripts of pTi (Willmitzer et al. 1983). The agropine Ri plasmid A4b has shown to possess 20 Kb T L -DNA but is related to any other characterized Ti plasmid, unlike the T R - DN A (Huffman et al. 1984).

4.2.3 Biology of Tumour Formation by Agrobacterium

The colonization and establishment of the virulence system by bacteria include various steps involved in the genetic transformation mediated by Agrobacterium. The next step is the formation of a T-DNA transfer complex, which helps to transfer T-DNA into the nuclear genome of host plant tissues and subsequently incorporate it. The entire T-DNA transfer mechanism begins with the production of phenolic compounds as a result of plant wound, which leads to a cascade of sensory signal transduction. First, the signal is received by virA, which acts together with ChvE, a monosaccharide transporter that senses the presence of a particular phenolic compound, as a periplasm antenna (Doty et al. 1996). In addition to an autophosphorylating, VirA also transphosphorylates the VirG protein, which is activated by phosphorylation, leading to the increase in levels of transcription of other genes of viral protein machinery (Jin et al. 1990a, b). The proteins VirD1 and VirD2 then nick both LB and RB sequences at the bottom of the T-DNA. In conjunction with VirD2, another T-DNA strand is coated by VirE2, which results in the formation of a T-complex, which is actually transported to the host plant genome. Different workers have demonstrated the ability of VirE2 to transfer to the plant cell in the absence of a T-strand (Vergunst et al. 2000), and it may be possible that T-strand is compounded with VirE2 protein either in the bacterial export passage or in the host plant cell. This single-stranded DNA-binding Agrobacterium protein VirE2, which protects the T-DNA from degradation when transported to the plant cell, has been

assigned a protective function. The VirD4 protein and the 11 VirB proteins form a membrane channel to support the successful transport of the T-complex to the host plant cells in which the Vir protein is linked, i.e. VirD4, makes processed T-complex is facilitated through the combined effort of dynein-like Arabidopsis protein DLC3 and VirE2 interacting protein2 (VIP1) (Tzfira et al. 2002). Another recently discovered protein of Arabidopsis, VIP2 (VirE2 interacting protein2), was also reported to be involved in the successful incorporation of T-DNA into the host plant cell's nuclear genome (Anand et al. 2007). The T-DNA is integrated in the host genome at random positions by the non-homologous recombination process.

4.2.4 Vectors Based on Ti and Ri Plasmid

The integration of the gene of interest into the T-DNA region for its transfer into the host plant involved the tedious task of genetic recombination of the gene of interest into the T-DNA region (Zambryski et al. 1983). The Agrobacterium Ti/Ri plasmid wild type cannot be used as gene cloning vectors due to its large size, presence of oncogenes and lack of unique sites of endonuclease restrictions and marker sites in T-DNA. Other problems with these plasmids include difficulty in isolating them, a low number of copies in bacteria, recalcitrant in vitro manipulation and the inability to replicate genetically transformed host within the preferred host. i.e. E. coli. The difficulty in using wild-type Ti plasmid for genetic transformation was overcomed with the development of binary and cointegrates vectors.

4.2.5 Binary Vector

The binary vector was introduced when the virulence region and the T-DNA region of pTi could be divided into different replicates (de Framond et al. 1983). The transfer of T-DNA is mediated by Vir proteins encoded in the vir region located on separate replicates, but present in the same cell of Agrobacterium. The binary vector therefore has two components: The first component is a disarmed (lack of oncogenes) Ti plasmid consisting of T-DNA, the origin (s) of E replication. Tumefaciens coli and agrobacterium, as well as antibiotic-resistant genes used to select binary vector bacteria. The second component of the binary vector is the helper Ti plasmid, which contains the viral genes that mediate the transfer of T-DNA in the other replica. To facilitate genetic manipulation studies, a large number of more sophisticated T-DNA bi nary vectors and vir helper plasmids have been developed over the last 25 years. The examples of some commonly used T-DNA binary vector series include pBINPLUS (van Engelen et al. 1995), B IBAC (Hamilton 1997), pGreen (Hellens et al. 2000), pGD (Goodin et al. 2002), pS ITE (Chakrabarty et al. 2007), pMSP (Lee et al. 2007) and many more. Moreover, some of the frequently used disarmed Agrobacterium vir helper strains are C58-Z707 (Hepburn et al. 1985), AGL-1 (Lazo et al. 1991), EHA 105 (Hood et al. 1993), NT1 (pKPSF2) (Palanichelvam et al. 2000), etc.

4.2.6 Co-integrate Vectors

These vectors are also called hybrid Ti plasmid, in which the same vector contains both T-DNA and virulence regions. For the construction of co-integrated vectors, two component vectors are required, the disarmed pTi vector and the intermediate vector. The oncogenic region of T-DNA was exchanged with the gene of interest in the disarmed Agrobacterium pTi. The two examples of these vectors include: (a) SEV series in which the RB sequences and the oncogenic region of T-DNA have been replaced by the bacterial gene resistant to antibiotic kanamycin. The LB sequences and the adjacent minor part of the left segment (T L) of novel T-DNA called as left inside homology (LIH) are left intact. (b) pGV series in which a part of pBR322 vector is used to replace oncogenic region of pTi. The conserved regions of these vectors include LB and RB sequences as well as the nopaline synthase gene of the pTi.

4.2.7 Intermediate Vectors

This consists of E. Coli plasmids (small plasmids based on pBR322) with border sequences of T-DNA and vir area. Replicate the intermediate vectors in E. Coli, but cannot replicate ate in Agrobacterium and are transmitted by conjugation to Agrobacterium. They carry DNA segments with disarmed T-DNA homology. Therefore, both the intermediate and disarmed pTi plasmids have some common sequences (pBR322), which help to recombine and integrate two plasmids in a homologous way. The newly formed cointegrate vector therefore has both the disarmed T-DNA with the desired gene and virulence area, e.g. pGV2260.

4.2.8 Direct Delivery Methods

4.2.8.1 Biolistics or Microprojectiles or Gene Gun or Particle Bombardment Method

This method was developed to achieve success in the genetic transformation of monocots or other plants recalcitrant to Agrobacterium-mediated transformation, and the technique was used in a number of plant species to produce transgenic lines (Breitler et al. 2002). The use of this gene transfer method overcomes the limitations on transgenic size, cell type, species or genotype. Approximately 149 hits between 1987 and 1995, 500 hits between 1995 and 2002, 200 hits between 2002 and 2004, 945 hits between 2005 and 2010 and nearly 1225 hits between 2011 and 2015 were recorded on the basis of the literature database available on the Web of Science for citations of microprojectile method in plants. Sanford and coworkers developed the first particle delivery method using PDS-1000/He machine. In this technique gold or tungsten particles of about 0.6–1.0 mm diameter known as microcarriers are coated with the DNA of interest followed by their acceleration at an elevated speed so as to get integrated inside the target cell.

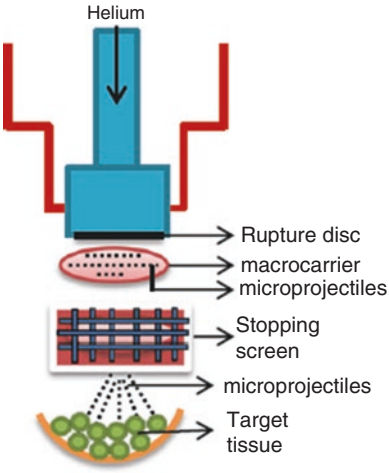

Fig. 4.2 Diagrammatic representation of particle bombardment method

These microcarriers are evenly dispersed on the macro-carrier consisting of circular plastic film, and the whole unit is then kept in the main vacuum chamber in the machine under the rupture disk. Underneath the macrocarrier is placed a wire mesh called a stop screen to retain the macrocarrier and allow the microcarrier to pass through it, as well as to place the target tissue underneath the entire system. The various types of rupture disks that burst at different pressures ranging from 450 to 2200 psi are now available. The microprojectile is fitted red under a partial vacuum and the gas acceleration tube is fitted with helium gas (He), which increases the necessary pressure. Macrocarriers are retained by the wire mesh during this process while microcarriers pass through it and hit the target cell at high speed. Microcarriers penetrate through the cell wall into the host cell and release DNA leading to the formation of transformed cells (Fig. 4.2). The advantages of this method include the absence of biological constraints, the ability to deliver DNA to different types of cells, the absence of vector requirements, the simultaneous transformation of multiple genes and the presence of high molecular weight DNA that can be delivered to the target cells. In addition, only particle bombardment technique has achieved mitochondrial transformation until now (Johnston et al. 1988). The transgenic rice lines having Xa21 gene which shows resistant against bacterial blight disease as well as Bt cry1Ab–cry1Ac fusion gene for lepidopteran insect resistance have been produced by gene gun method at IRRI. These resistant Bt lines have been tested in field in China (Tu et al. 2000a, b) as well as in India (Datta et al. 2002).

4.2.9 Electroporation -Mediated Genetic Transformation

In this method, the target cells and tissues are applied with a short electrical pulse of high field strength, which causes certain types of structural changes in the host cell membrane, thus increasing the permeability of the cell membrane. The most used

use of the electroporation technique is now the introduction of DNA in vitro into the target cells. This method was developed earlier to transform protoplasts, but this method could also transform intact plant cells. In this method, protoplasts or intact cells in an ionic solution containing vector DNA are suspended between the electrodes. In a specially designed electroporation chamber, which alters the permeability of the cell membrane allows the absorption of suspended vector DNA from its surrounding solution, 25 mV voltages and 0.5 mA current are generally applied for a period of about 15 min. The surface concentration of DNA and the tolerance of cells to membrane permeation affect the efficiency of the electroporation. However, higher transformation rates could be achieved with the electroporation method by adding polyethylene glycol after the addition of DNA, giving a heat shock to protoplasts at 45 °C for only 5 min before the addition of DNA and using linear DNA instead of a circular form. There is a lot of monocot and dicot plants have been successfully transformed with protoplasts. The first successful fertile transgenic plant of rice was developed by the use of embryogenic protoplasts through this method (Shimamoto et al. 1989). One of the limitations of this method of delivery is the use of protoplasts in most cases and the absence of regeneration of protoplasts in plantlets in most plant species. However, transformed intact plant cells and tissues could also be obtained using the same electroporation principles as those required for protoplasts, and the first transgenic plants were produced in barley in this regard (Salmenkallio-Marttila et al. 1995). The gene was also transferred to intact sugarcane meristem tissue by electroporation (Seema et al. 2001). The thick cell walls of intact tissues are generally the key barrier in the electroporation method. This method is cheap and easy as compared to gene gun method but has lower transformation efficiency with success only in a few plant species.

4.2.10 PEG/Liposome- Mediated Genetic Transformation

By using chemical compounds such as polyethylene glycol (PEG), direct DNA delivery to target protoplasts can also be stimulated. The desired DNA and protoplast are mixed in this method, and the addition of polyethylene glycol facilitates the absorption of DNA by the protoplast. Higher PEG concentrations, i.e. 15–25%, precipitate DNA and stimulate endocytosis without damaging protoplasts. The transformed protoplast is then selected to produce transformed plantlets and regenerated. This method is very simple because no specialized machinery is required, but this technique achieves a lower transformation frequency due to the inability of protoplast to regenerate into whole plants. The transgenic maize and barley plants have been produced by this method (Daveya et al. 2005). Liposomes are referred to in small spherical lipid bags that contain a large number of plasmids and are formed by phospholipid hydration. The desired DNA is introduced into the target protoplast by the protoplasts through the endocytosis of liposome-containing DNA. In general, PEG helps induce liposome fusion with protoplasts. The positive charge of the liposome is attracted to the DNA and cell membrane, which are both negatively charged entities (Gad et al. 1990). The process begins with the adhesion of liposomes on the

protoplast surface with the subsequent merging of liposomes with protoplast at the site of their union and finally the discharge of plasmids into the protoplast. The positive charge of the liposome is attracted to the DNA and cell membrane, both of which are negatively charged entities (Gad et al. 1990). The process begins with the adhesion of liposomes on the protoplast surface with the subsequent merging of liposomes with protoplast at the site of their union and the discharge of plasmids to the target cell. The method of lipofection-PEG was used to transform intact YACs into tob accounts (Wordragen et al. 1997). The advantages of this technique are the protection against the digestion of nucleic acids by nucleases, lower levels of cell toxicity, stability of nucleic acids due to liposome encapsulation and wide range of applications for all cell types. Nonetheless, this method is very tedious and has very low transformation efficiency, as there are very few fruitful reports on the applicability of this procedure in plant transformation.

4.2.11 Microinjection/Macroinjection

Microinjection involves the direct as well as accurate DNA delivery inside the cells, protoplast or nucleus through glass microcapillary injection pipette of 0.5–1.0 μm diameter (Crossway et al. 1986). In this technique, the target cells are immobilised under the microscope and agar with low melting point is positioned under the microscope, and two micromanipulators, one holding the micropipette and the other holding a microcapillary needle, are used to penetrate the small amounts of desired DNA solution inside the cell membrane or nuclear membrane. This method is generally exploited to transform meristem, immature embryos and pollen, excise ovules and suspended embryogenic cells. The process is very time consuming and tedious, and expensive micromanipulator device along with highly skilled and experienced personnel are required. In addition, the transformation efficiency of microinjection technique is ten times lower than that of biolistics. Despite certain disadvantages, the precise nature of the delivery of this technique proved to be extremely effective, and genetic transformation of bacco (Crossway et al. 1986), petunia (Griesbach 1987), rape seed (Neuhaus et al. 1987), soya bean (Chee et al. 1989) was achieved using this method. In addition to inserting plasmids, this technique can also be used to introduce an intact chromosome into the plant cell genome (Griesbach 1987). Hypodermic needles with a diameter greater than the cell diameter are used to transfer DNA to the target cells in the macroinjection technique. This method is generally applied by conventional syringe to cereal plants in which DNA is injected into the section of the plant developing floral tillers. The area above the plant tiller node is injected with 0.3 ml of DNA solution until many droplets of solution emerge from the top of the young inflorescence (Jogdand 2006). It is important that the time of injection of DNA is 14 days before meiosis. The formation of chimeric plants is the main disadvantage of this technique, which transforms only part of the plant. But transformed plants from single cells could be subsequently produced from this chimeric plant. This procedure has also been used to transform other plant species, for example, rye (de la Peña et al. 1987), cotton (Zhou et al. 1983), rice (Xie et al. 1990), watermelon (Chen et al. 1998) and soy bean (Hu and Wang 1999).

4.2.12 Silicon Carbide (SiC) Method

In order to deliver DNA to maize and tobacco plants, the silicon carbide (SiC) method was first used (Kaeppler et al. 1990). SiC whiskers are able to puncture cells due to their physical and chemical characteristics without damaging the target cells. In this method, small needle-type SiC whiskers are mixed with plasmid DNA, which has a gene of interest, together with the suspension of callus /cell clusters/immature embryos. These contents are then mixed with the help of shaker or vortex (Kaeppler et al. 1992). The SiC whiskers pierce the cells and create small cell membrane holes through which DNA-coated fibers enter the target cells (Kaeppler et al. 1990).

The size of the fiber, the time required for vortexing, the type and speed of vortexing, the shape of the vessels used and the cellular characteristics of the host plant, such as the thickness of the cell wall, are the various parameters governing the efficiency of this technique (Mizuno et al. 2004). The elongated fiber with a length of 10–80 mm and a diameter of 0.6 mm is most frequently used in this method. The negative charge of SiC fibers and DNA molecules at neutral pH (Appel et al. 1988) leads to a minor rejection of plasmid DNA and SiC fibre. The transformation efficiency of earlier shaking of fibers with DNA has been shown to not increase on earlier shaking of fibres with a DNA suspension (Yamagishi et al. 2007). Therefore, it could be concluded that the fibers are not involved in the transport of DNA within the cells; instead, their perforation and abrasion mechanisms facilitate the transfer of DNA (Wang et al. 1995). Carborundum, silicon nitrate and glass with similar properties of silicon carbide fiber can also introduce DNA into plant cells; however, their transformation efficiency is lower. The SCMT is considered a simple and easy way to carry out transformations on a larger scale, as no sophisticated machinery or other costly resources or qualified engineers are required. The SCMT technique allows the stable transformation of various plants, including maize, rice (Takahashi et al. 2000), wheat (Sawahel and Saker 1997), tobacco (Kaeppler et al. 1990), etc. In addition, silicone carbide fibers have been reported to increase the efficiency of the Agrobacterium-mediated method of transformation (Singh and Chawla 1999). The disadvantage of this technique is its low transformation efficiency and the cell damage also reduces its impact. Furthermore, SiC fibres can produce extreme respiratory hazard, so the laboratory staff should take precautions to avoid inhaling fibres (Svensson et al. 1997). Recently, with the help of SiC fibers, the rate of callus transformation in rice is increased by 30–50% (Nagatani et al. 1997). The mesoporous silica nanoparticles formed after the reaction of tetraethyl orthosilicate with a micellar rod template (Nandiyanto et al. 2009) were also used for the transfer of DNA and other compounds within the cellular genome of the plant and whole leaves (Torney et al. 2007).

4.2.13 Ultrasonication-Mediated Transformation

The incorporation of exogenous DNA into the interior of target cells also known as sonication by ultrasound (high frequency sound above 20 kHz) is one of the other

potential techniques of genetic transformation. It has been reported earlier that ultrasound can change the transient permeability of cell membranes (Tachibana et al. 1999), allowing large molecules such as DNA to enter cells (Wyber et al. 1997). The breakdown of cell membranes can be induced to medium-frequency sounds such as clinical shock waves and ultrasounds with frequency in MHz by acoustic cavitations bubbles generated from sounds with a lower frequency, i.e. in kHz (Miller et al. 2002). Ultrasonic waves with a frequency exceeding 20 kHz propagate in aqueous media as longitudinal pressure waves. Acoustic cavitations are the phenomenon in which rapid pressure change leads to the development of microscopic gas bubbles with their subsequent collapse (Frizzel 1988). The first possible mechanism of acoustic cavitation-induced absorption of DNA may be the generation of high pressure and temperature shock waves resulting from the violent collapse of cavitation bubbles, which leads to plasmalemma rupture and subsequent absorption of exogenous DNA, followed by the restoration of membrane integrity. The second hypothetical mechanism is the electromechanical cal model (Zimmermann et al. 1974), which states that there is a critical hydrostatic pressure at which the intrinsic membrane potential is sufficiently large to induce mechanical disruption of the plasma membrane. The collapse of microbubbles carrying DNA leads to the release of DNA trapped in microbubbles or layered into plant cells on the surface of microbubbles (Unger et al. 2001). The cavitation method is more effective for lower plants, which do not carry flowers such as mosses, lichens and algae, in which the ducts and fibres are absent, since cavitation is governed by gas bodies. The explants are suspended in a sonic medium (few mm) in a microcentrifugal tube, followed by the addition of plasmid DNA (perhaps carrier DNA). The above sample is also used after rapid mixing for sonication. The cavitation phenomenon is not only influenced by the exposure time, strength and main frequency, but also by the application type, such as continuous or pulsed, the pulse rate and the duty cycle (Santarem et al. 1998). The stable transformation in tobacco was reported for 30 min by sonicating leaf tissue of approximately 4–8 mm at 0.5 W/cm^2 (Zhang et al. 1991). The intensity used to sonicate leaf tissue was approximately similar to the intensity used to sonicate protoplasts, but the exposure time increased to 1500–2000 times. This technique is mainly used in tissues in conjunction with the biological method of transformation of plant cells or tissues (Weber et al. 2003), i.e. sonic agrobacterium-mediated transformation (SAAT). The target tissues are exposed to short ultrasound periods in the presence of Agrobacterium in this technique, thereby improving the transformation efficiency by hosting a great number of micro-wounds into the host plant cells or tissues (Subramanyam et al. 2011).

4.2.14 Gene Expression in Transgenic Plants

The transformed cells are selected using a marker gene (scorable and selectable marker), which may be linked to the gene of interest (as part of the cassette) or

unlinked, as in the case of co-transformation. The gene products of selectable markers (herbicide or antibiotic resistance, antimetabolite marker) and scorable markers (luciferase, GUS, GFP, acetyltransferase chloramphenicol, anthocyanin) should not induce variation or affect the performance of the plant. In addition, molecular analysis is carried out to confirm the transgenic status of regenerants in which the transformed status is indicated by the PCR amplification of the marker gene or transgen indicates the transformed status. Further the successful incorporation of desired foreign gene into the genome of target plant is confirmed by Southern hybridisation, which is also helpful in revealing the number of independent insertions of introduced genes (Potrykus 1991). In order to assess the expression of the introduced gene, other techniques such as RT-PCR and northern and western hybridization are used. The functionality of the transgenic product can also be evaluated in the bioassays available. In primary transgenics, however, somaclonal and transgenic effects are confused, so progeny analysis is recommended. The presence of a single copy of the transgene, which is separated as a Mendelian trait and expressed uniformly from one generation to the next, is the characteristic of perfect transformants. The production of ideal transformants is a difficult task, whose success is to some extent governed by the transformable plant material as well as nature and transgenic complexity. In addition, variability from one transgenic plant to another is often observed due to the random integration of genes into the genome phenomenon known as' position effect variation (Vaucheret et al. 1998).). Sometimes high levels of introduced gene expression have been observed, as the introduced gene is close to an enhancer element. The transgenes lodged in the subtelomeric region could have a positive effect on the position, since it is known that these regions are highly expressed (Topping et al. 1991). The sufficient production of transgenic plants and finding out some transgenics with the desired level of expression could overcome this problem. Another problem that is mainly caused by increased DNA methylation or homology-dependent gene silencing is partial or complete inactiveation of transgenes often referred to as gene silencing is another problem, which is mainly caused by increased DNA methylation or homology-dependent gene silencing or transgene suppression by its antisense counterpart or RNA interference. Efforts are made to achieve stable expression and inheritance of transgenes, thereby eliminating the random integration of transgenes. Scaffold attachment regions could achieve this, which could protect the transgene from the influence of its surroundings. Information on gene expression control elements may come from genome sequencing. The ability to target integration could also lead to transgenic expression control (Puchta 1998). Site-specific recombinases are expected to help in this effort (Ow 1996). In order to produce selectable markers free transgenic plants, the cotransformation strategy in which markers and genes of interest are placed on two separate T-DNAs in a single plasmid or on separate plasmids in one or more agrostrains could be used. The selectable marker is segregated from the gene of interest in the next generation. The other method includes the removal of marker genes by transposases in

which either the marker gene is placed on a mobile element lost after transposition, or the mobile transgene is transferred to a new chromosomal position.

4.2.15 Engineering Plants for Useful Agronomic Traits

The genetic transformation approach has provided an important platform for increasing the efficiency of the crop production system, firstly by producing transgenic plants with useful phenotypes, which could not be achieved by conventional plant breeding, and secondly by correcting any shortcomings of cultivars more effectively than conventional breeding, or by allowing the capture of commercial valves. Production of "transgenic crops of the first generation," i.e. commercially improved herbicide, insect cultivars, viruses or postharvest deterioration resistance foreign genes, as well as the accumulation of modified and highly useful storage products have resulted in meeting one of the expectations (Shah et al. 1995).). Plants are modified to increase resistance to biotic stresses such as insect, viral, fungal and bacterial diseases, which have caused severe losses in crop yields. Tobacco (Vaeck et al. 1987) and tomato (Fischhoff et al. 1987) first reported resistance to insects. Several strategies for insect control have been proposed, the most effective of which is Bt. The first feature introduced in crop plants was resistance to virus infection. The most important molecular strategy for increased virus resistance in plants using a transgenic approach includes cross-protein coat protection, which was first shown in transgenic tobacco mosaic virus (TMV) coat protein showing resistance to TMV (Powell Abel et al. 1986). Genes that encode antimicrobial protein have now been identified and cloned to help respond to plant defence. These antimicrobial proteins include hydrolytic enzymes (chitinase, glucanase and other proteins related to pathogenesis (PR), proteins that inactivate ribosomes (RIP), antifungal proteins, biosynthetic enzymes for the production of antimicrobial phytoalexins, etc. Abiotic stresses (drought, low temperature, salinity, and alkalinity) have caused severe losses in crop productivity (10–20%), which has become a major challenge, especially in developing countries. Genetic transformation approaches to abiotic stress resistance include improving or reducing stress protection. For the production of transgenic plants resistant to stress, genes encoding enzymes for the production of osmoprotectants, late embryogenesis abundant proteins (LEA), antifreeze proteins, chaperons and detoxification proteins, as well as proteins involved in the transcription of stress-responding genes were identified and used. Increasing photosynthetic efficiency is another important application of genetic transformation. The intact phosphoenolpyruvate kinase enzyme from maize has been transferred to C3 rice plants, and the phosphoenolpyruvate kinase enzyme activity in transgenic rice plants has increased two to three times compared to maize (Ku et al. 1999). Most recently the researches are undertaken to increase the development strategies for molecular stacking of many desired traits in a single transgene locus. Potato line containing seven transgenes developed by Monsanto Company (APHIS Application 98-069-23 N) is an interesting example in this respect. Amongst seven genes, one is Colorado potato beetle resistant cry gene (cry IIIA Bt); other three are selectable

markers, viz. npt II, gus and CBI. Another CBI gene serves to provide resistance against Verticillium results in changed metabolic carbohydrate pathway as well as improved resistance in bruising. Virus coat protein gene and replicase gene are the remaining two genes, which provide resistance against two viral diseases. However, the production of efficient lines through transformation technology, having required phenotype without any unwanted side effects, governs the extent of meeting the other commercial or practical expectations of plant transformation.

4.2.16 Implications of Plant Genetic Transformation

Specific cultivation conditions are required for each crop species to be transformed. However, in the last two decades, numerous methods for gene transfer to target cells have been developed for various plants, among which Agrobacterium and particle bombardment are now standard laboratory techniques that have been sufficiently used to transform essentially any plant species. Despite this progress, the use of this technology is limited by the recalcitrant nature of many economically important crops and tree species. However, efforts are being made to efficiently integrate foreign genes to produce stable transgenics using both agrobacterial and biolistic transformation and improved tissue regeneration. In order to produce genetically transformed plants with desired characteristics, new techniques are still being developed (Veena 2008). The lower frequency of transformation combined with the high frequency of undesirable genetic change and unpredictable transgenic expressions constitute two major limitations in the practical transformation of many plant species. These problems require costly transformation and screening programs on a large scale to produce useful transformants. Another problem that needs to be addressed is the presence of different selectable marker genes along with the gene of interest, which requires the future development of marker-free transgenic plants. More progress in genomics, cloning technology and vector design is therefore needed in the future to eliminate the need for a selectable bacterial marker gene. A clearer understanding of the various events that occur during gene transfer through Agrobacterium is also required. The different questions raised in the use of Agrobacterium-mediated transformation include whether transient expression is a satisfactory test for Agrobacterium-mediated transformation, or whether another convenient test for the rapid detection and optimization of this key event is necessary. Do cell types influence Agrobacterium-mediated transformation and, if so, what are the key characteristics of gene transfer determination in these favored cells? Can these features be imparted to cell types that are highly regenerable? Stable transportation is observed when the naked DNA is transferred into many actively dividing and regenerable cells using direct gene transfer experiments offers a unique advantage of gene stacking with the production of multivalent vaccines in a single transformation step. Also, there is no concern about gene silencing in plastid transformation. Zinc finger nuclease technology (ZFN) is another promising technique that can be used in basic and applied agricultural biotechnology. The gene functions in plants could also be determined by ZFN-assisted gene targeting and

chromatin re-modeling studies. Today, however, different resources are used to develop zinc finger nuclease technology in various plant species. The introduction of mini-chromosome technology provides a solution to gene stacking technology in which large DNA sequences containing multiple genes could be integrated into the targeted genome of plants leading to genetic engineering advances. Hence, there is a hope that the GM plants would provide a solution to meet the world's demands for food, feed, fibre and fuel by the production of improved crop species with minimal genomic modifications (Chapotin and Wolt 2007).

4.3 GM Crops: History and Scope

Recombinant DNA technology is the combination of DNA molecules from two different species to produce new genetic combinations in a host organism. In medicine, agriculture and industry, the recombinant organisms have value and promise. Recombinant DNA technology allows a specific protein to be isolated from a segment of DNA or gene. The nucleotide sequence can be determined with this fragment, the transcripts can mutate the sequence in very specific ways if necessary and the modified sequence can be reinserted into a living organism. This technology has significantly benefited both agriculture and medicine (Slater et al. 2008). It should be noted that the timeline of plant science and the improvement of our crops is one more step. Genetically modified organisms or GMOs are currently referred to as crops or foods modified by modern genetic technology. GMOs have simply altered their genetic composition, so they can code for a new property. To turn it on, the gene needs a mechanism. This is called the promoter segment on the switch. One of the most commonly used promoters is 35S. When a new GMO with a new feature has been developed, the resulting gene construct is called an event with regular events. Before approval for use, these events are subject to various regulatory and security reviews. One area that unfortunately is growing is the development of unapproved events (James 2006; James 2015). Genetically modified (GM) foods were first approved for human consumption in the United States in 1994, and approximately 90% of maize, cotton and soybeans planted in the United States were GM by 2014–2015. GM crops covered more than ten million km^2 (3.86 million square miles) of land in 29 countries around the world by the end of 2010. Most GM crops in the Americas have been grown. The technique was applied to soybeans in the agricultural arena in 1988, paving the way for one of the most successful crops tolerant to glyphosate soy (FAO 2015). Although this development was of significant importance for commercial agriculture, very few consumers were aware of it. The introduction of tomato "Flavr Savr" in 1994 was probably the first GMO crop many saw. In the 1980s there was anecdotal information that the enzyme polygalacturonidase was a key since it dissolved cell wall pectin. A group from Celgene suggested the development of an antisense gene to limit this enzyme. The researchers hoped this would delay maturation and allow it to stay firm longer. Calgene identified and cloned the tomato fruit pg gene in 1987 and submitted a petition to the FDA in 1992 and in 1994 approved the addition of a kanamycin resistance gene

for the creation of PG-antisense tomato. Work continued and tomatoes from Flavr-Savr were introduced at the end of 1994. Demand was high and remained high, but also high production costs and the product was not profitable. Although it may have been a technological success, it has been a commercial failure and has not done anything for the cause of biotechnology in general, the use of biotechnology and transgenic food has become the focus of commercial agriculture. There is currently a significant amount of food grown with approx. DNA recombinant technology. Eighty five percent of maize grown in the US is GMOs and nearly 90 percent of soybeans comprise a significant percentage. GMO crops have different characteristics such as two of the most common crops with their associated characteristics. Demand was high and remained high, but the cost of production was also high and the product was not profitable. Although it may have been a technological success, it has been a commercial failure and has not done anything for the cause of biotechnology in general, the use of biotechnology and transgenic food has become the focus of commercial agriculture. There is currently a significant amount of food grown with approx. DNA recombinant technology. Eighty five percent of maize grown in the US is GMOs and nearly 90% of soybeans comprise a significant percentage. GMO crops have different characteristics such as two of the most common crops with their associated characteristics. Roundup ready to contain soybeans contains proteins that interfere most with the EPSPS pathway. Round Up known as glyphosate is a general-purpose pesticide used not only in agriculture but also in homes to eliminate weeds. Although it is good to eliminate weeds, healthy crops such as flowers, crops and ornamentals are also eliminated. In the case of Roundup Ready Soy, the GMO feature enables the farmer to use Round Up to remove weeds without killing soy. In addition, a farmer can be more productive if tedious weeding is eliminated. The second example is that BT maize has been encoded with a gene that eliminates the maize borer, which allows more maize per acre. The production of 170 million hectares, including 312 events in 29 species with 3497 approvals in 59 countries, was based on data from the end of 2012.

The use of Agrobacterium as a vector to insert the new DNA into a plant was one of the earlier techniques used to insert genes into plants. Agrobacterium tumefaciens in plants causes a disease known as a disease of the crown gall. A tumor-like growth or gall in the infected plant characterizes Crowngall. The transfer of a segment of DNA from the bacterial tumor-inducing plasmid initiates tumors. The plasmid T-DNA is semi-randomly integrated into the host cell genome in which the tumor morphology genes are expressed on the T-DNA, causing gall formation (Francis and Spiker 2004). In biotechnology, in particular genetic engineering for plant improvement, the ability of Agrobacterium to transfer genes to plants and fungi is used. A modified plasmid of Ti or Ri can be used. The plasmid is “disarmed” by deleting the genes that induce tumors; the only essential parts of the T-DNA are its two small border repeats (25 base pairs), at least one of which is necessary for plant transformation. Marc Van Montagu and Jozef Schell at the University of Ghent (Belgium) discovered the mechanism for gene transfer between Agrobacterium and plants, which led to the development of methods for the transformation of Agrobacterium into an efficient system for gene engineering in plants

(Schell and Van Montagu 1977; Joos et al. 1983). This work laid the basis for the insertion of specific genes into a plant using Agrobacterium. One can also argue that the gene transfer has been going on for a very long time and we have learned to use it effectively for specific crop improvements. The genes to be introduced into the plant are cloned together with a selectable marker into a plant transformation vector containing the bacterial plasmid T-DNA region. In conjunction with the other desired genes, an antibiotic marker gene was often incorporated into the plasmid to allow the selection of successfully transformed plants. Plants are grown on media containing antibiotic following transformation, and those that do not have the T-DNA integrated into their genome will die. Transformation with Agrobacterium can be accomplished by incubating either protoplasts or leaf discs with the Agrobacterium to cause the plasmid insertion. From the callus that results, whole plants regenerated using plant tissue culture. Agrobacterium does not infect all plant species, but other plant transformation techniques, one of which is the gene gun, have been used. A genetic weapon is a biolistic particle delivery system that was originally designed to transform plants by injecting genetic material into cells. The plasmid DNA is coated on heavy metal elementary particles. The genetic weapon can transform almost any cell type, including plants, and is not limited to the nucleus' genetic material: It can also transform organelles, including plastids. Gene insertions intended to transform prokaryotic genomes generally have an interesting gene or genes, at least one sequence of promoters and terminators and a reporter which is a gene used to ease detection or removal of those cells which didn't integrate the construct into their DNA. These genes may each have their own promoter and terminator, or they may be grouped together to produce multiple gene products from a single transcript, in which case binding sites for translation machinery should be placed between them in order to ensure maximum translation efficiency. In any case, regions called border sequences, which are similar in sequence to locations within the genome, flank the entire construct; this allows the construct to target a specific point in the existing genome (Slater et al. 2008). A gene gun often targets a callus of undifferentiated plant cells growing in a Petri dish on a gel medium. The gel and callus are largely after the gold particles have affected the dish the gel and callus are largely disrupted. However, some cells are not killed in the impact, and have incorporated enveloped a DNA coated gold particle, which eventually migrates to and integrates into a plant chromosome. The term "genetic modification" and "genetically modified organisms" is often misused. All types of agriculture (organic, conventional) modify plant genes so that they have desirable characteristics. Traditional breeding forms indirectly change the genetics of the plant by selecting plants with specific characteristics, while genetic engineering changes the characteristics by directly modifying the DNA. Crosses are made relatively uncontrolled in traditional breeding. The breeder selects the parents to cross in conventional plant breeding, the results are unpredictable because the parents 'DNA recombines randomly. Genetic engineering, by contrast, enables highly precise gene transfer, rapid and efficient gene tracking in new varieties. This ultimately leads to increased efficiency in the development of new and desirable crop varieties (Popping 2010).

4.3.1 Scope

The first GMO crop to be introduced to the market was the introduction of "Flavr Savr" tomato in 1994. Anecdotal information was available in the 1980s that the enzyme polygalacturonidase (PG) was key to softening tomato fruit because it dissolved pectin in the cell wall. By developing an antisense gene, Calgene proposed to limit this enzyme. The goal was to delay the maturation so that the tomatoes could stay firm longer. Calegene identified and cloned the PG gene for tomato fruits in 1987 and submitted a petition to the FDA in 1992. In 1994, the FDA approved the addition of a gene construct for kanamycin resistance to the creation of tomato PG-antisense (FAO 2015). Work continued and the Flavr-S continued in late 1994 was introduced. Although it may have been a technological success, it has been a commercial failure and has not done anything for the cause of biotechnology in general, the use of biotechnology and transgenic food has become a major issue in agriculture. There is currently a significant amount of food produced with approx. DNA recombinant technology. Eighty five percent of maize grown in the US is GMO and nearly 90% of soybeans. GMO crops have different characteristics. There are examples of two of the most common crops with their associated characteristics. Roundup Ready Soybeans contain a protein that interferes most with the EPSPS pathway. Round Up, known as glyphosate, is a pesticide used to eliminate weeds not only in agriculture, but also in homes. While it is good to remove weeds, healthy crops such as flowers, crops and ornamentals are also eliminated. In the case of Roundup Ready Soy, the GMO feature allows the farmer to use Round Up to remove weeds without killing soy. A farmer can also be more productive in eliminating tedious weeding (James 2015). The second example is that BT maize has been encoded with a gene that eliminates the maize borer, which allows more maize per acre. The production of 170 million hectares, including 312 events in 29 species with 3497 approvals in 59 countries (NAS 2016), was based on data from the end of 2012. Approximately 12 genetically modified crops were used in 2015 (FAO 2015; James 2015). Nine food crops, three non-food crops and two types of flowers were commercially available for production in 2015. Maize and soybean were the genetically modified crops most widely grown. Since its first commercial release in 1996, the production of genetically modified maize has increased substantially to 53.7 million hectares by 2015. Genetically modified soybean rapidly increased from its introduction in 1996 to more than 92 million hectares in 2015 (James 2015). The seven other food crops of which GE varieties were grown in 2015 were apple (*Malus domestica*), canola (Brassica napus), sugar beet (Beta vulgaris), papaya (Carica papaya), potato, squash (Cucurbita pepo), and eggplant (*Solanum melongena*) (James 2015). The contribution of GE varieties to the production of those crops was small, except for canola; GE varieties of canola constituted 24% of the 36 million hectares planted in 2015 (James 2015) rd of all land planted to maize worldwide that year (James 2006, 2015). Herbicide resistance, insect resistance and virus resistance are the most economically important crop changes to date. Herbicide resistance introduces a crop's ability to resist the use of certain weed control herbicides. For nine different herbicides, herbicide-resistant traits have been developed and

introduced into eight herbicide-resistant traits for soybeans, six for cotton, three for canola, three for maize, two for sugar beet and one for alfalfa. Some varieties of crops with stacked resistance to two herbicides (e.g. glyphosate and 2,4-D or glyphosate and dicamba). Glyphosate has been introduced for soybeans since 1996 while as glyphosate resistance has been introduced in alfalfa, cotton, canola, maize, and sugar beet by 2015 (FAO 2015).

Insect-resistant (IR) characteristics include insecticidal properties produced internally by a plant. An example of insect resistance is the transfer of gene coding from the soil bacterium Bacillus thermogenesis for a crystalline (Cry) protein. When the insect feeds the plant, the Cry is toxic to the target insect. Cry proteins can control many insect pests-moths, beetles and flies in particular (Höfte and Whiteley 1989). Cotton, eggplant, maize, poplar and soybean insect-resistant varieties were commercially produced in 2015 (NAS 2016). The resistance of the virus prevents the susceptibility of a plant to specific viral diseases. The resistance of the virus in crops targets the targeted virus 'coat-protein gene. The transgene prevents the virus from successfully replicating in the host plant. In 1998, commercially grown varieties of papaya resistant to viruses were first introduced in Hawaii. In the late 1990s NAS, 2016, virus-resistant squash was also marketed in the United States.

4.3.2 Testing

The ability to determine whether a crop has been genetically modified is important because consumers and regulators need this information. On selected commodities, there are two basic types of testing: Protein and DNA. The new gene is sandwiched between two segments in the development of the gene sequence for a crop, a promoter and a terminator. There are a number of promoter and terminator segments that are easily identified from a new source. 34S and 35S, which come from the Cauliflower Mosaic Virus (CaMV), and the Figwort Mosaic Virus (FMV), are two of the most common promoter segments. Nopaline Synthase is a relatively common terminator marker. There are two approaches to testing GM content. In the first approach, an ELISA or immunochromatography method can be used to test the expressed protein. For decades, ELISA tests have been used for a large number of compounds. While these are useful, the number of possible proteins to be tested is limited and the levels of proteins are very low. A second approach is to test fragments such as 34S, 35S and NOS using PCR or RT-PCR using several commercial test protocols with kits for testing the specific insert (Slater et al. 2008). Samples must obviously be extracted and prepared for analysis using one of several available techniques before any of these techniques. In qualitative PCR, the DNA polymerase specificity is used to amplify target sequences. Two pairs of primers are used in standard PCD with one being a sequence of senses and the other antisense. These sequences are multiplied by approximately a million times. These segments can be separated by electrophoresis of agarose gel after amplification, but other techniques such as HPLC have been used. The alternative approach to qualitative PCR is quantitative real-time PCR, in which fragment separation is performed automatically.

Should an organization not choose to perform testing, there are several contract labs that can perform this assay (Ahmed 2002). Although the various technologies involved in GMO testing are of interest, a new phenomenon has emerged in recent years, which is GMO verification services, the most visible of which is the non-GMO verification project.

4.4 Environmental Implications of GM Crops

The debate on the impact of GM crops on the environment has focused on questions such as: What are the potential environmental risks of GM crops? If we market genetically modified crops, how far will it have unwanted effects on non-target species? First, toxicity produced by chemicals used in GM crops is a major challenge for both the environment and hereditary plants (De Schrijver et al. 2015). Second, such crops may be toxic to non-target species, in particular to "friendly" species such as beetles, bees and butterflies (Yu et al. 2011). Generally speaking, the effect of subsistence, organic or intensive agriculture on the environment is evident, which demonstrates strongly that GM crops must have implications on the environment. The International Council for Science (ICSU), the GM Science Review Panel and the Nuffield Council on Bioethics (www.nuffieldbioethics.org), among many environmental protection platforms, approve that GM crops have a positive or negative impact on the environment, depending on how and where they are used. The role of genetic engineering is plausible in more sustainable crop production and conservation of natural resources, including biodiversity. Its role in accelerating the harmful effects of agriculture cannot be avoided, however. The issue of basic environmental impacts in relation to the release of transgenic commercial crops is particularly relevant (Domingo 2011). Direct impacts include gene transfer, non-target species trait effects and wildlife, invasiveness, weediness and genetic recombination of free DNA in the environment. By contrast, indirect effects include harmful and adverse effects of chemical control, i.e. reduced efficiency of pest control, disease and weed control, effects on water and soil, and global biodiversity decline (Tutelyan et al. 2010). The most debatable environmental implications are discussed below.

4.4.1 Direct Impact of Transgenes on Environment

4.4.1.1 Gene Flow

Gene flow is considered to be a major evolutionary force, leading to changes in gene frequencies, mutation, genetic drift and selection (Lu and Yang 2009). Gene flow can affect the environment by reducing population differentiation and increasing the diversity of people in a population (Mertens 2008). One of the effects of gene flow is also the structure of genetic diversity (Gepts and Papa 2003). The introduction of non-native GMOs into ecosystems poses potential long-term risks to the environment and its consequences are quite difficult to predict. Scientists from different streams around the world are concerned with the possibility of transferring

transgenic sequences to related wild species or weeds through horizontal gene transfer (HGT) or hybridization. There is no doubt that the environmental effects of gene flow are variable, but some of the effects of gene flow could be generalized on the basis of general findings in many cases, such as the development of superweeds, the development of new viral pathogens, the instability of transgenes in the environment, the creation of GD, the development of pests and pathogens with resistance to new compounds (Egan et al. 2011). At the same time, it is also necessary to address the secondary effects of gene flow, including effects on non-target species, disturbance of biodiversity, displacement and extinction of species, disturbance in the microenvironment of the soil and species of environmental concern (Layton et al. 2015). The possibility of new species evolution cannot be ignored and could also lead to an infinite number of biotic interactions (Beusmann and Stirn 2001). It is implicitly expected that gene flow from GM crops will be considered, as it has occurred between sexually compatible species for millennia (Keese 2008). However, this expectation is based on certain basic concepts, such as the distance between compatible plant species, the synchronization of flowering time, the ecology of the recipient species and sexual compatibility off course (Han et al. 2015). Some transgenic features make them more suitable for introgressing into wild counterparts, such as dominance, no association with harmful crop alleles, and location on shared genomes and/or homologous chromosomes. To predict the possibility of gene transfer through this mechanism, mathematical models of pollen movement are being developed (Dale et al. 2002. Examples of such investigations are reported in rapeseed, maize, cotton, wheat, barley, beans and rice (Han et al. 2015). The transfer of the pollen-mediated gene depends solely on the biology of the plant's pollination, the amount of pollen produced, the matching system between donor and recipient species, the excess rate, the relative density of the donor and recipient species, vector types, wind, air turbulence, water current, temperature, humidity and light intensity (Hancock 2003). A recent investigation by Dong et al. (2016) reported that the wind direction significantly affected a pollen-mediated gene flow. In addition, an increasing distance from the pollen source in WYMV-resistant transgenic wheat N12-1 reported a drastic decrease in pollen-mediated gene flow. In transgenic corn, canola and creeping bentgrass, pollen transfer rate decreased rapidly when the distance was increased just by 30 m, 20 m and 20 m respectively (Van de Water et al. 2007). In creeping bentgrass and rigid ryegrass, the highest frequency of gene flow was also reported as a result of pollen flow with a pollen donor only 2000 and 3000 m away (Van de Water et al. 2007). In self-pollinated crops, comparatively low frequency of gene flow was observed than in cross-pollinated crops (Warwick et al. 2009), as in the case of direct and indirect pollen-mediated gene flow from rice to red rice and vice versa <1%. Two other possible mechanisms for gene flow are seed mediation and vegetative propagulate mediation (Lu 2008). The transmission of seed-mediated genes is supported by human error in the seeding, harvesting or post-harvesting of adventitious plants (Schulze et al. 2014). In maize, wheat and canola, adventitious presence of herbicide resistance genes was observed in farm-harvested seed (Friesen et al. 2003). The transgenic transmission of vegetative propagules is caused by vegetative plant organs or by different animals (Schulze et al. 2014).

Scientists argue that whether or not such a transgenic flow really matters and what would be the consequence if it really matters? As discussable, such events occurred in nature between conventional crops and land races without constitution of any environmental problem. Introduction of new traits and novel genes into ecosystems as a result of genetic engineering raises additional concerns allowing flow of genes into diverse crops with variable outcrossing potentials (Ellstrand 2003). Here we discuss the detailed impacts of gene flow on the environment accompanied with relevant underpinning research.

4.4.2 Transgene X Wild Hybridization

Because of the ability of plants to hybridize with sexually compatible species and the release of hybrids into the environment and the spread of transgenic contamination, scientists recognize the possibility of transgenic flow. Ecosystem disturbance can be attributed to the persistence of a possible transgenic hybrid with a competitive advantage over the wild population. In theory, a rare hybridization event would be sufficient to develop such a hybrid under natural conditions (Cruz-Reyes et al. 2015) and the developed hybrid could be more fit than its parents. Fitness is a hybrid's relative ability to survive and reproduce thereafter in an environment (Haygood et al. 2003). The development of such a hybrid depends on certain factors, such as the synchronization of the flowering period, the hybrid's reproductive fitness and survival rate (Lu and Yang 2009). Fitness may be reduced in the first hybrid progeny F1, but is recovered in the next hybrid progeny as seen in sunflowers resistant to imidazolinone (IMI) (Presotto et al. 2012). In Brassica rapa/Brassica napus F1 hybrids and both parental species, ample fitness differences were observed. In regions where the crop species originated and had wild relatives, the risk of unintended gene transfer is greater (Lu and Snow 2005). Detection of the terminator of NOS (Nopaline Synthase) and the promoter of CaMV (Cauliflower Mosaic Virus) 35S in Mexican maize populations has strengthened the idea of gene transfer from GMO to land races and wild relatives (Pineyro-Nelson et al. 2009). Some factors such as hybrid vigor, selection and heterosis will play a role in determining the frequency of transgenes in wild populations after transgene flow to host plant genomes. Hybrid fitness depends solely on the ability to cross wild counterparts or related species, the life cycle of hybrids and their parents, fertility, changes in the survival rate of seed banks, seed persistence and seed dormancy (Lu and Snow 2005). Fitness costs in wild plants and crops must be different due to their diverse genetic background and the possible causes are pleiotropy, the physiological costs of new features or the effects of specific insertion sites in the genome and genetic changes in plant genomes as a result of mutagenesis (Schnell et al. 2015). The fitness of crop wild sunflower hybrids was higher in relative competitive wheat intercropping conditions compared to crop lines and was greatly affected by the interactions between the genotype environment (Mercer et al. 2014). The influence of the above random

and unintended effects on other associated characteristics is not negligible, but may remain unnoticed until the establishment of transgenes as wild populations; one such example is the transgenic sugar beet × swiss chard hybrids for their bolting pattern (Ellstrand 2003). The evidence of GM/wild interspecific hybridization was presented by a collection of triploid individuals in commercial canola fields in Chile (Prieto 2006). In Arabidopsis thaliana, Gressel (2000) also hypothesized a fitness penalty resulting from the resistance to the target site and increased ability to donate pollen to nearby non-GM mothers. Such gene flow cases are always accompanied by selection pressure against herbicides, insecticides, abiotic stress or pathogens. However, even if selection pressure introgression is not present, the persistence of transgenes in wild populations is still possible due to the recovery of selective fitness through successive backcrossing (Wang et al. 2001) and was noticed by Schulze et al., (2014) who reported the presence of glufosinate-resistant (particularly, events MS8 × RF3, MS and RF3) feral plants of oilseed rape in Switzerland even if there was no transgenic oilseed rape in the surrounding area at the time of sampling. On the other hand, genetic bridge is also responsible for gene flow, as crop hybrids and a sexually compatible wild plant can also provide transgenes directly to non-hybridizing species (Lu and Snow 2005). The Poaceae and Brassicaceae families have been reported to have a maximum number of natural hybrids among the target families of transgenic introgression (European Food Safety Authority 2016). Ellstrand (2002) reported that in different agro-ecological areas of the world at least 44 cultivated plants could cross with one or more wild relatives. Twenty-eight cultivated species, including 22 world food crops, have seen natural hybridization with one or more wild associations. He further confirmed hybridization with related wild plants for 83 species, due to the presence of sympatry, it was evident that 48 species had something more than just morphological intermediaries. Recently, the outcrossing potential of 11 GM crops with vascular flora in Chile was documented by Sanchez et al., (2016). 810 of 3505 introduced species and 824 of 4993 native species had interrelationships either based on genus or species correspondence.

In addition, the progeny of hybrids of GM/sexually compatible species may carry hemizygous allelic conditions that may not be expressed at the phenotypic level unless the condition is homozygous as a result of additional self-pollination or cross-pollination events (Sanchez et al. 2016). The GM Science Review Panel (2003) confirmed the absence of such hybrids, which in the UK could have become wildly invasive. In addition, the transgenic transfer of maize, cotton, canola and soybean has not been documented (Heuberger et al. 2010). In the case of B, though. Transgenic herbicide resistance napus transferred to its relative wild weed type B. Québec rapa and its persistence for the following 6 years, it was observed that no herbicide selective pressure in natural conditions occurred (Warwick et al. 2008). Based on the above reports it is obvious that hybrids may develop by introgression of GMO with its wild relatives and hence the possibility of transformation of resistant genes exists.

4.4.3 Transgene Stacking

With the development of transgenic plants with improved resistance to herbicides and insect pests, the use of GM crops worldwide is increasing. The area covered by single transgenic characteristics, such as glufosinate tolerance, is still high, but the relative percentage of GM crops with stacked characteristics (herbicide tolerance, insect resistance, restauration of fertility, male sterility, mannose metabolism, visual marker and antibiotic resistance) has increased. In 2012 alone, 43.7 million hectares were planted with biotech characteristics, with an average annual increase of 31% (www.isaaa.org). Many businesses such as Bayer Crop Science, Syngenta, Pioneer, Monsanto and Dow Agro Sciences are pursuing to achieve GM crops with stacked traits. The environmental and ecological consequences of transgenic stacking must also be taken into account. Transgenic contamination may include approved transgenic constructs and sequences and constructs not approved in a given country (De Schrijver et al. 2007). Kok et al. (2014) classified three possible risk scenarios from stacked plants. These include gene stability, gene expression changes and synergistic or antagonistic effects. Initially, the probability of stacked gene escape may be low, but multiple transgenes are likely to occur in wild plant populations in the long term (De Schrijver et al., 2007). Nuclear-coded and in rare cases, nuclear-encoded and plastid-encoded genes may even be combined (Halpin 2005). Accidental stacking, as well as intentional breeding between sexually compatible GM plants, may lead to accumulation of many genes in the same area. Consecutive generations of related and sexually compatible weed species would be able to receive transgenes with a wide range of action modes, such as pest resistance, various stresses, herbicide tolerance, etc., and would persist more forcefully in the environment (Mertens 2008). Recent developments in plastid genetic engineering have allowed multiple genes to be expressed in a single operation. On the contrary, gene escape from such events paves the way for introgressing perhaps the entire stack of transgenes that are often linked to a single metabolic pathway. As a result of this gene flow, environmental risks could only develop resistant and tolerant weeds in one generation (Bock 2007). In comparison to a single event or conventional counterparts, significant changes in endogenous gene expression and protein levels can be observed in GM plants with stacked characteristics. The expression of two stacked genes (Enolpyruvulshikimate-3-phosphate synthase and cry genes) led to changes in maize energy/carbohydrate and detoxification. In comparison to single event hybrids, both stacked genes had a 34% lower expression (Agapito-Tenfen et al. 2014). Some reports indicated that these reduced expressions could lead to the development of resistance in target insect pests (De Schrijver et al. 2015). The synergistic and antagonistic effects of stacked transgenes can present risks at two levels. Firstly, the interaction of proteins or stacked event components at the level of the GM plant may affect certain pathways, such as high oleic acid GM soybean, which may have a synergistic or antagonistic effect on other components of the oleic acid pathway. Secondly, the effect can be expected at the cellular level, where the expression of transgenes may affect the levels of cell components (Kok et al. 2014). At the cellular level, however, the risk cannot only be associated with stacked

characteristics, since transgenes of single events could also present the same risk. Open pollinated crops are at higher risk of developing polygenic transgenic characteristics as a result of the recombination of multiple transgenes compared to self-pollinated crops. What would be the possible impact on the environment and biodiversity of such gene flows? The most important thing is weed management and stacked volunteers of transgenes. Stacked transgenic volunteers have been resistant to various herbicides in Canada (oilseed rape) (Dietz-Pfeilstetter and Zwerger 2009). The question is, how can such a threat to the environment be managed? Orson (2002) suggested that such volunteers in the field of volunteering are inevitable to practice such volunteers in the field of agriculture. De Schrijver et al. (2015) proposed theoretical scenario tests to estimate the effect of stacked Bt proteins on non-target invertebrate species. He stressed that current knowledge of interactions with Bt toxins is limited and should be evaluated using more precise data. Schuppener et al. (2012) reported that lepidopteran and chrysomelidae were not significantly affected by stacked maize (Cry1A.105 andCry2Ab2) in European agricultural landscapes. Another, study involving Bt11 ut MIR604 maize, which expressed Cry1 Ab andmCry3A proteins, revealed unbelievable results that the cultivation of stacked GM maize did not differ more than single maize events (Raybould et al. 2012). In milk cows, beef heifers, swine, laying hens, broiler chickens and rodents, the combined toxicological impact of Cry1F and phosphinothricin acetyltransferase (PAT) proteins from TC1507 maize was considered. The report showed negligible or no allergic or toxic effects on humans or any of the organisms studied. There was no detection of gene flow and HGT (Baktavachalam et al. 2015).

4.4.4 Horizontal Gene Transfer

Stable transfer of genes to offspring other than parents (sexual/asexual) is considered to be horizontal gene transfer (HGT) (Keese 2008). The transfer takes place through the passage of genetic material from donors across cell boundaries, followed by heritable incorporation into the recipient organism's genome. The most popular strategy for genetic transformation is The result of HGT is agrobacterium tumefaciens (Conner et al. 2003). In addition to transduction, transformation and conjugation, many different mechanisms are naturally involved in the absorption and establishment of genetic material. With the advent of genetic engineering, the possibilities of HGT question the risks associated with the environment and biodiversity. The role of HGT in the evolution of microorganisms and macroorganisms under natural circumstances has already been recognized, and it is well understood that mechanistic HGT has no direct adverse effects, but changes in the fitness of the recipient organism have a drastic effect (Conner et al. 2003). Keese (2008) explained in detail the risks associated with HGT and possible factors that play a role in gene transformation. HGT from genetically modified plants raised further concerns about the possibility of transgenic transfer to another organism. Such gene flow could constitute a potential risk to humanity and the micro and macro environment (Conner et al. 2003). Possible HGT cases may include the transfer of transgenic

antibiotic resistance to pathogens and transgenic flow to viruses and/or humans (Ho et al. 2000). Such gene transfers could occur in soil, water and a human or animal gastrointestinal tract. These cases are still highly speculated, however, and detailed experimental evidence is expected. It is important to consider the interplay of alleles between bacterial communities with special consideration for HGT, which highlights the possibility of overcoming ecological barriers to the transfer of alleles among bacterial communities with special consideration of HGT, which highlights the fact that ecological barriers to allele transfer could be surpassed in different ways. Many bacterial species adopt such a strategy to maintain genetic similarity in the population, but this characteristic phenomenon poses a threat to the environment when considered at microclimate level in the context of genetically modified plants and bacterial interaction. Another major concern is the acquisition of multiple antibiotic resistances in a wide range of bacterial populations due to the widespread use of antibiotics in humans and animal medicine (Lawrence and Retchless 2009). Transgenic transfer from genetically modified plant roots and leaves to microorganisms was demonstrated by Tepfer et al. (2003), and such studies confirmed that Arabidopsis, oilseed rape, tobacco, alfalfa and carrot could transfer genes (nptII gene system as a marker) to Acinetobacter spp. Many experiments have shown that intact tobacco leaves with plastid transgenes can consistently produce bacterial transformants. Some factors were considered important in HGT, such as the size of the transgene, nuclear or plastic transgene, sequence mosaicism, selective pressure, transgene copy number, the genome size of the recipient species, the use of codons between the donor and the recipient, the type of promoter used in the insert, compatibility of RNA and protein synthetic machinery. (Tepfer et al. 2003; Daniell et al. 2001). Natural GM sweet potato (*Ipomoea batatas* (L.) Lam.) harboring many A. tumefaciens DNA (particularly two T-DNA regions i.e. IbT-DNA1 and IbT-DNA2) sequences strengthen the hypothesis that HGT can be a possible route of transgene movement from microflora to GM plants and vice versa (Kyndt et al. 2015). During evolution, when A. tumefaciens infected sweet potato these regions were transferred naturally. Recent investigations targeted at the transfer of CaMV-P35S promoter from a GM diet to blood in liver and brain of male Wistar albino rats suggested that this promoter have affinity of incorporation. The report suggested that larger segments had a higher incorporation frequency than shorter sequences and affinity increased with the increase of feeding duration (Oraby et al. 2015). Many researchers are in a debate that HGT frequency from plants to prokaryotes is as low as $2 \times 10–17$, while some scientists argue that 10 recombinants per 250 m^2 could be predicted considering a transgene transmission frequency of 10–17 (Mertens 2008).). Matthews et al. (2011) predicted HGT of *Rhodnius prolixus* less than $1.14 \times 10–16$ per 100,000 generations with 99% certainty level. Apart from traditional marker transgenes, novel transgenes having no natural counterparts i.e. those genes which are being engineered for production of pharmaceuticals, chemicals and vaccines, necessitate investigation in relation to HGT which may frequently include

unique combinations of toxin protein domains and regulatory elements, derived from diverse species which will probably differ considerably from those arising by natural evolution. HGT of dsRNA from GM crops to other related organisms should also be accounted for (Heinemann et al. 2013).

4.4.5 Structure of Genetic Diversity

Gene flow can affect the environment by reducing population differentiation and increasing the diversity of people in a population. The structure of GD or so-called "domestication bottleneck" is also a result of gene flow and can be determined by taking into account the history of life and demographic factors of domesticated crops (Lu and Yang 2009). Those crops that domesticated from a small initial crop population show a reduction in genetic variation known as the bottleneck of domestication. The main driving force for partial restoration of GD and GD is the natural flow of genes from wild to domesticated crops and new alleles and introduction (Marri et al. 2007). Such gene flow also plays an important role in development. By the advent of modern genetic engineering and plant breeding, characteristics including resistance to many pests and pathogens and quantitative quality and yield characteristics have been incorporated into crop plants grown on a commercial scale. The flow of these transgenes from GM crops to wild families reduces GD and sometimes completes the genetic extinction of wild populations (Gepts and Papa 2003). The frequency of genes is primarily affected by mutation, selection, genetic drift and migration (Papa and Gepts 2004). Migration of gametes to wild relatives through the movement of pollen between GM plants could be a strong factor in reducing GD between subpopulations. With such transgenic migration, gene frequencies in the entire genomes of the recipient species will be disturbed mainly by genetic recombination on target loci (Cruz-Reyes et al. 2015). In the GM cropping system, GD of rhizosphere bacteria can also be affected. There has not yet been such a detailed report, however. In major rhizospheric bacterial groups such as Proteobacteria, Actinobacteria, Chloroflexi and Firmicutes in the root zone of MON810 maize, no significant genetic variations were detected (Ondreickova et al. 2014). Overall, the possibility of controlling disturbance in GD is considered to be the decision of the farmer by compensating crop production with non-GM crop plants instead of agreeing with current scenarios of widespread GM monocrop crops. In any particular case, the extent and quality of gene flow unfolds the possible risks associated with it. It is now clear from the above discussion that gene flow is a strong evolutionary force and strongly demands that special containment strategies be developed to reduce it as much as possible (Ellstrand 2003). Possible strategies include (1) isolation zones or border areas (2) trap crops (3) molecular strategies such as limiting the opening of the flower, chloroplast engineering, male-sterility, genome incompatibility, seed sterility, apomixes, transgene excision and cleistogamy (Husken et al. 2010).

4.4.6 Fate of Naked DNA

In the natural environment, nDNA encoding a resistance or tolerance feature may persist (Barnes and Turner 2016). There are several possible sources of nDNA to be transferred, such as compost of GM plants and manures of animals with GM fodder (Gulden et al. 2005). There is another possibility of transgenic movement of meat and milk from animals fed with GM diets to natural habitats. Naked dsRNA from GM plants produced by dsRNA silencing may pose additional risks in addition to nDNA (Heinemann et al. 2013). Once nDNA has escaped, its persistence in the environment depends only on certain factors, i.e. transgenic size, DNA type (plastid/nuclear), kind of mineral or particle in soil to which DNA will bind, physiological state of recipient micro/macro-organism, stress on recipient microbe as well as availability of nutrients, pH of soil, amount of humic acid and soil temperature (Dale et al. 2002). The size of naked and degraded DNA (possibly transgenic and its regulatory sequence) and its facilitating sequence of flanking DNA are key factors for successful integration. In order to gain a perspective on the impact of nDNA on the environment, let us consider the amount of such DNA added to the environment. In contrast to immense amounts of DNA from non-GM plants added to the environment by pollen, leaves, fruits and compost and decaying plants, the relative amount of DNA from GM plants is relatively low (Dale et al. 2002). Once nDNA has escaped from a GM host and reached the environment, what damage to the environment could be possible and what is the risk? This DNA can create interruption in ecosystems? Well, the risk from such events is not negligible. First, such naked-extracellular DNA could be a source of the gene pool for microbial communities in the vicinity, especially bacteria and fungi with natural intake of DNA. Secondly, viral pathogens residing in microflora that could receive nDNA could be the most devastating danger. Third, there is another possibility of gene transfer from bacteria residing in GM crops to microbes in the intestines of animals feeding on GM crops (Dale et al. 2002). The intake of GM DNA in dairy cows fed transgenic Bt maize was 0.000094% of the total intake of DNA, which was nearly 54 μg/day. Although it was found that the daily intake of non-GM DNA in cows was 54–57 g/day (Phipps et al. 2002). Although the possibility of such a transfer is quite negligible due to nucleases in the intestine of the animal, nDNA would degrade (Flachowsky et al. 2005). Fragments of degraded DNA of 680 bp were detected in maize cob silage within 28 days, while only 194 bp were detected in whole plant silage for up to 35 days (Einspanier et al. 2004). In response to different acids, endonucleases and microbial activities, this fragmented DNA was immediately degraded in the animal digestive tract. A case study to detect CP4EPSPS in sheep fed with Round Ready canola detected fragments of 527 bp after 2 min (Alexander et al. 2004). The likelihood of risk in the digestive tract of an animal is quite negligible. However, it is possible that microbes residing in animal intestines can endocytise these small fragments and can be incorporated into host microbial genomes. Third, highly degraded segments of DNA may introduce amino acid substitutions or indels to bacterial genomes by transposition or homologous recombination, apart from the fact that these highly degraded segments are unlikely to transfer new protein encoding

capabilities (Van Hoek et al. 2011). Finally, if the decomposed GM material is exposed to aquatic ecosystems, it may be in aquatic animals 'gastrointestinal tract of aquatic animals and fish, fungal species could possibly up take nDNA (Mullany 2000). Persistence of nDNA from Bt corn (event MON863) containing Bt3Bb1 and nptII genes and DNA from plasmid Pns1 in water was reported to decrease by two orders of magnitude within >4 days (Zhu 2006). As far as the persistence in agricultural ecosystems concern, the possibility of nDNA perseverance is not zero. The persistence of nDNA in root zones of Roundup Ready GM corn and soybean is for a very short duration of 26.7 h if temperatures are high (> 15 °C) while, its persistence increases when temperatures are <15 °C and frequent rainfalls can distribute their DNA into various soil layers and across the agricultural fields (Gulden et al. 2005).

4.4.7 Weediness

Another growing concern that has severe and irreversible effects on biodiversity is the change in invasiveness or persistence of crops in agricultural and natural habitats. The establishment of a transgenic or transgenic hybrid as a weed is referred to as weediness in other fields or other habitats. Weediness is one of the possible effects of herbicide-resistant crops (HR) (Ammann et al. 2000). The ICSU, GM Science Review Panel agreed that domesticated crops are at low risk of weed establishment because domesticated characteristics are often less fit in the wild. Recent studies, however, support domesticated crops as it can escape cultivation (ferality) and turn into a potential weed. Features such as rapid growth rate, self-compatibility (crop features) could promote weediness (Ellstrand 2012). Increased herbicide resistance by hybridization with GM plants could lead to its persistence in agricultural habitat (Guan et al. 2015). Scientists have a contradiction about the establishment of transgenic recipients as weeds in the environment. For example, Williamson et al. (1990) reported that small genetic modification of domesticated crop hybrids by GMOs could cause major environmental changes. On the other hand, Luby and McNichol (1995) argued that it is unlikely to establish a crop as a weed by adding a single transgen. Based on the risk of increased fitness, some characteristics are strong candidates who can increase the chances of competitiveness, such as herbicide tolerance, stress resistance, pathogens and pests and characteristics responsible for increased growth (Yang et al. 2012). In view of the dispersal, plants with perennial, robust, prolific and competitive characteristics and the ability to withstand a variety of natural habitats could be regarded as plants with high impact (Mertens 2008). Furthermore, the rate of weediness through gene flow relies on the frequency of hybridization and net selective effects of target transgenes (Lu and Yang 2009). Certainly, weeds and crops exist in some plant species (Ammann et al. 2000). What could be the risk of such species? A change in habitat could obviously put potential pressure on the development of a weed from a cultivar or from a closely related feral plant. Plants can develop several mechanisms of herbicide resistance, such as herbicide detoxification, changes in the intracellular compartmentation of herbicides,

insensitivity to target sites, reduced entry of herbicides and translocation of herbicides and overproduction of target sites (Guan et al. 2015). According to the GM Science Review Panel, "there have been detailed field experiments in a variety of environments on several GM crops in a range of environments have demonstrated that the transgenic traits do not significantly increase the fitness of the plants in semi-natural habitats". Resistance to disease or pests are characteristics that could give weeds a fitness advantage and could have negative environmental penalties, but the possibility is little as present evidence shows. Current evidence is insufficient to determine this probability and more experimental investigations and field surveys are needed. The hybrid progeny had limited fitness advantages in the case of reduced ambient selection pressure of selective insects in Bt/CpTI GM rice in the intensive cultivated agricultural area (Yang et al. 2012). The herbicide resistance transgene from GM soybean to its wild counterpart (i.e. glycine soy) can still persist with zero herbicide selection pressure, escaped herbicide resistance transgene from GM soybean to its wild counterpart (i.e. Glycine soja) can still persist in nature (Guan et al. 2015). A notable case of amaranth (Amaranthus palmeri; cotton weed), first reported in Georgia in 2004, spread to 76 countries in the next 7 years (Gilbert 2013). This report also revealed that after release of many RT crops since 1996, 24 glyphosate tolerant weeds have been identified. Interestingly, from 1996 to 2011, PG Economics reported an 8.9% improvement in the environmental impact quotient. WeedScience (Ondreickova et al. 2014) published a chronological increase in resistant weeds on a global scale from 1955 to 2014. The report describes that around 145 plant species have become resistant to eight herbicide groups including acetolactate synthase (ALS) inhibitors, triazines, Acetyl-CoA Carboxylase Inhibitors, synthetic auxins, bipyridiliums, glycines, ureas, amides, and dinitroanilines. Current GM crops undergo the most extensive risk assessment studies so that the likelihood of invasiveness of these crops tolerant to herbicides in natural or agricultural habitats could be speculated (Dale et al. 2002). Although the risk of pervasiveness or invasiveness is considered relatively low, there are possible biological changes that could lead to weediness, such as tolerance to extreme temperature regimes, water and soil salinity, changes in the characteristics of seed propagation and dormancy, and the introduction of pest or pathogens resistance (Mertens 2008). In response to competition, an increase in the fitness of a cropwild hybrid was reported in wild sunflower hybrids and most importantly to the application of the herbicide (Mercer et al. 2014). However, the competitive fitness of susceptible and resistant common cocklebur against acetolactate synthase was not significantly different suggesting that case-by-case risk assessment studies are needed before approval of any GM crop for commercial cultivation (Crooks et al. 2005).

4.4.8 Chemical Toxicity

Plants naturally use toxins to fight threats such as pests and pathogens. Such chemicals cause biotic and abiotic environmental factors toxicity. Toxins such as glycoalkaloids, ricin and endotoxins from delta are of greater risk and are thoroughly

investigated. In most GM plants, Bt delta endotoxins were targeted and the effects of their proteins on the environment and friendly organisms were extensively studied (Yu et al. 2011). Bacteria are the most common sources of transgenes, while fungi, plants, animals and humans are also used as sources of different transgenes. Transgenes are used for plant codon from these hosts are used either for plant codon usage or for direct molecular evolution (so called molecular breeding) (Keese 2008). Direct gene transfer expresses the desired proteins in the recipient organism, while numerous parental genes are fragmented and reassembled through molecular breeding in order to express new proteins that are not present in nature. In Escherichia coli, for example, a new carotenoid was expressed by shuffling DNA coding for a pair of enzymes involved in the pathway to carotenoid biosynthesis (Schmidt-Dannert et al. 2000). There are therefore risks associated with natural and novel toxins in the body of the plant. Natural toxins could be assessed on the basis of certain developed models. However, new toxins can affect life both target and non-target. We are concerned with the risks from both natural and novel toxins. In some negative interactions, engineered toxins responsible for growth or stress resistance could have unintended effects on the ecosystem. The environmental impact of herbicide tolerance toxins and resistance to insects/pests is analyzed below.

4.4.9 Herbicide Toxicity

The risks of herbicide toxicity can be regarded as a qualitative estimate, including the possibility and severity of immediate or delayed adverse effects on the environment, human health and the economy of the farmer. However, there are some factors associated with the probability and severity of each toxic effect, such as crop and characteristics, local weed flora, farm management practices and climatic conditions (Madsen et al. 2002). The cultivation of herbicide-tolerant GM crops is associated with potential threats to farmland and wild habitats. Eighty percent of transgenic crops grown in laboratories or in commerce have transgenes expressing glyphosate, glufosinate and glyphosate tolerance and/or stacked with insect resistance. There is also the possibility of toxicity to other forms of life in addition to toxicity to plants themselves. Johal and Huber (2009) explained in detail the direct weakening and increased pathogen virulence of plant defense induced by glyphosate. Glyphosate inhibits the defense and structural barriers of the plant and immobilizes micronutrients such as manganese (Mn), which play a key role in disease resistance. The metabolism of plant nitrogen is modified in response to applied glyphosate in a manner similar to changes caused by high temperatures. By modifying the nitrogen and carbohydrate metabolism, the transient resistance of soybean and wheat rust was reduced. Some reports confirmed lethal effects of roundup on amphibians, larval amphibians, fish, tadpoles, snails, insect predators, small arthropods, fungi and bacteria (Relyea 2005). There was almost a complete mortality (96–100%) rate of post-metamorphic amphibians and North American tadpoles in response to direct application of roundup (Relyea 2005). Even, concentrations below environmental protection agency (EPA) levels harmed Pacific Northwestern Amphibian larval

community when exposed to 0–5.0 mg dilutions (King and Wagner 2010). Herbicide stratification was directly linked to temperature stratification and implicated the habitat choice in ectotherms (Jones et al. 2010). Application of roundup on rice has proven the increase of mortality in water weevil (Lisorhoptrus oryzophilus) in terms of 20% reduced larval incidence on herbicide treated rice (Tindall et al. 2004). Liver congestions, necrosis (2.5–5.5 times higher) and sever nephropathies (1.3–2.3 times higher) was found in male Sprague-Dawley rats fed with roundup applications in drinking water and GM maize diet (DKC 2678 R-tolerant NK603) for 2 years. The noticeable point is that even lower concentration than field application rates was also tested and found to be of concern. In the case of female rats, mortality increased two to three times and pre-mature death was observed whilst, mammary tumors appeared more frequently (Seralini et al. 2014). Antimicrobial activity of glyphosate and glufosinate is another rising concern (Samsel and Seneff 2013) as Kruger et al. (2013) clearly stated that glyphosate disrupts intestinal bacteria in cattle and poultry. Some scientists suggested altered defense response of plants against microflora (Benbrook 2016). Increase in bacterial biomass, enhanced activities of urease, alkaline phosphatase, and invertase have been observed in the rhizosphere of Basta-tolerant oilseed rape grown with the application of Basta (glufosinate) and Butisan S (metazachlor) depicting that GM plants and applied herbicides modify activities of the associated microflora (Sessitsch et al. 2005). Decreased activity of *Bradyrhizobium japonicum* (a nitrogen-fixing bacteria), *Azotobacter chroococcum, A. vinelandii* and entomopathogenic bacteria have been reported (Morjan et al. 2002). Such decreased activities of microorganisms especially of nitrogen-fixing bacteria indirectly reduced soybean yield by 8–10% because of inhibition of nodule formation, reduced nodule biomass and reduced nitrogen fixation (King et al. 2001). Alteration of Cytochrome P450 raised another affiliated risk of glyphosate use. Suppression resulted in a synergistic effect with intestinal bacteria and disrupted aromatic amino acid biosynthesis and could be a pathway to many modern diseases (Samsel and Seneff 2013). Apart from such effects on other life forms, the health of GM plants itself is another issue. Frequent application of glyphosate could possibly increase the susceptibility of crop plants by increasing the incidence of microflora in the rhizosphere. For example, Fusarium solani was reported to have higher incidence after glyphosate application (Njiti et al. 2003). Increased disease severity is a common hypothesis among plant pathologists in terms of weakening plant defense mechanisms and increasing the population of casual organisms. This can be indirectly linked to the immobilization of disease-related micronutrients, impeded plant growth, altered physiology and changes in soil microflora behavior (Johal and Huber 2009). In response to GM crop cultivation and cultural practices, Kremer et al. (2005) documented that microbial components of GM soybean and maize rhizospheres have been altered. In a comprehensive review, Duke et al. (2012) concluded that the balance of minerals in herbicide tolerant plants is not significantly affected and disease incidence is negligible after using glyphosate and the fact that current amount of evidence in insufficient in this context. The reduced levels of aromatic amino acids, i.e. phenylalanine and tyrosine in RT crops, resulted in a reduced effectiveness of the plant defense mechanism against abiotic stress and

pathogens (Benbrook 2012). The continuous use of herbicides causes a differential expression of transgenes in specific tissues, such as cotton, in which reproductive tissues have higher glyphosate concentrations (Pline et al. 2002). If a plant part with a higher accumulation of glyphosate is used for food or feed, the health risk to humans and animals will increase depending on the part of the genetically modified plant to be consumed and the level of expression of the transgene in that part of the plant. Bohn et al. (2014) investigated compositional differences in GM soybeans and reported high residues of glyphosate and aminomethylphosphonic acid in glyphosate tolerance GM soybeans. Young et al. (2015) presented a detailed report on the role of glyphosate in human endocrine disruption and cytotoxicity to human cells. Such increased concentrations will also affect pollination problems in the plant itself, reduced pollen viability, retention of bolls and abortion of bolls (Pline et al. 2002). In addition to direct toxic effects, the indirect effects of herbicide tolerance include disturbed biodiversity of weeds, arthropods inhabiting weeds, parasitoids, predators and decomposers, which may lead to disturbances in symbiotic relationships, a decrease in the population of beneficial insects and rapid changes in the food chain of agricultural land (Schutte and Schmitz 2001). In conclusion, the cultivation of GM crops with resistance to herbicides, influences host plants and non-target soil life, weeds and farmland biodiversity depending upon the degree of adoption. Despite extensive laboratory, greenhouse and farmland studies, there are still significant gaps in knowledge about the potential induced toxicity of herbicides. For more information on the toxicity of mammalian herbicides based on glyphosate, see Mesnage et al. (2015). Glyphosate will prevail in the coming years as the herbicide of choice worldwide and the quantification of its effects on human health and ecological consequences will thrive (Benbrook 2016).

4.4.10 Insecticide Toxicity

In the development of a resistant GM plant, the most challenging consideration is to identify a resistance gene and direct its product to appropriate plant tissues so that it targets only the pest without any side effects on friendly organisms. Apart from proteinase inhibitors, α-amylase inhibitors, avidin, chitinases and lectinases, Bt delta endotoxins are the most important examples of engineered insect resistance (Dale et al. 2002). Previously, toxin-based bacterial formulations were used to directly spray targeted insects. Preferences have been shifted to the expression of toxins in transgenic plants, which seemed to be relatively efficient and safe at eliminating insect pests (Schutte and Schmitz 2001). GM plants produce toxins throughout their lives, but sprayed formulations are used for a certain period of time. Although the Bt toxins expressed differ from natural toxins, less specific but sprayed natural toxins are rapidly disintegrated in natural conditions. The marketing of GM plants expressing Bt toxins has been rapidly adopted by the farming community and the area of GM plants is increasing every year, so that a broad community of researchers is questioning the ultimate potential target and non-target impacts of transgenic toxins. Many laboratory studies have been carried out to answer the

question: "Does Bt toxins kill monarch butterflies? Well, the answer to the question is inconsistent. The first report on the mortality of monarch butterfly caterpillars in response to pollen from commercial Bt maize has shown that Bt toxins pose a potential risk to non-target life forms (Losey et al. 1999). This has been followed by numerous studies that have also reached agreement on toxicity concerns raised by Losey and colleagues (Obrycki et al. 2001). However, later investigations concluded that toxicity to the host plant and non-target species depends on a variety of factors such as pollen, weather conditions, local fauna and flora, alternative host species for non-target insects, event of transformation, promoter, level of expression of toxin, the tissue of GM plant where transgene is being expressed, likelihood of exposure and routes of exposure (Fontes et al. 2002). The hazards of Bt and other toxins on lacewings, earthworms, herbivores, honeybees, human fetuses are reported in numerous farms and laboratories (Aris and Leblanc 2011). In response to Bt-maize pollen, no significant risks were associated with larval survival and the prepupal weight of honey bees. Delayed growth and reduced weight gain were observed in herbivores feeding on sublethal doses of Bt (Agrawal 2000). Higher mortality, reduced egg production and a lower proportion of females reaching maturity were observed in Daphnia magna; a crustacean arthropod, when fed with Cry1Ab maize (Dekalb 818 YG) (Szenasi et al. 2014). Bt doses could then possibly affect tri-trophic interactions (i.e. plant-herbivores-their natural enemies) in synergistic, additive, or antagonistic ways. Effects of Bt toxins on other trophic-levels including vertebrate predators preying on lepidopteran pests are yet to be considered (Clark et al. 2005). The presence of Bt toxins in aphid (Myzus persicae) samples detected by a double enzyme-linked immunosorbent test confirmed the potential effects of these toxins on food chains and trophic levels of natural herbivore enemies (Burgio et al. 2007). In contrast, many researchers reported that non-target species were not toxic due to shorter persistence or degradation of Bt toxins in the soil (Oraby et al. 2015). However, the combined effect of Cry1Ab and Cry1Acas is not inert, as well as in response to 1–200,000 ppm was confirmed. Cry1Ab concentration of 100 ppm resulted in the death of human embryonic kidney cells (Mesnage et al. 2012). Domingo (2000, 2007, 2011, 2016; Domingo and Giné Bordonaba 2011) reviewed the adverse effects of GM crops on health and summarized the published studies and reported that GM crops have the same health effects as their counterparts with few exceptions which clearly indicates that it is difficult to consider GM food and feed safe due to the presence of controversial experimental results. Given the chemical toxicity, we can conclude that most of the chronic and sub-chronic studies that have been carried out so far to test the toxicity of genetically modified (GM) organisms used as food and feed do not show any potential health effects (Domingo 2016), but have many limitations, such as the exposure period, which is too short to assess the long-term effects and endpoints. Classical toxicological studies do not take into account the whole area of interactions that may occur in real life exposure between genetically modified organisms and other chemicals exposed to humans every day, even at doses below or around regulatory limits, which could lead to synergistic and potential effects (Hernandez et al. 2013). In addition, these types of single compound studies do not focus on various types of long-term toxicity, for

which neurotoxicity, cardiotoxicity, nephrotoxicity, genotoxicity, hepatotoxicity and endocrine disruption are currently of particular concern. The fact is that the international regulatory authorities have also begun to recognize the need for this cumulative risk assessment and new methodologies are being developed, but only for commercial artificial mixtures (EFSA journal, 2013; Regulation 1272/2008/EC 2015). For non-commercial artificial mixtures that represent the real scenario of real life exposure, no regulatory provisions have been taken. As for pesticides and other chemicals to which consumers are exposed during their lifetime, it is also necessary for genetically modified organisms to pass from a single compound risk assessment to cumulative risk assessments, which threaten the long-term exposure to low doses of chemical mixtures, which simultaneously monitor different endpoints associated with the investigation of systemic mechanistic pathways such as oxidative.

4.4.11 Indirect Impact of Transgenes on Environment

The environmental impact of transgenic crops is evident in response to changes and changes in current agronomic practices or agricultural practices in general. Indirect effects of GM crops include soil, water, and biodiversity of wildlife and reduced weed, insect and pest control efficiency. The level of risk depends primarily on the nature of changes in agricultural practices (ICSU, GM Science Review Panel). Nevertheless, it must be decided whether the overall impact of such a modified use of pesticides has positive or negative prospects, but there are reports that establish the concept that changing agricultural practices have disturbed the habitat of the fauna and flora of farmland.

4.4.12 Effect on Soil and Water

Scientists and farming communities continue to debate the effects of the introduction of GM crops on groundwater and water reservoirs. This debate is directly linked to the extent and extent of the use of herbicides in GM crops. GM crops are known to be herbicide tolerant and invite wide-spectrum use of herbicides (Benbrook 2012). This increase in the use of herbicides was indirect, i.e. the replacement of more toxic herbicides that persist with glyphosate in the environment (Duke et al. 2012). There is a general decrease in the use of toxic herbicides and an increase in herbicides based on glyphosate (Benbrook 2016). Glyphosate is probably the world's most common herbicide. Glyphosate can reach the soil by direct interception of spray in early season or post-harvest applications, by removal or leaching of herbicide from vegetation and by exudation from roots or death and decomposition of plant material (Duke et al. 2012; Kremer et al. 2005). The addition of glyphosate to agricultural water and ultimately to aquatic ecosystems and their impact on aquatic life is evident. However, due to a shorter half-life compared to many other herbicides and strong adsorption to the soil matrix, the risk of glyphosate toxicity to non-target soil biota is often considered to be marginal. Zabaloy et al. (2016) showed

no negative effects on soil microbial communities in fields that were exposed to glyphosate. This study suggests that glyphosate use at recommended rates poses a low risk to microbiota (Duke et al. 2012). The antimicrobial activity of glyphosate is a matter of debate too, because large scale applications of glyphosate would certainly disturb microbial communities at farm scale (Samsel and Seneff 2013).

At the same time, the transfer of Bt toxins from GM crops to soil and water has many possible routes, including pollen deposition during anthesis, root exudates and residues from GM plants (Yu et al. 2011). There is evidence that Bt toxins bind to clay and humic substances to biodegrade proteins (Clark et al. 2005). Once the protein is bound to the particles of clay, its susceptibility to degradation decreases, as Stotzky (2004) observed, with a special reference to Cry1Ab, Cry1Ac and Cry3A in the root exudates of GM maize, potato, rice, canola and cotton. But the unintended effects of these proteins on organisms residing in the soil have not been consistent and have not been taken up by non-GM roots. Statistically, non significant pH levels under Cry1Fa2 GM maize were observed as compared to soils under non-GM maize (Liu et al. 2010a, b). Most studies have suggested that Bt proteins from transgenic plants break down relatively rapidly in the early stage after entering the soil and that only a small amount of them can remain for a long time period, so that Bt proteins do not bio-accumulate in soil (Yu et al. 2011).). However, the persistence of Bt toxins in the soil depends largely on the type of toxin and type of soil, not on the number of expressed transgenes (Rauschen et al. 2008). In South Africa (Bennett et al. 2003), as a result of less chemical pesticides being sprayed on cotton, demonstrable health benefits for farm workers have been documented.

4.4.13 Effect on Biodiversity

Widespread commercial cultivation of GM crops, in particular herbicide-tolerant crops, poses serious threats to the complexity of the ecosystem and biodiversity reduction. Contrary to yield loss and contamination, weeds are ecofriendly in a sense too; consider the reduction of soil erosion by weeds and provision of habitat to a range of beneficial organisms (Mertens 2008). Studies have also shown that, contrary to conventional systems, the diversity, density and biomass of the seed bank in farmland are obviously lower in GM systems (Bohan et al. 2005). UK Farm Scale Evaluations (FSE) reported a reduction in weed seed banks of 20–36% (Andow 2003). However, the report found that weeds of dicot were more susceptible than monocots). Rapid changes in habitat destruction will have a significant impact on changes in food webs and food supplies. In addition to the impact on beneficial organisms, the balance of the predator-prey systems becomes even more critical. Of course, this will not result in disturbed tri-trophic interactions and symbiotic associations leading to complicated disruption in the food web. It is clear that such disturbances in the management of weeds, insects and pests will result in increased use of pesticides (Schutte and Schmitz 2001). In most cases, this change

in accessibility of resources has knock-on effects on higher trophic levels. The free-style foraging behavior can also be adapted by the frequent application of herbicides as in the case of glyphosate application where spiders moved to superfluous cricket killing behavior (Marchetti 2014). Other consequences are moving from herbivore to detritivore in the food web. The application of glyphosate resulted in an increase in fungal biomass in relation to bacterial biomass, which paves the hypothesis of a change in the food web on the basis of slower nutrient turnover and harnessed enrichments; based on resources of the carbon and nitrogen ratio (C: N ratio) (Powell et al. 2009). Types of herbicides and insecticides used, degree of adoption, frequency of use, timing of application of herbicides or insecticides, target crops, rotational and agronomic practices, local fauna and flora, alternative hosts for friendly insects, microclimate conditions, history of management and surrounding habitats (Merte) The emigration of agrobiont wolf spider (Pardosa milvina) was reduced when Baccaneer ® Plus (glyphosate) was used to indicate that there is a disturbance in the predator-prey relationship in food webs across the eastern United States (Wrinn et al. 2012). Bt crops were also questioned for their potential threats to biodiversity in parallel with herbicide-tolerant GM crops. Pesticides are often transported beyond crop fields and can have a significant impact on land and aquatic ecosystems or plant populations near crop fields. Most prominent targets are mammals and birds, and many studies have shown little or no evidence of Bt toxicity (Flachowsky et al. 2005; Aris and Leblanc 2011). In a broader sense, it can be concluded that the cultivation of HR GM crops has a negative impact on biodiversity (Bohan et al. 2005). The discussed dangers to biodiversity could be possibly observed on a long-term basis and of course, risks could not be left out of the equation. However, one short-term food web assessment (a 2-year investigation) in response to the cultivation of GM maize revealed the presence of stable and complex food webs and their persistence was not compromised. The study included GM maize having resistance against Coleoptera, Lepidoptera and glyphosate and mainly focused on arthropod food webs with an experimental population of 243,896 individuals (Szenasi et al. 2014).

4.4.14 Reduced Efficiency of Pest, Disease and Weed Control

The effects of changes in agronomic practices in response to the introduction of GM crops are few of the frequent crop swooping, increased use of broad-spectrum herbicides and increased impetus for minimal cultivation/zero-tillage agricultural systems (Dale et al. 2002). Of course, there are many advantages associated with changed agricultural practices such as soil erosion, less disturbance to earthworms and minimal disturbance to the microclimate of the soil, especially in the case of zero laying. In contrast, many indirect risks are also associated, such as the development of RT weeds, weed population shifts, cross-resistance development and multiple resistance and resistance to Bt toxins.

4.4.15 Evolution of Herbicide Resistance

The appearance of RT weeds is inevitable because weed species have a remarkable ability to develop herbicide tolerance in the weed gene pool (Agapito-Tenfen et al. 2014). Weeds can develop herbicide resistance in approximately 3 years, as polygenic herbicide resistance was reported in the progeny of F1, F2 and backcross in the case of low doses of diclofop methyl (Busi et al. 2013). Increased prominence of Asian dayflower (Commelina cumminus L), wild buckwheat (Polygonum convolvulus L) and common lambsquarters (Chenopodium albus L) were observed where there was significant selective pressure due to the concomitant use of herbicide and frequent cultivation of herbicide-resistant crops (Owen and Zelaya 2005). In the development of tolerance, different mechanisms could help the plant, such as target site over production, modification of intracellular herbicide compartmentation, minimal absorption and translocation of herbicides, herbicide detoxification and insensitivity to the target site (Brower et al. 2012). Although the probability of target-site resistance to a single herbicide is relatively low, but not negligible, i.e. one person in 10–5 to 10–10, while the frequency is nearly half when multiple-target site resistance is considered (Mortensen et al. 2012). As of February 2016, a total of 467 unique cases of RT weeds from 249 species (144 dicots and 105 monocots) have been recorded globally. These 249 species are resistant to 22 of the 25 known action sites for herbicides and 160 other herbicides (http://www.weedscience.org). The resistance of glyphosate and glufosinate from the weed gene pool is highly unlikely, mainly due to its chemical structure, no residual activity, limited glyphosate intake from soil plant roots, mode of action and persistence of near-zero soil (Baylis 2000). Few reports of the development of glyphosate resistance are annual rye grass in Australia and horseweed in the United States (Dale et al. 2002). This development of resistance may be over-expression of the target enzyme, reduced translocation of herbicides and different sensitivity of the target enzyme to glyphosate (Wakelin et al. 2004). Many independent evolutionary events could simultaneously interact with the emergence of herbicide resistance on a large geographical scale (Bonny 2016). Regular use of glyphosate in a significant proportion of GM crop fields makes the assumption of the development of glyphosate resistance a reasonable assumption. Weeds are not a poorer competitor than susceptible weeds, as no fitness difference between susceptible and resistant Lolium rigidum biotypes was detectable (Busi et al. 2013). Conclusively, although the development of biotypes of resistant weeds, the development of cross and multiple resistance and the shift in weed populations is inevitable, delay strategies for development could be strategies could though comprehend the herbicide resistance development (Schutte and Schmitz 2001).

4.4.16 Evolution of Insecticide and Pesticide Resistance

It has been shown that the control of pests by conventional and chemical techniques is challenging, as insecticide and pesticide resistance have developed in many cases

(Dale et al. 2002). In particular, due to the constitutive expression of Bt toxins in all plant tissue, the possibility of evolution of Bt-resistant insect pests cannot be negated imparts higher selection pressure on target species (Yu et al. 2011). The use of Bt bio-pesticides by organic farmers in Central America, Florida, Japan, the Philippines, Hawaii and China (Tabashnik et al. 2005, 2013). Gassmann et al. (2014) reported that Bt maize with a higher dose of toxin offers a higher selection pressure on the western corn rootworm, resulting in the development of cross-resistance between maize Cry3Bb1 and maize mCry3A. The selection of resistance to Bt toxins European maize borer, pink bollworm, cotton bollworms was also reported in many laboratory studies. The intensity of selection is an important driving force in determining the evolution of resistance, the size and arrangement of shelters, the mating behavior of insect pests, seasonal changes in habitat and population regulation by insecticides in GM crops and shelters (Caprio 2001). A decade-long report by Tabashnik et al. (2005) explained the presence of recessive alleles of the gene of cadherin (BtR) in the resistant strains of pink bollworm (Pectinophora gossypiella) associated with Cry1Ac resistance. Griffitts et al. (2001) reported a different resistance mechanism in nematodes and lack of encoded protein by bre-5 (a putative ß-1, 3-galactosyltransferase) in the Caenorhabditis elegans intestine resulted in no binding leading to resistance to the Bt toxin Cry5B. Tabashnik et al. (2013) surveyed 77 reports of developments in pest resistance to Bt toxins from five continents and confirmed the resistance of Bt toxins to fiel in 5 of 13 species. EPA has proposed two strategies to delay the evolution of resistance, i.e. high dose of toxins and high dose refuge (www.epa.gov). Dale et al. (2002) has also proposed a gene pyramiding strategy that delays the development of resistance in a much more effective way. Contrary to the development of resistance to Bt toxins and insecticides, the development of pathogen resistance is quite high because viruses, bacteria and fungi are known to adapt to selective forces very quickly. In principle, it is easy to overcome single gene-based resistance mechanisms. In addition, there have been frequent mutations in avirulence (Avr) genes of bacteria and fungi, so that resistance can be overcome through the integration of the corresponding resistance (R) gene. Other delaying strategies based on developments in the field include low initial frequency of resistance alleles, recessive inheritance, abundant refuge populations and the use of two-toxin Bt crops instead of single-toxin Bt crops (Tabashnik et al. 2013).

4.5 Safety Assessment of Genetically Engineered Crops

The modified crop is substantially equivalent to the unmodified parent, with the exception of one or a limited number of identifiable characteristics (such as the presence of proteins confering insect resistance or herbicide tolerance) resulting from the genetic change. In these circumstances, the safety of the new feature is sufficient to conclude that the modified crop or the food/feed products derived from it are safe. In most cases, the new feature was the presence of a specific protein and the safety considerations were addressed by determining the safety of this protein.

4.5.1 Protein Safety Evaluation

Protein safety assessment will be based on its structure, function, bioavailability, specificity and potential allergenicity. Proteins in the diet are generally not considered to pose a significant risk to human health, since proteases destroy almost all proteins ingested in the digestive tract. However, some adverse effects associated with proteins must be taken into account and specific safety assessment strategies must be taken into account. For example, proteins are some of the most powerful toxins known to humans (Rappuoli and Montecucco 1997). The other main adverse effects associated with proteins are, in addition to acute toxicity the other main adverse effects associated with proteins are anti-nutrient effects (e.g. soybean trypsin inhibitors), effects on the immune system (e.g. lectins) and allergenicity (Taylor and Lehrer 1996).

4.5.2 Protein Allergenicity

With the development of GM crop plants, there has been an increasing interest in available approaches to confirm the lack of allergenicity of new gene products or otherwise (Kimber et al. 2000). In a comprehensive analysis carried out by the International Life Sciences Institute (ILSI), the Institute for Allergy and Immunology and the International Food Biotechnology Council, the hierarchical decision tree (Metcalfe et al. 1996) was proposed for the evaluation of the allergenic potential of food derived from GM crops and was further recommended by the joint FAO/WHO expert consultation. If the food in question is in this scheme, containing gene from a source needs to be considered allergenic then the immunological identity of the novel protein with allergens deriving from the source material is determined. The purpose here is to protect those who have already been sensitized from accidental exposure to allergens induced. An example of the successful usefulness of this approach is the study of modified soybean expressing the storage protein Brazil nut 2S. Sera was found to contain IgE antibody reactive with 2S protein from eight of nine subjects with confirmed Brazil nut sensitization (Nordlee et al. 1996). However, if the protein of interest is a product of a gene derived from a source that is not normally associated with allergies, or if human consumption is not widespread, an alternative strategy is recommended. This is based on sequence homology considerations with known allergens and of protein stability (Gendel 1998). The homology of the linear sequence of eight or more contiguous amino acids between the test protein and one or more known human allergens (based on the minimum length of the peptide for immune recognition) is indicative of a sufficient immunological identity. If this linear sequence homology is identified and/or if other structural similarities exist between the test protein and known human allergens, the immunological identity as described above should be investigated. The stability (digestibility) of the protein in a simulated gastric fluid (SGF) containing the relevant protelotic enzymes is examined in the other approach (Astwood et al. 1996). The assumption is that rapidly digested proteins will not cause an immune response, and the

available data show that many food allergens are relatively resistant to SGF digestion (Astwood et al. 1996; Metcalfe et al. 1996). Since the correlations between the homology of sequences and stability are part of an overall safety assessment, the nether approach provides direct evidence of allergic potential. A number of laboratories are therefore developing suitable animal models (Atkinson et al. 1996). A recent review has resulted in a proposed amended strategy to predict allergens (FAO/WHO 2001).

4.5.3 Requirement for Animal Studies

If the food characterization indicates that the available data are insufficient for a thorough safety assessment, testing of animals may be considered necessary. This would be especially the case if the food is expected to make a significant dietary contribution, if the gene product is stable and if there is no history of consumption, or if the change affects several metabolic pathways. The studies should be designed to address specific safety aspects relating to the difference between transgenic and parental crops or their derived foods. The aim is to ensure that after prolonged consumption of GM crops and their derived foods, there is no concern for adverse health effects for humans or animals. Where toxicology studies are considered necessary to assess the safety of long-term food consumption in the diet, it is generally considered that a subchronic study of 90 days is the minimum requirement to demonstrate the safety of repeated food consumption in the diet. This may need to be preceded by a short-term pilot study to ensure that the diet is suitable for the test species and that the incorporation level of the test item is appropriate. The highest dose level used in any animal study should be the maximum possible without causing a nutritional imbalance, while the lowest level should be comparable to the expected intake of humans. The need for additional toxicological tests should be taken into account case by case, taking into account the results of the 90-day study and other studies. For example, proliferative tissue changes in the 90-day study may indicate the need for a long-term study of toxicity (FAO/World Health Organization 2000). In addition to animal studies specifically designed for safety assessment, nutritional or health tests can be carried out to determine whether the food or feed product of the GM crop poses any nutritional problems compared to the unmodified parent crop (Hammond et al. 1996). These studies involve the administration of the food or feed product in quantities representative of anticipated use to an appropriate test species. The best species are normally those that consume food or feed or can be selected due to particularly high growth rates, which would lead to an increased sensitivity to any nutritional problems. Studies would typically last 28 or 90 days and the end points are generally indices of growth and nutrition, such as food consumption, general condition, weight gain, yield and composition of milk (cattle), performance of laying (hens), or efficiency of food conversion and body composition (fish). Observations in some studies would also include simple pathological endpoints such as carcass quality and organ weights and their postmortem macroscopic appearance. Although these studies should not be confused with toxicology

studies, as they are not necessarily optimal for safety assessment, they provide useful data.

4.6 Viral Resistant Crops

Viral diseases are a major threat to crop production throughout the world. In developing countries, the problem is exacerbated, particularly in tropical areas where crop-free seasons are rare and plants are constantly under pressure from viruses transmitted by vectors from both cultivated and wild plants (Fargette et al. 2006). Africa suffers from major pandemics and epidemics of recurrent plant viruses in important crops. Known viral diseases caused by DNA viruses include cassava mosaic disease (CMD) caused by begomoviruses (members of the Geminiviridae family) (Zhou et al. 1997), banana bunchy top disease caused by babuvirus (member of the Nanoviridae family) (Blomme et al. 2013) and maize streak disease caused by maize streak virus (MSV), a mastrevirus (a member of the family Geminiviridae) (Thottappilly et al. 1993). Cassava brown streak disease is caused by two closely related RNA viruses (members of the genus Ipomovirus, family Potyviridae) (Alicai et al. 2007). In eastern Africa, maize suffers from lethal necrosis caused by co-infection by two RNA viruses, maize chlorotic mottle virus (a member of the genus Machlomovirus, family Tombusviridae) and the potyvirus sugarcane mosaic virus (a member of the family Potyviridae) (Mahuku et al. 2015). Sweetpotato virus disease (SPVD), the most devastating disease of sweetpotatoes, is also caused by co-infection by two RNA viruses, the crinivirus sweet potato chlorotic stunt virus (SPCSV, a member of the family Closteroviridae) and the potyvirus sweet potato feathery mottle virus (Karyeija et al. 2000).

Natural sources of resistance to many tropical viral diseases are known and used in breeding. However, resistance sources are often lacking, or the genetic complexity and difficulties of introgressing resistance genes to cultivars by crossing hamper crop enhancement efforts. The development and transfer of crop resistance by biotechnological means is therefore an attractive alternative. Powell Abel et al. (1986) studies have shown that the transformation of tobacco plants (*Nicotiana tabacum* L.) to express the tobacco mosaic virus coat protein has made the plants resistant to the virus. The transformation of plants into non-structural viral proteins (Golemboski et al. 1990) and truncated defective viral genes also protected against homologous viruses (Anderson et al. 1992). These findings have created a great deal of excitement and hope for a quick solution to problems with viral diseases in crop plants. With the discovery of post-transcription gene silencing (i.e., RNA silencing or RNAi), the resistance mechanism in genetically engineered plants became understandable as binary vectors expressing virus-specific inverted-repeat (hairpin) RNA to target the virus to degradation by RNAi (Waterhouse et al. 1998).

It was later found that some viral proteins suppress or interfere with antiviral RNAi (Anandalakshmi et al. 1998) and resistance derived from the virus could fail if the plant was infected with a virus that differed >15–20% at the sequence level from the donor of the transgene (Savenkov and Valkonen 2001). The use of chimeric

transgenes from pieces of genomes from viruses expected to infect the crop can overcome this problem (Chung et al. 2013). In developing countries, considerable efforts have been made to develop resistance to viruses affecting crops relevant to agriculture and food production. In addition to the above, the main target crops have been potato (*Solanum tuberosum* L.) (Orbegozo et al. 2016), tomato (*Solanum lycopersicum* L.) (Fuentes et al. 2006), peanuts (Arachis hypogaea L.) (Magbanua et al. 2000), sugarcane (Saccharum officinarum L.) (Ingelbrecht et al. 1999), peppers (Capsicum annuum L.) (Lee et al. 2009), rice (Oryza spp.) (Shimizu et al. 2009), papaya (Carica papaya L.) (Ferreira et al. 2002), passionfruit (Passiflora edulis Sims) (Trevisan et al. 2006) and soybean (Glycine max (L.) Merr.) (Reddy et al. 2001).

4.6.1 Transgenic Resistance to Potato Viruses

After rice and wheat, potato is the world's third-most cultivated food crop. The climate in highland areas is well suited to potatoes and Africa has grown rapidly. Root crops such as potatoes and sweet potatoes are also expected to be less affected by climate change than many other subsistence crops (Adhikari et al. 2015). The most common and devastating potato viruses in the world are potato viruses Y (PVY, a member of the genus Potyvirus; family Potyviridae), potato leafroll virus (PLRV, a member of the genus Polerovirus; family Luteoviridae) and potato virus X (PVX, a member of the genus Potexvirus; family Alpha flexiviridae). The PVY and PLRV transmitted by aphid may cause significant yield losses on their own. The PVX transmitted by contact becomes significant with PVY co-infection, which induces synergistic viral interactions leading to high PVX accumulation. In potato cv, virus-derived resistance was developed to PVY and PVX in order to solve this problem. Russet Burbank, which represents only the second example of genetically engineered resistance to the virus in crop plants (Kaniewski et al. 1990). In the late 1990s, "Russet Burbank" was also designed to resist PLRV, PVY and Colorado potato beetle (Leptinotarsa decemlineata Say) and approved for marketing under the name NewLeafTM (Lawson et al. 2001). Potatoes are propagated clonally and are therefore prone to viral infections over generations. In industrial countries, potato viruses are controlled by the planting of certified seed potatoes without viruses produced under special cultivation schemes. In combination with control of the aphid vectors using pesticides, this has reduced the prevalence of PLRV in the last 30 years. However, potato viruses are common in low-income countries and losses are severe, as healthy seed potatoes and pesticides are not frequently available or affordable (Valkonen et al. 2015). The introduction of virus resistance genes into new cultivars of potatoes is demanding and time consuming because of the highly heterozygous outcrossing and polyploid nature of potatoes. This combination of factors makes transgenic approaches to virus resistance especially appropriate for potato, as well as other major clonal crops such as bananas, cassava and yam (Dioscorea spp.).

Resistance to PLRV in "NewLeaf" reached commercial production in the USA, but the engineered variety only remained on the market a few years before it was

withdrawn due to the decision of major potato processing industries to refrain from the use of transgenic potatoes (Thornton 2003). The demand for "NewLeaf" was not high in the US market, as clean seeds can be purchased every year and pesticides are affordable, providing alternative means of controlling viruses. However, in low-income countries, virus-resistant potato varieties would be of great importance to prevent yield losses, as farmers rarely renew their seed potatoes. Resistance to both primary and secondary infections with PLRV has been achieved using efficient inverted repeat hairpin constructs (Orbegozo et al. 2016). High levels of resistance in transgenic plants expressing such hairpin constructs have also been obtained against PVY, PVX and the aphid-transmitted potato virus A (Missiou et al. 2004).

4.6.2 Viruses in Common Bean

Common beans (Phaseolus vulgaris L.) and vegetable crops such as tomatoes, peppers and cucurbits (Cucurbita spp.) are worldwide damaged by begomoviruses transmitted by whitefly (Leke et al. 2015), especially in Latin America (Morales and Jones 2004). The bean golden mosaic virus (BGMV) and the related bean yellow golden mosaic virus are one of Latin America's greatest constraints in bean production. The Brazilian Agricultural Research Corporation (EMBRAPA) was able to produce a transgenic line of common beans with high and stable levels of resistance to BGMV after almost two decades of work (Aragão and Faria 2009). In 2011, the EMBRAPA 5.1 transgenic line was approved for cultivation in 2011, and field trials for registration of several new cultivars developed from EMBRAPA 5.1 by breeding were initiated in 2012 (Faria et al. 2016). The resulting resistance is expected to allow the recovery of bean production in areas affected by BGMV, increase yields and quality, and reduce the need for vector control pesticides in Brazil. Due to differences between viruses and virus strains, these transgenic lines may not necessarily confer resistance to bean-infecting begomoviruses in other parts of the world. However, the approach to bean varieties that are resistant to the main begomoviruses found in other developing countries can be applied using an inverted repeat construct aimed at the viral replicase gene and a highly efficient transformation system.

4.6.3 Transgenic Virus Resistance and Impact on Low-Income Countries

Although no genetically engineered plants for virus resistance have been approved for cultivation in low-income countries, many efforts are ongoing, especially in Africa, where recurrent epidemics of viruses are a major constraint to crop production. These efforts are primarily focused on major staple and food safety crops such as manioc, sweetpotato, banana, rice and maize.

4.6.4 Cassava Mosaic and Cassava Brown Streak Diseases

As a subsistence crop in Africa, manihot esculenta Crantz is very important. The epidemic of manioc mosaics began and spread rapidly in East Africa in the mid-1990s, devastating manioc crops in many regions. The disease increased when African cassava mosaic virus and virulent recombinants co-infected cassava plants with other begomoviruses transmitted by whitefly, resulting in synergy, very severe symptoms, growth retardation and new virulent recombinants of the viruses (Zhou et al. 1997). More mosaic-resistant manioc germplasm was introduced to breeding programs in West Africa, and the new varieties eventually slowed the epidemic remains, and new means offered by biotechnology are being used in resistance breeding (Bart and Taylor 2017). As a subsistence crop in Africa, manihot esculenta Crantz is very important. The epidemic of manioc mosaics began and spread rapidly in East Africa in the mid-1990s, devastating manioc crops in many regions. The disease increased when African cassava mosaic virus and virulent recombinants co-infected cassava plants with other begomoviruses transmitted by whitefly, resulting in synergy, very severe symptoms, growth retardation and new virulent recombinants of the viruses (Zhou et al. 1997). More mosaic-resistant manioc germplasm was introduced to breeding programs in West Africa, and the new varieties eventually slowed the epidemic started in Tanzania, Uganda and Kenya in the mid-1990s (Alicai et al. 2007). Local resistance breeding programs have increased tolerance to symptom formation in new cassava varieties, but resistance to the brown streak viruses has not yet been achieved. However, transformation of the Ugandan farmer preferred cassava cultivar TME with a virus-derived inverted repeat construct appears effective against both brown streak viruses (Fondong 2017), but inadvertently resulted in the loss of resistance of CMD by the CMD2 gene, apparently as an unexpected consequence of the somatic embryogenesis process involved in regenerating transgenic plants (Beyene et al. 2016).

4.6.5 Sweetpotato Virus Disease

Sweetpotato (Ipomoea batatas Lam.) originates in South and Central America, but plays a particularly important role in Africa as a subsistence crop (Valkonen et al. 2015). During the above-mentioned CMD epidemic in the 1990s, the importance of sweetpotato increased. Sweetpotato is a crop that is generally healthy, with only a few diseases. More than 30 viruses infect sweetpotatoes, but most cause mild or no symptoms and only minor losses in yield. The main disease is SPVD, which has severe symptoms of leaf malformation and stunted growth of plants. Diseased plants may not produce for consumption any tuberous roots.

In sweet potato plants co-infected with SPCSV transmitted by whitefly and virtually any other sweet potato virus, SPVD develops (Cuellar et al. 2015). Targeting SPCSV with pathogen-derived resistance using different genomic regions of SPCSV as transgenes significantly reduces the accumulation of SPCSV in transgenic sweetpotato plants, but other sweetpotato viruses break down resistance and

cause severe symptoms (Kreuze et al. 2008). Studies show that SPCSV's double-stranded-RNA-specific RNase III enzyme suppresses antiviral RNAi by cutting small interfering RNAs used to target viral degradation of RNA (Cuellar et al. 2009).

4.6.6 Banana Bunchy Top Disease

Banana bunchy top virus (BBTV) is the world's most destructive viral pathogen of bananas and plantains. It comes from Asia, has probably been introduced to Africa from the South Pacific (Kumar et al. 2011 and Jooste et al. 2016) and has now reached most areas of sub-Saharan Africa (Cuellar et al. 2015). BBTV is currently a major concern for food security, as infected bunches are difficult for smallholder farmers to recognize and will not produce any usable fruit. By targeting the viral replicase gene with intron-hairpin RNA transcripts, Shekhawat et al. (2012) were able to generate high levels of resistance to BBTV by targeting the viral replicase gene with intron-hairpin RNA transcripts. This approach was also effective against other Nanoviridae family members of the virus. The challenge in introducing transgenic resistance to various banana cultivars is the sterility of vegetatively propagated cultivars and the need to separately transform each cultivar, which is not a trivial task. Since banana cultivars are essentially sterile, it is unlikely that transgenic flow to other cultivars or wild Musa species will occur. Since it is difficult to control the number of copies and integration sites of transgenes, the use of modern genome editing technologies could allow the targeting of specific host genes (Dale et al. 2017), for example, those that play a role in the susceptibility of viruses.

4.6.7 Rice and Maize Viruses

The rice yellow mottle virus (RYMV, a member of the Sobemovirus genus) causes a major disease in rice, which is used as an example of the key role of agricultural intensification in the emergence of plant viruses (Pinel-Galzi et al. 2015), and the virus can overcome recessive resistance genes in rice germplasm (Pinel-Galzi et al. 2007). However, as early as 1998, highly efficient resistance was achieved in transgenic lines of African rice varieties generated by the expression of RYMV's open reading frame 2. The resistance remained stable for at least three generations and conferred resistance to a wide range of RYMV isolates (Pinto et al. 1999), since rice is seed-propagated, transgenic resistance trait can be introgressed into local varieties via crossing. However, to our knowledge, these lines never progressed beyond the proof-of-concept stage. MSV causes maize streak disease, which is a major constraint in Africa's maize production (Shepherd et al. 2007). For the protection of crops from MSV, dominant and recessive natural resistance genes are available. In addition, the expression of a defective form of a viral gene involved in viral replication (Shepherd et al. 2010) is available in transgenic maize plants with engineered resistance to MSV and was the first transgenic crop plant developed in Africa. There

is a better system of transgenic expression that is only activated by MSV infection (Shepherd et al. 2014). Maize (corn) mortal necrosis caused by co-infection with maize chlorotic mottle virus and another potyvirus, maize mosaic virus maize dwarf mosaic virus was described in USA, but is currently ravaging East-Africa and threatening to spread further across the continent. The disease can be controlled by transgenic resistance against maize dwarf mosaic virus (Murry et al. 1993), which could provide a solution.

4.6.8 Naturally Transgenic Virus Resistance

Natural transgenes recently corresponding to Agrobacterium spp's transfer of DNA (T-DNA). The sweet potato genome was found to be integrated, raising the question of its possible role in host defense or crop domestication (Kyndt et al. 2015). Agrobacterium T-DNAs are also found in other plant species (Matveeva et al. 2012), similar to transgenic plants created by the transformation of Agrobacterium. However, viral sequences integrated into plant genomes are more commonly found, which is comparable to plants transformed by particle bombardment and leads to a random integration of DNA into the plant genome.

The integration of viral sequences in a plant genome was first carried out in bananas carrying fragments of banana streak virus, a Para retrovirus with a double-stranded DNA genome encapsulated in bacilliform particles (Harper et al. 1999), and later in other Para retroviruses. Petunia vein clearing viruses, for example, and tobacco vein clearing viruses-such as sequences are integrated into petunia and many solanaceous crops. In woody plants such as grapevine and fig, viral integrations have also been observed (Laney et al. 2012 and Bertsch et al. 2009). Some of the integrated sequences can be reactivated and cause disease if stress or other exceptional conditions affect plants.

However, para retroviruses are mostly dormant and the host plants are rather resistant to them; indeed, retroviruses integrated into the plant genome may confer resistance to infecting homologous viruses (Chabannes and Iskra-Caruana 2013) The most likely resistance mechanism is the silencing of RNA induced against endogenous sequences as a method to control viral expression. Sequences of other viruses of DNA and RNA are also found in plant genomes (Chiba et al. 2011). The integrated sequence of cucumber mosaic virus in soybean is structurally similar to the constructs of hairpin RNA designed to induce target-specific RNA silencing and resistance to viruses (da Fonseca et al. 2016).

4.7 Next Generation Quantitative Genetics in Plants

For almost a century, scientists have used quantitative trait loci (QTL) analysis to dissect the genetic architecture of quantitative traits in plants (Fisher 1918). These analyze associate genetic markers with the phenotypic variation of a quantitative trait in a segregating population. History has consistently improved the

techniques used to obtain markers and physiological phenotypes (Montes et al. 2007). The fall in prices of high-performance technologies has allowed plant researchers to quantify the general abundance of transcripts, proteins or metabolites in segregated populations (Drost et al. 2010). These studies show that there are multiple benefits in using "omic" technologies for QTL analyses, even when the goal is to characterize physiological phenotypic diversity. Firstly, molecular phenotypes are the first step towards the production of physiological phenotypes and their regulation is based on a great deal of phenotypic diversity (Stern and Orgogozo 2008). Secondly, the availability of information across the genome significantly increases the ability to identify candidate genes for QTLs (Jimenez-Gomez et al. 2010). Third, molecular characteristics measured at the system scale allow the estimation of the effect of QTLs on genetic pathways of interest, or the identification of other genetic networks altered by the loci responsible for the variation (Kliebenstein et al. 2006). Finally, molecular characteristics provide researchers with a better understanding of how mutation drives physiological variation and what are the evolutionary forces acting at primary levels. So it is clear that HTS will be the choice tool for QTL analyses very soon. An important limiting factor remains to be removed: Analysis of data. It requires long and computer-intensive pipelines to be customized for each specific experimental setup. An increasing number of new algorithms are constantly being released to the community, and the debate on which pipelines deliver the most accurate results continues. Comparing, combining and customizing these pipelines requires simple Unix or Linux commands and benefits greatly from knowledge in powerful statistical software such as R and in languages such as Perl or Python. For non-bioinformaticians, integrated solutions with convenient interfaces are becoming popular both from collaborative open projects and companies (Goecks et al. 2010). Www.seqanswers.com is a popular website that keeps an updated list of available software tools, where users and developers also discuss new technological advances and pipelines. The majority of tools are developed for Linux or Unix-based systems in terms of the computer equipment required for HTS data analysis. Although parts of the analysis can be carried out on any modern computer, machines with dozens of gigabytes of RAM are recommended in cases where reference sequences are available for the species concerned or with hundreds if no reference is available. An alternative option that is likely to be popular is to rent storage and computer power in specialist centers or "the cloud" (Stein 2010). Due to the rapid improvement in HTS, this review only aims to capture a snapshot of the opportunities it offers for the discovery, genotyping and molecular phenotyping of molecular markers in the segregation of plant populations. The purpose of this review is to help researchers who have not incorporated this technology into their work to think about HTS requirements and options. This review does not refer to all available experimental designs or analytical tools, and the solutions proposed here are merely suggestions that will soon be replaced by new and better ones.

4.7.1 Library Preparation

Sample preparation protocols are continually improved to use less biological material, to be completed faster and to reduce their output bias. For example, most current protocols allow multiplexing samples by adding a short sequence tag to all readings in a library, which is a convenient feature given the increasing number of readings per HTS run. The same companies that developed the HTS sequencers market library preparation protocols that are optimized for the most common experimental designs. Other companies also have kits that provide comparable results and can be more cost-effective. Finally, many researchers develop customized protocols for specific information such as the transcribed strand in RNA-seq experiments, the rate of RNA degradation, or the positions occupied by RNA polymerases, just to name a few (Parkhomchuk et al. 2009).

4.7.2 Quality Control and Pre-Processing

Detecting biases in the base composition, base quality and complexity of the sample assesses the quality of HTS readings. The quality of the sequences affects the reliability of the analysis 'biological interpretations (Dohm et al. 2008). The sample preparation protocols introduce parts of these biases, in particular during the synthesis of cDNA in RNA-seq experiments (Hansen et al. 2010) and PCR amplification (Aird et al. 2011). Further biases are specific to each HTS technology (Smith et al. 2008) or to each sequencer run (Auer and Doerge 2010). It is usually necessary to pre-process the readings by trimming low quality nucleotides and adapter sequences. At this stage, foreign sequences such as vectors or DNA from organisms contaminating the samples can also be removed.

4.7.3 Molecular Marker Discovery

Depending on the type of library, further pre-processing may be required, such as poly A or poly T tails and tails for terminal transferase in RNA-seq libraries. When multiplexing several libraries, reads should be separated by their barcode. With basic scripts written in Perl (Bioperl), R (Bioconductor) or Python, both quality control and pre-processing can be done easily. There are some convenient tools for non-programmers that can perform all or some of these tasks (Goecks et al. 2010; Schmieder and Edwards 2011). A cost-effective solution for obtaining molecular markers is the sequence of DNA or RNA from parental genotypes and polymorphisms from the resulting readings. These polymorphisms can later be used to design PCR markers or a genotyping test of high-performance for the entire population. This approach works remarkably well in diploid and polyploid species with a sequence of as low as 5, i.e. five times the genome size (Geraldes et al. 2011). A recent article reviews available methods and tools for the identification and genotyping of single nucleotide polymorphism (SNP) (Nielsen et al. 2011). In order to

align the readings with the reference, mapping software based on "seed methods" is preferred despite its slower nature because of its polymorphic strength. Before SNP, users can consider removing readings from the map to multiple locations in the reference and duplicate readings that may have been generated from PCR artifacts. A recent pipeline also recalibrates the quality of nucleotides in readings to correct high error rates in HTS, and realigns readings in complex genomic positions where rapid processing algorithms may have failed (Depristo et al. 2011). Commonly used indicators of the veracity of polymorphisms are based on the quantity and quality of readings showing polymorphism, frequency of observed alleles, alignment quality and/or proximity to other polymorphisms. There are some basic and popular options for calling polymorphisms from aligned reads (Depristo et al. 2011), tools for analyzing reads from specific sequencing platforms (Souaiaia et al. 2011), that have the ability to detect structural variation (Chen et al. 2008), or that have into account the quality of the reference in addition to the quality of the reads. High-performance sequencing sequences can be used to build the necessary reference to identify molecular markers if they are not already available. Although it is possible to assemble de novo a complete genome sequence with HTS, very deep sequencing and extensive bioinformatic analysis are required, especially given the relatively large size of most plant genomes. Sequencing mRNA is a more efficient option, which greatly reduces the complexity of the sample compared to genome sequencing and has the advantage of providing functional information such as polymorphism coding or levels of expression (Wei et al. 2011). A comprehensive compilation of the transcriptome assembly methods and tools has been recently published (Martin and Wang 2011). De novo assembly algorithms benefit greatly from long and paired readings, but are extremely sensitive to errors and polymorphisms and will not perform well during the assembly of mixed genotypes or highly heterozygous people. As the number of reads increases, the amount of new genomic positions detected in RNA-seq experiments decreases exponentially. The majority of medium and highly expressed transcripts in a sample are detected at low coverage, and increased coverage will mainly add non-coding RNAs and low expressed transcripts at very high costs (Tarazona et al. 2011). If the aim is to assemble complete transcriptomes, the sequencing depth is preferred to obtain samples from various tissues, time points and conditions. Even under the best possible conditions, RNA-seq reads will return only a subset of existing transcripts, many of which will be fragmented. This is expected due to the low expression of specific transcripts, the non-uniform reading coverage and the presence of various isoforms per gene. Researchers can use normalization protocols that deplete the most abundant transcripts from the samples to help assemble low-expressed transcripts (Christodoulou et al. 2011).). In any case, contigs resulting from *de novo* assembly can be effectively used as a reference for molecular marker detection and characterization of transcripts in un-sequenced genomes (Kaur et al. 2011). When comparing highly similar genotypes, RNA-seq may not be the best option, as it primarily targets less diverse coding regions than non-coding regions. In these cases, researchers can build reduced representation libraries by shearing DNA using endonucleases of restrictions and selecting the fragments to be sequenced. Readings from these libraries may be clustered by similarity and mined for polymorphisms near restriction

sites; or used to detect the presence of specific tags, indicating polymorphism at the restriction site itself (Etter et al. 2011). If a reference sequence is available, obtaining polymorphisms from reduced representation libraries is more efficient (Wu et al. 2010). However, researchers have already developed tools for genotype samples from these tags using a low number of readings from organisms without a reference (Ratan et al. 2010), or to reconstruct part of the targeted genome using paired-end sequencing (Willing et al. 2011). There are additional protocols for obtaining markers from reduced representation libraries in which different combinations of restriction enzymes are used for each of the genotypes involved (Hyten et al. 2010), or in which the DNA is not screened, but the readings are filtered for single copy sequences. The amount of reads necessary to perform this type of analysis depends on the size of the genome, the restriction enzymes used, and the availability of a reference (You et al. 2011).

4.7.4 Genotyping Populations

With the fall in prices of HTS technologies and the possibility of multiplexing samples, genotyping has become realistic for the entire population (Schneeberger and Weigel 2011). In the case of a sequenced system such as rice, the generation of readings from individuals in a population of 0.02–0.055 coverage allowed the genotyping of high density by comparison with parental genotypes (Huang et al. 2009), or by inferring the parental genotypes from the polymorphisms found in the population (Xie et al. 2010). Since erroneous calls for polymorphism are expected at low coverage, it is necessary to define more or less complex algorithms to correctly genotype each polymorphism in each individual (Huang et al. 2009;; Xie et al. 2010). In addition, a reference sequence can serve researchers to design enrichment essays that will target their preferred genomic locations, although at high cost (Kenny et al. 2011). For species where a genome sequence is not available, a very practical approach is to sequence reduced representation libraries as mentioned above (Hohenlohe et al. 2010).

4.7.5 Molecular Phenotyping

The list of molecular phenotypes that can be quantified with HTS is extensive and is rapidly increasing (Hawkins et al. 2010). Examples of these phenotypes are protein–RNA interactions, translation rates (Ingolia 2010), transcription rates (Churchman and Weissman 2011), protein–DNA interactions (Barski et al. 2007), RNA degradation rates (Addo-Quaye et al. 2008), RNA secondary structure (Underwood et al. 2010), transcription start positions (Plessy et al. 2010), chromatin accessibility (Boyle et al. 2008), methylation states (Cokus et al. 2008), natural antisense transcription (Parkhomchuk et al. 2009) or small RNA profiles (Lu et al. 2005). QTL analysis using these phenotypes as traits is an exciting field that remains un-explored. Therefore, the computational frameworks to quantitatively compare these phenotypes between individuals will need to be established.

Although many cases of phenotypic variation caused by coding polymorphisms have been documented, it has been shown that the variation in gene expression is the basis of much phenotypic diversity (Reviewed in Stern and Orgogozo 2008). One way to detect differences in expression between people using HTS is to sequence 26–27 tags of nucleotides from expressed transcripts (Hong et al. 2011). A recent study shows that this method reaches 6–8 million reads per sample saturation in mice (Hong et al. 2011). Its advantages over the sequencing of full transcripts are lower costs, higher sensitivity, reduced bias during amplification due to the fixed length of fragments and simplified statistical models to calculate differential expression. Methods based on tags, on the other hand, do not detect most coding polymorphisms and isoforms and require a sufficiently close reference sequence to extract biologically relevant results. Due to its simple preparation protocol, digital nature, large dynamic range and high sensitivity compared to previous technologies, RNA-seq is rapidly becoming a standard in expression profiling (Liu et al. 2010a, b). It can also be used to genotype people, identify new transcripts, characterize alternative splicing and quantify the specific expression of alleles (Reviewed in Costa et al. 2010). The novelty of the technique means that there is no consensus on the preparation of the sample preparation protocols presents fewer biases (Raz et al. 2011). Due to their increased precision, however, strand-specific methods could become a standard due to their ability to distinguish between sensory and antisense transcripts (Levin et al. 2010). As with any other type of genome-wide analysis (Auer and Doerge 2010), biological samples must be randomized and replicated in terms of experimental designs. There is little consensus on the sequence depth required for RNA-seq profiling. Recent estimates range from 30 million readings to compare the expression profiles of two samples, to 100 million readings to detect most transcribed genes and quantify isoforms, to 500 million readings to obtain accuracy including low expressed transcripts (Zhang et al. 2010). In any case, it is advisable to balance the number of reads between samples in the same experiment in order to perform accurate expression comparisons (Tarazona et al. 2011). The profiling of expression from HTS data sets is necessarily based on a reference sequence of the reads mapped to each transcript. If a reference genome or transcriptome is not available, at least one of the genotypes described above can be reconstructed using a de novo read assembly. The simpler and less computational intensive protocol for profiling expression is to map the RNA-seq reads to known transcripts (or de novo assembled) and a set of possible exon-exon junctions (when available) to detect alternative splicing. However, this protocol will not allow the detection of new exons, transcripts and isoforms in organisms with sequenced genomes this protocol will not allow detection of novel exons, transcripts, and isoforms. The preferred pipeline involves aligning the reads to the genomic reference using an alignment tool that splices the reads to detect intron–exon junctions (Wang et al. 2010; Lou et al. 2011). The need for robust quantification of readings generated from two or more alleles is a challenge for expression analyzes in samples from two unrelated persons. This implies that readings with the closer genotype to the reference are better aligned than readings from a more distant genotype, in which more polymorphisms can interfere with their mapping capability (Fontanillas et al. 2010). Aligners

based on seed methods will perform better in these cases than those based on the algorithm Burrows-Wheeler Transform (see Garber et al. 2011 for a review). Although most studies ignore this problem, the polymorphisms that cause these biases are identified and removed. (Degner et al. 2009), aligning the reads to all references from the genotypes involved (Bullard et al. 2010) or including the polymorphisms found in the references (Gan et al. 2011). When two references are used, a potential problem may arise from motifs that are more abundant in one reference with respect to the other if only uniquely mapped reads are counted. The use of longer reads and/or paired end reads greatly decreases the number of ambiguously mapped reads. In addition, there are robust methods to assign these multimapped reads to a single location (Wang et al. 2010; Ji et al. 2011). There are a number of tools to count the number of reads aligned to each transcriptional unit to calculate expression, most of which require knowledge of Perl, Phyton, Linux/Unix, or R (Anders and Huber 2010 and Morgan and Pagès 2010). Some alignment tools can directly calculate the number of reads per transcript and/or a measure of expression based in the reads (or fragments) per gene size in kilobases per million reads mapped, called RPKM (or FPKM; Mortazavi et al. 2008; Trapnell et al. 2010). However, these expression units show biases depending on the length, number, abundance of the transcripts present in the samples, or because of technical replication (Mcintyre et al. 2011). For this reason researchers have developed dedicated R/Bioconductor packages to calculate differential expression between samples based on raw read counts per transcript (Anders and Huber 2010). In addition, there are software packages that take into consideration the biases inherent to RNA-seq when calculating expression or performing downstream analyses such as gene ontology over-representation studies (Young et al. 2010; Zheng et al. 2011).

High-performance sequencing data sets allow expression quantification for each isoform separately, resulting in significantly more accurate estimates than gene expression calculation (Wang et al. 2010). Users must first identify splicing events from reads that align to exon-exon junctions for this purpose. Quantifying isoform expression is complicated because most reads can not be assigned to a single isoform in an alternative spliced transcript. The most promising methods of addressing this complex problem use the information provided by paired end and/or unambiguously mapped reads (Trapnell et al. 2010; Nicolae et al. 2011). One advantage of the complex process of identifying alternative splicing is that it can also be used as a feature for QTL analysis (Lalonde et al. 2011).

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Stress Management: Sustainable Approach Towards Resilient Agriculture

5

Abstract

The improvement of crop performance by increasing osmotic potential-adjusting ability is more significant in roots than other plant parts to avert stress. The role of osmotic adjustment in root elongating zone is to maintain turgor pressure to continue root elongation and root growth in drying soils, which enable the plant to maintain its transpiration by exploiting a greater volume of soil or utilize available water in a given soil volume more efficiently. In this chapter the various tolerance mechanism and diversity among the plants to combat is documented with numerous illustrations. In this last section role of plant metabolites for abiotic stress management is detailed out.

Keywords

Stress · Metabolites · Tolerance · Abiotic · Salt · Drought

5.1 Osmotic Adjustments and Crop Improvements

Osmotic adjustment is a mechanism in plants to tolerate osmotic stress by lowering the osmotic potential by the accumulation of solutes (Radin 1983), and to maintain the volume of protoplast and turgor pressure (Santakumuri and Berkowitz 1991). Osmotic adjustment is a function of either an increase in the net osmoticum deposition rate in the growing region and/or a reduction in the rate of tissue volume expansion (Sharp et al. 1990). The former is more likely to represent an adaptive response that could contribute to growth maintenance (Sharp et al. 2004). Osmotic adjustment occurs in leaves, hypocotyls, roots, floral apex and spikelets under osmotic stress conditions (Turner and Jones 1980). In leaf mature zone, osmotic adjustment plays an important role for plant cell survival (Flower and Ludlow 1986), facilitates higher stomatal conductance (Düring and Dry 1995) and leaf expansion (Westgate and Boyer 1985) to sustain photosynthesis under water stress condition (Turner and

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Jones 1980). Turner and Jones (1980) stated that osmotic adjustment in the root has a different role from that in the shoot, and Serraj and Sinclair (2002) stated that osmotic adjustment in root might be important in relation to crop production. As in leaf growth, root elongation is related with the turgor pressure in root elongation zone (Westgate and Boyer 1985; Frensch and Hsiao 1994). The role of osmotic adjustment in root elongating zone is to maintain turgor pressure to continue root elongation and root growth in drying soils, which enable the plant to maintain its transpiration by exploiting a greater volume of soil or utilize available water in a given soil volume more efficiently. The significance of osmotic adjustment in the elongated zone of the root is to promote desiccation tolerance and to initiate lateral roots that are responsible for efficient absorption and transport of water (Azhiri-Sigari et al. 2000). Therefore, improving crop performance by increasing the ability to adjust osmotic potential in roots may be more important than other parts of the plant (Serraj and Sinclair 2002), although the process in roots has not been studied as extensively as in leaves. There are few reports in the root of osmotic adjustment. Sharp and Davies (1979) showed that even if osmotic potential decreased in conditions of water stress, turgor potential and root growth were maintained (Sharp and Davies 1979; Westgate and Boyer 1985). On the other hand in leaf (Sharp and Davies 1979) and stem (Westgate and Boyer 1985), extension rate and the development of leaf area were reduced by water deficit, due largely to the reduction in leaf turgor pressure. In order to reduce the osmotic potential, the accumulation of compatible solute (Voetberg and Sharp 1991) and the decrease in the rate of influx of water in the tissue and expansion of the tissue volume (Sharp et al. 1990) contributed to the seminal root of maize. Turgor was recovered faster by osmotic adjustment in the cells deep inside the tissue compared to cells near the surface of the root, which showed that the phloem was a possible source of osmotic adjustment compounds (Frensch and Hsiao 1994). The root is the first organ exposed to deficit in water. The growth of leaves is highly sensitive to water stress and can be inhibited by a slight reduction in water potential in the tissue (Hsiao and Xu 2000). Therefore, it is assumed that osmotic adjustment in the root occurs before osmotic adjustment in the leaf to increase turgor pressure for continued root growth and water and nutrient absorption. The osmotic adjustment in the root is therefore expected to delay the onset of shooting water deficit, which reduces the activity of stomatal conductance and photosynthetic activity. In order to clarify the role of root osmotic adjustment in maintaining plant growth in a water deficit condition, it is important to examine the transient changes in the concentration of solvents under stress.

5.1.1 Yield and Osmolyte Accumulation

Despite the widespread suggestion that osmotic accumulation (OA) is beneficial for increasing crop yields under conditions of water deficit, experimental data provide little support. Typically, crop yields of high osmotic-adjusting lines were compared to low osmotic-adjusting lines. The data published by Morgan on wheat (Morgan 1995) and by Ludlow on sorghum (Ludlow et al. 1990) are the ones usually cited as

the critical references for the putative benefits of OA on crop yield. Morgan (1983) worked with wheat and initiated his yield and OA studies by evaluating different wheat lines for osmotic adjustment under greenhouse conditions using estimates of relative water content (RWC) at a given water potential value (−2•5 MPa) to select high osmotic adjustment lines. Unfortunately, no direct measurements of osmotic adjustment were made either in the greenhouse or in the field, and osmotic adjustment was inferred from indirect correlations, which neglected possible variations in the elasticity module value. Babu et al. (1999) recently showed that different OA measurement methods do not necessarily yield consistent results. In the field of wheat lines selected for possible differences in osmotic adjustment (Morgan 1983), the grain yields were 44 g m^{-2} for lines identified with high OA and 29 g m^{-2} for lines with low OA. These results probably reflected an advantage during severe phase III survival in these particular experimental conditions. The difficulty is that these yield levels were so low that growers would consider even yield at 44 g m^{-2} to be a failed crop. In industrial agriculture, yields of at least 150 g m^{-2} and more likely 200–400 g m^{-2} are likely to be required for viable production under dryland conditions. Morgan (1995) also reported comparisons of the yield of wheat grain under drought for low and high osmotic adjustment lines from 5 years of field experiments. Of the nine comparisons presented, only three pairs had a significantly higher yield in high osmotic adjustment lines which was limited to severe water deficits and very low grain yield. Three other comparison pairs had a small non-significant advantage for high osmotic-adjusting lines, and three pairs had a lower but non-significant yield for the high osmotic-adjusting group. Blum, Zhang and Nguyen (1999) recently reported comparisons of OA and yield between ten spring wheat cultivars, including two Morgan lines. Ludlow et al. (1990) reported a positive association between a high capacity for osmotic adjustment and grain yield in sorghum after anthesis. Apparently the higher yield was due to more and larger grains and was associated with a higher harvest index and distribution index. Interestingly, in this study, osmotic adjustment had almost no effect on dry matter at maturity. The main physiological effect of OA was then interpreted as turgor maintenance in the panicle, which could lead to continued metabolic activity during grain filling and thus a higher harvest index. This study may have reflected the particular case in which OA may have extended physiological activity in the panicle prior to the rescue of the crop by rainfall. Results were also reported from the same sorghum lines as above for the contribution of osmotic adjustment to grain yield when subjected to severe pre-anthesis water deficits (Santamaria et al. 1990). The overall response was a higher average grain yield for high osmotic adjustment lines, mainly due to a higher number of grains and a higher harvest index. However, one pair of lines showed a non-significant yield advantage for the low osmotic-adjusting line compared to the three pairs, one pair showed that osmotic adjustment was associated with more water extraction, and the third pair showed that osmotic adjustment was associated with better panicle exertion. In a greenhouse test, the osmotic adjustment capacity of seven pea genotypes was measured and compared to the yield of grains obtained in field trials (Rodríguez-Maribona et al. 1992). Correlation between the capacity of osmotic adjustment and yield was only significant in the case of dry years, but not in a rainy year when drought was

only moderate and higher yields were achieved. The same results were obtained with chickpea (Morgan et al. 1991), in that lines produced greater yields with high osmotic-adjustment capacity only when grown in environments of greatest stress where yields were low. In addition to the frequently cited studies, which are usually used as references to illustrate the beneficial effect of OA on grain yield, numerous reports do not show any effect of osmotic adjustment or even report negative effects on crop yields. Quisenberry et al. (1984) reported a significant negative correlation between the weight of the cotton shooting and the osmotic adjustment estimated by the osmotic potential of zero turgor. They concluded that a reduced growth potential could result if selection pressure is aimed at improving osmotic adjustment during drought. Grumet et al. (1987) reported that barley lines selected for high osmotic adjustment had slower growth, lower dry matter production and grain yield than lines with low osmotic adjustment. No yield benefit was found with osmotic adjustment in four sorghum cultivars under severe drought (Flower et al. 1990). Recently, Subbarao et al. (2000) reported that OA was positively correlated with grain yields at 72 and 82 days after sowing (DAS) whereas OA at 92 DAS contributed negatively to the yield. Bolaños and Edmeades (1991) showed that correlations between osmotic adjustment and performance of tropical maize populations under drought were weak, inconsistent and non-significant. The CIMMYT maize program has apparently made the same assumption (Guei and Wassom 1993). A large amount of rice research has failed to demonstrate the benefit of OA in crop yield (Fukai and Cooper 1995). Overall, the exceptional results in the published literature show a positive correlation between osmotic adjustment and yield, which is usually achieved under severe drought stress when yields are too low to be of practical value.

5.2 Mechanism of Salt Tolerance in Plants

Depending on the severity and duration of the stress, salinity stress involves changes in different physiological and metabolic processes and ultimately inhibits crop production (Flowers 2004; Munns and Tester 2008; FAO 2009). Initially, soil salinity in the form of osmotic stress is known to suppress plant growth, followed by ion toxicity (Rahnama et al. 2010). During the initial phases of salinity stress, the ability to absorb water from root systems decreases and the loss of water from leaves is accelerated due to the osmotic stress of high salt accumulation in soil and plants, and therefore salinity stress is also considered to be hyperosmotic stress (Munns 2005). Osmotic stress in the initial phase of salinity stress causes various physiological changes, such as membrane disruption, nutrient imbalance, impairment of the ability to detoxify reactive oxygen species (ROS), differences in antioxidant enzymes and reduced photosynthetic activity, and decrease in stomatal aperture(Munns and Tester 2008; Rahnama et al. 2010). Also considered a hyperionic stress is salinity stress. The accumulation of Na^+ and Cl^- ions in tissues of plants exposed to soils with high NaCl concentrations is one of the most harmful effects of salinity stress. The entry of Na^+/Cl^- into the cells causes severe ion

imbalance and excessive absorption may cause significant physiological disorders. High concentrations of Na^+ inhibit the absorption of K^+ ions, which is an essential element for growth and development, leading to lower productivity and even death (James et al. 2011). ROS production, such as singlet oxygen, superoxide, hydroxyl radical and hydrogen peroxide, is increased in response to salinity stress (Apel and Hirt 2004; Mahajan and Ahmad et al. 2010; Ahmad and Prasad 2012). The formation of salinity-induced ROS can lead to oxidative damage in various cellular components such as proteins, lipids and DNA, which interrupts vital plant cellular functions. There are genetic variations in salt tolerance, and the degree of salt tolerance varies from species to species. Barley (*Hordeum vulgare*) is more tolerant to salt than rice (*Oryza sativa*) and wheat (*Triticum aestivum*) among the main crops. In the case of dicotyledons ranging from Arabidopsis thaliana, which is very sensitive to salinity, to halophytes such as *Mesembryanthemum crystallinum*, *Atriplex* sp., *Thellungiella salsuginea* (formerly *T. halophila*) (Abraham et al. 2011; Pang et al. 2010), the degree of variation is even more pronounced. Sumptuous research has been carried out in the last two decades to understand the mechanism of salt tolerance in model plant Arabidopsis (Zhang and Shi 2013). Genetic variations and differential responses to salinity stress in plants differing in stress tolerance enable plant biologists to identify physiological mechanisms, sets of genes, and gene products that are involved in increasing stress tolerance and to incorporate them in suitable species to yield salt tolerant varieties.

5.2.1 Physiological and Biochemical Mechanisms of Salt Tolerance

To survive in soils with high salt concentrations, plants develop various physiological and biochemical mechanisms. The main mechanisms include, but are not limited to (FAO 2009), homeostasis and compartimentation of ions (Flowers 2004), transport and absorption of ions (Munns and Tester 2008), biosynthesis of osmoprotectants and compatible solutes, (James et al. 2011), activation of antioxidant enzymes and synthesis of antioxidant compounds (Rahnama et al. 2010), synthesis of polyamines, (Munns 2005). The research progress that clarifies these mechanisms is discussed below.

5.2.2 Ion Homeostasis and Salt Tolerance

The maintenance of ion homeostasis by ion absorption and compartimentation is not only crucial for normal plant growth, but is also an essential process for salt stress growth (Xiaomu et al. 1995; Serrano et al. 1999; Hasegawa 2013). Glycophytes and halophytes cannot tolerate high concentrations of salt in their cytoplasm, regardless of their nature. The excess salt is therefore either transported to the vacuole or sequestered in older tissues, which are eventually sacrificed, protecting the plant against salinity stress (Reddy et al. 1992; Zhu 2003).

The main form of salt in the soil is NaCl, so the study of the transport mechanism of Na^+ ion and it is the main focus of research is the study about the transport mechanism of Na^+ ion and its compartmentalization. The Na^+ ion that enters the cytoplasm is then transported to the vacuole via Na^+/H^+ antiporter. Two types of H^+ pumps are present in the vacuolar membrane: vacuolar type H^+-ATPase (V-ATPase) and the vacuolar pyrophosphatase (V-PPase) (De Lourdes Oliveira Otoch et al. 2001; Wang and Ratajczak 2001; Dietz et al. 2001). Of these, V-ATPase is the most dominant H^+ pump present within the plant cell. During nonstress conditions it plays an important role in maintaining solute homeostasis, energizing secondary transport and facilitating vesicle fusion. Under stressed condition the survivability of the plant depends upon the activity of V-ATPase (Dietz et al. 2001). In a study performed by De Lourdes Oliveira Otoch et al. (2001) in hypocotyls of *Vigna unguiculata* seedlings, it was observed that the activity of VATPase pump increased when exposed to salinity stress but under similar conditions, activity of V-PPase was inhibited, whereas in the case of halophyte Suaeda salsa, V-ATPase activity was upregulated and V-PPase played a minor role (Wang and Ratajczak 2001). Increasing evidence demonstrates the roles of a Salt Overly Sensitive (SOS) stress signalling pathway in ion homeostasis and salt tolerance (Hasegawa et al. 2000; Sanders 2000). The SOS signalling pathway consists of three major proteins, SOS1, SOS2, and SOS3. SOS1, which encodes a plasma membrane Na^+/H^+ antiporter, is essential in regulating Na^+ efflux at cellular level. It also facilitates long distance transport of Na^+ from root to shoot. Overexpression of this protein confers salt tolerance in plants (Shi et al. 2000; Shi et al. 2002). SOS2 gene, which encodes a serine/threonine kinase, is activated by salt stress elicited Ca^+ signals. This protein consists of a well-developed N-terminal catalytic domain and a C-terminal regulatory domain (Liu et al. 2000). The third type of protein involved in the SOS stress signalling pathway is the SOS3 protein, which is a myristoylated Ca^+ binding protein and contains a myristoylation site at its N-terminus. This site plays an essential role in conferring salt tolerance (Ishitani et al. 2000). C-terminal regulatory domain of SOS2 protein contains a FISL motif (also known as NAF domain), which is about 21 amino acid long sequence, and serves as a site of interaction for Ca^{2+} binding SOS3 protein. This interaction between SOS2 and SOS3 protein results in the activation of the kinase (Guo et al. 2004). The activated kinase then phosphorylates SOS1 protein thereby increasing its transport activity, which was initially identified in yeast (Quintero et al. 2002). SOS1 protein is characterised by a long cytosolic C-terminal tail, about 700 amino acids long, comprising a putative nucleotide binding motif and an autoinhibitory domain. This autoinhibitory domain is the target site for SOS2 phosphorylation. Besides conferring salt tolerance it also regulates pH homeostasis, membrane vesicle trafficking, and vacuole functions (Oh et al. 2010; Quintero et al. 2011). Thus with the increase in the concentration of Na^+ there is a sharp increase in the intracellular Ca^{2+} level which in turn facilitates its binding with SOS3 protein. Ca^{2+} modulates intracellular Na^+ homeostasis along with SOS proteins. The SOS3 protein then interacts and activates SOS2 protein by releasing its

self-inhibition. The SOS3-SOS2 complex is then loaded onto plasma membrane where it phosphorylates SOS1. The phosphorylated SOS1 results in the increased Na^+ efflux, reducing Na^+ toxicity (Martínez-Atienza et al. 2007). Many plants have developed an efficient method to keep the ion concentration in the cytoplasm in a low level. Membranes along with their associated components play an integral role in maintaining ion concentration within the cytosol during the period of stress by regulating ion uptake and transport (Sairam and Tyagi 2004). Different carrier proteins, channel proteins, antiporters and symporters carry out the transport phenomenon. Maintaining cellular Na^+/K^+ homeostasis is pivotal for plant survival in saline environments. Ma et al. (2012) have reported that Arabidopsis NADPH oxidases AtrbohD and AtrbohF function in ROS-dependent regulation of Na^+/K^+ homeostasis in Arabidopsis under salt stress. Plants maintain a high level of K^+ within the cytosol of about 100 mM ideals for cytoplasmic enzyme activities. Within the vacuole K^+ concentration ranges between 10 mM and 200 mM. The vacuole serves as the largest pool of K^+ within the plant cell. K^+ plays a major role in maintaining the turgor within the cell. It is transported into the plant cell against the concentration gradient via K^+ transporter and membrane channels. K^+ transporters mediate high affinity K^+ uptake mechanisms when the extracellular K^+ concentration is low, whereas K^+ channels carry out low affinity uptake when the extracellular K^+ concentration is high. Thus uptake mechanism is primarily determined by the concentration of K+ available in the soil. On the other hand a very low concentration of Na^+ ion (about 1 mM or less) is maintained in the cytosol. During salinity stress, due to increased concentration of Na+ in the soil, Na+ ion competes with K^+ for the transporter as they both share the same transport mechanism, thereby decreasing the uptake of K^+. A large number of genes and proteins, such as HKT and NHX, encoding K^+ transporters and channels have been identified and cloned in various plant species. During salt stress expression of some low abundance transcripts is enhanced which are found to be involved in K^+ uptake. This was observed in the halophyte *Mesembryanthemum crystallinum* (Yen et al. 2000). Transporters located on the plasma membrane, belonging to the HKT (histidine kinase transporter) family, also play an essential role in salt tolerance by regulating transportation of Na^+ and K^+. Class 1 HKT transporters that have been identified in Arabidopsis protect the plant from the adverse effects of salinity by preventing excess accumulation Na^+ in leaves. Similar results were observed in the experiment which was carried out with rice where class 1 HKT transporter removes excess Na^+ from xylem, thus protecting the photosynthetic leaf tissues from the toxic effect of Na^+ (Schroeder et al. 2013). Intracellular NHX proteins are Na^+, K^+/H^+ antiporters involved in K^+ homeostasis, endosomal pH regulation, and salt tolerance. Barragan et al. (2012) showed that tonoplastlocalized NHX proteins (NHX1 and NHX2: the two major tonoplast-localized NHX isoforms) are essential for active K^+ uptake at the tonoplast, for turgor regulation, and for stomatal function. In fact more such NHX isoforms have been identified and their roles in ion (Na^+, K^+, H^+) homeostasis established from different plant species (e.g., LeNHX3 and LeNHX4 from tomato) (Galvez et al. 2012).

5.2.2.1 Compatible Solute Accumulation and Osmotic Protection

Compatible solutes, also known as compatible osmolytes, are a group of uncharged, polar and soluble organic compounds that do not interfere with cellular metabolism even at high concentrations. They include mainly proline (Hoque et al. 2007; Ahmad et al. 2010; Hossain et al. 2011; Nounjan et al. 2012; Tahir et al. 2012), glycine betaine (Khan et al. 2000; Wang and Nii 2000), sugar (Bohnert et al. 1995; Kerepesi and Galiba 2000), and polyols (Ford 1984; Dopp et al. 1985; Ashraf and Foolad 2007; Saxena et al. 2013). Organic osmolytes are synthesised and accumulated in varying amounts amongst different plant species. For example, quaternary ammonium compound beta alanine betaine's accumulation is restricted among few members of Plumbaginaceae (Hanson et al. 1994), whereas accumulation of amino acid proline occurs in taxonomically diverse sets of plants (Saxena et al. 2013). The concentration of compatible solutes within the cell is maintained either by irreversible synthesis of the compounds or by a combination of synthesis and degradation. The biochemical pathways and genes involved in these processes have been thoroughly studied. As their accumulation is proportional to the external osmolarity, the major functions of these osmolytes are to protect the structure and to maintain osmotic balance within the cell via continuous water influx (Hasegawa et al. 2000). Amino acids such as cysteine, arginine and methionine, which constitute approximately 55% of total free amino acids, decrease when exposed to salinity stress, while the concentration of proline increases in response to salinity stress (El-Shintinawy and El-Shourbagy 2001). The accumulation of proline is a well-known measure for salinity stress relief (Matysik et al. 2002; Ben Ahmed et al. 2010). Intracellular proline, which accumulates during stressful salinity, not only provides stress tolerance, but also serves as an organic nitrogen reserve during stress recovery. Proline is either glutamate or ornithine synthesized. The primary function in osmotically stressed cell glutamate functions as the primary precursor. Two major enzymes are the biosynthetic pathway, pyrroline carboxylic acid synthetase and pyrroline carboxylic acid reductase. These two regulatory steps are used to overproduce plant proline (Sairam and Tyagi 2004). It works as an O_2 quencher revealing its antioxidant capability (Matysik et al. 2002). Ben Ahmed et al. (2010) observed that proline supplements increased salt tolerance in olives (Olea europaea) by improving some antioxidant enzyme activity, photosynthetic activity and plant growth and maintaining the appropriate status of plant water in salinity conditions. It has been reported that proline improves salt tolerance in *Nicotiana tabacum* by increasing the activity of enzymes involved in antioxidant defence system (Hoque et al. 2008). Deivanai et al. (2011) also showed that the growth of rice seedlings from seeds pretreated with 1 mM proline during salt stress improved. Glycine betaine is an amphoteric quaternary ammonium compound found ubiquitously in microorganisms, higher plants and animals, and is electrically neutral over a wide range of pH levels. It is highly soluble in water, but also contains non-polar moiety in 3-methyl groups. It interacts with both hydrophobic and hydrophilic domains of the macromolecules, such as enzymes and protein complexes, due to its unique structural characteristics. Glycine betaine is a non-toxic cellular osmolyte that increases the osmolarity of the cell during the stress period and therefore plays an important

role in mitigating stress. Glycine betaine also protects the cell by osmotic adjustment (Gadallah 1999), stabilizes proteins (Makela et al. 2000), and protects the photosynthetic apparatus against stress damage (Cha-Um and Kirdmanee 2010) and the reduction of ROS (Ashraf and Foolad 2007; Saxena et al. 2013). Glycine betaine accumulation occurs in a wide range of plants from different taxonomic backgrounds. Glycine betaine is either choline or glycine synthesized in the cell. Synthesis of glycine betaine from choline is a 2-step reaction involving two or more enzymes. In the first step choline is oxidised to betaine aldehyde, which is then again oxidised in the next step to form glycine betaine. In higher plants the first conversion is carried out by the enzyme choline monooxygenase (CMO), whereas the next step is catalysed by betaine aldehyde dehydrogenase (BADH) (Ahmad et al. 2013). Another pathway, which is observed in some plants, mainly halophytic, demonstrated the synthesis of glycine betaine from glycine. Here glycine betaine is synthesized by three successive N-methylation and the reactions are catalysed by two S-adenosyl methionine dependent methyl transferases, glycine sarcosine N-methyl transferase (GSMT), and sarcosine dimethylglycine N-methyl transferase (SDMT). These two enzymes have overlapping functions as GSMT catalyses the first and the second step while SDMT catalyses the second and third step (Ahmad et al. 2013). Rahman et al. (2002) reported the positive effect of glycine betaine on the ultrastructure of *Oryza sativa* seedlings when exposed to salt stress. Under stressed condition (150 mM NaCl) the ultrastructure of the seedling shows several damages such as swelling of thylakoids, disintegration of grana and intergranal lamellae, and disruption of mitochondria. However, these damages were largely prevented when seedlings were pretreated with glycine betaine. When glycine betaine is applied as a foliar spray in a plant subjected to stress, it led to pigment stabilization and increase in photosynthetic rate and growth (Cha-Um and Kirdmanee 2010; Ahmad et al. 2013). Polyols are compounds with multiple hydroxyl functional groups available for organic reactions. Sugar alcohols are a class of polyols functioning as compatible solutes, as low molecular weight chaperones, and as ROS scavenging compounds (El-Shintinawy and El-Shourbagy 2001). They can be classified into two major types, cyclic (e.g., pinitol) and acyclic (e.g., mannitol). Mannitol synthesis is induced in plants during stressed period via action of NADPH dependent mannose-6-phosphate reductase. These compatible solutes function as a protector or stabilizer of enzymes or membrane structures that are sensitive to dehydration or ionically induced damage. It was found that the transformation with bacterial mltd gene that encodes for mannitol-1-phosphate dehydrogenase in both Arabidopsis and tobacco (*Nicotiana tabacum*) plants confer salt tolerance, thereby maintaining normal growth and development when subjected to high level of salt stress (Binzel et al. 1988; Thomas et al. 1995). Pinitol is accumulated within the plant cell when the plant is subjected to salinity stress. The biosynthetic pathway consists of two major steps, methylation of myo-inositol, which results in formation of an intermediate compound, ononitol, which undergoes epimerization to form pinitol. Inositol methyl transferase enzyme encoded by imt gene plays major role in the synthesis of pinitol. Transformation of imt gene in plants shows a result similar to that observed in the case of mltd gene. Thus it can be said that pinitol also plays

a significant role in stress alleviation. Accumulation of polyols, either straight-chain metabolites such as mannitol and sorbitol or cyclic polyols such as myo-inositol and its methylated derivatives, is correlated with tolerance to drought and/or salinity, based on polyol distribution in many species, including microbes, plants, and animals (Bohnert et al. 1995). Accumulations of carbohydrates such as sugars (e.g., glucose, fructose, fructans, and trehalose) and starch occur under salt stress (Parida et al. 2004). The major role played by these carbohydrates in stress mitigation involves osmoprotection, carbon storage, and scavenging of reactive oxygen species. It was observed that salt stress increases the level of reducing sugars (sucrose and fructans) within the cell in a number of plants belonging to different species (Kerepesi and Galiba 2000). Besides being a carbohydrate reserve, trehalose accumulation protects organisms against several physical and chemical stresses including salinity stress. They play an osmoprotective role in physiological responses (Ahmad et al. 2013). Sucrose content was found to increase in tomato (*Solanum lycopersicum*) under salinity due to increased activity of sucrose phosphate synthase (Gao et al. 1998). Sugar content, during salinity stress, has been reported to both increase and decrease in various rice genotype (Alamgir and Ali 1999). In rice roots it has been observed that starch content decreased in response to salinity while it remained fairly unchanged in the shoot. Decrease in starch content and increase in reducing and non-reducing sugar content were noted in leaves of *Bruguiera parviflora* (Parida et al. 2004).

5.2.3 Antioxidant Regulation of Salinity Tolerance

Abiotic and biotic stress in living organisms, including plants, can cause chloroplasts and mitochondria to overflow, deregulate or even disrupt electron transport chains. Under these conditions, molecular oxygen (O_2) acts as an electron accepter, which results in ROS accumulation. Single oxygen (O_2), hydroxyl radical (OH^-), superoxide radical (O^{2-}) and hydrogen peroxide (H_2O_2) are all highly oxidizing compounds and thus potentially harmful to the integrity of cells (Groß et al. 2013). Antioxidant metabolism, including antioxidant enzymes and non-enzymatic compounds, play a key role in the detoxification of salinity stress-induced ROS. Salinity tolerance is positively correlated with the activity of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidise (GPX), ascorbate peroxidase (APX), and glutathione reductase (GR) and with the accumulation of nonenzymatic antioxidant compounds (Asada 1999; Gupta et al. 2005). Gill et al. (2013) and Tuteja et al. (2013) have recently reported a couple of helicase proteins (e.g., DESD-box helicase and OsSUV3 dual helicase) functioning in plant salinity tolerance by improving/maintaining photosynthesis and antioxidant machinery. Kim et al. (2013) showed that the application of silicone (Si) to the root zone of rice influenced hormonal and antioxidant responses to salinity stress. The results showed that Si treatments increased the growth of rice plants significantly compared to salinity stress controls. Si treatments reduced the accumulation of sodium resulting in low electrolytic leakage and lipid peroxidation

compared to salinity stress control plants. In control plants, the response to enzymatic antioxidants (catalase, peroxidase and polyphenol oxidase) was more pronounced than in Si-treated plants under stress of salinity. Anthocyanin is a flavonoid whose accumulation has been largely documented in plants exposed to salt stress. Van Oosten et al. (2013) isolated the anthocyanin-impairedresponse-1 (air1) mutant that is unable to accumulate anthocyanins under salt stress. The air1 mutant showed a defect in anthocyanin production in response to salt stress but not to other stresses such as high light, low phosphorous, high temperature, or drought stress. This specificity indicated that air1 mutation did not affect anthocyanin biosynthesis but rather its regulation in response to salt stress. The discovery and characterization of AIR1 opens avenues to dissect the connections between abiotic stress and accumulation of antioxidants in the form of flavonoids and anthocyanins. Ascorbate is one of the major antioxidants present within the cell. Pea plants grown under saline (150 mM NaCl) stress showed an enhancement of both APX activity and Snitrosylated APX, as well as an increase of H2O2, NO, and S-nitrosothiol (SNO) content that can justify the induction of the APX activity. Proteomic data have shown that APX is one of the potential targets of PTMs mediated by NO^-derived molecules (Begara-Morales et al. 2014). Using recombinant pea cytosolic APX, the impact of peroxynitrite ($ONOO^-$) and S-nitrosoglutathione (GSNO), which are known to mediate protein nitration and S-nitrosylation processes, respectively, was analysed. While peroxynitrite inhibits APX activity, GSNO enhances its enzymatic activity. The results provide new insight into the molecular mechanism of the regulation of APX, which can be both inactivated by irreversible nitration and activated by reversible S-nitrosylation (Begara-Morales et al. 2014). Exogenous application of ascorbate mitigates the adverse effects of salinity stress in various plant species and promotes plant recovery from the stress (Agarwal and Shaheen 2007; Munir and Aftab 2011). Another antioxidant in stress mitigation is glutathione, which can react with superoxide radical, hydroxyl radical, and hydrogen peroxide, thereby functioning as a free radical scavenger. It can also participate in the regeneration of ascorbate via ascorbate-glutathione cycle (Foyer et al. 1997). When applied exogenously glutathione helped to maintain plasma membrane permeability and cell viability during salinity stress in Allium cepa (Aly-Salama and Al-Mutawa 2009). Application of glutathione and ascorbate was found to be effective in increasing the height of the plant, branch number, fresh and dry weight of herbs and flowers, and the content of carbohydrates, phenols, xanthophylls pigment, and mineral ion content when subjected to saline condition (Rawia Eid et al. 2011). Many studies have found differences in levels of expression or activity of antioxidant enzymes; these differences are sometimes associated with the more tolerant genotype and sometimes with the more sensitive genotype. Munns and Tester (2008) suggested that differences in antioxidant activity between genotypes may be due to genotypic differences in degrees of stomatal closure or other responses that alter the rate of CO_2 fixation and differences that lead to processes that avoid photoinhibition and for which the plant has an abundant capacity (Munns and Tester 2008). In their recent review, Roy et al. (2014) argued that plants have three main features that help them adapt to salinity stress: ion exclusion, tissue tolerance and salinity tolerance. Antioxidants appear to play a role in the mechanism of tissue and salinity tolerance.

5.2.4 Roles of Polyamines in Salinity Tolerance

Polyamines (PA) are small, low molecular weight, all encompassing, polycationic aliphatic molecules widely distributed throughout the plant kingdom. Polyamines play a variety of roles in normal growth and development, such as the regulation of cell proliferation, somatic embryogenesis, differentiation and morphogenesis, tubers and seed germination, flowers and fruit development, and senescence (Panicot et al. 2002; Knott et al. 2007; Gupta et al. 2013a, b). It also plays a crucial role in the tolerance of abiotic stress, including salinity, and polyamine levels are correlated with stress tolerance in plants (Kovacs et al. 2010). The PA biosynthetic pathway has been thoroughly investigated in many organisms including plants and has been reviewed in details (Rambla et al. 2010). PUT is the smallest polyamine and is synthesised from either ornithine or arginine by the action of enzyme ornithine decarboxylase (ODC) and arginine decarboxylase (ADC), respectively (Hasanuzzaman et al. 2014). N-carbamoyl-putrescine is converted to PUT by the enzyme N-carbamoyl-putrescine aminohydrolase (Alcazar et al. 2010). The PUT thus formed functions as a primary substrate for higher polyamines such as SPD and SPM biosynthesis. The triamine SPD and tetramine SPM are synthesized by successive addition of aminopropyl group to PUT and SPD, respectively, by the enzymes spermidine synthase (SPDS) and spermine synthase (SPMS) (Alcazar et al. 2006). ODC pathway is the most common pathway for synthesis of polyamine found in plants. Most of the genes involved in the ODC pathway have been identified and cloned. However there are some plants where ODC pathway is absent; for instance in Arabidopsis polyamines are synthesized via ADC pathway (Kusano et al. 2007). All the genes involved in polyamine biosynthesis pathways have been identified from different plant species including Arabidopsis (Ge et al. 2006). Polyamine biosynthesis pathway in Arabidopsisinvolves six major enzymes: ADC encoding genes (ADC1 and ADC2); SPDS (SPDS1 and SPDS2) and SAMDC (SAMDC1, SAMDC2, SAMDC3, SAMDC4) (Hanzawa et al. 2002). On the contrary SPM synthase, thermospermine synthase, agmatine iminohydrolase and N-carbamoylputrescine amidohydrolase are represented by single genes only (Urano et al. 2004). When the plant is exposed to salinity stress, an increase in endogenous polyamine levels has been reported. Polyamine catabolism regulates the level of intracellular polyamine. Polyamines are oxidatively catabolized by amine oxidases, including copper binding diamine oxidases and FAD binding polyamine oxidases. These enzymes play an important role in the tolerance of stress (Takahashi and Kakehi 2010). Changes in the level of cellular polyamine due to stress may have effects on stress, but do not demonstrate their role in counteracting stress. To understand, therefore, whether polyamines actually protect cells from damage caused by stress, the exogenous use of polyamines, which is expected to increase endogenous polyamine, has been investigated before or during stress. Application of exogenous polyamine has been found to increases the level of endogenous polyamine during stress; the positive effects of polyamines have been associated with the maintenance of membrane integrity, regulation of gene expression for the synthesis of osmotically active solutes, reduction in ROS

production, and controlling accumulation of Na^+ and Cl^- ion in different organs (Roychoudhury et al. 2011). It was observed that plant deficient in ADC1 and ADC2 is hypersensitive to stress (Hussain et al. 2011). In Arabidopsis, expression of ADC and SPMS increases when exposed to salinity stress, whereas mutants of polyamine biosynthetic genes show sensitivity to salinity (Yamaguchi et al. 2006). Overproduction of PUT, SPD, and SPM in rice, tobacco, and Arabidopsis enhances salt tolerance (Roy and Wu 2002). Salt stress regulates polyamine biosynthesis and catabolism by acting as a cellular signal in hormonal pathways thereby regulating abscisic acid (ABA) in response to stress (Shevyakova et al. 2013). Additionally, SPM and SPD are regarded as potent inducers of NO an important signaling molecule (Moschou et al. 2008a, b) and its involvement in salinity tolerance is discussed below. It has been reported that exogenous application of polyamines could alleviate salt-induced reduction in photosynthetic efficiency, but this effect depends on polyamine concentration and types and level of stress (Duan et al. 2008). When the seedling of Sorghum bicolor treated with 0.25 mM SPM is subjected to salt stress it shows improvement in growth and partial increase in the activity of peroxidase and glutathione reductase enzyme with a concomitant decrease in the level of membrane lipid peroxidation (Chai et al. 2010). Li et al. (2013) performed 2-DE gel electrophoresis and MALDITOF/TOF MS with cytosolic proteins to understand the effect of exogenous SPD on proteomic changes under normal and NaCl stress of 3 days old cucumber seedling leaves. Many changes were observed in the levels of proteins involved in energy and metabolic pathways, protein metabolic, stress defense, and other functional proteins. They observed that increased salt tolerance by exogenous SPD would contribute to higher expressions of proteins involved in the SAMs metabolism, protein biosynthesis, and defense mechanisms on antioxidant and detoxification. Li et al. (2013) also argued that the regulation of Calvin cycle, protein folding assembly, and the inhibition of protein proteolysis by SPD might play important roles in salt tolerance.

5.2.5 Roles of Nitric Oxide in Salinity Tolerance

Nitric oxide (NO) is a small volatile gaseous molecule, which is involved in the regulation of various plant growth and developmental processes, such as root growth, respiration, stomata closure, flowering, cell death, seed germination and stress responses, as well as a stress signalling molecule (Zhao et al. 2009). NO directly or indirectly triggers expression of many redox-regulated genes. NO reacts with lipid radicals thus preventing lipid oxidation, exerting a protective effect by scavenging superoxide radical and formation of peroxynitrite that can be neutralised by other cellular processes. It also helps in the activation of antioxidant enzymes (SOD, CAT, GPX, APX, and GR). Exogenous NO application has been found to play roles in stress mitigation (Hossain et al. 2010), but the effects depend on NO concentration. Exogenous application of sodium nitroprusside (SNP), a NO donor, on Lupinus luteus seedlings subjected to salt stress enhanced seed germination and root growth. Seed germination was promoted at concentrations between 0.1 and

800 μM SNP in a dose-dependent manner. The stimulation was most pronounced after 18 and 24 h and ceased after 48 h of imbibition. The promoting effect of NO on seed germination persisted even in the presence of heavy metals (Pb and Cd) and NaCl. Kopyra and Gwóźdź (2003) further showed that the pretreatment of *L. luteus* seedlings for 24 h with 10 μM SNP resulted in efficient reduction of the detrimental effect of the abiotic stressors on root growth and morphology. Pretreatment of maize seedlings with 100 μM SNP increases dry matter of roots and shoots under salinity stress; however, when the concentration of SNP was increased to 1000 μM shoot and root dry weight decreased (Zhang et al. 2006). Thus, this experiment highlighted both the protective effects of low NO concentration and the toxic effect of high NO concentration on plants. The positive effects of NO on salinity tolerance or stress mitigation have been attributed to antioxidant activities and modulation of ROS detoxification system. Improved plant growth under salinity stress by exogenous application of NO was associated with increases in antioxidant enzymes such as SOD, CAT, GPX, APX, and GR and suppression of malondialdehyde (MDA) production or lipid peroxidation. Effects of NO on salinity tolerance are also related to its regulation of plasma membrane H^+-ATPase and Na^+/K^+ ratio (Bajgu 2014). NO stimulates H^+-ATPase (H^+-PPase), thereby producing a H^+ gradient and offering the force for Na^+/H^+ exchange. Such an increase of Na^+/H^+ exchange may contribute to K^+ and Na^+ homeostasis (Zhang et al. 2006). Although NO acts as a signal molecule under salt stress and induces salt resistance by increasing PM H^+-ATPase activity, research results from Zhang et al. (2007) with calluses from *Populus euphratica* also indicated NO cannot activate purified PM H^+-ATPase activity, at least in vitro. They initially hypothesized ABA or H_2O_2 might be downstream signal molecules to regulate the activity of PM H^+-ATPase. Further results indicated H_2O_2 content increased greatly under salt stress. Since H2O2 might be the candidate downstream signal molecule, Zhang et al. (2007) tested PM H^+-ATPase activity and K to Na ratio in calluses by adding H_2O_2. The results suggested that H_2O_2inducing an increased PM H^+-ATPase activity resulted in an increased K to Na ratio leading to NaCl stress adaptation.

5.2.6 Hormone Regulation of Salinity Tolerance

ABA is an important phytohormone whose application to plant ameliorates the effect of stress condition(s). It has long been recognized as a hormone, which is unregulated due to soil water deficit around the root. Salinity stress causes osmotic stress and water deficit, increasing the production of ABA in shoots and roots (Cabot et al. 2009). The accumulation of ABA can mitigate the inhibitory effect of salinity on photosynthesis, growth, and translocation of assimilates (Jeschke et al. 1997). The positive relationship between ABA accumulation and salinity tolerance has been at least partially attributed to the accumulation of K^+, Ca^{2+} and compatible solutes, such as proline and sugars, in vacuoles of roots, which counteract with the uptake of Na^+ and Cl^- (Gurmani et al. 2011). ABA is a vital cellular signal that modulates the expression of a number of salt and water deficit-responsive genes.

Fukuda and Tanaka (2006) demonstrated the effects of ABA on the expression of two genes, HVP1 and HVP10, for vacuolar H^+-inorganic pyrophosphatase, and of HvVHA-A, for the catalytic subunit (subunit A) of vacuolar H^+-ATPase in Hordeum vulgare under salinity stress. ABA treatment in wheat induced the expression of MAPK4-like, TIP 1, and GLP 1 genes under salinity stress (Keskin et al. 2010). Some other compounds having hormonal properties, such as salicylic acid (SA) and brassinosteroids (BR), also participate in plant abiotic stress responses (Fragnire et al. 2011). Under salinity stress endogenous level of SA increased along with the increase in the activity of salicylic acid biosynthetic enzyme in rice seedling (Sawada et al. 2006). Jayakannan et al. (2013) have recently shown that SA improves salinity tolerance in Arabidopsis by restoring membrane potential and preventing salt-induced K^+ loss via a guard cell outward rectifying K^+ (GORK) channel. Arabidopsis seedling pretreated with SA showed up regulation of H^+-ATPase activity, thereby improving K^+ retention during salt stress; SA pretreatment did not prevent accumulation of Na^+ in roots but somehow helped to reduce the concentration of accumulated Na^+ in the shoot (Jayakannan et al. 2013). The application of SA also promoted salinity tolerance in barley, as manifested by increases in the content of chlorophyll and carotenoid and maintaining membrane integrity, which was associated with more K^+ and soluble sugar accumulation in the root under saline condition (El-Tayeb, 2005). Nazar et al. (2011) have argued that SA alleviates decreases in photosynthesis under salt stress by enhancing nitrogen and sulfur assimilation and antioxidant metabolism differentially in mung bean cultivars. The negative effects of salinity may also be mitigated by BR (El-Mashad and Mohamed 2012). Application of BR enhanced the activity of antioxidant enzymes (SOD, POX, APX, and GPX) and the accumulation of nonenzymatic antioxidant compounds (tocopherol, ascorbate, and reduced glutathione) (El-Mashad and Mohamed 2012). Both BRs and SA are ubiquitous in the plant kingdom, affecting plant growth and development in many different ways, and are known to improve plant stress tolerance. Ashraf et al. (2010) have reviewed and discussed the current knowledge and possible applications of BRs and SA that could be used to mitigate the harmful effects of salt stress in plants. They have also discussed the roles of exogenous applications of BRs and SA in the regulation of various biochemical and physiological processes leading to improved salt tolerance in plants.

5.3 Diversity in Germplasm Pool: Abiotic Stress Management

Producing sufficient food for the growing population is a major challenge, with climate change emerging as an additional threat to the food security and livelihood of millions of people (Abberton et al. 2016). Achieving significant yield gains in staple crops is essential because rising demand requires a twofold increase in crop production by 2050 (Tilman et al. 2011). The increasing frequency of droughts and heat stress is impacting crop productivity (Lesk et al. 2016), and the increased frequency and severity of flooding events may cause yield loss in regions such as

Asia, where prolonged flooding of rice fields already substantially reduces yields (Mackill et al. 2012). In order to meet the challenges of increasing demand in a changing climate, new and improved crop cultivars must be produced more quickly. The main components of human diet and animal feed are cereals and grain legumes. Grain legumes also enrich nitrogen soil and improve the texture of the soil for other crops (Graham and Vance 2003). The discovery of semidwarfing genes has led to a sharp increase in yields in rice and wheat production worldwide (Trethowan et al. 2007). However, dependence on a narrow range of elite cultivars has likely resulted in some negative effects on the productivity of agroecosystems (Dwivedi et al. 2016), although this assumption is present.

More recent evidence also suggests that productivity of major food crops is either stagnating or not increasing at the rate needed to ensure food security (Ortiz 2015). Accelerated progress in plant breeding is required to better harness crop genetic resources and produce higher-yielding, climate-resilient cultivars. As the methods to assess functional diversity in crops have become more sophisticated during the last 100 years, our understanding of the mechanisms underlying this diversity has grown. Functional diversity refers to a component of biodiversity related to what organisms do in communities and ecosystems (Petchey and Gaston 2006). The decreasing cost of highthroughput DNA sequencing has facilitated the recent rise of genome-wide methods such as genotyping by sequencing (Scheben et al. 2017a) for assessing functional diversity of crops using single nucleotide polymorphisms (SNPs) (Huang and Han 2014). Common targets of breeding are yield-related traits such as abiotic stress tolerance, pest resistance and flowering time. The potential yield gains are substantial, considering that abiotic stress can reduce average yields of major crops by 50% (Bray et al. 2000) and pests can cause 26–40% yield losses. The assessment and use of functional diversity in flowering time control pathways is also important for yield, especially as crop development control can improve adaptation to the predicted impact of climate change. The genomics era led to a rapid increase in sequence data capturing the genetic diversity underlying heritable target characteristics in elite cultivars, land breeds and wild relatives of crops. Although more than 100 plant genomes were already available in 2015 (Michael and VanBuren 2015), more than half of which were crops, the functions of the vast majority of plant genes remain unknown (Rhee and Mutwil 2014). Powerful and high-performance forward and reverse genetic techniques are needed to help clarify these unknown gene functions in order to support targeted breeding. Genetic mapping approaches also play an important role in the association of phenotypic genomic regions. Vast improvements in our understanding of the functional knowledge of crop genomes are an important prerequisite for targeted approaches to genome editing to access new breeding programs to diversity often limited by the natural diversity found in germplasm resources (Scheben et al. 2017b). It is necessary to understand and shape the functional diversity of crops using genomic technologies will be necessary to ensure continuing yield increases to keep pace with growing global food demand.

5.3.1 Plant Architecture and Edible Yield in Cereals

Domestication and subsequent artificial selection by humans has dramatically changed plant architecture, phenology and components of grain yield in many cereals, largely to address agronomic needs and to adapt the crops to various stress-prone environments. Candidate genes and SNPs associated with crop phenology, plant architecture, and yield-attributing traits are known in cereals. Several unique candidate gene regions related to plant growth and development and grain yield have been identified in maize (Farfan et al. 2015). Bouchet et al. (2017) found 34 and 6 QTL for individual or combinatorial trait combinations in maize, respectively. They identified a QTL cluster in a 5 Mb region around Tb1 associated with tiller number and ear row number. The latter was positively correlated with flowering (days to anthesis for male and female flowering and anthesis to silking interval measured in days) and negatively correlated to grain yield. Kn1 and ZmNIP1 have been identified as candidate genes for tillering, along with ZCN8 for leaf number and Rubisco Activase 1 for kernel weight. A more upright leaf in maize has been shown to be influenced by variation in liguleless genes (Tian et al. 2011). A large GWAS study in rice detected 42 significant genotype– phenotype associations for plant morphology, grain quality, and root architecture traits, which in most cases were co-localized with QTL and candidate genes controlling the phenotypic variation of single or multiple traits (Biscarini et al. 2016). Several SNPs in rice were associated with plant and panicle architecture, biomass and yield (Rebolledo et al. 2016), while candidate genes in pathways regulating plant architecture overlap with QTL associated with panicle architecture traits (Rebolledo et al. 2016). In wheat, candidate genes associated with SNPs were involved in carbohydrate metabolism, floral fertility, spike morphology and grain number, providing valuable targets for selection (Guo et al. 2016). Significant marker-trait associations also provided insight into genetic architecture of flowering, plant height and grain weight in barley (Pasam et al. 2012). Individual QTL accounted, however, only for a small portion of phenotypic variation. In sorghum, several SNPs were associated with plant and inflorescence architecture traits, with many located within previously mapped QTL (Zhao et al. 2016). Candidate genes KS3 (associated with seed number) and GA2ox5 (associated with plant height) were also reported (Zhao et al. 2016). A QTL with a major effect corresponded to the priori known photoperiod response gene Ppd-H1 (Maurer et al. 2015).

5.3.2 Abiotic Stress Adaptation in Soybean

Multiple SNPs are reported to be associated with tolerance to drought and heat stress in soybean. Dhanapal et al. (2015) reported 39 SNPs associated with carbon isotope ratio (δ 13C), which is a surrogate trait to measure water use efficiency. The genomic distribution of these SNPs revealed that several are co-located and likely tag the same locus, suggesting that markers for δ 13C can be identified in soybean using GWAS. Dhanapal et al. (2016) reported 52 unique SNPs for total chlorophyll

content tagged on 27 loci across 16 chromosomes. While many of these putative loci were near genes previously identified or annotated as related to chlorophyll traits (Hao et al. 2012), numerous SNPs marked chromosomal regions with unknownfunction genes. Under abiotic stress conditions, non-photochemical quenching (NPQ) protects plants from heat when more light is absorbed than photosynthesis can be used (Li et al. 2009). The reflectance of the canopy measured as a photochemical reflection index (PRI), suitable for high-performance field phenotyping, is a substitute for the measurement of NPQ (Gamon et al. 1992). Thirty-one PRI-specific SNPs may provide an opportunity to improve photosynthesis in soybean in 15 loci on 11 chromosome-harboring candidate genes associated with NPQ, photosynthesis and sugar transport (Herritt et al. 2016).

5.3.3 Abiotic Stress Adaptation in Cereals

Cereal crops have been extensively investigated for SNPs and candidate genes associated with abiotic stress adaptation. Ethylene levels have been linked to yield penalty under heat stress in wheat, largely due to reduction in spike fertility and grain weight (Hays et al. 2007). Valluru et al. (2017) reported 5 and 32 significant SNPs associated with spike ethylene, and 22 and 142 significant SNPs associated with spike dry weight, in greenhouse and field studies, respectively. Some of these SNPs are close to SNPs associated with plant height, suggesting associations between plant height and spike related traits. This opens the possibility of gene discovery and breeding of wheat *Aegilops tauschii* has potential as an excellent source of abiotic stress tolerance. Qin et al. (2016) reported 25 SNPs and several putative candidate genes (enzyme, storage protein, and drought-induced protein) associated with drought adaptation, while Liu et al. (2015) found 13 SNPs and putative candidate genes related to P-deficiency tolerance. A major Al-tolerance gene SbMATE on chromosome 3 has been shown to be associated with grain yield in sorghum, where SbMATE specific SNPs under –P conditions contributed up to 16% genotypic variance (Leiser et al. 2014). Forty-eight genomic regions associated with Al tolerance were reported in rice, four of which co-localized with a priori known candidate genes, and two co-located with previously identified QTL (Famoso et al. 2011). In barley, a genomic region on chromosome 2H was associated with grain yield under heat stress, a region on chr 7H with grain yield, and a region on chr 4H and chr 7H with elevated CO_2 under two factor treatments (high temperature and elevated CO_2). None of the SNPs associated with single factor treatments were retrieved under two factor treatments, thus emphasizing the importance of multifactor treatments (Ingvordsen et al. 2015). Genic SNPs associated with environmental variations (but independent of geographical location) predicted genotype × environment interactions for drought stress and aluminum toxicity in sorghum (Lasky et al. 2015). Wissuwa et al. (2015) reported several SNP loci associated with phosphorus use efficiency (PUE) in rice on chromosomes 1, 4, 11, and 12. A minor indica-specific haplotype on chromosome 1 and a rare aus-specific haplotype on chromosome 11 displayed the highest PUE, and could have potential for targeted introgression while

breeding for rice under P-limited cropping systems. Emerging evidence suggests that responses to stress combinations cannot be reliably predicted from the responses to individual stresses (Makumburage et al. 2013). An integrated approach is therefore needed to model the genetics of responses to a range of single and combined stresses. For example, association analysis report QTL with contrasting and with similar responses to biotic versus abiotic stresses, and below-ground versus above-ground stresses. There is a need to conduct multi-trait GWAS to identify robust candidate genes for multiple stresses (Thoen et al. 2016). The proliferation of genome wide association analyses has led to identification of candidate loci (often co-located with major QTLs or candidate genes) associated with abiotic stress adaptation, phenology and plant architecture, and edible yield. The identification of such loci can facilitate genomics-assisted breeding in cereal and legumes.

5.4 Integrated Metabolome Analysis for Abiotic Stress Management

Environmental stresses such as stresses on biotics and abiotics are serious threats to agricultural production (Lobell et al. 2014). Abiotic stresses such as drought, salinity, cold, high light/UV-B, heat, air pollution, heavy metals, mechanical wounds and nutritional deficiencies (Vickers et al. 2009) lead to a global reduction in crops, leading to global economic costs(Suzuki et al. 2014). To understand and improve the stress responses and tolerances of crops, researchers have focused on the perception of signals, transcriptional regulation and expression of functional proteins in plants' stress response mechanisms against abiotic stress (Hirayama and Shinozaki 2010). In addition, posttranslational, posttranscriptional and epigenetic regulations have been studied. The accumulation of small molecules with antioxidative activity in vitro has often been discussed with respect to the role they play in mitigating the accumulation of reactive oxygen species (ROS) induced by abiotic stresses. This discussion has progressed under the conjecture that the reaction in vitro may occur in vivo. Integrated 'omics' analysis centered on metabolomics (integrated metabolomics) can be a powerful technique to identify the functions of genes involved in the metabolic processes of plants (Saito 2013). Analytical methodologies for narrowing down potential genes and identifying their functions are relatively mature; transcriptome coexpression analysis and (un) targeted analysis in metabolomics using mutant lines have become commonplace (Saito et al. 2008).

The development of aerobic organisms, including plants, depended on the development of effective mechanisms to mitigate the damage of highly reactive and toxic ROS caused by abiotic stress, i.e. singlet oxygen, radicals of anion superoxide, hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH) (Mittler et al. 2004). Since plant cells and organelles containing them (e.g. chloroplasts, peroxisomes, cytosol, mitochondria and vacuoles) are exposed to ROS (Miller et al. 2010), plants have developed two different biological processes to cope with ROS: Prevention or prevention of ROS formation and scavenging of ROS by enzymatic and

non-enzymatic processes, such as the accumulation of low-enzymatic processes, such as accumulation of low-enzymatic processes.

Ascorbic acid (AsA), glutathione (GSH), a-tocopherols, amino acids (e.g. proline (Signorelli et al. 2014), sugars (Nishizawa et al. 2008), carotenoids (Havaux 2014) and quinic acid derivatives (e.g. chlorogenic acid (Niggeweg et al. 2004) are antioxidants presumed to function in planta; however, it is unclear why plants produce such a wide variety of antioxidants. Along with the accumulation of metabolites exhibiting antioxidative activity, abiotic stresses also induce the production of various kinds of specialized metabolites. Of these, it has also been suggested that saponins (Okubo and Yoshiki 2000), glucosinolates (Natella et al. 2014), phenolamides (Velikova et al. 2007), phenylpropanoids (Shahidi and Chandrasekara 2010) and flavonoids (Agati et al. 2012) act as antioxidants in vivo on the basis of their in vitro antioxidative activity.

5.4.1 Integrated Metabolomics Experimentally Identifies Flavonoids as Antioxidants in Planta

Flavonoids that are common in the plant kingdom (Tohge et al. 2013) are responsive to almost all abiotic stresses. Given that flavonoid aglycones (e.g. phenylchroman-based and flavilium-based structures) generally exhibit antioxidative activity in vitro, flavonoids are assumed to function as antioxidants in vivo. It has been suggested that there is a spatio-temporal correlation between flavonoid accumulation and oxidative stress (Hernandez et al. 2009). In comparisons of natural varieties and mutant lines, integrated metabolomics is useful for identifying the functions of genes performing the biosynthesis of target specialized metabolites. The utilization of single gene knock-out/knock-down lines and overexpressing lines clarify the correlation between gene expression and metabolite accumulation, enabling the identification of the gene's function in the integrated analysis oftranscriptomic and metabolomic data Saito et al. 2013). These genetic lines can be also used in identifying the in vivo functions of target metabolites by investigating the phenotypes of plants. Recently, the antioxidative function of flavonoidsin planta was experimentally identified using a series of transgenic and mutant Arabidopsis lines (Nakabayashi et al. 2014). Wild-type Col-0 (Columbia-0), single overexpressors of MYB12/PFG1 (Production of flavonol glycosides1) or MYB75/PAP1 (Production of anthocyanin pigment1), double overexpressors of MYB12 and PAP1, and flavonoid-deficient MYB12 or PAP1 overexpressing lines — obtained by crossing tt4 (transparent testa4) and the individual MYB overexpressor — were subjected to an extensive integrated analysis using transcriptomics, hormonomics and metabolomics. This study excluded the possible effects of the overexpression of the MYBs, the expression of stress-related genes, and the alteration of phytohormones and additional metabolites other than flavonoids on enhancing stress tolerances. The enhanced stress tolerance in this report was solely due to the antioxidative chemical character of over accumulated flavonoids. It is inferred that the flavonoid

accumulation in accordance with abiotic stress exposure is a late response implemented to protect plants (Kusano et al. 2011).

5.4.2 Role of Flavonoids in the Vacuole

The hypothetical insights regarding the role of antioxidative metabolites, particularly flavonoids, may be further extended to understanding their role in the vacuole. H_2O_2 in the cytosol can easily enter into the vacuole (Bolouri-Moghaddam et al. 2010). H_2O_2-dependent class III peroxidase near the inner side of tonoplasts catalyzes the reaction that converts H_2O_2 to OH, which is known to react with almost all metabolites. AsA and GSH present in the vacuole (e.g. 4.19 mM AsA in Catharanthus roseus (Ferreres et al. 2011) and 0.03–0.70 mM GSH in Arabidopsis are general scavengers of H_2O_2 and OH (Queval et al. 2011). In addition, sugars and water-soluble specialized metabolites that are stored in the vacuole are utilized as important antioxidants in plants (Peshev et al. 2013). The in vitro research on the OH-scavenging capacity indicates that sugars and water-soluble specialized metabolites may play a role in radical reactions occurring near the inner side of the tonoplast and in the vacuolar rumen (Peshev et al. 2013). Interestingly, in Arabidopsis, the over accumulation of galactinol and raffinose, which are abiotic stress-responsive vacuole components (Obata and Fernie 2012) and display OH-scavenging activity in vitro, enhanced oxidative stress tolerance in vivo (Keunen et al. 2013). It has been hypothesized that flavonoids mediate the previously unknown key role of the vacuole in maintaining cellular H_2O_2 homeostasis despite the concentration of H2O2 in the vacuole being much lower than in other cell components (Agati et al. 2012). It is estimated that the concentration of rutin (100 mM) in the vacuole is capable of reducing H_2O_2 at a rate of 0.045 mM s^1, which is close to the rate of H_2O_2 generation. Specialized metabolites in the vacuole have been partially qualified and quantified — 1.39 mM total chlorogenic acid (caffeoyl quinic acids) and 1.57 mM total phenolics in *C. roseus* (Ferreres et al. 2011) — suggesting that these highly accumulated compounds play an ROS-mitigating role in the vacuole. Reports of the in vitro antioxidative activity of chlorogenic acid, flavonol glycosides, and anthocyanins against H_2O_2 and OH also suggest their role as antioxidants in the vacuole (Bi et al. 2014).

5.4.2.1 Chemical Challenges

Metabolomics can theoretically qualify and quantify vacuole metabolites at the same time. Recently, metabolomic profiling using gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) enabled the simultaneous detection of 259 putative metabolites in barley vacuoles (*Hordeum vulgare*), including primary and specialized metabolites (Tohge et al. 2011). The metabolic dynamism between vacuolar and extravacuolar compartments was determined by a study using isolated vacuoles using the giant cells in algae *Chara australis* (Oikawa and Saito 2012). These research studies show that the vacuole metabolome can be characterized by combining metabolomics with

state-of - the-art technologies and gain further insights into its functionality. However, they also indicate the difficulties associated with the qualification and quantification of the metabolome, in particular with regard to specialized metabolites, since, due to the specialization of their metabolites, reference materials (i.e. standard compounds or MS/MS spectra) are often not available for chemical assignment. Metabolomic strategies are improved in order to assign putative structural information to detected metabolites using a combination of retention times, exact masses, UV spectra and MS/MS patterns of known metabolites, according to the guidelines of the alphanumeric metrics of identification (Sumner et al. 2014), the plant science community (Fernie et al. 2011) or the Metabolomics Standards Initiative (MSI) (Sumner et al. 2007). According to levels 1 and 2 of the MSI guideline, chemically assigned metabolites are often used in integrated metabolomics studies. For unknown metabolites, MS/MS, UV and isotope analysis are performed to characterize metabolite features such as substructure, metabolite group or elemental composition. Utilizing the patterns of electrospray ionization-based MS/MS spectra on known metabolites in the positive and negative ion modes allows the chemical assignment of unknown metabolites (Morreel et al. 2014). The large amount of data on flavonoid Oglycosides (Afendi et al. 2012), flavone C-glycosides (Yang et al. 2014), phenolamides (Handrick et al. 2010), saponins (Pollier et al. 2011) and glucosinolates (Bottcher et al. 2008) yield a certain pattern of MS/MS fragmentation for each group of compounds. Characteristic UV spectra distinguish these metabolite groups (Oleszek 2002). Applying the information of exact mass and natural abundance to isotopes also allows the chemical assignment of elemental composition to unknown metabolites. Using the differences in exact mass and natural abundance of 32S and 34S, S-containing metabolites were profiled in Arabidopsis (Glaser et al. 2014) and onion (*Allium cepa*) (Nakabayashi et al. 2013). The unambiguous elemental compositions determined using the sulfur and carbon numbers in stable isotope labeling is powerful information that can be utilized in further experiments on chemical assignment. The precise information concerning the generally accepted metabolites assignments can be the precursor of their isolation and structure identification/elucidation to obtain standard compounds for quantification and qualification using general or recent technologies (Nakabayashi et al. 2013). So far, metabolomics-oriented and phytochemical genomics oriented studies have revealed 78 metabolites in Arabidopsis and rice (Oryza sativa) (Yonekura-Sakakibara et al. 2014). Recently, the preparative LC–MS and LC–solid phase extraction– nuclear magnetic resonance–MS (LC–SPE–NMR–MS) systems have been made available for high-throughput analysis (Sturm and Seger 2012). The combination of empirical and computational approaches is expected to streamline the identification of metabolite structures (Nakabayashi et al. 2013).

5.4.2.2 Biological Challenge

Genome sequencing using next-generation sequencers is a great promise for the generation of biosynthesis and vacuolar metabolite roles of mutants. For the manipulation of primary and specialized metabolisms, gene expression and genome engineering technologies have recently been available. Virus-induced gene silencing

techniques (VIGS) are used for biosynthetic gene mutants that define the metabolic pathways of g-aminobutyric acid in tomatoes (*Solanum lycopersicum*) (Bao et al. 2014) and vindoline in *C. Besseau* et al. 2013). Transcription-like effector nuclease (TALEN) technology has been used to disrupt a biosynthetic cholesterol gene, sterol side chain reductase 2, in potatoes (*Solanum tuberosum*) (Sawai et al. 2014). In plants and crops, the clustered regularly interspaced short palindromic repeat (CRISPR) /CRISPR-related protein 9 (Cas9) technology (Belhaj et al. 2013) was used to edit the tt4 gene in Arabidopsis (Mao et al. 2013). In primary and specialized metabolisms, fusion proteins consisting of nuclease dead Cas9 and activator/repressor domains are expected to regulate targeted gene expression (Mahfouz et al. 2014). To identify the roles of not only flavonoids in the vacuole, but also other specialized metabolites, a series of mutants are required. Moreover, consideration of the evolution of the path would result in increasingly interesting information, especially with regard to gene clusters of certain specialized metabolites (Nutzmann and Osbourn 2014).

5.5 Plant Metabolites and Abiotic Stress Tolerance

Responses to environmental stresses alter the metabolism of plants in a variety of ways, including the production of compatible solutes (e.g., proline, raffinose and glycine betaine), which can stabilize proteins and cellular structures or maintain cell turgor by osmotic adjustment, and redox metabolism to remove excess levels of ROS and restore the balance of cell redox(Janska et al. 2010). Glycine betaine improves stress tolerance caused by chilling, frost, salt, drought and high levels of light (Chalker-Scott 2002). Under different adverse environmental conditions, non-protein amino acid c-amino butyric acid (GABA) rapidly accumulates to high levels (Kinnersley and Turano 2000). In many plant species, proline accumulates due to various environmental stresses, including drought, high salinity and heavy metals (Kavi-Kishor et al. 2005). Flavonoids are one of the largest classes of plant phenolics that carry out various functions in plant systems, including pigmentation, defense and scavenging ROS as antioxidants (Harborne and Williams 2000). In particular, the response to UV light stress tends to increase flavonoids (Lavola et al. 2000). In this section, the role of plant metabolites with particular reference to secondary compounds during abiotic stress tolerance is presented in the published literature.

5.5.1 Drought Stress

Tolerant plants initiate defense mechanisms against water shortages to deal with drought (Chaves and Oliveira 2004). Plants show a variety of physiological and biochemical responses to the prevailing drought stress at the cellular and whole organism levels (Farooq et al. 2009). These mechanisms include osmotic adjustment by accumulation of compatible solutes such as proline, betaine glycine, polyols, sugar alcohols and soluble sugars (mannitol, sorbitol, sucrose, fructans, glutamate

and oligosaccharides). Plants have also developed enzymatic antioxidant systems to cope with the stress of drought and to prevent oxidative damage. Low-molecular osmolytes, including glycine betaine, proline and other amino acids, organic acids and polyols, are essential for cellular functions under drought. Plant growth substances such as salicylic acid, auxins, gibberellins, cytokininine and ABA modulate the response to drought in plants. Polyamines, citrulline and several enzymes act as antioxidants and reduce water deficit adverse effects (Chaves and Oliveira 2004).

5.5.2 Salt Stress

Salinity is one of the most important abiotic factors in many arid and semi-arid environments around the world limiting productivity (Msanne et al. 2011). A major threat to global food security is soil salinity. Up to 20% of the irrigated land of the world, which produces a third of the world's food, is affected by salt. In addition, salinity stress is a major worldwide problem for the soil ecosystem (Sima et al. 2009). Plants respond to these stresses through various biochemical and physiological processes, including reduced stomatological performance, carbon fixation and efficiency of light harvesting mechanisms, cell growth repression, and increased respiration and accumulation of osmolytes and proteins involved in stress tolerance (Szabados and Savoure 2010). Plants must synthesize compatible organic solutes such as proline, glycine betaine, trehalose, sorbitol, mannitol, pinitol and sucrose in cytosol to combat osmotic stress imposed by high salinity (Liang et al. 2008). In order to counteract the negative effects of salinity stress, plants have developed stress management strategies involving antioxidants such as ascorbic acid, glutathione, vitamin E, flavonoids, carotenoids(Ahmed 2009) and antioxidant enzymes, such as SOD, CAT, guaiacol peroxidase, APX, monodehydroascorbate peroxidase, and dehydroascorbate peroxidase (Arora et al. 2002). Exogenous use of ascorbic acid has been reported to mitigate the effect of salinity in different crops. Proline is considered to act as an osmolyte, a ROS scavenger and a molecular chaperone that stabilizes the protein structure, thereby protecting cells against damage caused by salt stress(Hale and Orcutt 1987).

5.5.3 Temperature Stress

Plant temperature stress can be divided into the effects of high-temperature, chilling and freezing damage caused by temperature (Arora et al. 2002). Temperature is an important factor in the survival of living organisms, and when water, the biological solvent, freezes to ice, living species face significant challenges. High-temperature stress is often associated with reduced water availability under field conditions. Increased heat stress leads to overproduction of various organic and inorganic osmolytes and accumulation. These osmolytes protect plants against stress by cellular osmotic adjustment, ROS detoxification, biological membrane protection

and enzyme/protein stabilization (Verbruggen and Hermans 2008). The production of ROS, causing oxidative damage to cells and tissues, is one of the main effects of heat stress (Simoes-Araujo et al. 2003). The production of phenolic compounds such as flavonoids and phenylpropanoids is caused by high temperature stress (Morrison and Stewart 2002). Similarly, the accumulation of soluble sugars under heat stress in sugarcane has been reported, which has a significant impact on heat tolerance. Heat stress disturbed the relationship between leaf water and the conductivity of the root (Morales et al. 2003). HSPs are exclusively involved in the response to heat stress. The expression of stress proteins is an important adaptation to the stress of the environment. In addition, other proteins such as glycine betaine, an amphoteric quaternary amine, play an important role in high-temperature plants as compatible solutes (Sakamoto and Murata 2002). GABA acts as a compatible solution, among other osmolytes. Anthocyanins, a subclass of flavonoid compounds, are highly modulated by high temperatures in plant tissues (Shaked-Sachray et al. 2002).

5.5.4 Cold Stress

Cold stress affects plant growth and development adversely. Most temperate plants gain freezing tolerance through a cold acclimation process (Thomashow 1999). Low temperature stress causes the accumulation of phenolic compounds that protect chilled tissues from damage caused by free oxidative stress induced by radicals. Cold stress also increases the amount of water-soluble phenolics and their subsequent incorporation into the cell wall as suberin or lignin (Ippolito et al. 1997). Many researchers report the effects of low temperature on phenolic metabolism and have shown that under chill stress, phenolic metabolism is improved. The accumulation of sucrose and other simple sugars caused by cold acclimation also helps to stabilize the membrane and protects the membranes against freezing damage (Ippolito et al. 1997). Fructans reduce the freezing point during cold stress due to their high concentration in vacuoles, contributing to the change in osmotic potential and increasing plant resistance (Van den Ende et al. 2002). The accumulation of fructane in non-freezing conditions (cold acclimatization) was usually correlated with an increase in freezing tolerance (Pontis 1989). Freezing tolerance is the ability to withstand the formation of extracellular ice and prevent intracellular ice. Extracellular freezing results in freezing dehydration due to the removal of water from the cytoplasm to the growing ice crystals. As freeze-dehydration continues, the content of the cells is increasingly concentrated (Levitt 1980).

5.5.5 Chilling Stress

Chilling (low but non-freezing temperature) is one of the world's most severe abiotic stress factors that restrict plant growth and productivity. In addition to ultrastructural changes, chilling also leads to a series of physiological, biochemical and

molecular changes, such as photosystem I photo inhibition (Kudoh and Sonoike 2002) and increased accumulation of hydrogen peroxide (H2O2) in chilled leaves(Zhou et al. 2004). Endogenous phytohormones, including ABA (Anderson et al. 1994), as well as polyamines and their biosynthetic or responsive genes (Moschou et al. 2008a, b), have been modulated in order to allow plants to adapt to chilling stress. In addition, the expression of some cold-regulated genes is one of the most successful strategies developed by plants to adapt to chilling stresses. In chilling stress, polyamines are involved (Moschou et al. 2008a, b). Zhang et al. (2009) reported on the effect of treatment with chilling on cucumber polyamines. The spermidine content in leaves increased markedly in cucumber during chilling. Chilling damage in response to cold may be prevented by the accumulation of polyamine (He et al. 2002). In addition, agmantine and putrescine have also been reported in seedlings of Pringlea antiscorbutica (Hummel et al. 2004).

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Soil Health and Food Security

6

Abstract

Food security is a flexible concept as reflected in the many attempts at definition in research and policy usage. One more crucially important, factor in modifying views of food security was the evidence that the technical successes of the Green Revolution did not automatically and rapidly lead to dramatic reductions in poverty and levels of malnutrition. The forecast of 2050 global crop demand and then quantitatively evaluate the global impacts on land clearing, nitrogen fertilizer use, and GHG release of alternative approaches by which this global crop demand might be achieved. The role of soil microbial community for improving plant growth and development for keeping the pace with the global food demand and sustainable agriculture is documented here. A general perception about genetic engineering and public intervene and sustainable agricultural intensifications and food production is discussed in the preceding sections.

Keywords

Food demand · Security · Genetic engineering · Plant growth agriculture

6.1 Concept of Food Security

In many attempts at definition in research and policy usage it has been reflected that food security is a flexible concept as there were about 200 definitions in published writings before a decade (Maxwell and Smith 1992). Whenever the concept is introduced in the title of a study or its objectives, it is necessary to look closely to establish the explicit or implied definition, (Maxwell 1995). In the mid-1970s, the concept of food security originated in the discussions of international food problems at a time of global food crisis as its preliminary focus of attention was mainly on food supply problems of assuring the availability and to some degree the price stability of basic foodstuffs at the national and international level. The crisis had

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been precipitated for global food economy at institutional and international set of concerns reflected the changing organization. A process of international negotiation followed, leading to the World Food Conference of 1974, and a new set of institutional arrangements covering information, resources for promoting food security and forums for dialogue on policy issues. The issues of famine, hunger and food crisis were also being extensively examined, following the events of the mid 1970s. The outcome was a redefinition of food security, which recognized that the behavior of potentially vulnerable and affected people was a critical aspect. One more crucially important, factor in modifying views of food security was the evidence that the technical successes of the Green Revolution did not automatically and rapidly lead to dramatic reductions in poverty and levels of malnutrition. These problems were recognized as the result of lack of effective demand.

The continuing evolution of food security as an operational concept in public policy has reflected the wider recognition of the complexities of the technical and policy issues involved. The most recent and careful redefinition of food security is that negotiated in the process of international consultation leading to the World Food Summit (WPS) in November 1996. The contrasting definitions of food security adopted in WFS 1974 and WFS 1996, along with those in official FAO and World Bank documents of the mid 1980s are set out below with each substantive change. A comparison of these definitions highlights the considerable reconstruction of official thinking on food security that has occurred over 25 years. These statements also provide signposts to the policy analyses, which have re-shaped our understanding of food security as a problem of international and national responsibility.

A procedure of global exchange pursued, prompting the World Food Conference of 1974, and another arrangement of institutional courses of action covering data, assets for advancing sustenance security and discussions for discourse on approach issues. In the mid 1970s, after the occasions, issues like starvation, hunger and sustenance emergency were additionally being widely analyzed. The result was a redefinition of nourishment security, which perceived that the conduct of conceivably helpless and influenced individuals was a basic perspective. One all the more critically essential, factor in changing perspectives of nourishment security was the proof that the specialized accomplishments of the Green Revolution did not consequently and quickly lead to sensational decreases in neediness and dimensions of lack of healthy sustenance. These issues were perceived as the consequence of absence of successful interest. The proceeding with development of food security as an operational idea in broad daylight approach has mirrored the more extensive acknowledgment of the complexities of the specialized and arrangement issues included. The latest and watchful redefinition of nourishment security is that consulted during the time spent universal conference prompting the World Food Summit (WPS) in November 1996. The differentiating meanings of nourishment security embraced in WFS 1974 and WFS 1996, alongside those in authority FAO and World Bank reports of the mid 1980s are set out underneath with every substantive change. A correlation of these definitions features the impressive remaking of authority thinking on nourishment security that has happened more than 25 years. These

announcements additionally give signposts to the arrangement examinations, which have re-molded our comprehension of nourishment security as an issue of global and national duty. The underlying focus, mirroring the worldwide worries of nourishment security, was on the volume and soundness of sustenance supplies. The idea was characterized in the 1974 World Food Summit as: "Accessibility consistently of sufficient world sustenance supplies of essential foodstuffs to continue an unfaltering development of nourishment utilization and to counterbalance variances underway and costs" (UN 1975). Further the FAO extended his idea to incorporate verifying access by powerless individuals to accessible supplies, inferring that consideration ought to be adjusted between the interest and supply side of the sustenance security condition. It is characterized as, "Guaranteeing that all individuals consistently have both physical and monetary access to the essential nourishment that they need" (FAO 1983).

FAO (1983) has amplified idea of food security to incorporate the accompanying parts:

(a) The severe goal of world food security must to be to guarantee that all individuals consistently have both physical and financial access to needed sustenance.
(b) Food Security ought to have three essential points, guaranteeing creation of satisfactory nourishment supplies, boosting solidness in the stream of provisions, and guaranteeing access to accessible supplies with respect to the individuals who need them.
(c) Action will be required on a wide front including all factors that have a bearing on the limit of the two nations and individuals to deliver or buy foods, while grains will keep on being the primary focal point of consideration, activity should cover all fundamental nourishment stuff essential for wellbeing, farming and rustic advancement, food production, food holds, the working of national and worldwide cereal showcase.

The outside trade needs of bringing in nations, exchange progression and fare profit, the buying intensity of most unfortunate strata of the populace, money related assets and specialized help and the stream of sustenance help and courses of action to address crisis issues. This more extensive idea of food security is like that embraced by the World Bank 3 years after the fact in its position paper Poverty and Hunger: Issues and Options for Food Security in developing countries. It presented the generally acknowledged qualification between chronic food frailties, related with issues of auxiliary neediness and low earnings, and transient food insecurity, which included times of increased weight brought about by catastrophic events, monetary breakdown. This idea of food security is additionally expounded regarding: "Access surprisingly consistently to enough nourishment for a functioning, sound life". The most broadly utilized meaning of food security is that of the World Bank: 'Access by all individuals consistently to enough nourishment for a functioning, sound life'. The expression "access" here is comprehensive of both the supply side (accessibility) and the interest side (privilege).

By the mid-1990s food security was perceived as a noteworthy concern, traversing a range from the person to the worldwide dimension. Nonetheless, get to now include adequate sustenance, demonstrating proceeding with worry with protein-vitality unhealthiness. In any case, the definition was expanded to join nourishment wellbeing and furthermore wholesome parity, reflecting worries about food synthesis and minor supplement necessities for a functioning and sound life. Food inclinations, socially or socially decided, presently turned into a thought. The possibly high level of setting particularity suggests that the idea had both lost its straightforwardness and was not itself an objective; however an intermediating set of activities that add to a functioning and sound life.

The UNDP Human Development Report propelled the work of human security, including different part points, of which food security was only a solitary. This thought is immovably related to the human rights perspective on enhancement that has, in this way, affected talks about food security. The more broad examination concerning the activity of open action into battling longing for and hardship, found the same spot for sustenance security as a dealing with framework for action. Or maybe, it focused on an increasingly broad form of institutionalized funds, which has various undeniable portions including, clearly, prosperity and sustenance, (Dreze and Sen 1989). The World Food Summit (1996) grasped a still progressively grow definition: "Food security, at the individual, nuclear family, national, nearby and overall measurements is cultivated when all people, reliably, have physical and money related access to satisfactory, ensured and nutritious sustenance to meet their dietary needs and sustenance tendencies for a working and sound life" (FAO 1996). This definition is again refined in The State of Food Insecurity Report 2001: "Food security is a situation that exists when all people, reliably, have physical, social and monetary access to satisfactory, ensured and nutritious sustenance that meets their dietary needs and nourishment tendencies for a working and strong life" (FAO 2002). This new accentuation on usage, the premium side and the issues of access by frail people to sustenance, is most solidly identified with the key examination by Amartya Sen and focused on the capabilities of individuals and nuclear families (Sen 1981).

These inexorably expansive explanations of shared objectives has acknowledged by the global network, as its commonsense reaction has been to concentrate on straightforward and thin goals around which to compose worldwide and national open activity. The pronounced essential goal in universal improvement arrangement talk is progressively the decrease and disposal of neediness. The 1996 WFS exemplified this heading of approach by making the essential target of universal activity on sustenance security, dividing of the quantity of ravenous or undernourished individuals by 2015. Basically, nourishment security can be portrayed as a wonder identifying with people. It is the nourishing status of the individual family unit part that is a definitive center, and the danger of that sufficient status not being accomplished or getting to be undermined. The later hazard portrays the weakness of people in this unique situation. As the definitions explored above infer, powerlessness may happen both as a ceaseless and brief marvel. Helpful working definitions are depicted underneath.

Food security exists when all individuals, consistently, have physical, social and financial access to adequate, sheltered and nutritious sustenance which meets their dietary needs and sustenance inclinations for a functioning and sound life. Family sustenance security is the utilization of this idea to the family level, with people inside families as the focal point of concern. Guaranteeing Food Security involves meeting two conditions. One condition is guaranteeing that there are sufficient nourishment supplies accessible, through residential creation or imports. The other is guaranteeing that family units whose individuals experience the ill effects of under nourishment can get sustenance, either in light of the fact that they produce it themselves or on the grounds that they have the salary to procure it (Reutlinger 1985).

All individuals consistently to the sustenance required for a sound life characterize nourishment security in its most essential structure as access. Food security contrasts from appetite in that sustenance security is an issue that a network in a nation state, city or neighborhood encounters (Conway 1997a, b). A more extensive meaning of food security consolidates what is regularly eluded in the personal satisfaction pointers. As needs, food security suggests employment security at the dimension of every family and all individuals inside, and includes guaranteeing both physical and monetary access to adjusted eating routine, safe drinking water, ecological sanitation, essential instruction and fundamental social insurance. It is envisioned that food security includes-

(a) Financial development, particularly access to assets.
(b) Instruction particularly training of ladies.
(c) Populace programs
(d) Common habitat.
(e) Participation and responsibility are the characteristic count reactants to starvation and ailing health (Gittinger et al. 1987).

6.2 Soil Microbial Community and Plant Growth

Schmidt et al. (2014) reported that there has been an upsurge in phytomicrobiome publications; this community of microbes is now seen as key to the growth and health of plants and there is still a great deal to be learned about the composition and nature of interactions among members of this community, and its interactions with the host plant. Microbes associate with the phyllosphere (epi- and endophytes, of leaves and stems), rhizosphere and reproductive structures such as flowers, fruits and seeds. In grape, *Pseudomonas* and *Bacillus* spp. colonize the epidermis and xylem of the ovary and ovules, while *Bacillus* spp. colonize berries and seed cell walls (Compant et al. 2010a, b). Nitrogen-fixing plant growth promoting rhizobacteria (Quecine et al. 2012) e.g., *Acetobacter diazotrophicus, Pantoea agglomerans*) associate with plant roots (Pisa et al. 2011), and stems of sugarcane (Velázquez et al. 2008), residing in the apoplast in a low-nitrogen, high-sucrose environment (Dong et al. 1994). Other nitrogen-fixing bacteria (*Azotobacter*, *Azospirillum, Azoarcus, Burkholderia, Bacillus Enterobacter*, *Klebsiella*, *Herbaspirillum* and

Gluconacetobacter) are found in grasses such as rice and maize (Santi et al. 2013). Phyllomicrobiome communities influence the plant development and ecosystem function, while the host controls aspects of phytomicrobiome composition and function. Within the plants the biosynthesis of many metabolites is known to alter by environmental factors; specific members of the rhizomicrobiome also alter plant development, composition and growth. Badri et al. (2013) reported that specific phyllomicrobiome components suppress the feeding of leaves by insect larvae. There is a random distribution and community composition of microbes in the phyllosphere, whereas plants create niches in the rhizosphere and endosphere to accommodate specific microbial communities (Lebeis 2015).

Amongst diverse root endophytes as some are PGPRs, which are comprised of rhizomicrobiome (Gaiero et al. 2013). Rhizomicrobiome is dynamic in time and space, the presence of other soil organisms, soil physical conditions, in response to environmental conditions plant species and genotype and interactions between a specific microbe and a specific plant type. The best characterized microbes in the rhizomicrobiome are the PGPR which include bacteria in the soil near plant roots, in spaces between root cells or inside specialized cells of root nodules, on the surface of plant root systems; they stimulate plant growth through a wide range of mechanisms (Mabood et al. 2014), such as: (1) nutrient solubilization (P) (Trabelsi and Mhamdi 2013), (2) N- fixation (Drogue et al. 2012), (3) production of metal chelating siderophores, (4) production of phytohormones, (5) production of volatile organic compounds, (6) production of 1-aminocyclopropane-1-carboxylate deaminase (ACC) (7) induction of systemic resistance [induced systemic resistance (ISR) and systemic required resistance (SAR) – Jung et al. 2008], and (8) Antibiosis (Spence et al. 2014). Lee et al. (2009) showed that "signal" compounds produced by bacteria in the phytomicrobiome stimulate plant growth particularly in the presence of abiotic stress (Prudent et al. 2015). In the broadest sense PGPR include legume-nodulating rhizobia. PGPR reside outside plant cells (extracellular – ePGPR) or, like rhizobia, live inside them (intracellular – iPGPR; Gray and Smith 2005). Application of PGPR to crops, except for rhizobia, has met with mixed results in the field, causing increased growth sometimes and not others (Nelson 2004). Elements of the phytomicrobiome also assist plants in dealing with abiotic stress. The *Arabidopsis* phytomicrobiome, for instance, can sense drought stress and help the plant maintain productivity (Zolla et al. 2013). Further, mycorrhizal associations enhance crop salinity tolerance (Ruiz-Lozano et al. 2012). At a time when we are looking to crop plants to provide biofuels and other bioproducts while still feeding the world's growing population, against a background of climate change, understanding and developing technologies that can increase overall plant productivity is imperative (Orrell and Bennett 2013).

Newer deployments of PGPR and arbuscular mycorrhizal fungi (AMF) consortia that promote crop productivity by mimicking, or partially reconstructing, the phytomicrobiome are being developed. Application of a PGPR consortium (*Bacillus amyloliquefaciens* IN937a, *Bacillus pumilus* T4, AMF *Glomus intraradices*) to greenhouse tomato resulted in full yield with 30% less fertilizer (Adesemoye et al. 2009). Co-inoculation of *B. japonicum* 532C, RCR3407 and *B. subtilis* MIB600

increased biomass for two soybean cultivars (Atieno et al. 2012). Co-inoculation of *B. japonicum* E109 and *Bacillus amyloliquefaciens* LL2012 improved soybean nodulation efficiency. Phytohormone production by *B. amyloliquefaciens* LL2012 improved nodulation efficiency for *B. japonicum* E109 (Masciarelli et al. 2014). A consortium of *B. megaterium*, *Enterobacter* sp., *B. thuringiensis* and *Bacillus* sp., plus composted sugar beet residue, on *Lavandula dentata* L. helped restore soils by increasing phosphorus availability, soil N- fixation and foliar NPK content (Mengual et al. 2014).

6.2.1 Signaling in the Phytomicrobiome

The complex community formed by the plant and its phytomicrobiome is carefully cautiously coordinated; there is signal exchange among the various microbes involved, and also between the host plant and the microbe community as these signals regulate aspects of each other's activities and the community overall (Engelmoer et al. 2014). Microbial chemical signals can help plants initiate immune responses to harmful pathogens or allow the entry of beneficial endophytes (Hartmann et al. 2014). Microbe associated molecular patterns (MAMPs) play a key role in plant immune response and antibiotic secretion in microbes. Plant associated *Bacillus* strains have been shown to down-regulate MAMP-regulated immune response including antibiotic secretion in the presence of plant root exudates to better facilitate root infection (Lakshmanan et al. 2012). Bacteria can also interfere with signaling between plants and other microbial strains. LCOs are similar in structure to chitin and can be cleaved by bacterially produced chitinases, thus interfering with plant microbe symbioses (Jung et al. 2008). Other aspects plant–microbe symbiosis follows pathways similar to pathogen infection (Barea 2015).

Signaling compounds produced by plants include a variety of root exudates such as primary metabolites (carbohydrates, proteins, organic acids, etc.) and secondary metabolites (flavonoids, phenol, phytohormones, etc.). Plants often excrete more of these signaling compounds in response to stress. PGPR-to-plant signaling compounds include phytohormones, acyl homoserine lactones, phenols and peptides and can also act as microbe to microbe signals (Barea 2015). Root exudates signal and recruit specific microbial communities. Secretion of malic acid in *Arabidopsis thaliana* in response to foliage pathogen attack stimulates the formation of beneficial biofilms in the rhizosphere (Rudrappa et al. 2008).

That plants and microbes use signal compounds to communicate during establishment of beneficial plant-microbe interactions (Desbrosses and Stougaard 2011), is well-described for the legume-rhizobia nitrogen fixing symbiosis (Oldroyd 2013), and somewhat elucidated for mycorrhizal associations (Gough and Cullimore 2011). In the legume-rhizobia relationship the plant releases flavonoid signals to rhizobia (Hassan and Mathesius 2012) or, in some cases, jasmonate signals (Mabood et al. 2006, 2014), followed by rhizobial production of lipo-chitooligosaccharides (LCOs) as return signals (Oldroyd 2013). The LCOs are bound by LysM receptors, which have kinase activity (Antolin-Llovera et al. 2012), changing root hormone

profile (Zamioudis et al. 2013) and triggering development of root nodules. Plants also communicate with, or otherwise influence the phytomicrobiome, affecting its composition and structure (Evangelisti et al. 2014). Bacteria also communicate among themselves (Cretoiu et al. 2013); quorum sensing via *N*-acyl homoserine lactone (Teplitski et al. 2000) is well characterized, and there are likely other, as of yet unknown, mechanisms (Lv et al. 2013). Quorum sensing signals can trigger immune responses and changes in hormone profiles in plants, leading to growth responses. Quorum sensing in the phytomicrobiome will be the subject of upcoming Frontiers in Plant Science theme volume (Plant responses to bacterial quorum sensing signal molecules, topic editors Schikora A, Hartmann A, and Munchen HZ). This sort of signaling almost certainly occurs in the phytomicrobiome. Plants also detect materials produced by potential pathogens and respond by activating response systems (Tena et al. 2011). Phytomicrobiome intercommunication in the rhizosphere dictates aspects of aboveground plant architecture and above-ground symbiotic/pathogenic microbial communities (Tena et al. 2011). Similarly, pathogen or herbivore attacks above ground can effect microbial community composition in the rhizosphere. Above ground injury has been shown to stimulate the production of signaling compounds in plant roots (Lakshmanan et al. 2012). Greater photosynthetic rates under elevated CO_2 conditions have been shown to change microbial community composition in the rhizosphere (He et al. 2012). Understanding plant responses to microbial signals via proteomics (Rose et al. 2012) and metabolomics (Zhang et al. 2012) studies has added valuable knowledge toward developing effective low-cost and eco-friendly practices to reduce fossil-fuel dependent crop inputs, leading to interest in phytomicrobiomes engineered to enhanced plant growth under variable soil and climatic conditions, improving global crop productivity.

Surprisingly, LCOs are also able to stimulate plant growth directly (Wang et al. 2012); confirmed by Oláh et al. (2005) for root growth in *Medicago truncatula*, Chen et al. (2007) for accelerated flowering (a typical response to stress) and increased yield in tomato, and stimulation of early somatic embryo development in Norway spruce (Dyachok et al. 2002). Enhanced germination and seedling growth, along with the mitogenic nature of LCOs, suggest accelerated meristem activity. Products based on LCOs are now used to treat seed sown into several 10s of million ha of crop land each year, largely corn and soybean. A similar jasmonate product is now available. The effects of LCOs are much greater when stress (salt, drought, cold) is present than under optimum conditions (Prudent et al. 2015). Thuricin 17, a bacteriocin produced by *Bacillus thuringiensis* NEB17 isolated from soybean roots, improves plant growth and resilience to stress (Subramanian 2014). Inhibition of legume nodulation, and of overall plant growth, by stressful conditions can be overcome by LCOs (nodulation – Zhang and Smith 1995; plant growth – Prudent et al. 2015); Estévez et al. (2009) showed that at least one rhizobial strain produce different LCOs when grown under salt stress, and that salt stress itself can induce the *nod* genes of this strain (Guasch-Vidal et al. 2013).

6.3 Global Food Demand and Sustainable Agriculture

With increasing in global population, which is propelled by a 2.3 billion person, increase results more demand for agricultural crops (Godfray et al. 2010). In order to meet the demands for food, as activities like land clearing and more intensive use of existing croplands is a common practice, which possesses environmental impacts, and tradeoffs of these alternative paths of agricultural expansion are unclear. Dirzo and Raven (2003)reported that agriculture already has major global environmental impacts includes land clearing and habitat fragmentation threaten biodiversity as about one-quarter of global greenhouse gas (GHG) emissions result from crop production, land clearing and fertilization (Burney et al. 2010), and these practices can harm terrestrial ecosystems, freshwater and marine (Vitousek et al. 1997). Quantitative assessments are required to achieve greater yields with lower impacts in order to fulfill future demands for food.

The conjecture of 2050 worldwide yield request and after that quantitatively assess the worldwide effects land clearing, nitrogen fertilizer use, and GHG release of elective methodologies by which this worldwide harvest request may be accomplished. To do these examinations, we aggregated yearly rural and populace information for 1961–2007 acquired from the FAOSTAT database (Food and Agriculture Organization of the United Nations; http://faostat.fao.org/) and different hotspots for every one of 100 substantial countries that contained 91% of the 2006 worldwide populace. At that point we determined net national interest for harvest calories and yield protein for every country for every year dependent on national yearly yields, generation, imports, and fares of 275 noteworthy yields (those yields utilized as human nourishments or domesticated animals and fish bolsters). The resultant per capita interest for calories or protein from all nourishment or feed crops joined envelops yearly human harvest utilization, crop use for animals and fish creation, and all misfortunes (waste and deterioration amid sustenance and yield generation, stockpiling, transport, and assembling). To decide long haul worldwide patterns and better control for financial contrasts among countries, countries were collected into seven monetary gatherings going from most astounding (Group A) to least (Group G) national normal per capita genuine (inflation-adjusted) (GDP).

6.3.1 Worldwide Crop Demand

Examinations uncover a straightforward and transiently reliable worldwide connection between per capita GDP and per capita interest for yield calories or protein. Over all years, per capita harvest use was correspondingly subject to per capita GDP both inside and among the seven monetary gatherings. The size of this reliance is shockingly expansive. In 2000, for instance, per capita utilization of calories and protein by the most extravagant countries (Group A) were 256% and 430% more noteworthy, individually, than use by the most unfortunate countries (Groups F and G).

These expansive contrasts in harvest request somewhat result from more prominent dietary meat utilization at higher pay (Poleman and Thomas 1995; Keyzer et al. 2005) and the low effectiveness with which a few sorts of animals convert crop calories and protein into eatable sustenances (Smil 2002). These analyses forecast that global demand for crop calories would increase by 100% ± 11% and global demand for crop protein would increase by 110% ± 7% (mean ± SE) from 2005 to 2050. This projected doubling is lower than the 176% (caloric) and 238% (protein) increases in global crop use that would occur if per capita demands of all nations in 2050 reached the 2005 levels of Group A nations. Any projection of future worldwide yield creation involves numerous components of vulnerability and of need underscores some possibly causative factors over others. Our conjecture of a 100–110% expansion in worldwide yield creation by 2050 is bigger than the 70% expansion that has been anticipated for this equivalent period (Tilman et al. 2002).

6.3.2 Quantification of Yield, Input, and Climate Relationships

The natural effects of multiplying worldwide crop production will rely upon how expanded generation is accomplished (Foley et al. 2011). Food generation could be expanded by agrarian intensification (that is, clearing extra land for harvest creation) or escalation (i.e., accomplishing higher yields through expanded data sources, enhanced agronomic practices, enhanced harvest assortments and different developments). The worldwide effects ashore clearing, GHG emanations, and nitrogen treatment of option pathways of agrarian advancement that meet the 2050 worldwide harvest generation is evaluated. Specifically, blends of present or enhanced farming innovations, upgrades to soil ripeness, and land clearing that could meet our anticipated 2050 worldwide caloric interest and what their natural effects would be assesed. For quickness, results for protein are not introduced here but rather are comparative. Due to information accessibility, we use past Nitrogen treatment rates as quantitative proportions of soil richness upgrade, yet we accentuate that dirt fruitfulness can likewise be improved by vegetables, spread harvests, and different methods and that yields could increment with less Nitrogen manure than before if Nitrogen use productivity builds (Chen et al. 2011). The numerous relapses to evaluate how country to-country and year-to-year contrasts in caloric yields have been identified with Nitrogen preparation power (N ha^{-1}) and different factors that are thought to affect yields. We found that caloric yields were at the same time identified with Nitrogen treatment power, precipitation, potential evapotranspiration, soil pH, rise, time (year), and monetary gathering. A less complex relapse that included just Nitrogen preparation power, precipitation, monetary gathering, and time gave comparative outcomes. Two generally comparative relapses utilized only 2005 information. These four relapses demonstrate that ∼80% of national-level variety in caloric yields was measurably clarified by a couple of fundamental factors. We utilize these fitted connections to measure situations, investigating the potential impacts of changes in these factors on caloric yields and the earth. We do as such with the proviso that the fitted connections need not be

characteristic of causation, while taking note of that fits are steady with different examinations of controls of yields (Foley et al. 2011). Subsequent to controlling for Nitrogen treatment, atmosphere, soil, and rise in these relapses, we will, for curtness, allude to the remaining yield contrasts attributed to financial gatherings as for the most part reflecting mechanical and framework variations among the monetary gatherings, and we will allude to the lingering yield contrasts that are credited to time (year) as essentially reflecting innovative upgrades from 1965 to 2005.

6.3.3 Alternative Pathways of Agricultural Expansion

These relapses can assess the reliance of worldwide yields on Nitrogen use (soil fruitfulness upgrade) if future technological advances were to proceed with observed temporal patterns to 2050 (innovation enhancement), on the off chance that under yielding countries were to beat mechanical differences by adjusting and, at that point receiving the high-yielding advances of Group A countries (innovation exchange), or if both innovation enhancement and innovation exchange were to happen. Specifically, we utilized our relapse results to measure bends characterizing the reliance of worldwide caloric yields on worldwide Nitrogen use for four cases that all meet our anticipated 2050 crop caloric demand forecast. For all cases, we accepted that the as of now substantial abberations among countries in farming powers (estimated here as Nitrogen ha^{-1}) were wiped out by 2050. We call this adjustment of Nitrogen utilize key N use, since it gives a bigger increment in worldwide harvest generation per unit of Nitrogen than would happen from more noteworthy Nitrogen use in countries as of now applying Nitrogen at high rates.

6.4 Genetic Engineering and Public Perception

As per Biotech Survey, different attitudes towards a total of ten concrete applications of genetic engineering and this selection reflects a wide extent of technical applications of genetic engineering in the fields of human, animal, plant and microbe genetics. On summarization off the assessments of these applications, a similar picture is obtained to the general assessment of genetic engineering where we are confronted with prevailing ambivalence. Only a marginal proportion of a little over 2% of the people interviewed assesses all applications consistently positively or negatively. By and large, the general population interviewed with endorses of around four applications and about 3.7 applications are opposed. This overwhelming undecided attitude design to a great extent blurs while thinking about the frames of mind towards the individual utilizations of genetic engineering. Therapeutic utilizations of genetic engineering meet with the most elevated endorsement. Three out of four individuals interviewed with endorse of this application, just 7% have a negative judgment. Uses of genetic engineering for the treatment of cell ailments meet with a correspondingly positive evaluation, they are affirmed of by 70%. The utilization of genetic engineering in the creation of antibodies and for the generation of

genetically altered microorganisms for the debasement of oil contamination in soil meets with to some degree less endorsement (Beck 1986; Rayner 1992). The two applications are evaluated decidedly by just about 66% of the general population met. Additionally, the utilization of genetic strategies for finding so as to analyze physical or mental sicknesses in unborn kids is evaluated all the more decidedly. An indemnity to this pervasively positive evaluation of restorative genetic engineering is the wide objection to the reproducing breeding of laboratory animals with certain hereditary deformities. Pretty much consistently individual met determinedly rejects this application (Zapf et al. 1987). The general population interviewed with responded all the more fundamentally to alleged 'green' genetic engineering, the use of hereditary building techniques in farming. In the utilization of genetic engineering for enhancing the obstruction of harvests against insects or plant ailments ('resistance breeding'), endorsement exceeds objection by a restricted edge of 36% to 33%. In any case, the utilization of genetic engineering as a development quickening agent in harvests is seen substantially more fundamentally: just 20% of the general population interviewed with affirms of these applications; the greater part of the general population talked with reject this application; while of this extent of chose rivals, 35% delineates thorough dismissal. The two last applications demonstrate that in fact fundamentally the same as utilizations of genetic engineering are evaluated diversely relying upon the application objective and its goal. The utilization of genetic engineering in the field of foodstuffs, for the alteration of support, to expand time span of usability or enhance the outward appearance of foodstuffs is surveyed amazingly fundamentally.

6.4.1 Assessment of Genetic Engineering and Its Applications

The very separated appraisal of genetic engineering by people most importantly demonstrates that, in view of a general evaluation drawing an equalization about genetic engineering, one can't finish up deterministically with regards to the appraisal of a solitary solid application (Hampel and Renn 1999). Both the rearing of transgenic animals so as to expand their agrarian value and hereditarily built foodstuffs are rejected (67% and 59%, individually) even by a dominant part of those affirming of genetic engineering, though, then again, half of genetic engineering rivals support of the utilization of genetic modified microscopic organisms for the corruption of oil contamination in soil and for the clinical determination of incurable sicknesses. How strong does the power of explanation of attitudes towards specific applications of genetic engineering become if all of them together are used to explain the overall assessment of genetic engineering? A multiple linear regression reveals an accounting for a proportion of 25% of the variance of the overall assessment of genetic engineering. The strongest influence on the assessment of genetic engineering is exerted by the attitudes towards genetic therapy, those towards increasing resistance in crops, towards clinical diagnosing and towards increasing yield in crops. Thus, both positively and negatively assessed applications of genetic engineering are used for the overall assessment of genetic engineering.

The ambivalent assessment of genetic engineering can be interpreted as a consequence of this cognitive dissonance (Habermas 1969; Brosius 1998). One of first lead is the summarizing of positive, ambivalent and negative assessments of genetic engineering across all specific applications. This additive index method leads to a distinctly higher proportion of the accounted-for variance of the overall assessment of genetic engineering in general ($r^2 = 0.57$). The cause of this is a sufficient concurrence of positive (55%) and negative (58%) attitudes towards genetic engineering. Ambivalent attitudes, however, are consistent to only about 46%.

6.4.2 Assessment of Genetic Engineering Via Social Dimension

Frames of mind are inactive subjective factors reliant on social determinants. The attitudes objects are associated as genetic engineering with past involvement, conceivable concern, and respond with fluctuating interest, or pay regard to the assessment of others regarding the matter (Gaskell et al. 1998; Peters 1999). Intrigue is a pertinent psychological precondition for the age of a frame of mind. Intrigue mirrors our worry and the significance of the mentality article to us. Genetic engineering is viewed as a vital subject; in any case, here we should proclaim a disparity between the foreseen social noteworthiness of the subject and the individual significance. In 75% of the considerable number of individuals interviewed with, we met with a high foreseen significance; a high social noteworthiness is, in reality, accepted by 90% surprisingly met. Notwithstanding, at about 65%, the individual enthusiasm for genetic engineering isn't articulated, with simply 20% of the general population interviewed with considering the subject as 'fascinating'. The rather restricted significance of communication within the social network (friends, acquaintances, relatives, colleagues at work) also speaks against a high personal significance. In the weeks preceding our review, just 40% of the general population met had discussed or examined genetic engineering with other individuals. On the off chance that those individuals are incorporated who had sooner or later recently talked in any event once about genetic engineering in their interpersonal organization and a more extended timeframe prior, this extent increments to 45%

Notwithstanding, the correspondence about hereditary designing is emotionally seen as serious. Of the general population met, 64% who examined hereditary building in their informal organization expect that this correspondence likewise prompt changes in demeanor. The greater part of the general population talked with see themselves here in the situation of supposition pioneer. Another applicable capacity of an interpersonal organization is its use as a wellspring of data, which, in any case, requires the nearness of very much educated individuals in the system concerned. In our examination, 27% of the general population solicited avows the nearness from such a 'genetic engineering master or source'. Be that as it may, just somewhat more than half of these individuals (55%) have really conveyed about genetic engineering. These findings likewise show the main moderate essentialness of genetic engineering as a point of regular correspondence. At the point when individuals are talking about genetic engineering in their informal communities, the

likelihood of their appraisals being affirmed are moderately high. With estimations of r = 0.57 (partners at work), r = 0.55 (companions) and r = 0.41 (relatives) the connections between's their very own evaluation and the apparent propensities of assessment in the informal organizations are certainly critical.

Around half of the general population interviewed with who in any capacity imparted about hereditary designing in their interpersonal organization expect that their very own mentalities are met with endorsement in their system. Just little the general population interviewed with move in a hereditary building related offensive system, where advertisers convey in systems to a great extent incredulous of genetic engineering or where commentators impart in informal communities to a great extent strong of hereditary building. Of the general population met, those with a conflicted disposition toward genetic engineering demonstrate the most reduced relationship (decided with the assistance of a file speaking to the propensities of sentiment in the general informal community) between their own appraisal and the apparent inclinations of supposition in their interpersonal organization (r = 0.20). Restricted to that, advertisers live in increasingly consistent systems (r = 0.38). The most noteworthy simultaneousness between their very own conclusion and that predominant in their informal organization is to be found with genetic engineering adversaries (r = 0.47).

As contrasting to supporters of hereditary designing, commentators of hereditary building don't just will in general live in systems where their feelings are shared; they additionally observe themselves more in concurrence with the popular conclusion. The appraisal of popular sentiment with respect to the general population enquired is unmistakably more wary than the individual supposition range. Genetic engineering has an unmistakably negative picture in general society. Around 80% of the advertisers accept that conflicted (38%) or dismissing (42%) mentalities will in general win among the overall population. In any case, just 40% of the faultfinders of genetic engineering trust that their own appraisal goes amiss from the popular feeling.

6.5 Sustainable Agricultural Intensifications and Food Production

In wider range of sustainable agriculture, the desire to produce more food without environmental impairment, or even positive contributions to natural and social capital has been reflected as for a 'doubly green revolution' (Conway 1997a, b), for an 'evergreen revolution' for 'alternative agriculture' (NRC 1989), for 'greener revolutions' (Snapp et al. 2010) and for 'evergreen agriculture' (Garrity et al. 2010), for 'agroecological intensification' (Milder et al. 2012), for 'green food systems' (DEFRA 2012). On recommendation, that agricultural and uncultivated frameworks should never again be considered as isolated from one another. In light of the requirement for the division likewise to contribute specifically to the goals of worldwide social– ecological difficulties, there have additionally been calls for

nutrition-sensitive (Thompson and Amoroso 2011), atmosphere keen (FAO 2013) and low-carbon (Norse 2012) farming. Sustainable generation frameworks should show various key traits at the creation end of food systems (Royal Society 2009). They should: Agricultural frameworks accentuating these standards will in general showcase various wide highlights that recognize them from the procedure and results of customary frameworks. In the first place, these frameworks will in general be multifunctional inside landscapes and economies (IAASTD 2009). They together produce nourishment and different merchandise for agriculturists and markets, while adding to a scope of esteemed open products, for example, clean water, natural life and living spaces, carbon sequestration, flood insurance, groundwater revive, scene comfort esteem, and recreation and the travel industry openings. In their setup, they gain by the collaborations and efficiencies that emerge from complex biological communities, social and monetary powers (NRC 2010).

(a) Utilize crop assortments and domesticated animals breeds with a high proportion of efficiency to utilization of remotely and inside determined sources of information
(b) Avoid the superfluous utilization of outside sources of information
(c) Harness agro ecological procedures, for example, supplement cycling, organic nitrogen obsession, allelopathy, predation and parasitism;
(d) Minimize utilization of advancements or practices that impact nature and human wellbeing
(e) Make gainful utilization of human capital as learning and ability to adjust and develop and of social funding to determine normal scene scale or framework wide issues
(f) Minimize the effects of framework the executives on externalities, for example, GHG outflows, clean water, carbon sequestration, biodiversity, and dispersal of bugs, pathogens and weeds.

Still, these frameworks are differing, synergistic and custom fitted to their specific social– natural settings. There are numerous pathways towards rural supportability, and no single setup of advancements, inputs and biological administration is bound to be generally relevant than another. Agrarian manageability suggests the need to fit these components to the particular conditions of various rural frameworks (Horlings and Marsden 2011). Difficulties, procedures and results will likewise differ crosswise over rural segments: in the UK, for instance, Elliot et al. (2013) found that animals and dairy activities progressing towards maintainability had specific troubles in lessening contamination while endeavoring to build yields. Thirdly, these frameworks frequently include progressively complex blends of tamed plant and creature species and related administration systems, requiring more noteworthy abilities and learning by ranchers. To build generation effectively and reasonably, ranchers need to comprehend under what conditions agrarian sources of info (seeds, manures and pesticides) can either supplement or negate organic procedures and biological community benefits that intrinsically bolster horticulture (Royal Society

2009). In all cases, ranchers need to see with their own eyes that additional multi-faceted nature and expanded learning sources of info can result in considerable net advantages to efficiency. Fourthly, these frameworks rely upon new arrangements of social capital, involving relations of trust typified in social associations, flat and vertical organizations among foundations, and human capital containing authority, resourcefulness, the executive's aptitudes and ability to advance. Rural frameworks with large amounts of social and human resources can advance despite vulnerability (Friis-Hansen 2012), and agriculturist to-rancher learning has been appeared to be especially essential in actualizing the setting explicit, information concentrated and regenerative practices of feasible strengthening (Rosset and Martínez-Torres 2012).

Regular reasoning about agricultural maintainability has frequently expected that it suggest a net decrease in info use, in this way making such frameworks basically broad (requiring more land to deliver a similar measure of sustenance). Natural frameworks regularly acknowledge lower yields per territory of land so as to decrease input use and increment the positive effect on common capital. Be that as it may, such natural frameworks may at present be effective if the board, learning and data are substituted for bought outer sources of info. Ongoing proof demonstrates that effective horticultural manageability activities and undertakings emerge from movements in the components of rural creation- for instance from utilization of manures to nitrogen-settling vegetables; from pesticides to accentuation on regular adversaries; from furrowing to zero-culturing). A superior idea is one that fixates on increase of assets, improving utilization of existing assets (for example land, water and biodiversity) and innovations (IAASTD 2009; Royal Society 2009; NRC 2010).

Similarity of the terms 'economical' and 'heightening' was indicated during the 1980s and after that initially utilized related in a paper inspecting the status and capability of African farming (Pretty 1997). Until this point, 'heightening' had turned out to be synonymous for a kind of agribusiness that definitely caused damage while delivering sustenance (for example Conway and Barbier 1990). Similarly, 'supportable' was viewed as a term to be connected to everything that can possibly be great about horticulture. The mix of the terms was an endeavor to show that attractive finishes (more nourishment, better condition) could be accomplished by an assortment of methods. The term was additionally advanced by its utilization in various key reports: Reaping the Benefits (Royal Society 2009), The Future of Food and Farming (Foresight 2011) and Save and Grow (FAO 2011). Supportable strengthening (SI) is characterized as a procedure or framework where yields are expanded without unfriendly ecological effect and without the development of more land (Royal Society 2009). The idea is therefore generally open, in that it doesn't expressive or benefit a specific vision of rural generation (Smith 2013). It underscores closes as opposed to implies, and does not pre-decide advancements, species blend or specific structure segments. Feasible strengthening can be recognized from previous originations of 'agricultural escalation' because of its unequivocal accentuation on a more extensive arrangement of drivers, needs and objectives than exclusively efficiency upgrade.

6.5.1 Sustainable Intensification: Emergent Criticisms

Currently, the evolving conceptual and theoretical field of SI has been shaped by a number of debates as Garnett and Godfray (2012) reviewed key contentions and debates surrounding SI, classifying these into three groups. The first relates to the vision and mode of SI, wherein the term is assumed to set down particular forms of agriculture deemed incompatible for various reasons. The second questions the justification for SI, and a third set of questions relates to the theoretical basis of SI: which is more important, 'sustainable' or 'intensification', and how do they relate to each other?

One conflict identifies with the potential for SI to be deciphered just as a 'productivist' venture. Much analysis of regular agribusiness center around worries over vast scale mechanical monocultures concerned just with expanding yields and the gross profitability of frameworks. Notwithstanding, a great horticulture would likewise be proficient in its utilization of assets, and impartial in giving access to its nourishment created (Foresight 2011). In partner SI with an account that proposes creation is the main key standard for agribusiness, a few pundits have asked whether the idea speaks to an adequately extreme takeoff from 'the same old thing'. Some have featured unmistakable and contending 'solid' and 'frail' translations of SI. 'Frail' elucidations might be available to the charge of advancing 'an obvious paradoxical expression' (Lang and Barling 2012) that may basically be utilized as a 'greenwash'. Such a view is exemplified by the ongoing declaration of a UK MP who communicated worry that '… is there not a threat that it [SI] will be utilized as a Trojan pony for the individuals who need us to have parcels more biotech and GM, etc.? … is there a potential clash between how this thought may be utilized and the eventual fate of little scale cultivating?' (Lucas 2011). Certain in the 'Trojan pony' contention is the idea of a relationship between 'expansive scale' and specific innovations, and a qualification between the estimations of 'huge' and 'little', with an understood inclination for just the last mentioned. This focuses to a pressure between various originations of what is great in farming, and uncovers a portion of the intricacy that SI must explore. Garnett and Godfray (2012) feature the center standards of the term, which has a transparency that 'means a yearning of what is to be accomplished as opposed to a depiction of existing generation frameworks, regardless of whether this be regular high-input cultivating, or smallholder agribusiness, or methodologies dependent on natural strategies. Practically speaking, it may not be anything but difficult to recognize approaches. For instance, protection farming (CA) and coordinated nuisance the board (IPM) can both be thought of differently as SI, as agro ecology, as 'atmosphere brilliant horticulture', as 'environmental escalation' or basically as a 'greener agribusiness' (Kassam et al. 2009). These terms ponder contrasting needs horticultural information sources and yields yet 'all should draw in with the truth that there are hard exchange offs between various alluring results and awkward decisions for all partners' (Garnett and Godfray 2012). Going past privileging a specific rural innovation, concentrating just on alluring social– natural results, there is a need to assess any innovation, approach or practice even-mindedly and observationally, and judge it on its benefits: does it produce

more nourishment per unit of asset; and does it do as such without damage to the earth? It stays clear, however, that better agrarian and nourishment frameworks could be envisioned by decreasing sustenance squander, expanding network commitment and lessening imbalance, paying little respect to the types of creation in fields and ranches (Stock 2015). As essential in horticultural frameworks to agriculturists and specialists are comes back to work, and the conveyance of advantages among ladies and men.

Notwithstanding, even the transparency of SI tosses some troublesome inquiries into alleviation. Characterizing 'maintainability' has dependably been hard. Similarly as with various adaptations of supportability, it is conceivable to contend that SI has 'light' and 'dim' green understandings. Characterizing limits – among horticulture and other financial divisions or around units of scene (ranches, watersheds, scenes) or around time ranges (5-year designs, decades, crosswise over ages) – is additionally troublesome as a result of fragmented learning, consistently advancing conditions and various human qualities (Garnett and Godfray 2012). Once more, results are critical: social and political changes might be expected to guarantee that yield increments conveyed through SI really decrease appetite and neediness (Holt-Giménez and Altieri 2013). Wording can shroud varieties by and by, and regularly manageability results. For instance, IPM establishes a wide scope of strategies, practices and advancements accessible to decrease irritation, weed and sickness dangers. A few methodologies focus on agroecological the executives and territory configuration, utilizing the administrations of biodiversity on and off homestead. Others focus on booking of pesticides. Jacobsen et al. (2013) contend, numerous contentions about nourishing the world expect that we need a greater amount of our current, western eating routine, yet it ought to be clear that the total populace can all the more likely be bolstered, both agronomically, naturally and concerning human wellbeing, with an eating regimen not the same as what is most basic in the created present reality.' It isn't constantly acknowledged that yields should be expanded (Tomlinson 2013). Elliot et al. (2013) argument out that in specific cases, SI 'may not be a suitable technique because other biological community capacities might be esteemed more exceedingly than increments in nourishment creation.

A typical protest made about numerous agroecological approaches for SI is their apparent requirement for expanded work (Tripp 2005). Be that as it may, maintainability concerns are exceedingly site explicit: at times more work isn't required; in others the additional work required is viewed as an important commitment to neighborhood economies (De Schutter and Vanloqueren 2011). In a few settings, work is profoundly constraining, particularly where HIV-AIDS has evacuated a vast extent of the dynamic populace; in different settings, there is copious work accessible as there is couple of other business openings in the economy. Effective frameworks of maintainable strengthening by definition fit answers for nearby needs and settings, thus consequently assess work accessibility. In Kenya and Tanzania, for instance, female proprietors of raised beds for vegetable generation utilize neighborhood individuals to deal with vegetable development and promoting (Muhanji et al.

2011). Work for yield and domesticated animals the board is in this manner not really a limitation on new innovations.

In Burkina Faso, work gatherings of young fellows have risen for soil protection. Tassas and zai planting pits are most appropriate to landholdings where family work is accessible, or where ranch hands can be employed. The system has prompted a system of youthful day workers who have aced this procedure. Attributable to the accomplishment of land recovery, agriculturists are progressively purchasing corrupted land for development, and paying workers to burrow zai pits and build the stone dividers and half-moon structures, which have changed efficiency. This is one reason why >3 Mha of land are currently restored and beneficial. In different settings, however, movements to manageable frameworks, for example, consolidating agroforestry into maize frameworks in Africa has prompted both diminished and expanded work prerequisites, contingent upon the neighborhood social and environmental setting.

6.5.2 Sustainable Intensification: Evidences and Impacts

Archiving and assessing proof from SI is muddled and at times argumentative. In the first place, applied decent variety and the inclusivity of the methodology imply that it is hard to 'bound' assessments. Agroecological approaches include numerous practices, adjusted from spot to put contingent upon farmer and community needs. There might be no unmistakable calculated, methodological or down to earth separating line among 'elective' and 'ordinary' practice. Contingent upon need and capacity, agriculturists may apply agroecological standards to industrial farms, or present the automation and inorganic contributions to generally agroecologically-overseen farms (Milder et al. 2012). Where studies seek to demonstrate simultaneous improvements to yields and environmental outcomes, results are highly sensitive to the variables and parameters selected to capture environmental improvements, the time scales involved and any weightings used (Elliot et al. 2013).

A few evaluations have been found to experience the ill effects of methodological flaws (Milder et al. 2012). In the first place, in spite of the heterogeneity of practices associated with any heightening procedure, appraisals regularly focus around yields from explicit, named methodologies –, for example, CA, agroforestry or IPM. Investigation of particular methodologies is likewise troublesome. For instance, proof on results from CA and the arrangement of rice escalation (SRI) is blended, and debate on the general relevance and adaptability of these methodologies has been 'prominent, continued and now and again bitter and emotive' (Sumberg et al. 2013). Furthermore, amalgamations, meta-examinations and reviews have so far concentrated essentially on yield increments as opposed to on various results and advantages (however observe Milder et al. 2012). At last, there is not yet adequate information on how extraordinary agroecological techniques may meet total territorial and worldwide objectives.

Halfway of the fact that SI is an umbrella term that incorporates a wide range of agrarian practices and advances, and on the grounds that it is more a methodology than an unmistakable arrangement of advances and procedures, the exact degree of existing SI practice is likewise obscure. Milder et al. (2012) estimate that all around somewhere in the range of 200 Mha of farming area are developed under some type of agroecological routine. Smallholder creation is especially reliant on sound biological systems nearby homesteads, and it has been assessed that a large portion of the world's smallholders practice some type of asset moderating agribusiness (IFAD and UNEP 2013).

Various amalgamations have featured expanded yields (among other positive social– environmental results) because of the use of agroecological techniques and update. These again have stressed the helpful results of both– and methodologies as opposed to either– or. Results are vital; pathways contrast. Yields, however, can be a rough proportion of the effective yields or effects of horticultural frameworks, especially where increasingly feasible frameworks are relied upon to impactsly affect the common segments of both farming and wild frameworks and territories. It is in creating nations that probably the most noteworthy advancement towards economical strengthening has been made in the previous two decades. The biggest examination involved the investigation of 286 undertakings in 57 nations (Pretty et al. 2006). Taking all things together, some 12•6 million ranchers on 37 Mha were occupied with changes towards farming supportability in these 286 activities (Pretty 2008).

The Government Office of Science, UK Foresight programme commissioned reviews and analyses from 40 projects in 20 countries of Africa where SI had been developed in the 2000s (Pretty et al. 2011, 2014). The cases comprised crop improvements, agroforestry and soil conservation, CA, IPM, fodder crop integration, horticultural intensification, livestock aquaculture, and novel policies and partnerships. These projects had documented benefits for 10·4 million farmers and their families and improvements on approx. 12·75 Mha by early on 2010. Across the projects, yields of crops rose on average by a factor of 2·13 (i.e. slightly more than doubled). The time scale for these improvements varied from 3 to 10 years. It was estimated that this resulted in an increase in aggregate food production of 5·79 Mt $year^{-1}$, equivalent to 557 kg per farming household. Milder et al. (2012) undertook a broad review of five sets of agro ecological systems, examining their contribution to yields, as well as nine distinct ecosystem services which were relevant to both on- and off-farm beneficiaries. In 1989, the US National Research Council (NRC) distributed the original Alternative Agriculture. Incompletely determined by expanded expenses of compost and pesticide contributions, in addition to developing shortage of common assets, (for example, groundwater for water system), and proceeded with soil disintegration, agriculturists had been embracing novel methodologies in a wide assortment of homestead frameworks. The NRC noticed that 'elective horticulture' was 'not a solitary arrangement of cultivating rehearses', that they were good with substantial and little ranches and that they were regularly broadened. Such option farming frameworks utilized harvest turns, IPM, soil and water rationing culturing, creature generation frameworks that underlined sickness counteractive action without anti-microbials, and hereditary enhancement of yields

to oppose nuisances and illness and use supplements all the more productively. Very much-estimated elective cultivating frameworks 'almost constantly utilized less manufactured compound pesticides, composts and anti-infection agents per unit of creation than practically identical ordinary homesteads' (NRC 1989). They likewise required 'more data, prepared work, time and the board aptitudes per unit of generation. The NRC (1989) dispatched 11 point by point contextual investigations of 14 cultivates as models of viable and distinctive ways to deal with accomplishing comparable points: monetarily fruitful homesteads with a positive effect on normal capital. The NRC (2010) led follow-up concentrates in 2008 on ten of the first homesteads. These included incorporated crop– animal's endeavors, foods grown from the ground ranches, one hamburger cows farm and one rice ranch. Following 22 years, regular highlights of ranches notwithstanding: In France, the IAD (2011) has required another European horticulture based around keeping up solid soil, biodiversity, suitable preparation and fitting plant assurance strategies. Testing 26 markers classed into seven subjects (financial suitability, social reasonability, input effectiveness, soil quality, water quality, GHG discharges and biodiversity) crosswise over 160 distinct kinds of ranch, the creators found that positive natural externalities can be both accomplished and estimated. Together, these pointers contain an extensive scorecard that can be connected to test advance towards the generation of positive environmental externalities just as support of profitability.

(a) All ranches underscoring the significance of keeping up and developing their characteristic asset base and augmenting the utilization of inner assets;
(b) All ranchers stressing the estimations of ecological maintainability and the significance of shut supplement cycles;
(c) The crop ranches accentuating watchful soil the executives, the utilization of harvest turns and spread yields; the domesticated animals ranches proceeding with the board rehearses that did not utilize hormones or anti-infection agents;
(d) More agriculturists taking an interest in non-customary item and direct deals markets (through ranchers markets as well as the web); some moving at a higher cost than normal with named attributes (for example natural, normally raised domesticated animals);
(e) Farms depending vigorously on relatives for work and the board; and
(f) The difficulties and dangers focused on rising area and rental qualities related with urban advancement weight, the accessibility of water and the spread of new weed species.

Farmers adopting SI approaches have been able to increase food outputs by sustainable intensification in two ways. The first is multiplicative – by which yields per hectare have increased by combining use of new and improved varieties with changes to agronomic–agro ecological management. The second is improved food outputs by additive means – by which diversification of farms resulted in the emergence of a range of new crops, livestock or fish that added to the existing staples or vegetables already being cultivated. These additive system enterprises included the following. Environmental externalities have been shown to be positive. Carbon

content of soils is improved where legumes and shrubs are used, and where conservation agriculture increases the return of organic residues to the soil. Legumes also fix nitrogen in soils, thereby reducing the need for inorganic fertilizer on subsequent crops. In IPM-based projects, most have seen reductions in synthetic pesticide use (e.g. in cotton and vegetables in Mali, pesticide use fell to an average of 0·25 L ha^{-1} from 4·5 L ha^{-1}: Settle and Hama Garba 2011). In some cases, biological control agents have been introduced where pesticides were not being used at all, or habitat design has led to effective pest and disease management (Royal Society 2009; Khan et al. 2011). The greater diversity of trees, crops (e.g. beans, fodder shrubs and grasses) and non-cropped habitats has generally helped to reduce runoff and soil erosion, and thus increased groundwater reserves. Projects across sub-Saharan Africa, where nutrient supply is a key constraint, have used a mix of inorganic fertilizers, organics, composts, legumes, and fertilizer trees and shrubs to improve nutrient availability, in conjunction with conservation tillage to improve soil health. Policy and institutional support has also been important. The Malawi fertilizer subsidy programme is a rare example of a national policy that has led to substantial changes in farm use of fertilizers and the rapid shift of the country from food deficit to food exporter (Dorward and Chirwa 2011). In this case, the importance of both bonding social capital between farmers in groups and linking social capital between national institutions and farmers was critical to rapid adoption.

(a) Aquaculture for fish raising (in fish ponds or concrete tanks) (Brummett and Jamu 2011).
(b) Small patches of land used for raised beds and vegetable cultivation (Muhanji et al. 2011).
(c) Rehabilitation of formerly degraded land (Sawadogo 2011).
(d) Fodder grasses and shrubs that provide food for livestock (and increase milk productivity) (Wambugu et al. 2011).
(e) Raising of chickens, and zero-grazed sheep and goats (Roothaert et al. 2011).
(f) New crops or trees brought into rotations with staple yields not affected, such as pigeonpea, soyabean, indigenous trees (Asaah et al. 2011).
(g) Adoption of short-maturing varieties (e.g. sweet potato, cassava) that permit the cultivation of two crops per year instead of one (Roothaert and Magado 2011)

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Nanotechnology and Sustainable Agriculture

7

Abstract

Nanoparticles interact with plants causing many morphological and physiological changes, depending on their properties. Their chemical composition, size, surface covering, reactivity, and most importantly the dose at which they are effective determine efficacy of Nanoparticles. Though their importance is immense and day-by-day due to the technological advancement their role is marking an impact in every sphere of living. Now a day's every science is served with Nano-techniques from nano-medicine to nano-Agriculture. In this context, the chapter is divided in several sections depending on the need of assessment of the topic particularly with reference to Agriculture sustainability.

Keywords

Nano pesticides · Microencapsulation · Nano fertilizers · Nanotechnology

7.1 Nanoparticles: Role and Functions

Nanoparticles communicate with plants causing frequent morphological and physiological changes, contingent upon their properties. Viability of Nanoparticles, is controlled by their synthetic arrangement, measure, surface covering, reactivity, and above all the portion at which they are compelling (Khodakovskaya et al. 2012). Specialists from their discoveries recommended both positive and negative consequences for plant development and improvement, and the effect of designed nanoparticles (Encapsulated Nanoparticles) on plants relies upon the creation, measure, focus and physical and synthetic properties of Encapsulated Nanoparticles just as plant species (Ma et al. 2008). Adequacy of Nanoparticles relies upon their fixation and shifts from plants to plants (Table 7.1). However, this review covers plausible role of Nanoparticles in seed germination, photosynthesis, roots, and plant development (root and shoot biomass).

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Table 7.1 Beneficiary concentration(s) of nanoparticles for plants

Nanoparticle(s)	Beneficiary concentration(s)	Plant	Part of plant/process
Nano-anatase TiO2	0.25% (foliar spray)	*Spinacia oleracea*	Rubisco activase (rca) mRNA expressions,
	0.25% (foliar spray)	*Spinacia oleracea*	Oxygen evolution, Rubisco carboxylation, Rubisco Activase, rate of photosynthetic carbon reaction
	0.25%	*Spinacia oleracea*	Several enzymes activities induction
Aluminum oxide Nanoparticles	400–4000 mg/L	*Arabidopsis thaliana,*	Root length
Alumina Nanoparticles	10 mg/L	*Lemna minor*	Root length
	0.3 g/L	*Lemna minor*	Biomass accumulation
nZVI (nanoscale Zero- Valent Iron particles) Iron oxide Nanoparticles	0.5 g/L	*Arabidopsis thaliana*	Root elongation
Iron oxide Nanoparticles	0.5–0.75 g/L	*Glycine max*	Yield and quality
	50 ppm (foliar spray)	*Vigna radiata*	Biomass
ZnFeCu-oxide Nanoparticles (suspension)	50 ppm (foliar spray)	*Vigna radiata*	Biomass
CeO_2 Nanoparticles	250 ppm	*Arabidopsis thaliana*	Biomass
CO_3O_4 Nanoparticles	5 g/L	*Raphanus sativus* L.	Root elongation
CuO Nanoparticles	500 mg/kg (sand culture)	*Triticum aestivum*	Biomass
Hydroxyapatite Suspension	100–2000 mg/L	*Lactuca sativa*	Root length
GNanoparticles	10 and 80 µg/mL	*Arabidopsis thaliana*	Germination
	10 and 80 µg/mL	*Arabidopsis thaliana*	Root length
	80 µg/mL	*Arabidopsis thaliana*	Shoot and root system (longer), early flowering, Yield
SilverNanoparticles	10–30 µg/mL	*Boswellia ovaliofoliolata*	Germination and seedling Growth
	60 ppm	*Phaseolus vulgaris* L., *Zea mays* L.	Root length
	60 ppm	*Phaseolus vulgaris* L., *Zea mays* L.	Shoot length
	60 ppm	*Phaseolus vulgaris* L., *Zea mays* L.	Dry weight of root and shoot
	100 µM	*Vigna radiata*	Antagonize inhibition by 2,4-dichlorophenoxyacetic acid (2,4-D) at 500 µM of plant Growth

(continued)

Table 7.1 (continued)

Nanoparticle(s)	Beneficiary concentration(s)	Plant	Part of plant/process
Sulfur Nanoparticles	500, 1000, 2000 and 4000 ppm	*Vigna radiata*	Dry weight
Silicon dioxideNanoparticles	15 kg/ha	*Zea mays L.*	Growth parameters
TiO2 Nanoparticles	400 mg/L	*Arabidopsis thaliana*	Root length
	60 ppm	*Foenicutum vulgare*	Germination
	Lower than 200 mg/L	*Lemna minor*	Plant growth
	1000 mg/L	*Triticum aestivum*	Chlorophyll content
	0.25%	*Spinacia oleracea*	Hill reaction, non cyclic photophosphorylation, protect chloroplasts from silvering
	0.05–0.2 g/L	*Lycopersicon esculentum Mill*	Net photosynthetic rate, conductance to H_2O, and transpiration rate, regulation of photosystem II (PSII)
Graphene oxide	400 and 800 mg/L	*Vicia faba* L.	Germination
Carbon nanotubes	40 μg/mL	*Lycopersicum esculantum*	Germination and seedling Growth
	75 wt% carbon nanotubes	*Medicsilvero saliva, Triticum aestivum*	Root elongation
	75 wt% carbon nanotubes impurities	*Medicsilvero saliva, Triticum aestivum*	Root elongation
SW carbon nanotubes	9, 56, 315, and 1750 mg/L	*Allium cepa, Cucumis sativus*	Root elongation
MULTI-WALLED carbon nanotubes	25–100 μg/mL	*Hordeum vulgare* L., *Glycine max, Zea mays*	Root elongation
	50 and 200 μg/mL	*Lycopersicon esculentum Mill*	Plant height and number of Flowers
	5 up to 500 μg/mL	*Nicotiana tabacum*	Growth
o-MULTI-WALLED carbon nanotubes	10–160 μg/mL	*Triticum aestivum*	Root growth, vegetative Biomass
ws carbon nanotubes	6.0 μg/mL	*Cicer arietinum*	Growth rate
MULTI-WALLED carbon nanotubes, dMULTI-WALLEDCNT	40 μg/mL	*Lycopersicon esculentum Mill*	Uptake nutrients (K, Ca, Fe, Mn and Zn)

(continued)

Table 7.1 (continued)

Nanoparticle(s)	Beneficiary concentration(s)	Plant	Part of plant/process
Pristine MULTI-WALLED carbon nanotubes	20 mg/L	*Zea Mays*	Nutrient transport, biomass
ZnO Nanoparticles	400 mg/kg	*Cucumis sativus* fruit	Micronutrients (Cu, Mn and Zn)
	1.5 ppm (foliar spray)	*Vigna radiata*	Biomass
	1000 ppm	*Arachis hypogaea*	Germination
	1000 ppm	*Arachis hypogaea*	Stem, root growth and yield
	500, 1000, 2000 and 4000 ppm	*Vigna radiate* L. Wilczek	Dry weight

Source: Siddiqui et al. (2015)

7.1.1 Silicon Dioxide Nanoparticles

Plant development and advancement begins from the germination of seeds pursued by root extension and shoot rise as the most prompt indications of maturity and improvement. Subsequently, it is essential to comprehend the course of plant development and advancement in connection to Nanoparticles. The announced information from different investigations recommended that impact of Nanoparticles on seed germination be also focused on secondary basis. In tomato, the lower concentration of nano-silicon dioxide enhanced seed germination (Siddiqui and Al-Whaibi 2014). As per Suriyaprabha et al. (2012), nano-silicon dioxide extended seed germination by giving better supplements accessibility to maize seeds, and pH and conductivity to the developing medium. Bao-shan et al. (2004) applied exogenous application of nano-SiO2 on Larix olgensis seedlings and found that nano-SiO2 improved seedling growth and quality, including mean height, root collar diameter, main root length, and the number of lateral roots of seedlings and also induced the synthesis of chlorophyll. Haghighi et al. (2012), in tomato and Siddiqui and Al-Whaibi (2014) in squash detailed that nano-silicon dioxide improved seed germination and animated the cell reinforcement framework under NaCl stress. Shah and Belozerova (2009) tried silica, gold, copper and palladium nanoparticles in their investigation and found that every one of these nanoparticles impact lettuce seeds. Exogenous use of nano-silicon dioxide and nano-titanium dioxide (nano-TiO2) enhances seed germination of soybean by expanding nitrate reductase and furthermore by upgrading seeds capacity to retain and use water and supplements (Zheng et al. 2005). Under salinity stress, nano-silicon dioxide enhances fresh and dry weight of leaves, chlorophyll substance and proline collection. An expansion in the collection of free amino acids, proline, substance of supplements, antioxidative enzyme activity due to the nano-silicon dioxide, accordingly enhancing the resilience of plants to abiotic stress (Kalteh et al. 2014; Haghighi et al. 2012). Wang et al. (2014) carried out an investigation on rice plant which were treated with

quantum spots (QDs), without QDs and with silica covered with QDs, and discovered silica covered with QDs advanced especially rice root development. Nanosilicon dioxide improves the plant development and advancement by increasing chlorophyll fluorescence parameters like transpiration rate, net photosynthetic rate, stomatal conductance, potential movement of PSII, electron transport rate, compelling photochemical productivity, legitimate photochemical effectiveness, and photochemical extinguish (Xie et al. 2011).

7.1.2 Zinc Oxide Nanoparticles

In various, investigations it has been found that these Nanoparticles impact lettuce seeds. As in soybean the exogenous utilization of nano-silicon dioxide and nanotitanium dioxide (nano-TiO2) enhances seed germination by expanding nitrate reductase and furthermore by upgrading seeds capacity to retain and use water and supplements (Zheng et al. 2005). Prasad et al. (2012) in peanut; Sedghi et al. (2013) in soybean; Ramesh et al. (2014) in wheat and Raskar and Laware (2014) in onion, reported that at lower concentration of Zinc oxide nanoparticles which exhibited beneficial effect on seed germination. However, at the higher doses of Zinc oxide nanoparticles impaired the process of seed germination. The effect of nanoparticles on germination depends on concentrations of these nanoparticles and significantly differs from plants to plants. de la Rosa et al. (2013) applied different concentrations of Zinc oxide nanoparticles on cucumber, alfalfa and tomato, and found that only cucumber seed germination was enhanced. Raliya and Tarafdar (2013) reported that Zinc oxide nanoparticles induced a significant improvement in *Cyamopsis tetrsilveronoloba* plant biomass, root area, chlorophyll and protein synthesis, shoot and root growth, rhizospheric microbial population, acid phosphatase, alkaline phosphatase and phytase activity in cluster bean rhizosphere. It is evident from the correlative light and scanning microscope, and inductive coupled plasma/atomic emission spectroscopy that seedling roots of Vigna radiata and Cicer arietinum absorbed Zinc oxide nanoparticles and promoted the root and shoot length, and root and shoot biomass (Mahajan et al. 2011). Nano Zinc Oxide along with MS media promoted somatic embryogenesis, shooting, regeneration of plantlets, and also induced proline synthesis, activity of superoxide dismutase, catalase, and peroxidase thereby improving tolerance to biotic stress (Helaly et al. 2014).

7.1.3 Carbon Nanotubes

The carbon nanotubes have gained a critical position because of their interesting mechanical, electrical, thermal properties among the available Nanoparticles. The accessible information uncovers that reviews on carbon nanotubes have chiefly centered on creatures and people (Ke et al. 2011; Tiwari et al. 2014). Relatively, there has been inadequate data accessible on carbon nanotubes and their connection with plants cells and plant digestion. The important property of carbon

nanotubes, that they can enter the cell divider and film of cells and furthermore give a reasonable conveyance arrangement of synthetic concoctions to cells. The single-walled- carbon nanotubes (SW carbon nanotubes) penetrate as nanotransporters for conveyance of DNA and color particles into plants cells (Srinivasan and Saraswathi 2010). As in various investigations researchers has revealed that multi-walled- carbon nanotubes have an enchantment capacity to impact the seed germination and plant development, and work as a conveyance arrangement of DNA and synthetic compounds to plants cells. Tiwari et al. (2014) reported that multi-walled-carbon nanotubes incite the water and basic Ca and Fe supplements take-up effectiveness that could upgrade the seed germination and plant development and advancement. Multi-walled carbon nanotubes added to sterile silvere medium invigorated seed germination of three imperative yields (grain, soybean, corn) because of the capacity of multi-walled carbon nanotubes to infiltrate the seed coats as the nanotube agglomerates were identified inside the seed coats utilizing Raman Spectroscopy and Transmission Electron Microscopy (Lahiani et al. 2013). Additionally, they detailed that multi-walled carbon nanotubes directed qualities articulation encoding a few kinds of water divert proteins in soybean, corn and grain seeds coat. The most extreme germination rate in tomato, half and half Bt cotton, Brassica juncea, Phaseolus mungo and rice was seen with multi-walled carbon nanotubes (Morla et al. 2011; Nalwade and Neharkar 2013). Likewise, numerous analysts affirmed the positive job of carbon nanotubes in seed germination and plant development and improvement. Khodakovskaya et al. (2012) announced that multi-walled carbon nanotubes go about as controllers for seed germination and development, and they exhibited that multi-walled carbon nanotubes can enlarge the development of tobacco cell culture by upregulating the marker qualities for cell divisions (CycB), cell divider arrangement (NtLRX1) and water transport (aquaporin, NNtPIP1). Wang et al. (2012) revealed oxidized multi-walled carbon nanotubes altogether improved cell stressing in the root framework and advanced dehydrogenase action. Regardless, a couple of researchers nitty gritty that multi-walled carbon nanotubes don't demonstrate a constructive outcome on seed germination in numerous plants notwithstanding when they got high centralization of multi-walled carbon nanotubes (Lin and Xing 2007). Multi-walled carbon nanotubes enhance the root and stem development and peroxidase and dehydrogenase movement might be because of essential take-up and accretion of multi-walled carbon nanotubes by roots pursued by the translocation from roots to leaves that could incite qualities articulation (Khodakovskaya et al. 2012; Lahiani et al. 2013). Tripathi and Sarkar (2014) affirmed the nearness of water dissolvable carbon nanotubes inside the wheat plants utilizing Scanning Electron and Fluorescence Microscope, and they revealed that carbon nanotubes instigated the root and shoot development in light and diffuse conditions. Additionally, multi-walled carbon nanotubes enhance water maintenance limit and biomass, blooming and organic product yield and increment restorative properties of plants (Husen and Siddiqi 2014). Notwithstanding, inhibitory impact of multi-walled carbon nanotubes on plants development has been accounted for by numerous specialists (Tiwari et al. 2014; Ikhtiar et al. 2013; Begum and Fugetsu 2012; Begum et al.

2014). In this way, the impact of Nanoparticles on plants differs from plant to plant, their development stages, and the idea of nanoparticles.

7.1.4 Gold Nanoparticles

Least efforts have been done on the association of Gold nanoparticle (Au Nanoparticles) with plants. A few researchers discovered Au Nanoparticles prompt harmfulness in plants by repressing aquaporin work, a gathering of proteins that assistance in the transportation of wide scope of atoms including water (Shah and Belozerova 2009). While as, Barrena et al. (2009) in lettuce and cucumber, Arora et al. (2012) in Brassica juncea; Savithramma et al. (2012) in Boswellia ovalifoliolata and Gopinath et al. (2014) in Gloriosa superba revealed that Au Nanoparticles enhance seed germination. Au Nanoparticles enhance the quantity of leaves, leaf zone, plant stature, chlorophyll substance, and sugar content that lead to the better harvest yield (Arora et al. 2012; Gopinath et al. 2014). Christou et al. (1988) brought neomycin phosphotransferase II quality into soybean genome through DNA-covered au particles. The beneficial outcome of AuNanoparticles in this manner needs further examination to investigate the physiological and atomic system. Kumar et al. (2013) detailed AuNanoparticles have a noteworthy job on seed germination and cell reinforcement framework in Arabidopsis thaliana and modified dimensions of microRNAs articulation that directs different morphological, physiological, and metabolic procedures in plants.

7.1.5 Silver Nanoparticles

As indicated by accessible information a substantial number of concentrates on silver nanoparticles (SilverNanoparticles) have been recorded on microbial and creature cells; be that as it may, just a couple of studies were done on plants (Krishnaraj et al. 2012). As we probably are aware about that Nanoparticle have both positive and negative consequences for plant development and improvement. As overdue, Krishnaraj et al. (2012) considered the impact of organically orchestrated Silver Nanoparticles on hydroponically developed Bacopa monnieri development digestion, and found that biosynthesized Silver Nanoparticles demonstrated a noteworthy impact on seed germination and incited the union of protein and sugar and diminished the all out phenol substance and catalase and peroxidase exercises. Additionally, organically blended SilverNanoparticles improved seed germination and seedling development of trees Boswellia ovaliofoliolata (Savithramma et al. 2012). Silver Nanoparticles expanded plants development profile (shoot and root length, leaf region) and biochemical qualities (chlorophyll, sugar and protein substance, cancer prevention capsulent chemicals) of Brassica juncea, regular bean and corn (Salama 2012; Sharma et al. 2012). In any case, Gruyer et al. (2013) revealed Silver Nanoparticles have both positive and negative impact on root prolongation relying upon the plants species. They announced that

root length was expanded in grain, yet was hindered in lettuce. Likewise, Yin et al. (2012) examined the impacts of SilverNanoparticles on germination of eleven wetland plants species (Lolium multiflorum, Panicum virgatum, Carex lurida, C. scoparia, C. vulpinoidea, C. crinita, Eupatorium fistulosum, Phytolaca Yankee folklore, Scirpus cyperinus, Lobelia cardinalis, Juncus effusus) and discovered SilverNanoparticles improved the germination rate of one animal varieties (E. fistulosum). SilverNP initiates root development by blocking ethylene motioning in Crocus sativus (Rezvani et al. 2012). The effect of Silver Nanoparticles on morphology and physiology of plants relies upon the size and state of Nanoparticles. Syu et al. (2014) considered the impact of 3 unique morphologies of Silver Nanoparticles on physiological and sub-atomic reaction of Arabidopsis and proposed that decahedral Silver Nanoparticles demonstrated the most elevated level of root development advancement (RGP); in any case, the round Silver Nanoparticles had no impact on RGP and set off the largest amounts of anthocyanin aggregation in Arabidopsis seedlings. The decahedral and round SilverNanoparticles gave the most minimal and most elevated qualities for Cu/Zn superoxide dismutase, individually. The three diverse size and state of SilverNanoparticles mancapsuled protein collections, for example, cell-division-cycle kinase 2, protochlorophyllide oxidoreductase, and fructose-1,6 bisphosphate aldolase and furthermore incited qualities articulation associated with cell occasions; for instance SilverNanoparticles instigated the quality articulation of indoleacetic corrosive protein 8 (IAA8), 9-cis-epoxycarotenoid dioxygenase (NCED3), and drying out responsive RD22. Likewise, SilverNanoparticles enacted the aminocyclopropane1-carboxylic corrosive (ACC)- determined restraint of root extension in Arabidopsis seedlings, just as diminished the statement of ACC synthase 7 and ACC oxidase 2, proposing that SilverNanoparticles went about as inhibitors of ethylene discernment and could meddle with ethylene biosynthesis.

7.1.6 Titanium Dioxide Nanoparticles

Like Silver Nanoparticles, various examines have concentrated on the effect of titanium dioxide nanoparticles (TiO2 Nanoparticles) on microscopic organisms, green growth, tiny fish, fish, mice, and rodents, however inquire about concentrating on the acknowledgment of the impacts of TiO2 Nanoparticles on plant stays deficient. TiO2 Nanoparticles improved seed germination and advanced radicle and plumule development of canola seedlings (Mahmoodzadeh et al. 2013). Jaberzadeh et al. (2013) detailed that TiO2 Nanoparticles increased wheat plant development and yielded parts submerged shortfall push condition. TiO2 Nanoparticles mancapsules chemicals action associated with nitrogen digestion, for example, nitrate reductase, glutamate dehydrogenase, glutamine synthase, and glutamic-pyruvic transaminase that causes the plants to assimilate nitrate and furthermore supports the transformation of inorganic nitrogen to natural nitrogen as protein and chlorophyll, that could expand the crisp weight and dry load of plant (Yang et al. 2006). TiO2 Nanoparticles

goes about as a photocatalyst and prompts an oxidation-decrease response. TiO2 Nanoparticles recognizably advances matured seeds' force and chlorophyll arrangement and animates Ribulose 1, 5-bisphosphate carboxylase (Rubisco) movement and builds photosynthesis, in this manner expanding plant development and improvement (Yang et al. 2006). TiO2 Nanoparticles builds light absorbance, hurry the vehicle and change of the light vitality, shield chloroplasts from maturing, and drsilver out the photosynthetic time of the chloroplasts (Yang et al. 2006). It might be expected to TiO2 Nanoparticles shields the chloroplast from unnecessary light by expanding the action of free radical generation, for example, catalase, superoxide dismutase, peroxidase, (Hong et al. 2005).

7.1.7 Role of Nanoparticles in Photosynthesis

As plants on the earth converts only 2–4% of the total incident light into chemical energy through the key process of photosynthesis and results promotion of plant growth. These days, researchers are endeavoring to enhance this low productivity of vascular plants by controlling strategies and quality controls. To accelerate the process of plant photosynthesis and turbocharged crops, researchers are working with Rubisco, an essential catalyst for photosynthetic procedure to catalyze the joining of carbon dioxide into natural mixes. As of delayed, Lin et al. (2014) developed new tobacco plants by substituting the Rubisco quality for carbon settling in tobacco plant, with two qualities of cyanobacterium Synechococcus prolongs; these new-built plants have more photosynthetic productivity than native ones. Likewise, in the field of nanobiotechnology, analysts need to create bionic plants that could have better photosynthesis effectiveness and biochemical detecting. Giraldo et al. (2014) detailed that inserted SW carbon nanotubes in the disconnected chloroplast enlarged multiple times higher photosynthetic action than that of controls, and improved greatest electron transport rates, and SW carbon nanotubes empowered the plants to detect nitric oxide, a flagging atom. They recommended that nanobionics way to deal with built plants would empower new and progressed useful properties in photosynthetic organelles. Likewise, they said that still broad research would be expected to see the effect of carbon nanotubes on definitive results of photosynthesis, for example, sugars and glucose. Additionally, Noji et al. (2011) announced that a nano-mesoporous silica compound (SBA) bound with photosystem II (PSII) and incited stable movement of a photosynthetic oxygen-advancing response, showing the light-determined electron transport from water to the quinone particles, and they recommended that PSII-SBA conjugate may have photosensors and counterfeit photosynthetic framework. Silicon dioxide Nanoparticles enhances photosynthetic rate by enhancing action of carbonic anhydrase and blend of photosynthetic colors (Siddiqui and Al-Whaibi 2014). Carbonic anhydrase supplies CO2 to the Rubisco, which may enhance photosynthesis. Nano-anatase TiO2 have a photocatalyzed trademark and enhances the light absorbance and the change from light vitality to electrical and synthetic vitality, and

furthermore prompts carbon dioxide osmosis. TiO2 Nanoparticles shield chloroplast from maturing for long time enlightenment (Hong et al. 2005; Yang et al. 2006). Nano-anatase TiO2 improves the photosynthetic carbon digestion by initiating Rubisco (complex of Rubisco and Rubisco activase) that could advance Rubisco carboxylation, along these lines expanding development of plants (Gao et al. 2006). Mama et al. examined the effect of nano-anatase on sub-atomic component of carbon response and proposed nano-anatase-prompted marker quality for Rubisco activase (rca) mRNA and upgraded protein levels and exercises of Rubisco activase brought about the enhancement of the Rubisco carboxylation and the high rate of photosynthetic carbon response. The exogenous use of TiO2 Nanoparticles enhances net photosynthetic rate, conductance to water, and transpiration rate in plants (Qi et al. 2013). As indicated by Lei et al. (2007) nano-anatase advanced firmly entire chain electron transport, photo reduction action of photosystem II, O2-developing and photophosphorylation movement of chlorophyll under both unmistakable and bright light. As per Govorov and Carmeli (2007), metal nanoparticles can incite the effectiveness of synthetic chemical energy production in photosynthetic frameworks. The chlorophyll in photosynthetic response focus ties to the AuNanoparticles and Silver nanocrystals, in this manner shaping a novel mixture framework that may create multiple times increasingly energized electrons due to plasmon reverberation and quick electron-opening division. The improvement components may help in the structure of artificial light-gathering frameworks.

7.2 Microencapsulation Techniques

Different systems are accessible for the embodiment of center materials. Comprehensively the techniques are separated into three sorts. Diverse kinds of microencapsulation strategies are.

1. Compound techniques
2. Physico-compound techniques
3. Physico-mechanical techniques

The previously mentioned procedures are generally utilized for microencapsulation of a few pharmaceuticals. Among these procedures, fluidized bed or air suspension strategy, coacervation and phase partition, spray drying and shower hardening, container covering and dissolvable dissipation strategies are broadly utilized. Contingent upon the physical idea of the core substance to be epitomized the method utilized will be differed.

7.2.1 Chemical Methods

7.2.1.1 Interfacial Polymerization (IFP)

In this method, the reactive multifunctional monomers will be polymerized to form the capsule shell will be at or on the surface of the droplet or particle as commonly used monomers are either individual particles or blend which includes multifunctional isocyanates and acid chlorides. The multifunctional monomer broke up in fluid center material and it will be scattered in watery phase containing dispersing agent. A co-reactant multifunctional amine will be added to the blend. This outcome in quick polymerization at interface and capsule shell happens (Scher 1983). On reaction of isocyanate with amine a polyurea shell will be formed, also polynylon or polyamide shell will be formed when acid chloride reacts with amine and when isocyanate reacts with hydroxyl containing monomer it produces a polyurethane shell. For instance, Saihi et al. (2006) embodied di-ammonium hydrogen phosphate (DAHP) by polyurethane-urea layer utilizing an interfacial polymerization technique. An elevated yield of blend (22%) of a powder of microcapsules was created with a fill substance of 62-wt% of DAHP as controlled by rudimentary investigation. The mean size of DAHP microcapsules is 13.35 mm. In addition, 95% of the measured particles have a width lower than 30.1 mm.

7.2.1.2 In Situ Polymerization

In polymerization, when monomers are added to the encapsulation reactor, it will lead to formation of capsule shell as like IFP; here no reactive agents are added to the core material, polymerization occurs exclusively in the continuous phase and on the continuous phase side of the interface formed by the dispersed core material and continuous phase. At first a low sub-atomic weight prepolymer will be shaped, over the long the prepolymer develops in size, it stores on the outside of the scattered center material there by creating a strong case shell (for example exemplification of different water-immiscible fluids with shells shaped by the response at acidic pH of urea with formaldehyde in watery media (Cakhshaee et al. 1985). Wang et al. (2013) prepared Carboxyl-functionalized magnetic microspheres by in situ polymerization of styrene and methyacrylic acid at 85C in the presence of nano-Fe3O4 in styrene, using lauroyl peroxide as an initiator.

7.2.2 Physico-Chemical Methods

7.2.2.1 Coacervation and Phase Separation

Bungenberg de Jong and Kruyt (1929) and Bungenberg de Jong (1949) defined this as partial desolvation of a homogeneous polymer solution into a polymer-rich phase (coacervate) and the poor polymer phase (coacervation medium). The term originated from the Latin ‘acervus’ meaning ‘heap’. This was the first reported process to be adapted for the industrial production of microcapsules. Currently, two methods for coacervation are available, namely simple and complex processes. The mechanism of microcapsule formation for both processes is identical, except for the

way in which the phase separation is carried out. In simple coacervation a desolvation agent is added for phase separation, whereas complex coacervation involves complexation between two oppositely charged polymers. The three basic steps in complex coacervation are: (i) formation of three immiscible phases; (ii) deposition of the coating; and (iii) rigidization of the coating. The first step includes the formation of three immiscible phases; liquid manufacturing vehicle, core material and coating material. The core material is dispersed in a solution of the coating polymer. The coating material phase, an immiscible polymer in liquid state, is formed by (i) changing temperature of polymer solution, e.g. ethyl cellulose in cyclohexane12 (N-acetyl P-amino phenol as core), (ii) addition of salt, e.g. addition of sodium sulphate solution to gelatine solution in vitamin encapsulation (iii) addition of non-solvent, e.g. addition of isopropyl ether to methyl ethyl ketone solution of cellulose acetate butyrate (Heistand et al. 1966) (methylscopalamine hydrobromide is core), (iv) addition of incompatible polymer to the polymer solution, e.g. addition of polybutadiene to the solution of ethylcellulose in toluene (The National Cash Register Co. 1963) (methylene blue as core material) and (v) inducing polymer–polymer interaction, e.g. interaction of gum Arabic and gelatine at their iso-electric point (Brynko et al. 1967). The second step includes deposition of liquid polymer upon the core material. Finally, the prepared microcapsules are stabilized by cross-linking, desolvation or thermal treatment (Fig. 7.1).

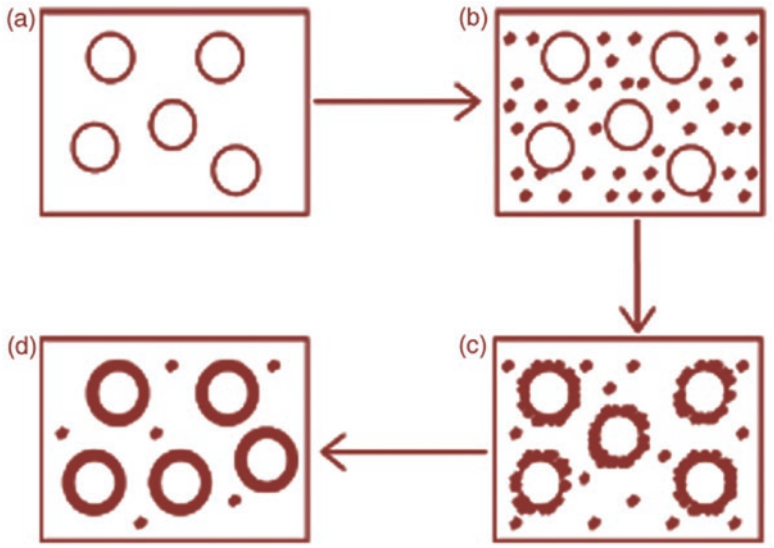

Fig. 7.1 Graphic Representation of the coa-cervation process (**a**) Core material dispersion in solution of shell polymer; (**b**) separation of coacervate from solution; (**c**) coating of core material by microdroplets of coacervate; (**d**) coalescence of coacervate to form continuous shell around core particles (Ghosh 2006)

During manufacturing or by a second reaction process, the primary monomers are polymerized as they are chemically crosslinked in order to make them insoluble which gives them a degree of cross-linked quantified molecular structure in terms of their cross-link density and have a profound impact on the swelling characteristics of the cross-linked system. For instance, derivatives of ethylene glycol di(meth) acrylate like, ethylene glycol diacrylate, di(ethylene glycol) diacrylate, tetra(ethylene glycol) diacrylate, ethylene glycol dimethacrylate, di(ethylene glycol) dimethacrylate, tri(ethylene glycol) dimethacrylate; derivatives of methylenebisacrylamide like N,N- Methylenebisacrylamide, N,NMethylenebisacrylamide, N,N-(1,2-Dihydroxyethylene) bisacrylamide (Klärner et al. 1999), glutaraldehyde, sodium tripolyphosphate, etc. Yin and Stöver (2003) prepared microspheres by poly (styrene-alt-maleic anhydride) partially grafted with methoxy poly(ethylene glycol) (SMA-g-MPEG) were prepared by reacting poly(styrene-alt-maleic anhydride) with a substoichiometric amount of MPEG lithium alcoholate. Aqueous solutions of the resulting SMA-g-MPEG formed complex coacervates with poly (diallyldimethylammonium chloride) (PDADMAC). These phase-separated liquid polyelectrolyte complexes were subsequently crosslinked by the addition of two different polyamines to prepare cross-linked hydrogel microspheres. Chitosan served as an effective cross-linker at pH 7.0, while polyethylenimine (PEI) was used as a cross-linker under basic conditions (pH 10.5). The resulting coacervate microspheres swelled with increasing salinity, which was attributed mainly due to the shielding of the electrostatic association within the polyelectrolyte complex. Huang et al. (2007) prepared microcapsules by using gelatine and gum Arabic by coacervation where the most frequently used cross-linking agent formaldehyde in the gelatin–acacia microencapsulation process was altered by glycerol and also it has been reported that the yield of gelatin–acacia microcapsules decreases at surfactant concentrations above or below the optimum. It has been investigated that the inhibition of coacervation due to high concentrations of surfactants and disturbance of microencapsulation due to high hydrophilic–lipophilic balance of (HLB) values. Generally the concentration of a surfactant required to increase the yield of microcapsules is too low to produce regular-sized droplets. The analysis of the size distribution shows that the microcapsules are multi-dispersed. In order to form negatively charged gelatin, the pH value of a continuous gelatin phase would be adjusted above its isoelectric point, which is able to create monodispersed droplets in the coacervation process. The positively charged gelatin is attracted to the negatively charged acacia to form coacervate droplets when the pH value is adjusted to below its isoelectric point. Therefore, theparticle size distributions of emulsion droplets are affected by the factors of pH adjustment, especially the adding rate of the acidifying agent. The report shows the indomethacin microcapsules had the slowest release rate when the coacervation pH was adjusted to the electrical equivalence pH value and not to the pH of maximum coacervate yield. It has been found that the gelatin is only stable at a pH 4–6 and this data shows that the alkalization caused the breaking of the wall of the microcapsule made by the cross-linking agent of glycerol. Not only is the purple-colour shikonin alkalized into a blue colour, but the saponification effects may also be undergone by the solvent (sesame oil) of extract containing shikonin reacting with sodium hydride.

However, this reaction would not be shown in the microcapsule made by the cross-linking agent of formaldehyde. This explains why the shell of the microcapsule made by formaldehyde is more rigid than that made by glycerol. In other words, the microcapsule made by glycerol has a more permeable shell than made by formaldehyde. The particle size of the microcapsule was not affected by the difference of cross-linking agents. Using the low concentration, 3% and 6% of plasticizer glycerol instead of formaldehyde, similar morphology results were obtained. Hence the mounting encapsulation ability has been achieved due to the amount of cross-linking agent. However, the results indicated that above 6% of glycerin, encapsulation ability decreases as the cross-linking agent increases due to the alteration of the mechanism and inability to integrate into the network even after the addition of an excess amount.

7.2.2.2 Polymer Encapsulation by Rapid Expansion of Supercritical Fluids

Supercritical fluids are highly compressed gasses that possess several advantageous properties of both liquids and gases. The most widely used being supercritical CO2, nitrous oxide (N2O) and alkanes (C2 to C4). At the critical point of supercritical fluids a small change in pressure or temperature causes a huge change in their density. Supercritical CO2 is widely used for its low critical temperature value, in addition to its non-toxic and non-flammable properties; it is also readily accessible, highly pure and commercial.

The most widely used methods are as follows:

(a) Rapid expansion of supercritical solution (RESS);
(b) Gas anti-solvent (GAS); and
(c) Particles from gas-saturated solution (PGSS).

(a) Rapid expansion of supercritical solution

During rapid expansion method, the active ingredient and the shell material are maintained at high pressure and then released at atmospheric pressure through a minute plunger in order to make supercritical solution. The shell material undergoes desolvation due to sudden drop in pressure which is then deposited around the active ingredient (core) and forms a coating layer (Fig. 7.2), but the drawback is that both the shell material and active ingredient must be very soluble in supercritical fluids. A least percent of polymers (e.g. polymethacrylates and polydimethylsiloxanes) are soluble in supercritical fluids such as CO_2, which possess low cohesive energy densities. As using can increase the solubility of polymers either the co-solvents or rarely non-solvents are used in supercritical fluids, but the shell materials do not dissolve at atmospheric pressure. Kiyoshi et al. very recently carried out microencapsulation of TiO2 nanoparticles with polymer by RESS using ethanol as a non-solvent for the polymer shell such as polyethylene glycol (PEG), poly(styrene)-b-(poly(methylmethacrylate)- copoly(glycidal methacrylate) copolymer (PS-b-(PMMAco-PGMA) and poly(methyl methacrylate).

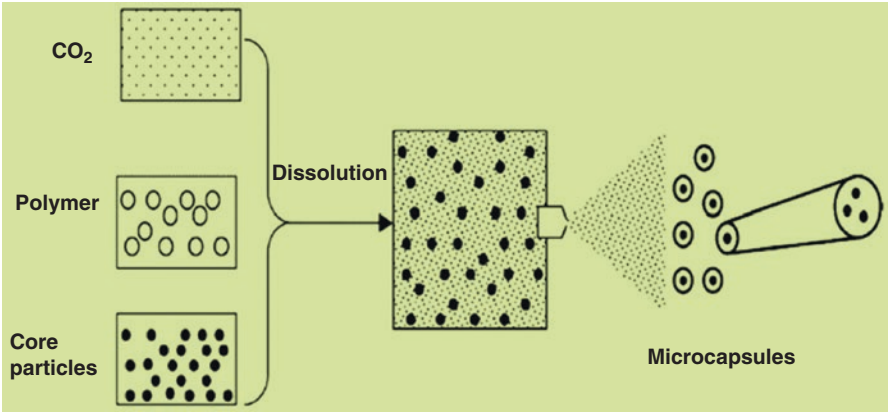

Fig. 7.2 Microencapsulation by rapid expansion of supercritical solutions (RESS) (Ghosh 2006)

(b) Gas anti-solvent (GAS) process

The Gas anti-solvent (GAS) process also referred as supercritical fluid anti-solvent (SAS) where supercritical fluid is added to a blend solution of shell material and the active ingredients at high pressure which will leads to a volume expansion of the solution that causes super saturation resulting into precipitation of the solute as the solute must be soluble in the liquid solvent, but not in the blend of solvent and supercritical fluid. While on the other side the supercritical fluid and liquid solvent must be miscible and this process is not suitable for the encapsulation of water-soluble ingredients, as water has low solubility in supercritical fluids.

(c) Particles from a gas-saturated solution (PGSS)

At high pressure, when core and shell materials are mixed to form the supercritical fluid as during this process supercritical fluid penetrates the shell material, causing swelling. After heating the mixture above the glass transition temperature (Tg), the polymer liquefies while the shell material is allowed to deposit onto the active ingredient slowly upon releasing the pressure and also the core and shell materials may not be soluble in the supercritical fluid.

7.2.3 Physico-Mechanical Process

7.2.3.1 Spray Drying and Congealing

Nowadays, a low-cost commercial process which is commonly used for the encapsulation of fragrances, flavors and oils is Microencapsulation by spray drying in which the core particles are dispersed in a polymer solution and sprayed into a hot chamber (Fig. 7.3). The shell material solidifies onto the core particles as the solvent evaporates such that the microcapsules obtained will be of matrix type or poly-nuclear.

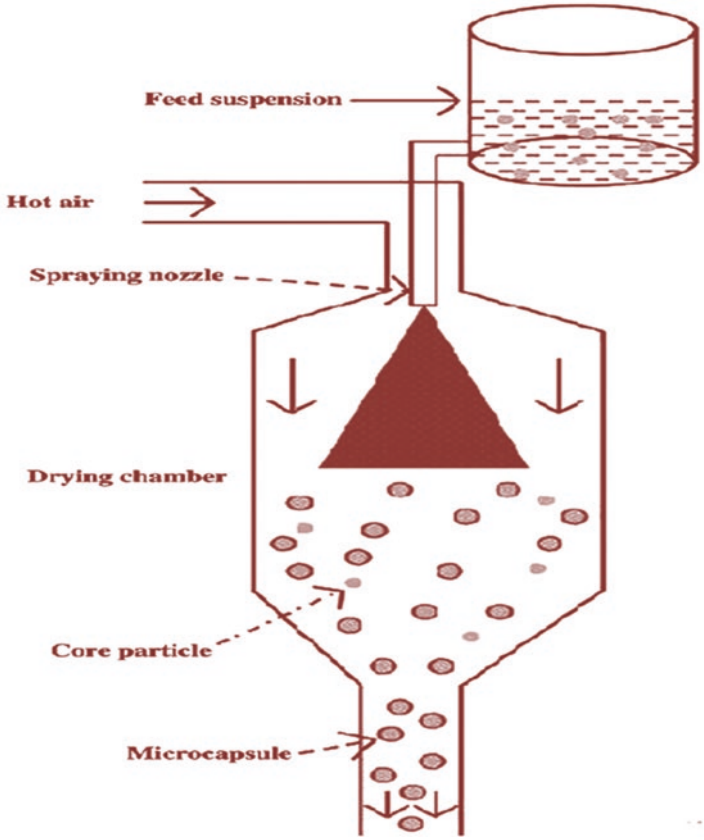

Fig. 7.3 Schematic illustrating the process of micro-encapsulation by spray drying. (Redrawn from Ghosh 2006)

Chitosan microspheres cross-linked with three distinctive cross-connecting specialists viz., formaldehyde (FA), gluteraldehyde (GA) and tripolyphosphate (TPP) have been set up by splash drying procedure. It has been widely analyzed that the impact of these cross-connecting operators on the properties of splash dried chitosan microspheres. The molecule size and epitome efficiencies of in this manner arranged chitosan microspheres went basically between 4.1–4.7 mm and 95.12–99.17%, separately. Surface morphology, rate disintegration, rate water take-up and medicate discharge properties of the shower-dried chitosan microspheres was amazingly impacted by the sort (substance or ionic) and degree (1 or 2% w/w) of cross-connecting operators. Splash dried chitosan microspheres cross-connected with TPP displayed higher swelling limit, rate water take-up, rate disintegration and medication discharge rate at both the cross-connecting degrees (1 and 2% w/w) when contrasted with those cross-linked with FA and GA. The globular and surface smoothness of the splash dried chitosan microspheres was lost when the cross-connecting degree was expanded from 1% to 2% w/w. Discharge rate of the medication from spray dried

chitosan microspheres diminished when the crosslinking degree was expanded from 1% to 2% w/w. The physical condition of the drug in chitosan-TPP, chitosan-FA and chitosan-GA lattices was affirmed by the X-beam diffraction (XRD) study and found that the medication stays in a crystalline state even after its embodiment. Arrival of the medication from chitosan-TPP, chitosan-FA and chitosan GA networks pursued Fick's law of dissemination (Desai and Park 2005).

Core material is dispersed in a coating material melt rather than a coating solution while as coating solidification is accomplished by spraying the hot mixture into cool air flow. At room temperature the substances, which are solids, like alcohols, waxes, fatty acids and polymers but they melt at their appropriate melting temperatures and are appropriate for spray congealing. The designed muco-adhesive microparticles is an innovative vaginal delivery system for econazole nitrate (ECN) to enhance the drug anti-fungal activity as the 7 different formulations were prepared by spraycongealing, a lipid-hydrophilic matrix (Gelucire ((R)) 53/ 10) was used as carrier and several muco-adhesive polymers such as sodium carboxymethylcellulose, chitosan, and poloxamers (Lutrol((R)) F68 and F127) were added (Albertini et al. 2008).

7.2.3.2 Fluidized-Bed Technology

As the outer layer on the particles can be achieved by spraying the liquid coating onto the particles which is accelerated by the rapid evaporation and also thickness and formulations of the coating can be obtained as per the requirements. Various types of fluid-bed coaters include bottom spray, top spray, and tangential spray (Fig. 7.4). While case of top spray system, the coating material is sprayed downwards on to the fluid bed in order to form encapsulated as by moving the solid or porous particles over the coating region. The opposing flows of the coating materials and the particles can be used for increased encapsulation efficiency and the prevention of cluster configuration. Dripping of the coated particles depends on the formulation of the coating material. Top spray fluid-bed coaters produce higher yields of encapsulated particles than either bottom or tangential sprays.

The bottom spray is also known as 'Wurster's coater' who has been named after its development by Wurster (1953). This procedure utilizes a coating chamber that has a tube shaped spout and a punctured base plate. The cylindrical nozzle is utilized for splashing the covering material. As the particles move upwards through the punctured base plate and pass the nozzle region, the covering material epitomizes them. The coating material adheres to the particle surface by evaporation of the solvent or cooling of the encapsulated particle. This procedure is preceded until the ideal thickness and weight is acquired. In spite of the fact that it is a tedious procedure, the multilayer coating technique helps in diminishing molecule surrenders. The tangential splash comprises of a pivoting circle at the base of the covering chamber, with indistinguishable breadth from the chamber. Amid the procedure the plate is raised to make a hole between the edge of the chamber and the circle. The tangential nozzle is placed above the rotating disc through which the coating material is release. The particles travel through the hole into the showering zone and are epitomized as they travel a base separation there is a higher yield of classified particles.

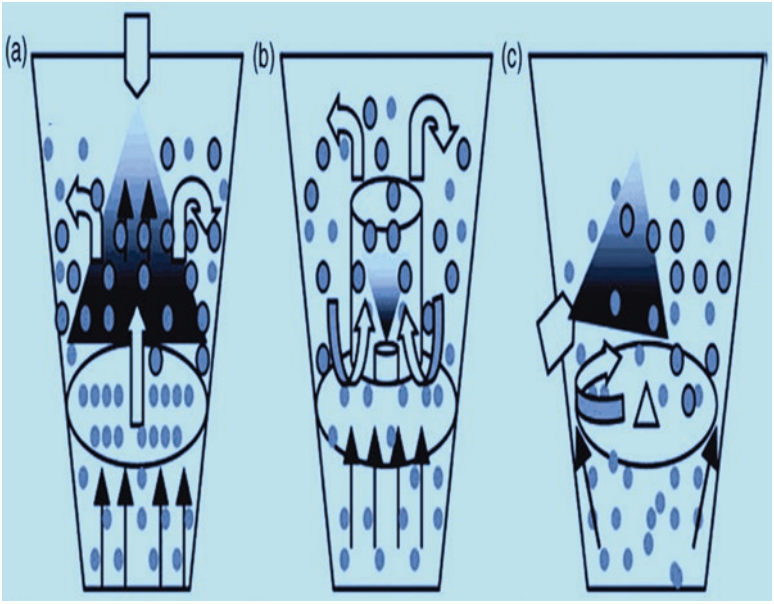

Fig. 7.4 Schematics of a fluid-bed coater. (**a**) Top spray (**b**) bottom spray (**c**) tangential spray. (Redrawn from Ghosh 2006)

7.2.3.3 Solvent Evaporation

Three phases are present in solvent evaporation process i.e., core, coat material and liquid manufacturing vehicle (LMV). At first, cost material will be dissolved in a volatile solvent, which is not soluble in LMV phase. A core material to be epitomized needs to be dispersed or dissolved in the coating polymer solution then this mixture is added to the liquid manufacturing vehicle phase with agitation, the blend is heated to evaporate the solvent for polymer. Here the coat material shrinks around the core material and encapsulates it. Microspheres of 5-fluorouracil have been prepared, using three grades of ethyl cellulose as wall forming materials, and utilizing a solvent evaporation technique under ambient conditions. An alcoholic solution of 5-fluorouracil and polymer was dispersed in liquid paraffin containing 33.3% n-heptane. The effect of stirring rate, time of stirring, and drug loading and polymer grade on drug release in two different media was evaluated. The drug-loaded particles were globular in shape having diameter range of 25–200 mm and were suitable for incorporating into a gel base. In aqueous media, the research related to drug release showed that acidic media provide a faster release rate than neutral media and an aqueous gel base preparation at pH 7.0 through a synthetic membrane was found to be promising for formulation of a gel-microsphere product for the treatment of skin lesions (Ghorab et al. 1990). Pseudoephedrine hydrochloride, a highly water-soluble drug, was entrapped within poly (methyl methacrylate) microspheres by a water/oil/water emulsification-solvent evaporation method. An aqueous drug solution was emulsified into a solution of the polymer in methylene chloride, followed by emulsification

of this primary emulsion into an external aqueous phase to form a water/oil/water emulsion. The middle organic phase separated the internal drug-containing aqueous phase from the continuous phase. Microspheres were formed after solvent evaporation and polymer precipitation. The drug content of the microspheres extended with expanding hypothetical drug stacking, increased measures of natural dissolvable, polymer and polymeric stabilizer and diminished with expanding mixing time, expanding pH of the consistent stage and expanded volume of the inside and outside aqueous stage (Rainer and Bodmeier 1990).

7.2.3.4 Pan Coating

Over the coating pan, the coating solution is applied as atomized spray to the solid core material and this coating solvent is removed by passing warm air over the coated material. Hence this method is employed to coat effectively the larger sized particles.

7.2.3.5 Factors Influencing Encapsulation Efficiency

As various parameters affect the encapsulation efficiency of the microcapsule, microparticle or microsphere, the factors influencing encapsulation efficiency has been documented as-.

7.2.3.6 Solubility of Polymer in the Organic Solvent

Mehta et al. (1996) investigated the effect of solubilities of different PLGAs polymers in methylene chloride, compared by measuring the methanol cloud point (Cs): Higher Cs meant that the polymer was more soluble in methylene chloride and, thus, required a larger amount of methanol to precipitate from the polymer solution. The PLGA polymer of a relatively high L/G ratio (75/25) had a higher solubility in methylene chloride than the other PLGA (L/G ratio ¼ 50/50). A lower molecular weight polymer had a higher solubility in methylene chloride than a higher molecular weight polymer. End-capped polymers, which were more hydrophobic than non-end-capped polymers of the same molecular weight and component ratio, were more soluble in methylene chloride. Diffusion of drugs into the continuous phase mostly occurred during the first 10 min of emulsification; therefore, as the time the polymer phase stayed in the no solidified (semi-solid) state was extended, encapsulation efficiency became relatively low. In Mehta et al.'s (1996) study, polymers having relatively high solubilities in methylene chloride took longer to solidify and resulted in low encapsulation efficiencies, and vice versa. Particle size and bulk density also varied according to the polymer. Since polymers having higher solubilities in methylene chloride stayed longer in the semi-solid state, the dispersed phase became more concentrated before it completely solidified, resulting in denser micro particles. Johansen et al. (1998) showed that the use of relatively hydrophilic PLGA, which carried free carboxylic end groups resulted in, significantly higher encapsulation efficiency compared to that of an end-capped polymer. A similar explanation as above applies to this observation: Hydrophilic PLGA is relatively less soluble in the solvent, methylene chloride, and precipitates more quickly than the end-capped one. High solidification rate might have increased the encapsulation

efficiency. On the other hand, the authors attribute the increase to the enhanced interaction between PLGA and the protein through hydrogen bonding and polar interactions (Johansen et al. 1998). Walter et al. (2001) also observed increased encapsulation efficiency from using relatively hydrophilic PLGA in DNA microencapsulation. The hydrophilicity of the polymer enhanced the stability of the primary emulsion and it contributed to such an increase.

7.2.3.7 Solubility of Organic Solvent in Water

Bodmeier and McGinity (1988) found that methylene chloride resulted in a higher encapsulation efficiency as compared with chloroform or benzene, even though methylene chloride was a better solvent for poly (lactic acid) (PLA) than the others. Methylene chloride is more soluble in water than chloroform or benzene. The 'high' solubility allowed relatively fast mass-transfer between the dispersed and the continuous phases and led to fast precipitation of the polymer. The significance of solubility of the organic solvent in water was also confirmed by the fact that the addition of water-miscible cosolvents such as acetone, methanol, ethyl acetate or dimethyl sulphoxide (DMSO) contributed to increase of the encapsulation efficiency. Knowing that the methanol is a non-solvent for PLA and a water-miscible solvent, it can be assumed that methanol played a dual function in facilitating the polymer precipitation: First, the presence of methanol in the dispersed phase decreased the polymer solubility in the dispersed phase (Jeyanthi et al. 1997). Secondly, as a water-miscible solvent, methanol facilitated diffusion of water into the dispersed phase. In order to explain the low encapsulation efficiency obtained with benzene, the authors mention that the benzene required a larger amount of water (non-solvent) than methylene chloride for precipitation of the polymer and the drug was lost due to the delayed solidification. However, given that benzene is a poorer solvent than methylene chloride for a PLA polymer, this argument does not agree with the widely spread idea that a poor solvent requires a smaller amount of non-solvent to precipitate a polymer. In fact, there could have been a better explanation if they had considered that the delayed solidification was due to the low solubility of benzene in water: As a poor solvent for a PLA polymer, benzene requires only a small amount of non-solvent for complete solidification of the polymer. However, since benzene can dissolve only a tiny fraction of water, it takes much longer to uptake water into the dispersed phase. That is, while solubility of a polymer in an organic solvent governs the quantity of a non-solvent required in precipitating a polymer, solubility of the organic solvent in the non-solvent limits diffusion of the non-solvent into the polymer phase. Thus, when a cosolvent system is involved, both solubility of a polymer in a solvent and solubility of the solvent in a non-solvent participate in determining the solidification rate of the dispersed phase.

Park et al. (1998) prepared lysozyme-loaded PLGA microparticles using the oil in water (o/w) single emulsion technique. Here, the authors used a co-solvent system, varying the ratio of the component solvents. DMSO was used for solubilization of lysozyme and PLGA and methylene chloride was used for generation of emulsion drops as well as solubilization of PLGA. Encapsulation efficiency increased and initial burst decreased as the volume fraction of DMSO in

the co-solvent system increased. Particle size increased and density of the microparticle matrix decreased with increasing DMSO. Overall, these results indicate that the presence of DMSO increased the hydrophilicity of the solvent system and allowed fast extraction of the solvent into the continuous phase, which led to higher encapsulation efficiency and larger particle size.

7.2.3.8 Concentration of the Polymer

Encapsulation efficiency increases with increasing polymer concentration (Mehta et al. 1996; Rafati et al. 1997; Li et al. 1999). For example, the encapsulation efficiency increased from 53.1% to 70.9% when concentration of the polymer increased from 20.0% to 32.5% (Mehta et al. 1996). High viscosity and fast solidification of the dispersed phase contributed to reduce porosity of the microparticles as well (Schlicher et al. 1997). The contribution of a high polymer concentration to the encapsulation efficiency can be interpreted in two ways. First, when highly concentrated, the polymer precipitates faster on the surface of the dispersed phase and prevents drug diffusion across the phase boundary. Secondly, the high concentration increases viscosity of the solution and delays the drug diffusion within the polymer droplets (Bodmeier and McGinity 1988).

7.2.3.9 Ratio of Dispersed Phase to Continuous Phase (DP/CP Ratio)

Encapsulation efficiency and particle size increase as the volume of the continuous phase increases (Mehta et al. 1996; Li et al. 1999). For eg. The encapsulation efficiency increased more than twice as the ratio of the dispersed phase to the continuous phase (DP/CP ratio) decreased from 1/50 to 1/300 (Mehta et al. 1996). It is likely that a large volume of continuous phase provides a high concentration gradient of the organic solvent across the phase boundary by diluting the solvent, leading to fast solidification of the microparticles. A relevant observation is described in the literature (Sah 1997). In this example, which utilized ethyl acetate as a solvent, the formation of microparticles was dependent on the volume of the continuous phase. When 8 mL of PLGA solution (o) was poured into 20 or 50 mL of water phase (w), the polymer solution was well disintegrated into dispersed droplets. On the other hand, when the continuous phase was 80 mL or more, the microspheres hardened quickly and formed irregular precipitates. This is because the large volume of continuous phase provided nearly a sink condition for ethyl acetate and extracted the solvent instantly. Due to the fast solidification of the polymer, particle size increased with increasing volume of the continuous phase. Microparticles generated from a low DP/CP ratio had a lower bulk density (0.561 g/cc at 1/50 vs 0.357 g/cc at 1/300), which the authors interpret as an indication of higher porosity of the polymer matrix (Mehta et al. 1996). On the other hand, a different example shows that a higher DP/CP ratio resulted in increased porosity, providing a large specific surface area (measured by the BET method) and the scanning electron microscope (SEM) pictures as evidence (Jeyanthi et al. 1997). This apparent discrepancy can be explained by the fact that low bulk density (Mehta et al. 1996) is not a true reflection of porosity but a result of large particle size. In fact, porosity increases with increasing DP/CP ratio, i.e. decreasing rate of the polymer precipitation.

7.2.3.10 Rate of Solvent Removal

The method and rate of solvent removal influence the solidification rate of the dispersed phase as well as morphology of the resulting microparticles (Mehta et al. 1994). In the emulsion-solvent evaporation/extraction method, the solvent can be removed by (i) evaporation, in which the solvent is evaporated around its boiling point, or (ii) extraction into the continuous phase. The temperature ramp or the evaporation temperature in the former and can control the rate of solvent removal by the volume of the dilution medium in the latter. PLGA microparticles containing salmon calcitonin (sCT) were prepared by emulsification, followed by different solvent removal processes (Mehta et al. 1994). In the temperature-dependent solvent removal process, the solvent (methylene chloride) was removed by increasing the temperature from 15 to 40 °C at different rates. The microparticles that resulted from this process had a hollow core and a porous wall. The core size and wall thickness were dependent on the temperature ramp. A rapid rise in temperature resulted in a thin wall and a large hollow core, whereas a stepwise temperature rise (15–25, then to 40 °C) resulted in a reduced core size. It is believed that the hollow core was due to the rapid expansion of methylene chloride entrapped within the solidified micro particles. In controlled extraction of the solvent, the solvent was removed gradually and slowly by dilution of the continuous phase, which left the micro particles in the soft state for a longer period of time. The resulting micro particles showed a highly porous honeycomb-like internal structure without a hollow core. In the later study, it was noted that the porosity was a function of the amount of water diffused into the dispersed phase from the continuous phase, which could only be allowed before the dispersed phase solidified completely (Li et al. 1995). In other words, the high porosity of the micro particles was due to the slow solidification of the micro particles. Even though it is generally assumed that fast polymer solidification results in high encapsulation efficiency, this does not apply to the observation of Yang et al. (2000). Here, the solvent evaporation temperature did not affect the encapsulation efficiency. It may be due to the different processing temperatures influencing not only the rate of polymer solidification but also the diffusivity of the protein and its solubility in water. While the high temperature facilitated solidification of the dispersed phase, it enhanced diffusion of the protein into the continuous phase, compromising the positive effect from the fast solidification.

7.2.3.11 Interaction Between Drug and Polymer

Interaction between protein and polymer contributes to increasing encapsulation efficiency (Boury et al. 1997). Generally, proteins are capable of ionic interactions and are better encapsulated within polymers that carry free carboxylic end groups than the end-capped polymers. On the other hand, if hydrophobic interaction is a dominant force between the protein and the polymer, relatively hydrophobic end-capped polymers are more advantageous in increasing encapsulation efficiency. For example, encapsulation efficiencies of more than 60% were achieved for salmon

calcitonin (sCT) microparticles despite the high solubility of sCT in the continuous phase (Jeyanthi et al. 1997). This is attributed to the strong affinity of sCT to hydrophobic polymers such as PLGA. On the other hand, such interactions between protein and polymer can limit protein release from the microparticles (Crotts and Park 1997; Park et al. 1998; Kim and Park 1999). In certain cases, a co-encapsulated excipient can mediate the interaction between protein and polymer ((Johansen et al. 1998). Encapsulation efficiency increased when gamma-hydroxypropylcyclodextrin (g-HPCD) was co-encapsulated with tetanus toxoid in PLGA microparticles. It is supposed that the g-HPCD increased the interaction by accommodating amino acid side groups of the toxoid into its cavity and simultaneously interacting with PLGA through van der Waals and hydrogen bonding forces.

7.2.3.12 Solubility of Drug in Continuous Phase

Drug loss into the continuous phase occurs while the dispersed phase stays in a transitional, semi-solid state. If the solubility of the drug in the continuous phase is higher than in the dispersed phase, the drug will easily diffuse into the continuous phase during this stage. For example, the encapsulation efficiency of quinidine sulphate was 40-times higher in the alkaline continuous phase (pH 12, in which quinidine sulphate is insoluble) than in the neutral continuous phase (pH 7, in which quinidine sulphate is very soluble) (Bodmeier and McGinity 1988).

7.2.3.13 Molecular Weight of the Polymer

Fu et al. (2005) studied the effect of molecular weight of the polymer on encapsulation efficiency and developed a long-acting injectable huperzine A-PLGA microsphere for the chronic therapy of Alzheimer's disease, the microsphere was prepared by using o/w emulsion solvent extraction evaporation method. The morphology of the microspheres was observed by scanning electron microscopy. A confocal laser-scanning microscope observed the distribution of the drug within microspheres. The results indicated that the PLGA 15000 microspheres possessed a smooth and round appearance with average particle size of 50 mm or so. The encapsulation percentages of microspheres prepared from PLGA 15000, 20,000 and 30,000 were 62.75, 27.52 and 16.63%, respectively. The drug release percentage during the first day decreased from 22.52% of PLGA 30000 microspheres to 3.97% of PLGA 15000 microspheres, the complete release could be prolonged to 3 weeks. The initial burst release of microspheres with higher molecular weight PLGA could be explained by the inhomogeneous distribution of drug within microspheres. The encapsulation efficiency of the microspheres improved as the polymer concentration increase in oil phase and PVA concentration decreased in aqueous phase. Reducing the polymer concentration could control the burst release. Evaporation temperature had a large effect on the drug release profiles. It had better be controlled under 30C. Within a certain range of particle size, encapsulation efficiency decreased and drug release rate increased with the reducing of the particle size (Fu et al. 2005).

7.3 Nanotechnology and Agricultural Sustainable Development

7.3.1 Nano Pesticides

In future, nanoparticles will be employed for crop production as well as for crop protection. NPs may play key role in the control of insect pests and pathogens as the insect pests are the predominant ones in the agricultural fields, which result in economic loss. As with advent of nano-encapsulated pesticide formulation, which has slow releasing, properties with enhanced specificity, permeability, solubility and stability (Bhattacharyya et al. 2016). In order to protect the active ingredients the encapsulation is necessary for premature degradation or to increase their pest control efficacy for a longer period of time. Nanoencapsulated pesticides formulation results in dosage reduction of pesticides and exposure of human beings to them, which is environmentally friendly for crop protection (Nuruzzaman et al. 2016). Therefore, to increase the global food production while reducing the negative environmental impacts; progress of non-toxic and promising pesticide delivery systems is necessary (de Oliveira et al. 2014; Kah and Hofmann 2014; Bhattacharyya et al. 2016; Grillo et al. 2016). The nanoencapsulation also known as microencapsulation is used to develop the quality of products of desired chemicals release to the target biological process. Few numbers of chemical companies openly promote nanoscale pesticides for sale as "microencapsulated pesticides" very recently as some products from Syngenta (Switzerland) such as Karate ZEON, Ospray's Chyella, Subdue MAXX, Penncap-M, and microencapsulated pesticides from BASF may fit for nanoscale (Gouin 2004). In Australia, Syngenta also markets some products such as the Subdue MAXX, Primo MAXX, Banner MAXX, etc. which confirms very thin interface between the term of microemulsion and nanoemulsion. This method is commonly employed for formulations of organic nanoparticles (Gouin 2004) containing active agrochemicals or substances of interest.

7.3.2 Nanoherbicides

Worldwide the agriculture has been affected by weed infestation which results in loss of huge quantity of crops as removal of weeds by conventional means are time consuming. As currently number of herbicides are available which eradicate the weeds but possess various threats to the biotic and aboitic components of environment. Hence, to adapt alternative method, which could be eco friendly like nanoherbicides as epitomization of polymeric nanoparticles, will help to solve the problem (Kumar et al. 2015; Pérez-de-Luque and Rubiales 2009). Prolonged use of chemical herbicides results in pile of soil residues, which damages the succeeding crops and also leads to weed resistance against same herbicide [Chinnamuthu and Boopathi 2009]. Effectiveness of nano zerovalent iron (nano ZVI) has been assessed

to dechlorinate herbicide atrazine (2-chloro-4ethylamino-6-isopropylamino-1, 3, 5-triazine) from atrazine-contaminated water and soil [Satapanajaru et al. 2008]. For delivery in roots of weeds, target specific nanoparticles used to load with herbicides as these molecules enter into the roots system of the weeds, translocate to cells and inhibit metabolic pathways such as glycolysis, which eventually leads to death of plants [Nair et al. 2010]. Toxicity of poly (ε-caprolactone) nanocapsules containing ametryn and atrazine against alga Pseudokirchneriella subcapitata and the microcrustacean Daphnia similis has been tested. Herbicides encapsulated in the poly (ε-caprolactone) nanocapsules resulted in lower toxicity to the algae (Pseudokirchneriella subcapitata) and higher toxicity to the microcrustacean (Daphnia similis) as compared to the herbicides alone (Clemente et al. 2014).

7.3.3 Nano Fertilizers

In the ongoing decade nanofertilizers are openly accessible in the market, yet especially the agricultural fertilizers are still not shaped by the major chemical companies (Table 7.2). Nanofertilizers may contain nano silica, iron, zinc and titanium dioxide, ZnCdSe/ZnS center shell QDs, Mn/ZnSe QDs, InP/ZnS center shell QDs, gold nanorods, center shell QDs, and so forth just as have to underwrite control discharge and enhance the its quality (Dimkpa 2014; Zhang et al. 2016) (Table 7.2).

Table 7.2 list of some commercial product of nano-fertilizers

Commercial product	Content	Company
Nano-GroTM	Plant growth regulator and immunity enhancer	Agro Nanotechnology Corp., FL, United States
Nano Green	Extracts of corn, grain, soybeans, potatoes, coconut, and palm	Nano Green Sciences, Inc., India
Nano-Ag Answer R	Microorganism, sea kelp, and mineral electrolyte	Urth Agriculture, CA, United States
Biozar Nano-Fertilizer	Combination of organic materials, micronutrients, and macromolecules	Fanavar Nano-Pazhoohesh Markazi Company, Iran
Nano Max NPK Fertilizer	Multiple organic acids chelated with major nutrients, amino acids, organic carbon, organic micro nutrients/trace elements, vitamins, and probiotic	JU Agri Sciences Pvt. Ltd., Janakpuri, New Delhi, India
Master Nano Chitosan Organic Fertilizer	Water soluble liquid chitosan, organic acid and salicylic acids, phenolic compounds	Pannaraj Intertrade, Thailand
TAG NANO (NPK, PhoS, Zinc, Cal, etc.) fertilizers	Proteino-lacto-gluconate chelated with micronutrients, vitamins, probiotics, Seaweed extracts, humic acid	Tropical Agrosystem India (P) Ltd., India

Source: Prasad et al. (2017)

7.4 Ecological Implications of Nanoparticles in Agriculture

The advancement of nanotechnologies has introduced significant degrees of manufactured NPs into the nature. So as to defend human wellbeing and plant from the intended opposing impacts of a wide scope of nanomaterials, an intensifying number of research have concentrated on the appraisal of the harmfulness of the nanoparticles typically utilized in industry (Yang and Watts 2005; Rana and Kalaichelvan 2013; Du et al. 2017; Tripathi et al. 2017a, b). Various factors like solubility, binding specificity to a biological site etc. determines the metal toxicity as metal nanoparticles exhibit antibacterial, anticandidal, and antifungal exercises (Aziz et al. 2016; Patra and Baek 2017). Depending on the charge at membrane surface, metal nanoparticles exert cytotoxicity and also the efficiency of nanotoxic effects of nanoparticles are certainly depending on structure of targeted cell-wall, thus the susceptibility order should be mould > yeast > Gram-negative > Gram-positive. Nanotoxicity may be accredited to electrostatic interaction between membrane and with nanoparticles and their accumulation in cytoplasm (Rana and Kalaichelvan 2011; Aziz et al. 2015, 2016).

The photochemically active nanoparticles include titanium dioxide, zinc oxide and silicon dioxide and Fullerenes as when they are exposed to light, the excited electrons are generated then form superoxide radicals in the presence of oxygen by direct electron transfer (Hoffmann et al. 2007). When organisms are simultaneously exposed to UV light and nanoparticles (particularly UV light has higher energy than visible light), the cells act in response to oxidative stress by increasing a number of protective enzymatic or genetic constitutions that can easily be measured (Kovochich et al. 2005; Vannini et al. 2014), thus the stress parameter is used to determine reactive oxygen species (ROS) that can be exploited in determination of the context of toxicity and ecotoxicity. Sayes et al. (2004) reported that in vitro studies on the toxicity of nanoparticles have confirmed the generation of ROS like fullerenes and titanium oxide while on other hand, some authors revealed that nanoparticles (fullerenes and silicon nanoparticles) may protect against oxidative stress (Daroczi et al. 2006; Venkatachalam et al. 2017). Significantly more inquires interactions among cells and nanoparticles just as robotic aspects of nanoparticles metabolism in organisms and specific cells are expected to clear up this division. Ecotoxicological research would progressively consideration on the ecological result of the materials and multifaceted nature of regular frameworks. Broad research would be important to determine delayed impacts of environmental exposure to nanoparticles and to help determine possible adaptive mechanisms (Cox et al. 2017; Singh et al. 2017). Nanoparticles in plants enter cell framework, translocate them to shoot and aggregate in different and accumulate in various aerial parts the likelihood of their cycling in the environment increments through different trophic dimensions. The rate of respiration, transpiration, photosynthesis, and interfere with translocation of food material have been affected by nanoparticles (Shweta et al. 2016; Du et al. 2017). The degree of toxicity is linked to this surface and to the surface properties of the nanoparticles and the ecotoxicity of nanoparticles is thus very important as it creates a direct link between the adverse effects of nanoparticles and the organisms

including microorganisms, plants, and other organisms including humans at various trophic levels (Rana and Kalaichelvan 2013). Reflection on what was referenced with respect to the examination of the conceivable results of nanotechnology and the factors utilized in the past investigations just as the examination of various researchers assessments drives one to social, financial, wellbeing, and social viewpoints as the components that are affected by nanotechnology. These outcomes can be positive or negative as the appropriately.

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Bioenergy: Plants and Products

8

Abstract

Agricultural ethanol has not been an optional fuel for petrol due to a big difference in prices. Nowadays purposefulness of developing alternative fuel production processes, including bioethanol production, is supported by both economic and environmental concerns. The role of secondary metabolites in development of plant growth and architect for enhancing the biofuel production. Besides now as days the main focus are on gene and gene products as they construct the main apparatus for the studying the plant genome. The sequencing technology and reduced cost of sequencing are accelerating the ability to discover new natural product pathways by identifying the underlying genes. In this chapter various plant products and substances are documented right from the classical biofules to gene products vis a vis gene clusters.

Keywords

Gene products · Clusters · Biofuel · Geneome · Alkaloids · Flavnoids

8.1 Bioproducts: Bioethanol

Ethanol production for food purposes and subsequently ethanol as lamp and lantern fuel component has been known since the beginning of nineteenth Century (Goettemoeller and Goettemoeller 2007). It was first used as fuel for Ford motorcars in 1930s (Kovarik et al. 1998). Furthermore, ethanol is commonly used as dissolvent and input substrate for production of various chemicals and their derivatives. For decades agricultural ethanol has not been an optional fuel for petrol due to a big difference in prices; neither has the lack of pro-environmental programmes contributed to that. Nowadays purposefulness of developing alternative fuel production processes, including bioethanol production, is supported by both economic and environmental concerns (Campbell and Laherrere 1998). Specific growth rate of

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bioethanol production was posted in the past 3 years of 2008, 2009 and 2010 when production of bioethanol regularly rose accounting for 132% and 157% and 177% of 2007 production volume, respectively, but the highest production volume growth rate was posted in the USA.

Bioethanol is a significant element of the fuel portfolio in the United States (corn) and Brasil (sugar cane) accounting for approximately 88% (including 57% in the USA) of the world's production output of this fuel. The aforementioned countries allocated at least 4% and 1% of arable land to bioethanol production (Goettemoeller and Goettemoeller 2007). The increase in the use of crops as resources (vegetable oils, sugar crops and starch crops) for production of liquid biofuels has global dimension; on the one hand it encourages diversification of energy sources and contributes to improvement of energy safety and on the other hand it may result in shortages of strategic food resources in the world. Use of energy crops may cause difficulties in balancing the world's grain market, fodder crops and oil crops. For instance, in the United States in 2007/2008 season the higher demand for corn from the bioethanol industry caused the crops to change to the disadvantage of soyabean – the basic fodder crop, which in turn caused the fodder prices to rise and consequently the food prices to rise (FAO 2008). Thus, obvious limitations to development of bioethanol production technology based on traditional saccharide and starch biomass – sugar cane, sugar beet, corn, wheat, rye, rice and potato, challenged contribution of this fuel to reduction of greenhouse gas emission (Farrell et al. 2006) as well as little price competitiveness in relation to petrol indicate purposefulness of searching for more effective resources that will not compete with man's consumer needs and requirements. Forest is such a traditional, abundant source of energy crops. However, in the case of forest stock there are limitations of both economic and environmental nature that result from this long time that is indispensable to restitute forest stand and high industrial value of timber, which is why mainly waste or residues are used for energy purposes and due to environmental concerns, the supreme position of woodland must be stressed in the circulation of global carbon. This is why more and more research is done to use resources originating from dedicated crops including the crops on low-productive land. In the world's literature a variety of interdisciplinary research output has been accumulated on the issue of lignocellulose production resulting in solid fuel used for power purposes, and there are more and more papers elaborating on the subject of lignocellulose conversion into bioethanol. It may be assumed that knowledge that has been compiled, has not reached the critical mass yet, which could result in technology advancement. Presently in the world dozen or so pilot biorefineries operate to produce ethanol from lignocellulose (timber, straw), however none of them has reached any competitiveness in relation to petrochemical biorefinery; furthermore none of them is based on alternative resource – originating from dedicated crops. However, the outburst development of lignocellulose biofuel is forecast to take place in the years 2020–2030 when production of first generation biofuel is going to be strongly reduced (IEA 2008, 2009). Hamelinck and Faaij (2006) as well as van Vliet et al. (2009) have estimated that lignocellulose bioethanol will become competitive in relation to petrochemical fuel when the price falls below USD 0.50 per 1

litre. As it has been mentioned, in lignocellulose biorefineries two ethanol conversion modes may be operated. The one is thermal-and-chemical through syngas into ethanol (that is out of this paper) and the other is biological or biochemical through hydrolysis, fermentation and distillation into bioethanol.

The agreed term "lignocellulose" defines the compound of three biopolymers: polysaccharides- cellulose and hemicellulose and polymer – lignin, that in conjunction with other chemical components (extracts, fats, proteins) are found in various proportions depending on species and crop habitats (Sluiter et al. 2010). Table 8.1 exhibits typical biopolymer content in agricultural residues and lignocellulose crops. Analysing numerous species of sugar crops, starch crops and lignocellulose crops Sanders (2009) indicated extensive volatile nature of chemical content.

Within the chemical content balance, lignocellulose crops such as Salix or millet – apart from saccharides compound – they also contain 1–5% of protein, and other crops having lignocellulose residue potential (straw) such as grain crops, grass or oil crops may contain 3–30% of fats. Lignocellulose-based biopolymers, particularly lignin, are hard to convert into simple compounds they are made from. The basic reason for that is essentially different sensitivity of those compounds to all and any thermal, biological and chemical processes. Thus cellulose and hemicelluloses content determine bioethanol capacity under biochemical process. In the case of cellulose, breaking glycoside bonds combining monosaccharides is a technological problem as the longer the saccharides chain is and the more branched it is, the more difficult the problem becomes. On the other hand in the case of hemicellulose, conversion into monosaccharides is easier, however those saccharides are more difficult to undergo fermentation. The specific nature of polysaccharides chemical bonding

Table 8.1 Biopolymer content in various agricultural residues

	Chemical content (% dry mass)		
Lignocellulose biomass source	Cellulose	Hemicellulose	Lignin
Corn cob	45	35	15
Corn stem	40	25	17
Rice straw	35	25	12
Wheat straw	30	50	20
Fibre residues from extracting sugar cane juice	40	24	25
Millet	45	30	12
Bermuda grass	25	35	6
Miscanthus	44	24	17
Pine tree(Pinus sylvestris)	40	29	28
Spruce (Picea glauca)	40	31	28
Eucalyptus (Eucalyptus camaldulensis)	45	19	31
Birch (Betula vernocosa)	41	32	22
Willow (Salix sp.), 1-year cycle crops	45	13	14
Willow (Salix sp.), 2-year cycle crops	48	12	13
Willow (Salix sp.), 3-year cycle crops	56	14	14
Poplar	42–48	16–22	21–29

Source: Gołaszewski et al. (2012)

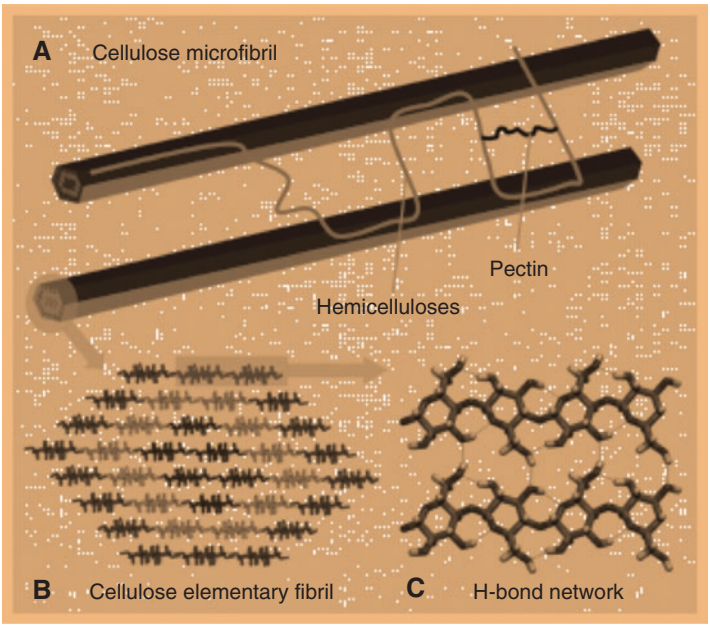

Fig. 8.1 Lignocellulose-based polysaccharides structure and interactions. (**a**) Simplified model of interactions among main polysaccharides in cell walls (no lignin whose interactions with cellulose and hemicelluloses are not well defined). In this model hemicelluloses are tightly connected with cellulose crystallites (micelle) building the network of microfibres. Pectines are polysaccharides "splicing" cell wall components. (**b**) Structure of elementary cellulose microfibrils. (**c**) Intrachain and interchain hydrogen bonding. Source: Himmel et al. (2007)

and mutual interactions are offered in Fig. 8.1. Troublesome decomposition and extensive volatility of chemical content in the case of lignocellulose crops in relation to genotypes and crops habitats cause development of economically efficient preliminary processing and hydrolysis to be a great challenge.

Sluiter et al. (2010) reviewed 15 methods of analysing content/division of structural hydrocarbons and lignin through sulphuric acid-based hydrolysis. Beginning from 1922 until 1993 the analytical process of a variety of lignocellulose resource (timber, soft timber, wood residues, wood pulp, and others) of various refinement degree and various extraction modes was regularly developed. Development of cellulose chemical hydrolysis-based processes was also contributed by Polish inventors, including Troszkiewicz and Bogoczek who in 1954 patented „the mode of hydrolysis of timber or other materials containing cellulose, sophisticated due to that the hydrolysed material mixes cold with smoking nitrogen acid and immediately distils the acid under lower pressure." In spite of many suggestions of specific arrangements, a number of contemporary research works have proven that for future arrangements the most promising will be inclusion of enzymatic hydrolysis but it bears noting that nowadays the cost of such a process has not become competitive yet (Yu and Zhang 2004).

Hahn-Hägerdal et al. (2006) analysing new technologies-related requirements and achievement of indispensable progress allowing for lignocellulose bioethanol to be commercially produced, have stated that optimization of the process engineering, fermentation technology, enzymes production and metabolic processes will be the real challenge for the next coming years. For successful biological lignocellulose conversion into bioethanol, a set of principal processes is indispensable for a technological line: (1) defibration (delignification) entailing release of cellulose and hemicellulose out of lignin – preliminary process, (2) depolymerisation of polysaccharides into monosaccharides – mineral acid-based hydrolysis/enzymatic hydrolysis, (3) hexose and pentose fermentation into ethanol, (4) ethanol refining by means of distillation and refinement. In the background of delignification process (stage 1), from among many various physical, chemical, biological and mixed methods (Table 8.2), the physical method „steam explosion" is referred to as a developing method, that entails treatment of disintegrated lignocellulose biomass with steam under a high pressure, and subsequent fast decompression causing the cells to break up and inducing easier enzyme penetration. Bonini et al. (2008) analysing various physical parameters of the steam explosion in the presence of various chemical compounds, that is to treat disintegrated biomass of two resources: a pine and corn stems, have stated inter alia that the thermal-and-chemical method with temperature of 200 °C applied for 5 min in the presence of 3% sulfuric acid results in the highest reclaim of lignin from corn stems, amounting to 65.08% of Klason lignin. Apart from the steam explosion, Zhu and Pan (2010) mention to two other technologies – Organosolv that entails lignin and hemicellulose organic solvents in the temperature of 140–220 °C and SPORL – Sulfite Pretreatment to Overcome Lignocelluloses Recalcitrance. The Organosolv technology is used in the paper industry but purposefulness of its use for lignocellulose crops and residues has been quite frequently reported. Alcaide et al. (2003) have examined the impact of ethanol, acetone and water mixture upon the features of a pulp from wheat straw stating inter alia that the process entailing the said solvents should be run in the temperature of 180 °C for 60 min in order to be effectively successful. The whole process covered three stages: 1 – Organosolv-based delignification reaction, 2 – lignin reclaim by means of pressurized dissolved air flotation, 3 – lignin used for polymer production. Zhu et al. (2009), reported that the pulp, obtained from SPORL entailing treatment of raw soft timber chips with acid sulphite (8–10%) and sulphuric acid (1.8–3.7%), was particularly efficient in the context of enzymes efficiency. In consequence hemicellulose is almost completely separated, delignification is done partially and lignosulfonian is obtained, and upon the lapse of 48-h enzymatic hydrolysis, cellulose is disintegrated in 90%. In the process of cellulose and hemicellulose enzymatic hydrolysis, kindred enzymes produced by a numerous group of microorganisms (fungi, bacteria, protozoons, and others) are used, the fungi being the best recognized group of microorganisms.

Cellulases disintegrating cellulose are classified according to the nature of catalyzed reaction: hydrolytic cellulases, including endocellulases, egzocellulases and

Table 8.2 Sources, chemical diversity and biological activity of four major families of alkaloids

Alkaloid family	Family (Plant species)	Active compounds	Pharmacological activity	Target
Monoterpene indole alkaloids (MIA)	Apocynaceae			
	Catharanthus roseus	Vinblastine, Vincristine	Anticancer	Tubulin
		Ajmalicine	Antihypertensive	α adrenergic receptor
	Rauvolfia serpentina	Ajmaline	Anti-arrythmic	Na + channels
	Nyssaceae			
	Camptotheca acuminata	Camptothecin	Anticancer	DNA topoisomerase I
Benzylisoquinoline alkaloids (BIA)	Papaveraceae			
	Papaver somniferum	Codeine	Antitussive, analgesic	Nicotinic acetylcholine (nACh) receptor
		Morphine	Analgesic, narcotic	μ3 opioid receptor
		Papaverine	Spasmolytic, vasodilators	c-AMP phosphodiesterase
	Eschscholzia californica	Sanguinarine	Antibacterial, proapoptotic	FtsZ (bacterial cytokinesis), mitoch.
	Ranunculaceae			
	Thalictrum flavum	Berberine	Antibacterial, antimicrobial	DNA
	Coptis japonica	Berberine, sanguinarine		
Tropane and nicotine alkaloids (TNA)	Solanaceae			
	Hyoscyamus niger	Hyoscyamine	Anticholinergic, narcotic, myorelaxant	Muscarinic receptor
	Datura stramonium	Scopolamine	Anticholinergic, narcotic, myorelaxant	Muscarinic receptor
	Atropa belladonna	Hyoscyamine	Anticholinergic, narcotic, myorelaxant	Muscarinic receptor
	Nicotiana tabacum	Nicotine	Neurostimulant, insecticide	nACh receptor

(continued)

Table 8.2 (continued)

Alkaloid family	Family (Plant species)	Active compounds	Pharmacological activity	Target
Purine alkaloids (PA)	Coffeeae			
	Coffea arabica	Caffeine	Central nervous system stimulant	Adenosine A1 & A2A receptors
	Theaceae			
	Camellia sinensis	Caffeine, theophylline	Central nervous system stimulant	Adenosine A1 & A2A receptors; phosphodiesterase
	Byttnerioideae			
	Theobroma cacao	Theobromine, caffeine	Central nervous system stimulant	Adenosine A1 & A2A receptors; phosphodiesterase

Source: Guirimand et al. (2010)

beta-glucosidases (disintegrating internal bonding of cellulose crystalline structure into single cellulose chains, subsequently into disaccharides (cellobiose) and finally into monosaccharides), oxidase cellulases and cellulose phosphorylases responsible for phosphate-based cellulose polymer degradation (Reddy and D'Souza 1998). Alcohol fermentation is a subsequent stage that is performed by microorganisms, during which simpler compounds are released, such as monosaccharides fermented into ethanol and carbon dioxide. Particularly preferred is a type of yeast *Saccharomyces cerevisiae* accumulating the largest quantity of ethanol? Number of types of bacteria, including pathogenic ones, for which the basis fermentation product is ethanol: *Clostridium sporogenes, Clostridium indoli, Clostridium sphenoides, Clostridium sordelli, Zymomonas mobilis, Spirochaeta aurantia, Spirochaeta stenostrepha, Spirochaeta litoralis, Erwinia amylovara, Escherichia coli, Leuconostac mesenteroides, Streptococcus lactis, Klesiella aerogenes*, *Mucor* sp., *Fusarium* sp. were reported (Dien et al. 2003).

Combining enzymatic hydrolysis (stage 2) and fermentation (stage 3) with the use of various types of yeast (Simultaneous Saccharification and Fermentation-SSF) was an innovative arrangement that was to improve efficiency of conversion of lignocellulose into ethanol, inter alia due to the enzyme cellulose produced by mutated strain of fungus Trichoderma reesei growing in the presence of hydrolysed glucose (normally glucose hampers this fungus from producing cellulose). The outcome problem with SSF technology arises from various optimal hydrolysis temperatures (45–50 °C) and fermentation (28–35 °C) and compatibility of microorganisms and enzymes (Lin and Tanaka 2006). Bothast and Saha (1997) have defined some economic minimum of the conversion of lignocellulose substrate into

ethanol providing for high capacity of ethanol with approximately 3% of mass-volume concentration and substrate input above 10% as obtained in a relatively short time – shorter than 4 days. Krishna and Chowdary (2000) maintained that combining enzymatic hydrolysis and fermentation with the use of cellulose obtained from Trichoderma reesei QM-9414 cells and *Saccharomyces cerevisiae* NRRL-Y-132. Ferreira et al. (2010) analysing the processes of disintegrating the residues of sugar cane-based saccharides production, state that the majority of cellulose enzymes that are commercially available do not prove satisfactory activity under simultaneously performed processes of saccharification and fermentation. They also refer to a larger biotechnological saccharification and fermentation potential of recombinant strains of *S. cerevisiae* than in the case of *T. reesei* as far as the production of second generation bioethanol is concerned. Modifications of SSF hitherto have aimed at optimalization of environmental conditions for microorganisms responsible for both phases by means of running the processes in two reactors having various optimal temperatures – Non-isothermal SSF (Wu and Lee 1998) or biopolymer structure-oriented process- SSCF (Simultaneous Saccharification and CoFermentation), performing hexose and pentose co-fermentation (Olofsson et al. 2010). Effective production of bioethanol from lignocellulose is the subject of numerous research works and expected technological progress will be preconditioned by achievements in glucose conversion-oriented fermentation technology (out of cellulose) and xylose (out of hemicellulose) into ethanol, more effective production of hydrolysis and fermentation enzymes and intensified activity.

8.2 Secondary Metabolites and Their Role in Plant Growth

8.2.1 Alkaloids

Alkaloids are classified in a number of families with completely different biosynthetic pathways. This section discusses four major families monoterpene indole alkaloids (MIA), benzylisoquinoline alkaloids (BIA), tropane and nicotine alkaloids (TNA) and purine alkaloids for which the biosynthesis and the regulation are more thoroughly studied. Although alkaloids are chemical diverse, they commonly originate from primary metabolites such as amino acids or bases. There is no genome-sequencing project for the major alkaloid-producing plants except for *Nicotiana tabacum*. Classical biochemical and molecular biology studies have therefore identified most enzymatic steps. The continuous elucidation of some biosynthetic pathways illustrates the recruitment of enzymes from recurrent multigene families, such as cytochrome P450 monooxygenases, acetyl transfers or methyltransferases. Transcriptomic projects focusing on MIA-producing species recently expressed sequence tags (EST) (Murata et al. 2008; Shukla et al. 2006), BIA-producing species (Ziegler et al. 2006; Zulak et al. 2007) and TNA-producing species (Li et al. 2006) helped to identify some missing enzymatic steps. Recently, metabolic profiling studies have also been carried out on major alkaloid – producing

species (Hagel et al. 2008), sometimes in conjunction with transcriptomic studies (Ziegler et al. 2006; Zulak et al. 2007; Hagel and Facchini 2008).

These approaches together now enable tremendous progress to understand these complex alkaloid biosynthetic pathways. The purpose of this section is to provide a more detailed description of all these complicated biosynthetic pathways.

8.2.1.1 Biosynthesis of Monoterpene Indole Alkaloids (MIA)

The approximately 2000 MIA chemical structures described to date are widespread in a large number of plant species (Ziegler and Facchini 2008). Some of these molecules are of interest to human health, such as the anticancer drugs like vinblastine and vincristine and the antihypertensive drug ajmalicine produced specifically in Catharanthus roseus, the anticancer compound camptothecin produced mainly in Camptotheca acuminata or the anti-arrythmic ajmaline produced in Rauvolfia serpentina (Table 8.2). These molecules form part of the wide range of MIA produced by a single plant species as in C. roseus in which more than 130 MIA has been reported (van der Heijden et al. 2004). In three above-mentioned species, the elucidation of MIA biosynthetic pathways has undergone significant recent progress in identifying 42 clones corresponding to 31 enzyme steps. At C. Roseus alone has been studied with 27 enzymatic steps (25 clones of cDNA and two other enzymatic activities without a clone assigned). The pathways have a common origin in these species with strictosidine synthase (STR), which catalyzes the condensation of the indole precursor tryptamine with the terpenoid precursor secologanin to form the first MIA, strictosidine. These plant species also share the upstream biosynthesis of the indole precursor from the shikimate pathway via tryptophan and the terpenoid precursor from the methyl erythritol phosphate (MEP) pathway. Strictosidine b-glucosidase (SGD), which catalyzes the deglucosylation of strictosidine, is the last common enzyme for the biosynthesis of 2000 MIA, since the resulting aglycon is the starting point for many different lateral MIA pathways, with the observed possibility of harboring more than one of these pathways for a particular species (e.g. C. roseus, reviewed in van der Heijden et al. 20). There are still many enzymatic steps to be discovered. More details on these pathways and the identified enzymatic steps are available in recent reviews (van der Heijden et al. 2004; Mahroug et al. 2007; Ziegler and Facchini 2008).

8.2.1.2 Biosynthesis of Benzylisoquinoline Alkaloids (BIA)

BIA is a diverse class of more than 2500 compounds with strong pharmacological properties and socio-economic significance for some of them. More than 80 alkaloids have been identified in opium poppy alone (Papaver somniferum). BIA has also been extensively studied in other Papaveraceae members, such as Eschscholzia californica, or Ranunculaceae members, such as *Thalictrum flavum* and *Coptis japonica.* BIA biosynthesis begins by condensing two tyrosine derivatives to produce (S)-norcoclaurine. In order to produce (S)-reticuline, the central precursor of the five major BIA subpathways, it is necessary to produce four characterized enzymatic steps leading to palmatine, berberine, sanguinarine, laudanine and codeine/morphine (Ziegler and Facchini 2008).

8.2.1.3 Biosynthesis of Tropane and Nicotine Alkaloids (TNA)

In medicine, TNA is widely used as muscarinic antagonists that affect peripheral and central nervous systems. This alkaloid family is found mainly in Solanaceae species, such as Hyoscyamus niger, Datura stramonium, Atropa belladonna or Nicotiana tabacum, and accounts for more than 200 different compounds (Oksman-Caldentey 2007). TNA are amongst the most studied alkaloids and their pharmacological effects are well documented. However, their biosynthetic pathways are still only partially understood. TNA is derived from ornithine and arginine amino acids, and in several species, the early biosynthetic steps leading to the formation of N-methylputrescine were elucidated. N-methylputrescine's oxidative deamination leads to N-methylpyrrolium cation, which is a branching point towards tropane alkaloids and nicotine alkaloids. The final steps of both pathways are characterized in part, but there is still no molecular information about the central steps. Overall, seven enzymatic steps and four enzymatic steps of the nicotinic alkaloid biosynthetic pathway have characterized the tropane alkaloid biosynthetic pathway. More information on these pathways and the identified enzymatic steps can be found in recent reviews (Oksman-Caldentey 2007).

8.2.1.4 Biosynthesis of Purine Alkaloids (PA)

PA is a natural product of purine nucleotides (Ashihara et al. 2008). The main PA is caffeine and theobromine, which act as neurostimulants on the central nervous system and are synthesized by several plants, including Coffea arabica, Camellia sinensis or Theobroma cacao. The initial precursor of PA is xanthosine, which is supplied by at least four different pathways. The main caffeine biosynthetic pathway has four enzymatic steps, comprising three characterised S-adenosylmethionine-dependent N-methylation reactions and one uncharacterised nucleosidase reaction. However, according to structural studies of N-methyltransferases involved in the biosynthesis of caffeine, it was suggested that xanthosine 7 N-methyltransferase could be used for ribose hydrolysis (McCarthy and McCarthy 2007). This pathway's details have recently been reviewed (Ashihara et al. 2008).

8.2.1.5 Spatial Organisation of Alkaloid Biosynthesis

The spatial organization of alkaloid biosynthesis has recently been extensively investigated using methods of hybridization in situ and immunocytochemistry (Kutchan 2005; Mahroug et al. 2007; Ziegler and Facchini 2008). An amazing complexity has been uncovered that shows multicellular organizations as a common feature. These organizational types imply the need for intercellular translocation processes. In C. roseus, a series of publications showed the sequential involvement of internal phloem associated parenchyma (IPAP), epidermis and laticifers-idioblasts during MIA biosynthesis in aerial organs (Irmler et al. 2000; Oudin et al. 2007). The IPAP cells harbour the expression of genes involved in early steps of monoterpenoid biosynthesis, i.e. four MEP pathway genes and geraniol 10-hydroxylase (G10H, CYP76B6) encoding the first committed enzyme in monoterpenoid biosynthesis (Oudin et al. 2007; Guirimand et al. 2009). The intermediate steps leading to the synthesis of the two MIA precursors, tryptamine and

secologanin, and to their subsequent condensation to form the first MIA strictosidine, occur within the epidermis (Irmler et al. 2000). Finally, the last two steps in the biosynthesis of vindoline, one of the monomeric MIA precursors of the dimeric MIA vinblastine, are localised to specialised laticifer-idioblast cells. Recently, these results were elegantly completed by an RT-PCR analysis of laser capture microdissected C. roseus leaf cells (Murata and De Luca 2005) and by an EST analysis study of epidermis-enriched fractions obtained using an original carborundum abrasion technique (Murata et al. 2008). All these results suggest that the translocation of an unknown monoterpenoid intermediate from IPAP to epidermis and the translocation of an unknown MIA intermediate from epidermis to laticifer-idioblast cells must be considered in order to ensure continuity in the metabolic flux along the MIA pathway. The identification of these intermediates requires the location of two subsequent enzyme steps in two different types of cells. Similarly, in the roots of several solanaceous species, the shuttle of intermediate TNA between the pericycle and the endodermis is suggested by the specific localisation of early and late enzymatic steps to the pericycle, and the specific expression of an intermediate enzymatic step (tropinone reductase 1) within the neighbouring endodermis (Nakajima and Hashimoto 1999; Suzuki et al. 1999). In this case, given the proximity of conducting tissues, it is hypothesised that the precursor of TNA (arginine) could come from the phloem, and that final TNA products, such as scopolamine, could flow to the aerial parts of the plant through the xylem (Nakajima and Hashimoto 1999). Four models of the spatial organisation of BIA synthesis in opium poppy and in a species (T. flavum) of Ranunculaceae also illustrate the complexity of these compartmentations. In opium poppy, two different models have been proposed (Kutchan 2005; Facchini and St-Pierre 2005). A similar rationale for MIA and TNA biosynthesis is proposed in one model, since there is a cell-specific separation between the early and late steps of different branches of the BIA pathway. According to immunolocation studies conducted on five biosynthetic enzymes, this model suggested that BIA synthesis usually begins with (S)-reticuline synthesis in the phloem parenchyma. Different situations are then observed for three BIA subpathways leading to laudanine, codeine/morphine and scoulerine respectively. In the same phloem parenchyma cells, laudanine synthesis appears to occur, while scoulerine biosynthesis is located in the leaf idioblasts and root cortex cells. Finally, an intermediate step in the codeine pathway is also located in phloem parenchyma, while the final step is in laticiferous substances (reviewed in Kutchan 2005). However, Facchini and St-Pierre (2005) proposed a totally different type of compartmentation in the same species with a so-called tale of three cell types, implicating the transcription of seven BIA biosynthesis genes within companion cells, the immunolocalisation of the corresponding enzymes to the sieve elements, and the accumulation of BIA in neighbouring laticifers (Samanani et al. 2006; reviewed in Ziegler and Facchini 2008). The discrepancy between both models has been attributed tentatively to differences in cultivars and/or in developmental stages. The last example of BIA synthesis in *T. flavum* illustrates that several steps of a pathway may be compartmentalised in a different manner within different plant species (Samanani et al. 2005). Alkaloid biosynthesis also involves subcellular compartmentation of biosynthetic enzymes,

as is the case for most natural product biosynthetic pathways. Beside cytosol, organelles such as ER (either lumen or membranes), plastids (either stroma or thylakoids), mitochondria and vacuoles have been implicated in various alkaloid biosynthetic pathways (Facchini and St-Pierre 2005; Mahroug et al. 2007; Ziegler and Facchini 2008). These results are based on biosynthetic enzyme sequences and more formal experimental evidence in silico analysis. Part of these experiments corresponded to the analysis of density gradients and a few examples of the direct location of immunogold enzymes (Samanani et al. 2006; Oudin et al. 2007; Guirimand et al. 2009) and by GFP-fusion image analyses (Costa et al. 2008; Guirimand et al. 2009) have been published. This emphasizes that a systematic reevaluation of the subcellular location of all enzymes available in a given alkaloid pathway is a future challenge, which should enable the development of more complete spatial compartimentation models that integrate cellular and subcellular levels. Together, these results suggest the recurrent need for alkaloid biosynthesis for transmembrane and intercellular translocation processes. Members of the ATP binding cassette (ABC) transporter superfamily have been demonstrated to be able to recruit various alkaloids such as vinblastine, hyoscyamine, scopolamine or berberine (Shitan et al. 2003). The possibility that alkaloid intermediates and/or enzymes flow through the symplasm should also be considered, even though no experimental proofs are available. Finally, the organisation of clusters of biosynthetic enzymes in metabolic channels has also been purported in BIA synthesis (Samanani et al. 2006).

8.2.1.6 Role of Alkaloids

The proposed roles of alkaloids in plant metabolism, plant catabolism, or plant physiology are (1) end products of metabolism or waste products, (2) storage reservoirs of nitrogen, (3) protective agents for the plant against attack by predators, (4) growth regulators (since structures of some of them resemble structures of known growth regulators), or (5) substitutes for minerals in plants, such as potassium and calcium. Of these items, (2) and (5) appear to be the least promising, and are not considered further here, but the other items, as well as new concepts, will be discussed in this chapter.

8.2.1.7 Alkaloids as Growth Stimulators and Inhibitors

Some alkaloid structures' structural similarity to plant growth hormones stimulated the idea that at least some alkaloids can play a role as plant growth regulators. Although the idea is quite old, the methodological obstacles have been enormous and in only a few cases the role of alkaloids in growth regulation has been proven. The first difficulty a researcher meets while attempting to investigate the role of alkaloids as growth regulators is the choice of the experimental plant material. If he or she decides to use a plant rich in alkaloids, the plant already has alkaloids and is growing, so the addition of alkaloids can either exceed the optimal alkaloid level or prove to be inhibitory, or it can still be below the critical point and stimulating. It can have no effect either. On the other hand, if he or she decides to use an alkaloid-free plant, the experiment will be artificial, since the plant normally develops

without alkaloids; so it will react to the added alkaloid in the same general way as to other physiologically active, but not natural, substances. Therefore, if an alkaloid added to an alkaloid-free plant influences the growth, nothing is proved except that the compound is not without effect in the particular plant. If a compound does give a similar reaction in a number of different species of plants, its role can be extrapolated to the plant from which it originates. Still, some uncertainty will remain. As a rule, alkaloid-rich plants grow more slowly than related alkaloid-poor species, but this rule has a number of exceptions. In addition, it is quite common that alkaloid-rich species grow under unfavorable conditions and that the abundance of alkaloids is caused by other factors that have no effect on the growth, but this abundance Other factors that do not affect growth cause alkaloids, but this abundance preserves plants from herbivorous animals. The slow rate of growth is an adaptation to the unfavorable environment that does not allow for more luxurious growth. A plant in an optimal environment, if grazed, can quickly regenerate and produce seeds, while a plant growing in an arid area, especially if a high percentage of the foliage is destroyed, cannot recover. Growth and predation are so interwoven that it is difficult to judge which the cause is and which the result is. When comparing plants belonging to one family, we quite often encounter slow-growing species and genera that are alkaloid rich growing in unfavorable conditions; in contrast, relatives growing luxuriously in better environments are alkaloid-poor. For example, the alkaloid rich Genisteae are usually encountered in arid areas, while the alkaloid-free Vicieae are encountered in moist areas. One explanation is that the alkaloid-rich plant, finding less competition for sun, does not need to grow so rapidly to avoid being overshadowed, and therefore a slow-growing xerophytic type is selectively favored. Other plausible reasons could also be important, such as the availability of soil moisture and nutrient levels. Colchicine is the best-known example of an alkaloid that inhibits cell division in plants. In minute quantities, this alkaloid interferes with the formation of a cell carokinetic spindle; instead of dividing the cell into two daughter cells, a restitutive cell with a double set of chromosomes is formed. The alkaloid, while very active on cells of most species of plants, produces no effects in Colchicum autumnale, the most common source of this compound. Alkaloids of Senecio and Crotalaria can cause chromosome breakage in a number of organisms, mostly animals. But the species that produce them are harmless. Some alkaloids inhibit germination when added to water in which alkaloid-free plant seeds are soaked. These types of allopathic effects are quite common and are caused not only by alkaloids but also by a number of other secondary metabolites. The alkaloid prevents the germination of seeds of foreign species, thus preserving living space for its own progeny. However, it was found recently that some plants can absorb alkaloids from the soil without serious effects. Not only can foreign alkaloids from decaying plants be absorbed, but a number of synthetic alkaloid like chemicals, as well as some microbial products, can also be taken in. The foreign compound is sometimes converted into derivatives, while it is only accumulated at other times. In cases where it has been shown that an alkaloid is poisonous to a foreign plant, it can be assumed that the species producing alkaloids has developed a hormonal system that can function effectively despite the present alkaloid. Waller and Burstrom (1969) have shown

that certain diterpenoid alkaloids from Delphinium ajacis exhibit growth-inhibiting effects on pea cambium growth. The diterpenoid alkaloids are related in chemical structure to the gibberellins in that the AlB ring junction is similar in the two groups of compounds and is antipodal to that of most naturally Steroids occur. These results showed clearly that delcosine and delsoline inhibited both phloem and xyl tissue growth (Sastry and Waller 1971). The inhibitory or delaying effect on delsoline cambium initiation was particularly important. Ajaconin, by contrast, had no significant growth effect. This inhibition of growth can be explained in various ways. Delcosine and delsoline can compete for enzyme-active sites with gibberellic acids, which change their catalytic function. Such interaction may be at an active site or in the vicinity of an allosteric site. Another mode of action may be the control of delco sine and delsoline production of gibberellic acid(s). Each group of compounds is likely to come from a common intermediate diterpenoid pyrophosphate, whose structure is unknown. The end product controls its production in one of the initial steps in biosynthesis (such as the branch point) in the usual feedback control method. On the pathway's gibberellic acid biosynthesis branch, it is conceivable that diterpenoid alkaloid control could be exercised at another step. In further studies of the relationship between diterpenoid alkaloids and gibberellic acids (GA), Lawrence and Waller (1973a, b, 1975) showed that the diterpenoid alkaloids of Delphinium ajacis inhibited the action of GA3 in bioassays with cucumber hypocotyl. Comparison of the effects of diterpenoid alkaloids with those of abscissic acid (ABA) (an inhibitor of GA action) and 2-isopropyl-4-trimethylammonio-5-methylphenyl piperidine-lcarboxylate chloride (AMO-161S) (an inhibitor of gibberellic acid, GA3) synthesis were completed. The results indicated that certain responses occurred with tests requiring the presence of GAa when inhibited aamylase synthesis in germination seeds induced the elongation of the hypocotyl and stem internode of lettuce seedlings in the light and dark, and induced cucumber hypocotyl elongation; however, they did not consistently mimic the effects of either ABA or AMO-161S. These results suggest that diterpenoid alkaloids affect the development of plants by affecting the biosynthesis and/or transport of GA within the plant. Diterpenoid alkaloids may inhibit the formation of microtubules (Shibaoka 1974), synthesis of cellulose (Hogetsu et al. 1974), or translocation of auxins. It must be borne in mind that these nitrogenous bases can be isolated from plants of the genera Aconitum and Delphinium of the genera Ranunculaceae, the genus Garrya of the genus Garryaceae and the genus Inula royleana of the genus Compositae; therefore, although they may have an effect on plants endogenous to gibberellic acid on ABA, they cannot be regarded as general in the plant realm. These results suggest that diterpenoid alkaloids affect the development of plants by affecting the biosynthesis and/or transport of GA within the plant. Diterpenoid alkaloids may inhibit the formation of microtubules (Shibaoka, 1974), synthesis of cellulose (Hogetsu et al. 1974), or translocation of auxins. It must be borne in mind that these nitrogenous bases can be isolated from plants of the genera Aconitum and Delphinium of the genera Ranunculaceae, the genus Garrya of the genus Garryaceae and the genus Inula royleana of the genus Compositae; therefore, although they may have an effect on plants endogenous to gibberellic acid on ABA, they cannot be

regarded as general in the plant realm. The remarkable transfer of ricinine from senescent leaves to maturing seeds raises questions about the possible function of this alkaloid in the germination of seeds (Skursky and Waller 1972). In the second week of germination, rapid de novo ricinine synthesis was reported (Schiedt et al. 1962). In this study, N-demethylricinine was detected as a normal constituent of R. communis (castor beans) during dormancy and in the very first stages of germination. Shortly after germination began, the N-demethylricinine concentration temporarily increased, apparently related to ricinine biosynthesis; but this alkaloid disappeared almost completely quickly. There is not enough evidence to permit a proposal of any hypothesis yet. Efforts are in progress to determine if N-demethylricinine is a biologically active compound, with ricinine as the inactive form. In maturing seeds, N-demethylricinine is first detectable when pigmentation of the seed coat (testa) begins, i.e., when the development of the seeds has almost reached the final stage of maturity. The increase of ricinine and N-demethylricinine during the first day of germination might be caused by the release of ricinine from some bound form. Experiments performed on the nicotinic acid-ricinine relationship in sterile cultures of R. communis established clearly that (1) the relationship exists, and (2) the metabolism of ricinine can be spared by the presence of higher concentrations of nicotinic acid than normally found in the tissue (Waller and Nakazawa 1963). This economical effect of nicotinic acid on the use of ricinine suggests a metabolic relationship between vitamin and alkaloids not previously found in a plant system. Some alkaloid precursors such as indole derivatives, purines and nicotinic acid are powerful growth stimulators, and some are inhibitors, such as some derivatives of phenylalanine-tyrosine. The biosynthetic version of these compounds into alkaloids indicates a process of deactivation. Most plants in which alkaloids were introduced in physiological quantities (up to 1% of the plant's dry substance) had no effect at all, but even if growth inhibition or poisoning is observed, this is no indication of the effect. Datura, Papaver and Atropa varieties were produced that contained up to three times the normal alkaloid content (Mothes and Romeike 1954). Sometimes these plants grew slower and were more susceptible than normal plants to unfavorable environmental conditions. It was found in 1930 that alkaloid-poor lupine mutants were as vigorous as the wild type at first glance (Sengbusch 1934). However, a careful examination of the seed and straw yield indicated a lower coefficient of reproduction for the mutant. In this case, the mutation appears to cause the plant to weaken by unbalancing the fine balance between various compounds. Despite intensive breeding programs, even compared to unselected alkaloid-rich (bitter) forms, the alkaloid-poor (sweet) forms are still lower. This is in some ways a confirmation of the additional pleitropic side effect of the alkaloid-poor mutation, which is that the original sweet mutants are weaker individuals. The alkaloid, on the other hand, could act as a stimulant or growth regulator. Repeated sweet backcrossing with bitter shapes improves the former's performance. There are significant differences in the number of seeds produced. Diminished seed production is the most striking feature in the sweet forms. Mackiewicz (1958) analyzed the viability of sweet and bitter pollen grains; he was able to confirm that the sweet plants actually have a lower percentage of stainable (and likely viable) pollen grains.

In favorable environmental conditions, sometimes germinating sweet plants developed better than bitter plants, but later ontogenic plants became poorer than wild plants. Maysurian reported a sweet Lupinus polyphyllus isolation in 1957. This mutant was remarkable because when placed on petri dishes in plain water, the seeds did not germinate. When alkaloids were added from the bitter seeds of Lupinus angustifolius, the seeds of Lupinus polyphyllus were well germinated. Since Lupinus angustifolius has a relatively similar alkaloid spectrum to Lupinus polyphyllus, there was no indication of the need for a specific alkaloid to initiate germination. The alkaloid-free plant's seed progeny, however, germinated quite well. From seeds obtained from Maysurian in 1957, plants were grown in Poland and compared to bitter genotypes, but the reason for the inferiority of the plants could not be determined. Even if the original stock came from the same part of North America, the approximately 100 years of propagation in Europe could have caused the differences. Therefore, the two strains were crossed, and the F2 segregates were investigated. The difference of vitality of sweet and bitter plants was so striking that even without testing for alkaloids it was possible to tell the plants apart. Since L. polyphyllus is a predominantly cross-pollinated species, the sweet plants were spatially isolated from the bitter L. polyphyllus to prevent cross-pollination with the bitter forms. The space between the bitter and sweet plants (about 400 m) was seeded with different lupines from North America. Normally L. Poiyphyllus is not easily hybridized. However, the number of interspecific spontaneous hybrids in this partiCUlar case was incredibly high, which led experimenters to investigate the viability of pollen. Some plants have found that no viable pollen grains have been produced. Lamprecht (1964a, b), while investigating certain aberrant mutations that cause the formation of morphological character peculiar to different species (he suggests calling these types of mutations exomutations or interspecific mutations), found that an exomutation is always accompanied by reduced fertility of the mutant and that the segregation ratio is usually distorted by a deficit of the recessive homozygotes. An alkaloidpoor mutant in lupines can be regarded as a typical exomutation, because the mutated form is lacking character of not only the species but also of the tribe's metabolical peculiarity to synthesize and accumulate quinolizidine alkaloids. The mutations in other lupine species are not so drastic, and the level of the residual alkaloids is usually higher than in the sweet L. polyphyllus. Applying lupanine solution to leaves of sweet L. albus, Nowotny-Mieczynska and Zientkiewicz (1955) were able to show a growth. The property of the applied alkaloid stimulates: Nowacki (1958), on the other hand, could not find any significant differences between sweet and bitter L. angustiJolius growing on soil supplemented with sparteine and that grown on soil to which the alkaloid spectrum typical to the L. angustiJolius alkaloid mixture was added. The plants had absorbed at least a part of the added alkaloids, but the increase of dry matter production was too low to be attributed to the effect of alkaloids. In unpublished experiments in laboratories in Poland, some seeds of sweet lupines were soaked with salts of pure alkaloids in levels from 0.01% to 1% of dry substance of the seeds. The results were confusing, but some conclusions can be drawn: hydroxylated alkaloids like lupinine and

hydroxylupanine were slightly stimulating to growth, while sparteine and lupanine were inhibiting, as were most species of foreign alkaloids such as nicotine, quinine, cytisine, and a number of other compounds. While the sweet mutants were developing more poorly than the bitter forms, mutants with increased alkaloid levels also suffered (Mothes and Romeike 1954). Alkaloid-free seeds such as peas and kidney beans reacted likewise. Pöhm (1966) found that cytisine was quite poisonous to Phaseolus seedlings, while N-methylcytisine was harmless. Tomatoes and belladonna grafted upon Nicotiana stock suffered, and the leaves developed brown necrotic spots (Mothes and Romeike 1954).

It is important to recognize that not all plants have these processes. Together with the species group, an alkaloid common to a plant family or a similar taxonomic unit most likely originated. Since most plant families' age can be traced back to the upper Cretaceous era, the alkaloids must be of the same age. Therefore, in approximately 80 million years of selection, the character has shown some benefit to the family species and a balance of metabolites has been created. The alkaloid level can therefore hardly be changed without seriously affecting the vitality of the plants. However, when mutants are produced with altered alkaloid levels, they can grow under special conditions. The age of families rich in alkaloids is probably not the same; the time needed to develop the character was therefore different. In cases where the alkaloid character is very old and all members of the plant family are rich in alkaloids, the production of alkaloid-poor, viable mutants is hardly expected. Therefore, despite repeated attempts, no alkaloidless mutants have been found in Papaver and in some other alkaloid-rich genera.

8.2.1.8 Alkaloids as Plant·Protecting Compounds

Alkaloids are certainly not growth-regulating compounds, at least in the sense of normal growth regulators-a fact that requires scientists interested in the role of alkaloids to look for another role. Some alkaloids, which are randomly distributed throughout the Angiospermeae and often occur simultaneously in some species and not in others, have no selective value. However, since alkaloids are limited to a certain natural systematic unit and are present in all plants of the unit, without exception, only because of selective pressure, they play a role in plants by increasing their fitness. There are a number of supplied with alkaloids, alkaloid-free plants can grow and produce seeds; these plants can tolerate them, although they do not produce alkaloids. This is important in order to discuss the role of alkaloids in plants further. Since most alkaloids are not toxic to most plants, a mutation leading to alkaloid synthesis would have no effect on the plant and could only be determined by genetic drift as a neutral character. The possibility that genetic drift can completely eradicate and impose one neutral character is small. Therefore, it is justifiable to assume that alkaloids, without exception, in a natural higher taxonomical unit are or were of significant selective value for them. Errera believed that alkaloids protected plants from herbivorous animals and possibly pathogenic microorganisms as early as 1887. Since then, acquiring knowledge about alkaloid physiology in animals, fungi and bacteria have broadened the idea.

8.2.1.9 Protection Against Fungi and Bacteria

Some alkaloids, such as quinine and other Cinchona have been used for centuries as drugs for killing protozoa while having little effect on the mammals ingesting such alkaloids. There have been attempts to extrapolate the poisonous effects of alkaloids to other microorganisms (protists), but it has been difficult to do. It was found that alkaloid-rich plants are affected by parasitic fungi and by pathogenic bacteria as much as alkaloid-free species. Phytophthora will attack, with the same deadly effects, alkaloid-rich Nicotiana species as readily as the less alkaloid-rich tomatoes and potatoes. In solutions containing a number of alkaloids, Cladosporium develops as well as in media without alkaloids. A number of pathogenic and saprophytic fungi thrive in lupine alkaloids on media as well as in controls (Nowacki 1958). Fusarium isolated from infected lupine plants grew on spartein, quinine and a mixture of lupine alkaloids (up to physiological concentrations in the natural environment of plants). The low-concentration alkaloids even stimulated growth. *Aspergillus niger*, one of the saprophytic fungi, was not only grown in media rich in alkaloids, but also used alkaloids. Still, in some species, resistant forms can be clearly attributed to chemical differences. As an example, Virtanen (1958) found that Fusarium-resistant varieties of rye (Secale cereale) owe their resistance to a presence of an alkaloid-like (glycoside) fungistatic compound. Two substances, benzoxazolinone extracted from rye and 6-methoxy-2(3)-benzoxazolinone (Virtanen 1958; Virtanen et al. 1957), cause the resistance of corn (Zea mays) and wheat (Triticum spp.) toward certain fungi. They occur as their glycosides in the plants from which they are released by glycosidase, which becomes active during extraction. Present evidence suggests that they are not important in resistance because neither the glycosides nor the aglycones are very toxic. Since most alkaloids do not affect parasitic microorganisms, there are probably a number of parasites that attack only alkaloid-free species; consequently, it seems that many alkaloids have not in the past played a role of protecting compounds and that parasites acted as selection factors only in some rare instances. Savile (1954), comparing the distribution of Puccinia and other related pathogens, pointed out that the Pucciniaceae are older than the Angiospermeae, and, since the number of possible mutations in an organism with so short a life cycle and so enormous a number of spores can readily exceed the number of mutations of a higher plant, a development of alkaloid-based immunity seems to be hopeless. Even if a mutation of a higher plant has caused accumulation of a fungistatic alkaloid so that the mutant has a certain degree of immunity and replaces the susceptible original form, it will probably never achieve the final goal of total immunity. This is because, in the meantime, a mutation will occur in the infecting fungus, which will in tum be able to attack the temporarily immune form. The result will be a steady state involving the immune form, the original form, and both forms of the parasite. The difficulties in breeding rust-resistant varieties of cultivated plants are the best illustrations of this problem. The race between the development of immune forms of crop plants and the mutations of the parasite is a case in point, for every time a resistant form is produced; a mutation of the parasite diminishes its usefulness. Phymatotrichum omnivorum parasitizes and destroys roots of a wide variety of higher plants from many families; Taubenhaus and Ezekiel (1936) first suggested

that alkaloids are responsible for the resistance to this parasite. Swaseyi, two species resistant to *P. omnivorum*, found that they are high in berber in a continuous zone of cells under the peridermis of the roots in higher concentrations than those that prevent the growth of the parasite in cultivation. Similar evidence is that the alkaloids of a number of other resistant plants prevent the growth of *P. omnivorum* in cultivation at lower concentrations than in resistant tissues, e.g. sanguinarine prevents growth at 2–5 ppm and chelerythrine prevents growth at 50 ppm. Both substances are found in plant tissue (*SanRuinaria canadensis*) at much higher concentrations and are considered important in plant tissue. Wood (1967) observed that P. omnivorum was more susceptible to alkaloids, while those tested were the least susceptible to vascular wilt fungi. In monocotyledons, which are generally resistant to the parasite, it is believed that the high resistance of Amaryllidaceae members is due to their high alkaloid content (Greathouse 1939; Greathouse and Rigler 1940a, b). Invasion by Fusarium spp. in potato tubers may be prevented by alkaloids. The role of solanine in diseases caused by F. avenaceum and F. caeruleum is attributed to the fact that it occurs mainly in vacuoles of living cells and because F. avenaceum, unlike F. caeruleum, quickly kills and penetrates cells which it approaches. This means that F. The avenaceum is exposed to the alkaloid quickly, while F. caeruleum grows at first between cells of the tuber in intercellular spaces, so does not kill the cell at once (McKee 1955). The concentration of solanine and chaconine increases near mounds produced by F. caeruleum and this increase is accompanied by increase in resistance (McKee 1961). Solanine in concentrations of 500 ppm is a factor in the resistance of green tomato fruit to Colleotrichum phomoides. Tomatine and tomatidine are thought to play a part in resistance to wilt caused by Fusarium oxysporum f lycopersici (Kern 1952). Since in most cases the resistance is caused by factors other than alkaloids, such as differences in cell wall structure, accumulation of phenolic compounds, or an ability to form suberin layers around the infection point, it seems that alkaloids have never played an important role in protecting plants against fungal and bacterial infections. Most alkaloids are without effect on the development of adapted, as well as foreign, fungi in experiments in vitro. Therefore, it seems justified to assume that in cases in which an alkaloid-rich plant is not infected by a certain parasite, the reason for its immunity is not the presence of alkaloids.

8.2.1.10 Alkaloids and Virus Resistance

The resistance to a virus in a certain plant can be caused by two main factors: (1) the cell medium of the plant cannot provide adequate material for the replication of viral nucleic acids and protein, and (2) the plant is prevented by a vector, usually an insect, due to certain chemical or mechanical properties. We are going to deal with the first option. The host specificity of most plant viruses is known; still there is a broad spectrum of species and genera that may be attacked by the same virus. Very often the species and genera belong to a single family, but exceptions are common. The virus develops quite well on alkaloid-rich and alkaloid poor plants, but in some cases only plants with a defined alkaloid group suffer. While infecting Nicotiana tabacum and N. glauca with most strains of TMV (tobacco mosaic virus) is successful, the symptoms of the disease are very different in these species. *N. tabacum*

rapidly becomes sick, the infection spreads all over the plant, and the typical mosaic pattern is formed. In contrast, N. glauca develops only restricted necrotic spots and the plant remains apparently healthy; the development and production of seeds is not retarded. A similar case has been found in Mediterranean species lupines. *L. digitatus, L. albus, and L. luteus* were victims of the viral infection that causes the narrow leafiness in yellow lupines. The infected plants were found to have a severalfold increase in free arginine content. The plants usually died before being able to produce seeds, while the same virus produced no obvious symptoms in L. mutabilis, a South American species that has a different set of alkaloids (Nowacki and Waller 1973). In both cases, the Nicotiana and Lupinus, the virus was able to infect not only the species belonging to the given genera but a number of other not closely related host plants as well. Some Solanaceae plants were damaged in the case of tobacco mosaic virus (TMV). Oddly, only N. glauca seems to be resistant. In the case of the lupines, the virus used developed not only on lupines, but on a number of Papilionaceae: peas, beans, clovers, and alfalfa. There is no evidence that alkaloids were actually involved in the resistance, but this is quite possible. The evidence that certain alkaloids are active mutagenic, carcinogenic, and antitumor agents is sufficiently great to suggest that the alkaloid present in the cell may interfere with the replication of the virus nucleic acid molecules. One might wonder why the alkaloids are not interfering with the exact Plant DNA replication. This case appears to be sufficiently explained in the experiments and reports of Fowden (1974), in which he found that unusual amino acid homologues (or alkaloids, in the broader definition of alkaloids) were not incorporated into proteins in the plant species that produce them, while unrelated plants incorporate unusual compounds instead of proper ones. Dawson and Osdene (1972) hypothesized that nicotine and anabasin could be linked to a polynucleotide as an integral part of the DNA and RNA macromolecules. Although they present strong arguments for the occurrence of a nicotine and anabasin-containing DNA or t-RNA, no alkaloid was isolated from either type of macromolecule. In plants that tolerate a high concentration of alkaloids, there must be a system to discriminate between alkaloids and nucleic acid bases or, in other cases, between alkaloids and protein amino acids. The mutagenic action of a number of alkaloids in cells of plants, bacteria, insects, and mammals is well known, but in no instance does an alkaloid produce a mutation in the plant where it is native. This can be partly attributed to the fact that, as usual, the native alkaloid can be deposited outside the cell's active centres. Most of the alkaloids are deposited in vacuoles. In contrast, the artificially introduced alkaloids can be distributed in the cells differently. For example, in cells of plants rich in alkaloids, alkaloids can always be found on the way from the centers of active metabolism to the dumping areas, vacuoles, and when a virus infects the cell, sufficient alkaloids are available to confuse the replication system. Of course, this concept requires experimental verification, but the evidence promises that different alkaloid spectra will simultaneously exist in the infected and immune species in genera in which a number of species are virus-infected and others are apparently immune. There is impressive evidence of alkaloid activity in the DNA reproduction mechanism.

8.2.2 Flavonoids

Flavonoids are the most colorful pigments of flowers, fruits and seeds. These secondary metabolites, which are widely distributed in plants, are classified in six major subgroups: Chalcones, flavonols, flavandiols, anthocyanins and proanthocyanidins or condensed tannins (Fig. 8.2), and in some species, the aurones (Winkel-Shirley 2006), a seventh group is found. Legumes and a small number of nonlegume plants also synthesize specialized flavonoids such as the isoflavonoids (Wang 2011), while few species either produce 3-deoxyanthocyanins or phlobaphenes. Groups of unrelated species, including grape and peanut, synthesized stilbenes, chalcone-related compounds (Chong et al. 2009). More than 6000 flavonoids have been identified and this number will surely increase. The different flavonoids have diverse biological functions, including protection against ultraviolet (UV) radiation and phytopathogens, signaling during nodulation, male fertility, auxin transport, as well as the coloration of flowers as a visual signal that attracts pollinators (Bradshaw and Schemske 2003). Flavonoids are also responsible for the display of fall color in many plants, which may protect leaf cells from photooxidative damage, enhancing the efficiency of nutrient retrieval during senescence (Feild et al. 2001). Flavonols are probably the most important flavonoids involved in stress response; they are the oldest and most common flavonoids with a wide range of powerful physiological activities.

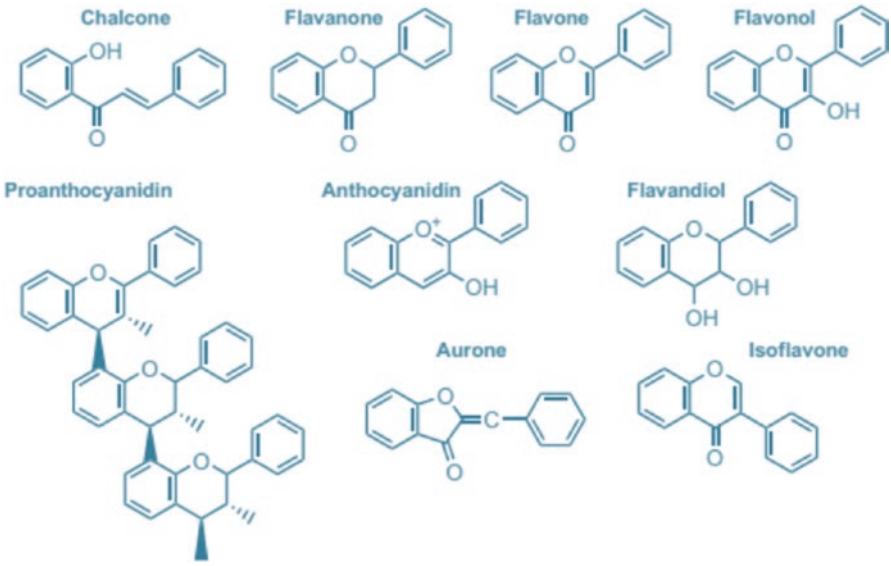

Fig. 8.2 Structure of the main classes of flavonoids

8.2.2.1 Flavonoids Biosythesis

A great deal of effort has been made to elucidate genetically the biosynthetic pathways of flavonoids. In a number of plant species, mutants affecting flavonoid synthesis have been isolated. Maize (Zea mays), snapdragon (Antirrhinum majus), and petunia (Petunia hybrida) were established as the first major experimental models in this system, leading to the isolation of many structural and regulatory flavonoid genes (Mol et al. 1998). More recently, Arabidopsis (Arabidopsis thaliana) has facilitated the analysis of the regulation and subcellular localization of the flavonoid pathway. An interesting aspect of using Arabidopsis to study flavonoid biosynthesis is that, with the exception of flavonol synthase (FLS), which is encoded by six genes, only two (FLS1 and FLS3) have demonstrated activity (Preuss et al. 2009). The genetic loci for both structural and regulatory genes were identified largely on the basis of mutations that abolish or reduce the pigmentation of the seed coat; the loci were therefore called transparent testa or tt mutants (Borevitz et al. 2000). Consequently, most structural genes have been correlated with specific mutant loci in Arabidopsis, as well as a number of regulatory genes. This species does not appear to be using flavonoids in the same way as some other species (e.g. in defense or male fertility), but these mutants help to define the roles of these compounds in essential processes such as UV protection and auxin transport regulation (Lewis et al. 2011). The phenylpropanoid pathway synthesizes flavonoids, which transforms phenylalanine into 4-coumaroyl-CoA, which finally enters the flavonoid biosynthesis pathway. Chalcone synthase, the first specific enzyme for the flavonoid pathway, produces chalcone scaffolds from which all flavonoids come. Although the central pathway for flavonoid biosynthesis is preserved in plants, the basic flavonoid skeleton is modified by a group of enzymes, such as isomerases, reductases, hydroxylases and several Fe2 + 2-oxoglutarate-dependent dioxygenases, leading to different subclasses of flavonoids (Martens et al. 2010). Finally, transferases modify the backbone of the flavonoid with sugars, methyl groups and/or acyl moieties, modulating the physiological activity of the resulting flavonoid by changing its solubility, reactivity and interaction with cellular targets (Ferrer et al. 2008). There is evidence that phenylpropanoid and flavonoid biosynthesis enzymes are organized into macromolecular complexes that can be associated with endomembranes (Kutchan 2005). Metabolic channeling in the secondary metabolism of plants allows plants to synthesize specific natural products effectively and thus avoid metabolic interference. The existence of metabolones associated with cytochrome P450 monooxygenases (P450s) has been shown: Direct and indirect experimental data describe P450 enzymes in phenylpropanoid, flavonoid, cyanogenic glucoside and other biosynthetic pathways (Ralston and Yu 2006). The transgenic tobacco plants expressing epitope-tagged versions of two isoforms of phenylalanine ammonia lyase (PAL1 and PAL2) and cinnamate-4-hydroxylase (Achnine et al. 2004) provided additional evidence for the channeling of intermediates between specific isoforms of phenylalanine ammonia lyase and cinnamate-4-hydroxylase. In addition, yeast-two hybrid experiments have proposed the existence of a multi-enzyme complex for the anthocyanin pathway in rice (Shih et al. 2008). Most of the flavonoid synthesizing enzymes are recovered in soluble

cell fractions; immunolocalization experiments suggest that they are loosely bound to the endoplasmic reticulum (ER), possibly in a multi-enzyme complex, whereas the pigments themselves accumulate in the vacuole (i.e., anthocyanins and proanthocyanidins) or the cell wall (i.e., phlobaphenes Winkel-Shirley 2006). Flavonol synthase1 has recently been localized in Arabidopsis nuclei (Kuhn et al. 2011), as well as chalcone synthase and chalcone isomerase (Saslowsky et al. 2005). Interestingly, Antirrhinum majus aureusidin synthase, the enzyme that catalyzes aurone biosynthesis from chalcones, was localized in the vacuole, while the chalcone 4′-O-glucosyltransferase is localized in the cytoplasm, indicating that chalcones 4-O-glucosides are transported to the vacuole and therein converted to aurone 6-O-glucosides (Ono et al. 2006). Moreover, a flavonoid-3′-hydroxylase has been recently localized in the tonoplast in the hilum region of the soybean immature seed coat (Toda et al. 2012). Two models have been proposed for the mechanism of anthocyanin transport from the ER to the vacuole storage sites: the ligandin transport and the vesicular transport (Zhao and Dixon 2010). The ligandin transport model is based on genetic evidence showing that glutathione transferase (GST)-like proteins are required for vacuolar sequestration of pigments in maize, petunia and Arabidopsis (AtTT19) (Alfenito et al. 1998). The vacuolar sequestration of anthocyanins in maize requires a multidrug resistance associated protein-type (MRP) transporter on the tonoplast membrane, which expression is co-regulated with the structural anthocyanin genes (Goodman et al. 2004). MRP proteins are often referred to as pumps for glutathione S-X (GS-X), as they carry a variety of glutathione conjugates. Since anthocyanin-glutathione conjugate(s) were not found, however, it is proposed that these GSTs could deliver their flavonoid substrates directly to the transporter, acting as a carrier protein or ligandin (Koes et al. 2005). This hypothesis is supported by the fact that Arabidopsis' GST (TT19), localized both in the cytoplasm and the tonoplast, can bind to glycosylated anthocyanins and aglycones but does not conjugate these compounds with glutathione (Sun et al. 2012). The vesicle-mediated transport model proposed is based on observations that anthocyanins and other flavonoids accumulate in the cytoplasm in discrete vesicle-like structures (anthocyanoplasts), and then they might be imported into the vacuole by an autophagic mechanism (Pourcel et al. 2010). Nevertheless, grape vesicle-mediated transport of anthocyanins involves a GST and two multidrugs and toxic compound extrusion-type transporters (anthoMATEs). Thus, these observations point out to the coexistence of both mechanisms of transports, in which the participation of GSTs and transporters would be specific to cell and/or flavonoid-type (Gomez et al. 2011).

8.2.3 Role of Flavonoids

A variety of derivatives of the initial phenylpropanoid scaffold serve vital roles in plant structural integrity, UV photoprotection, reproduction, internal regulation of plant cell physiology and signaling. Phenylpropanoids also act as key chemical modulators of plant communication with insects and microbes, either

as attractants or repellants, as phytoalexins against pathogens and herbivores, and as attractants to pollinators via flower color. They also induce root nodulation when excreted by symbiotic nitrogen-fixing rhizobia (Mandal et al. 2010). The biological functions of flavonoids are linked to their potential cytotoxicity and their capacity to interact with enzymes through protein complexation. Some flavonoids provide stress protection, for example, acting as scavengers of free radicals such as reactive oxygen species (ROS), as well as chelating metals that generate ROS via the Fenton reaction (Williams et al. 2004). Flavonoids are also involved in the resistance to aluminum toxicity in maize. Roots of maize plants that were exposed to aluminum exuded high levels of phenolic compounds such as catechin and quercetin; indicating that their ability of chelating metals can be an in vivo mechanism to ameliorate aluminum toxicity (Kidd et al. 2001). Evidence connects flavonoids to the control of auxin polar transport. This hormone is likely to play a role in responding to stress by controlling stomatal opening and allocating resources in poor growth conditions (Lewis et al. 2011). Flavonoids, such as quercetin, kaempferol, apigenin and other aglycone molecules synthesized in the first steps of the pathway of flavonoid biosynthesis, inhibit the transport of polar auxins and thus increase the accumulation of localized auxins in planta (Kuhn et al. 2011).

8.2.3.1 Roles of Flavonoids in Legume-Rhizobial Interactions During Nodulation

The nodulation process involves flavonoids. Nodules could be initiated by flavonoid-deficient roots of transgenic plants produced by chalcone synthase RNA interference (Wasson et al. 2006). The complementation of the nodulation and flavonoid deficiency in roots by exogenous naringenin and liquiritigenin, precursors converted to the usual end product in Medicago truncatula and soybean, indicated that the lack of flavonoids was the reason for nodulation deficiency. In addition, these flavonoid-deficient roots had also increased auxin transport and lacked local inhibition of auxin transport at the site of nodulation (Wasson et al. 2006). However, an isoflavone-hypersensitive Rhizobium strain that requires very low levels of isoflavones to induce the nod genes is able to normally nodulate isoflavone-deficient roots, indicating that nodule primordia can form even in the absence of auxin transport inhibition by isoflavones in soybean roots. In conclusion, isoflavone-mediated auxin transport inhibition is not always essential for soybean nodulation (Subramanian et al. 2006). By silencing different M. truncatulaflavonoid-biosynthesis enzymes, (isoflavone synthase, chalcone reductase, flavone synthase, and chalcone synthase), it was demonstrated that flavones and flavonols may act as internal inducers of rhizobial nodgenes and and auxin transport regulators during nodulation by Sinorhibium meliloti, respectively, (Zhang et al. 2009). In contrast to the increased auxin transport in isoflavone-null roots of soybean, isoflavone-null roots of *M. truncatula* showed unaltered auxin transport indicating that legumes use different flavonoid compounds to regulate auxin transport during nodulation (Zhang et al. 2009).

8.2.3.2 Flavonoids in Plant Defense

Responses Against UV-B Radiation

Flavonoids' UV-absorbing characteristics have long been regarded as evidence of the role of flavonoids in UV protection. Studies in a wide range of species, such as Ligustrum vulgare, Vitis vinifera, petunia and Arabidopsis, have shown new evidence that UV light induces flavonol compounds synthesis (Kusano et al. 2011). Because the presence of the OH group in the 3-position of the flavonoid skeleton is the main structural feature responsible in chelating metal ions such as iron, copper, zinc, aluminum, and hence, inhibiting the formation of free radicals as well as to reduce ROS once formed, it was suggested that flavonols might play yet uncharacterized roles in the UV stress response (Verdan et al. 2011). In addition, grass species such as *Deschampsia antarctica*, *Deschampsia borealis* and *Calamagrostis epigeios*, which grow in areas with high levels of solar UV-B radiation, have high levels of flavonoids such as orientin flavones and luteolin, which protect plants against this stress condition. Similarly, maize plants growing at high altitudes accumulate C-glycosyl flavones in leaves, maysin and its biosynthetic precursor rhamnosylisoorientin, flavones commonly found in silks, as a mechanism that prevents damage caused by high UV-B exposure (Casati and Walbot 2005). FLS genes are regulated by UV-B radiation in both high-altitude landraces and low-altitudes inbreds of maize. Higher transcript levels are present in high-altitude plants where there are high levels of UV-B radiation than at low-altitudes. Consequently, considering the protective role of flavonols to UV-B radiation, we hypothesize that the high transcript levels of ZmFLSgenes may also contribute to the adaptation to this stress condition with higher UV-B fluxes (Falcone Ferreyra et al. 2012).

Responses Against Infection

Flavonoids protect plants against pathogen and herbivores. According to the phytochemical co-evolution theory, the secondary metabolites are likely the most important mediators of plant-insect interactions. Thus, both plants and insect herbivores have evolved leading to the plant defense (i.e., plant secondary metabolites) and herbivore offense (detoxification ability) (Cornell and Hawkins 2003). Human-induced changes in abiotic environmental factors such as atmospheric CO2 and ozone (O_3) levels, UV light, changes in precipitation patterns or temperature may directly affect the concentration of secondary chemicals in plants, which in turn may influence levels of herbivory or pathogen. For example, UV-B radiation modifies the production of secondary metabolites with photoprotective qualities (Bassman 2004), such as anthocyanins, isoflavonoids, and flavonol glycosides. The induction of UV-absorbing chemicals is shared with plant responses to other stresses, such as herbivore or pathogen attack, and this induction may act either positively or negatively on the levels of phytochemical production. Genes associated with the response of Nicotiana longiflora plants to insect herbivory were also induced by UV-B radiation (Izaguirre et al. 2003). Conversely, there is evidence that the induction of the flavonoid biosynthesis pathway by UV light can be inhibited by pathogen-induced defense responses in parsley (*Petroselinum crispum*) (Logemann and Hahlbrock 2002).

In addition, UV light levels and herbivory are regulated by genes regulating the phenylpropanoid pathway leading to the synthesis of phenolic compounds such as flavonoids (Stratmann 2003). Arabidopsis plants therefore respond to the combination of biotic (bacterial elicitor flg22) and abiotic stress (UV-B radiation) by synthesizing defense-related compounds such as phytoalexins and lignin as structural barriers to restrict the spread of pathogen, and modify the expression of genes involved in the production of protective metabolites such as flavonols. This crosstalk involves antagonistic regulation of two MYB transcription factors, MYB12 and MYB4 positive and negative regulators (Schenke et al. 2011).

Genes expressed in incompatible and compatible plant-microbe interactions were identified by using a large-scale transcript profiling analysis of soybean and M. truncatula. There was a sharp and rapid up-regulation of genes encoding enzymes involved in the phenylpropanoid pathway, in particular for the synthesis of isoflavones and isoflavanones. The responses of soybean to avirulent and virulent strains of the bacterial pathogen *P. syringae* pv. glycinea, differing in the presence or absence of avrB, were investigated using a cDNA array (Zou et al. 2005). Decreased levels of transcripts specific to the anthocyanin branch of the flavonoid pathway were observed. Genes involved in the biosynthesis of flavone and isoflavone were the largest group of up-regulated genes. The opposite regulation of these branches is therefore suggested to increase the production of isoflavones that act as antioxidants and antimicrobial compounds compared to those responsible for color. The response to the fungal pathogen Fusarium solani f of susceptible soybean "Essex" and a partially resistant recombinant inbred line (RIL23). We spoke. The causative agent of Sudden Death Syndrome has been studied with glycines (Iqbal et al. 2005). In RIL23, several genes encoding enzymes in the phenylpropanoid pathway were observed, suggesting that the products of this pathway participate in the resistance to the syndrome of Sudden Death. After challenging susceptible soybean with the pathogen Phytophthora sojae, the transcription of phenylpropanoid metabolism genes was up regulated (Mo et al. 1992). Isoflavones are also important in R-gene-mediated resistance to *P. sojae*; RNAi down-regulated isoflavone synthase genes in soybean roots resulted in a 95% reduction in isoflavone accumulation and an enhanced susceptibility to the pathogen (Subramanian et al. 2005). Sustained up-regulation of genes involved in the metabolism of phenylpropanoids was associated with responses to R-gene resistance in *M. truncatula* with response to foliar pathogens, expression profile of two M's response. Genotypes of truncatula (one susceptible and one resistant) to fungal pathogen Colletotrichum trifolii have shown that both genotypes respond to infection by regulating genes encoding phenylpropanoid pathway enzymes (Torregrosa et al. 2004). Similarly, M's response has reported up-regulation of genes involved in the phenylpropanoid pathway, especially those leading to isoflavone and isoflavonoid compounds was reported in the response of M. truncatula to abiotrophic pathogen, *Erysiphe pisi*, the causal agent of powdery mildew (Foster-Hartnett et al. 2007).

Corn earworm, Helicoverpa zea, is a major pest of maize (Ortega et al. 1980). Thus, interest in achieving maize with resistance to corn earworm has increased. One type of natural resistance is associated with the presence in silks of a C-glycosyl

flavone: maysin, as well as related compounds: apimaysin and methoxymaysin (Snook et al. 1994). These compounds are insecticidal to H. zea larvae and are thought to interfere with the amino acid metabolism in the insect gut through their subsequent conversion to more toxic quinones. Quinones reduce the availability of free amino acids and proteins by binding to –SH and –NH2 groups (Byrne et al. 1997). Using flavone synthesis as a model trait locus (QTL) system, it was shown that in a population segregating for functional and nonfunctional p1 alleles, the p1 locus is the gene underlying the major QTL for maysin concentration and activity against the earworm (Byrne et al. 1997). Transgenic maize over-expressing the p1 gene had increased silk maysin level (Johnson et al. 2007). The transgenic plants were more resistant to earworm larvae, increasing insect mortality levels and decreasing mean weights of surviving larvae. Larval weight was significantly negatively correlated with maysin level in transgenic silks.

8.2.3.3 Flavonoids in Pollen: Roles in Plant Reproduction and Fertility

The unique structure and combination of different flavonoids in each species produce yellow pollen with a range of visible and UV reflection spectra that can be detected by the targeted insects and larger animals, facilitating successful pollination. The flavonoids impart a distinctive yellow color to pollen and can be 2–4% of the dry weight (Van Der Meer et al. 1992). The existence of "white pollen" has been reported in species as diverse as bristle cone pine and morning glory. The correlation between pollen fertility and flavonoids was first established in wind pollinated maize, with its numerous and well-characterized anthocyanin mutants (Mo et al. 1992). In maize and petunia, flavonoid-deficient mutants without chalcone synthase were produced to clarify the roles of flavonoids in pollen (Pollak et al. 1993). Not only were these mutants deficient in flavonoids, but they were also male sterile due to a failure to produce a functional pollen tube reversed by adding flavonol kaempferol at pollination (Mo et al. 1992). These mutant plants are conditionally male fertile, as flavonoid-deficient pollen does not function in self-crosses but it is partially functional on wild-type stigmas containing flavonols (Mo et al. 1992).

The silencing of chalcone synthase gene results in parthenocarpy in tomato, but it was not identified if the cause of this phenomenon was the lack of flavonan-3-ols and/or flavonols (Schijlen et al. 2007). The silencing of a FLS in tobacco causes production of less-seeded fruits, and silenced lines had lower flavonol and anthocyanidins levels, while the flavan-3-ol content is increased. In addition, the pollen of these silenced lines was unable to produce functional pollen tubes. This capacity can be reversed with quercetin (in vivo and in vitro); implying that flavonols (in particular quercetin) have essential roles in pollen germination and consequently in plant fertility (Mahajan et al. 2011). Arabidopsis mutant plants in the chalcone synthase gene, which are completely free of flavonols in flowers and stamens, are fertile and have no aberrations in pollen tube growth, indicating that flavonols are not universally necessary for the fertility of pollen (Burbulis et al. 1996). Polyketide synthases of Arabidopsis (LAP5 and LAP6) are required for the development of pollen and biosynthesis of sporopollenin (Kim et al. 2010). Single and double

mutants in LAP5/6 have reduced the development of anthers to undetectable levels of various flavonoids and lack of exine deposition that causes male sterility. Pollen grain cell walls defective in lap mutants may therefore be deficient in the deposition of flavonoid – containing extracellular pollen coat triphine. The reduced levels of flavonoids can therefore be an indirect result of reduced deposition of triphine containing flavonoids.

8.2.4 Terpenes and Terpenoids

The structural diversity associated with at least 40,000 compounds is one of the most impressive examples of the different evolution of plant chemicals in the terpenoid class. This compound class's evolutionary success is partly based on the simplicity of constructing various size molecules. All terpenoids are derived from the universal five – carbon building blocks, isopentenyl diphosphate (IPP) and its allylic isomer dimethylallyl diphosphate (DMAPP), according to the isoprene rule recognized by Wallach and Rutzicka at the end of the nineteenth and mid – twentieth century's (Kubeczka 2010). The intermediates of prenyl diphosphate built by condensation of these five-carbon units are used as precursors for the biosynthesis of terpenoids with fundamental growth and development functions and for the formation of a large number of terpenoid compounds with more specialized roles in the interaction of plants with their environment. The latter group of terpenoids is characterized by its enormous structural diversity as a result of the different evolution of the biosynthetic gene. Specialized terpenoids have a long history of use as flavors, fragrances, pharmaceutical products, insecticides and industrial compounds, many of which are covered in this book. In view of the growing need for sustainable platforms for the production of plant – based drugs and the emerging use of terpenoids in the production of alternative fuels, significant progress has been made in the design of biosynthetic pathways for terpenoids in microbes and plants (Zhang et al. 2011). Advanced approaches to functional genomics provide unlimited access to the biosynthetic genes and molecular regulators of plants producing terpenoids, while providing a deeper insight into the complexity of the metabolism and regulation of terpenoids. I provide an overview of the organization of the early and core metabolic pathways of terpenoids in this chapter and update the regulation and functional diversification of their genes and enzymes. In addition, before addressing the various roles of terpenoids in plant-environment interaction, I summarize the function of terpene synthases and describe aspects of their coordinated and tissue-specific regulation in specialized metabolism prior to addressing the diverse roles of terpenoids in plant–environment interactions.

8.2.4.1 Core Terpenoid Biosynthetic Pathways and their Regulation

The successful engineering of terpenoid products in plants is critically dependent on the flux of precursors supplied by the biosynthetic pathways of the core isoprenoid and therefore on the dynamic regulation of these biosynthetic pathways. The successful engineering of terpenoid products in plants is critically dependent on the

flux of precursors supplied by the biosynthetic pathways of the core isoprenoid and therefore on the dynamic regulation of these biosynthetic pathways. Plants use two independent pathways to produce IPP and DMAPP: Mainly cytosolic mevalonic acid (MVA) and plastidial methyl phosphate methylerythritol (MEP). The MVA pathway provides predominantly precursors for the cytosynthesis of sesquiterpenoids, polyprenols, phytosterols, brassinosteroids and triterpenoids, as well as for the biosynthesis of terpenoids in mitochondria (e.g., ubiquinones, polyprenols) and derived five-carbon units derived from the MEP pathway are preferably used for the biosynthesis of monoterpenoids, diterpenoids, carotenoids, hemiterpenoids (e.g., isoprene), and their breakdown products, chlorophyll, cytokinins, tocopherols, gibberellins,and plastoquinones. Both pathways are clearly heavily regulated at multiple levels, as discussed in two recent reviews by Hemmerlin and coworkers (Hemmerlin et al. 2012; Hemmerlin 2013). In addition to the transcriptional regulation of MVA and MEP pathway genes and their various paralogues, isoprenoid-pathway fluxes are controlled at post-transcriptional / translational levels and by feedback regulation. Recent studies have provided a more global view of the dynamics and networks of core isoprenoid pathways and the regulation of metabolic flux during the development of plants and in response to external stimuli (Vranova et al. 2013).

8.2.4.2 MVA and MEP Pathways: A Brief Summary of their Biosynthetic Steps

The MVA pathway in plants consists of six steps and begins with the condensation of two acetyl-CoA molecules to acetoacetyl-CoA (AcAc-CoA), which is catalyzed by acetoacetyl-CoA thiolase (AACT). AcAc-CoA is combined with a third molecule of acetyl-CoA in the subsequent aldol condensation reaction catalyzed by HMG-CoA synthase (HMGS) to form the C6-compound S-3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). A key function of HMGS in the MVA pathway is the response to various stresses, feedback regulation and the role of HMGS in sterol metabolism (see below). HMG-CoA reductase (HMGR) catalyzes the conversion of S-HMG-CoA to R-mevalonate in two steps of reduction dependent on NADPH in the following rate-limiting step. All HMGR proteins of the plant are membrane-bound with two sequences covering the membrane and a highly conserved catalytic C-terminal domain. The presence of ER-specific retention motifs indicates that the membrane-spanning domain is primarily associated with the ER, while the ends of the N-terminal and C-terminal are located on the cytosolic side (Vollack et al. 1994). The association of HMGR with membranes appears to negatively regulate its activity, thus limiting the accumulation of end products of terpenoids such as sterols (Holmberg et al. 2003). The critical role of HMGR in the biosynthesis of phytosterols, triterpenoids and sesquiterpenoid phytoalexins has been reported in many studies, although flux control often involves additional downstream enzymes such as sesquiterpene synthases (Suzuki et al. 2004; Ohyama et al. 2007). The MVA produced by HMGR is finally converted into IPP by three enzymatic steps: Two phosphorylation steps dependent on ATP, catalyzed by mevalonate kinase (MK) and phosphomevalonate kinase (PMK), and a decarboxylative

elimination catalyzed by mevalonate diphosphate decarboxylase (MVD or MPDC) driven by ATP. The MEP pathway which occurs in all photosynthetic eukaryotes and cyanobacteria, apicomplexan protozoa and most eubacteria (Lichtenthaler 1998; Lange et al. 2000), consists of seven enzymatic steps. 1-deoxy-d-xylulose5-phosphate (DXP) is formed by DXP synthase (DXS) from thiamine diphosphate (hydroxyethyl), which is derived from pyruvate, and glyceraldehyde-3-phosphate (GAP) in a condensation similar to transketolase. Plant DXS enzymes have a highly conserved binding domain of thiamine phosphate and are divided into enzymes of type Class I with primary expression in photosynthetic and floral tissues and enzymes of type Class II with more distinct roles in specialized metabolism (see below). Numerous studies have shown that DXS functions in the biosynthesis of plastidial terpenes as an important regulatory and rate-limiting enzyme (Estevez et al. 2001). Therefore, albino phenotypes are present in DXSmutants such as those from the single functional Arabidopsis class-I gene DXS (DXS1) (Estevez et al. 2000).

The enzyme1-deoxy-d-xylulose 5-phosphate reductoisomerase (DXR) catalyzes the second stage of the MEP pathway, in which DXP is converted into 2-C-methyl-d-erythritol4-phosphate (MEP) by an intramolecular rearrangement of DXP into 2-C-methyl-d-erythrose4-phosphate, followed by a reduction dependent on NADPH (Carretero-Paulet et al. 2002). The reaction can be specifically inhibited by fosmidomycin, an analog structure of the DXR substratum (Steinbacher et al. 2003), which blocks the biosynthesis of biosynthesis of downstream plastidial terpene (Huang et al. 2010). In some cases, the DXR catalyzed reaction is considered a rate-limiting step depending on the species, tissue and stage of development. In Arabidopsis, DXR1 is expressed in various plant organs (Carretero-Paulet et al. 2002) and dxr mutants show an albino phenotype and deficiencies in the biosynthesis of gibberellin and abscisic acid (ABA) similar to DXS1 (Xing et al. 2010).

MEP is further converted by the enzyme 4-diphosphocytidyl-2-C-methyl-d-erythritol (MCT or IpsD) in a CTP-dependent reaction (MCT or IpsD) (Rohdich et al. 2000a). The phosphorylation of CDP-ME by the enzyme 4-diphosphocytidyl-2-C-methyl-d-erythritol kinase (CMK, IspE) leads to the development of 4-diphosphocytidyl-2-C-methyl-d-erythritol2-phosphate (CDP-ME2P) (Rohdich et al. 2000b), which is subsequently cyclicated by 2-C-methyl-d-erythritol 2,4-cyclodiphosphate synthase (MDS, IspF) into 2-C-methyl-d-erythritol 2,4-cyclodiphosphate (MEcPP) upon loss of CMP. In the last two steps of the MEP pathway, the enzyme 4-hydroxy-3-methylbut-2-enyl diphosphate synthase (HDS, IspG) first converts MEcPP in a two-electron reduction to 4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP). In a final branching step, HMBPP is converted by 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR, IspH) to a mixture of IPP and DMAPP with a ratio of 5 to 6:1 (Tritsch et al. 2010).

Mutants of MCT, MDS and CMK have similar albino phenotypes and photosynthetic gene downregulation (Hsieh et al. 2008). Hds and hdr-1 mutants also have defects in the development of chloroplast (Hsieh and Goodman 2005). It is interesting that a partial loss of function mutant of Arabidopsis HDS, hds-3 (csb3), has been

shown to be more resistant to biotrophic pathogens, which suggest a link between the MEP pathway and the response to plant defense (Gil et al. 2005).

8.2.4.3 Differential Expression of MVA and MEP Pathway Isozymes

Several enzymes of the MVA and MEP pathways, in particular those with important regulatory roles, are encoded by small gene families that enable functional redundancy and divergence (Hemmerlin 2013). Paralogues for AACT, HMGS, HMGR and MPDC have been identified in the MVA pathway, while the MEP pathway enzymes DXS, DXR, MCT, CMK, MDS or HDR have been found to be encoded by two or more isogenes (Hemmerlin et al. 2012). The different roles of many isozymes of the MVA and MEP pathway depend on their expression in specific cell tissues and are often divided into essential functions for terpenoid precursors in primary metabolism, growth and development and more specific functions. In Brassica juncea, for example, a family of four genes represents HMGS. Two genes are highly expressed at the early stages of floral development and play a role in reproduction, as shown in Arabidopsis for the single HMGS gene (Ishiguro et al. 2010), while the expression of the two other paralogues is limited to leaves. In particular, HMGR paralogues exhibit different patterns of developmental and tissue specific expression and can be distinguished by their response to endogenous molecules such as phytohormones and sterol metabolites, as well as external stimuli including light, wounding, elicitor treatment, and pest and pathogen attack (Hemmerlin 2013). The differential expression of HMGR isozymes, as demonstrated by early studies of HMGR gene families in Solanaceous plants (tomato, potato) (Choi et al. 1992), is important for channeling and counterbalancing the flow of carbon into the various downstream pathways of response or development to stress. However, this functional differentiation does not appear to occur in all plants, since both HMGR genes in Arabidopsis do not respond to stress, but are essential for the production of cell elongation sterols, senescence, development of gametophytes and fertility (Suzuki et al. 2009).

However, this functional difference does not appear to occur in all plants, since both HMGR genes in Arabidopsis do not respond to stress, but are essential for the production of sterols for cell elongation, senescence, development of gametophytes and fertility (Suzuki et al. 2009). In the DXS gene family, functional divergence of MEP pathway genes was primarily observed. DXS genes of type II respond to biotic interactions and are induced by mycorrhizal colonization in legumes and other plant families in the biosynthesis of apocarotenoids (Paetzold et al. 2010). Several studies have also shown that genes of type II DXS are induced in response to pathogen and herbivore attacks in conjunction with the production of specialized metabolites (Hemmerlin 2013).

8.2.4.4 Metabolic Regulation and Networks

The role of pathway intermediates and downstream metabolites in the regulation of the core biosynthetic steps of terpenoids at transcriptional and post-translational levels is clear. For AACT and HMGR and for HMGS enzyme products, inhibition of free CoA feedback has been demonstrated (Soto et al. 2011). In addition, plant

MKs responds to inhibition of feedback from prenyl diphosphates, IPP, DMAPP, geranyl diphosphate (GPP) and farnesyl diphosphate (FPP), which modulate the activity of enzymes by acting as competitive ATP inhibitors (Schulte et al. 2000). Similarly, IPP and DMAPP found in vitro inhibition of feedback on a DXS protein from poplar and a structural analysis suggested possible binding of the prenyl diphosphates to the enzyme in competition with its thiamine pyrophosphate substrate (Banerjee et al. 2013). This feedback inhibition has also been supported in vivo by recent metabolic flux studies in poplar (Ghirardo et al. 2014).

The complexity of the regulatory network is also evident when metabolic disturbances and changes in metabolic flux resulting from over expression or reduced expression of genes in the core isoprenoid pathways promote feedback or feed-forward signals that change the expression of upstream or downstream genes. For example, overexpression of B. juncea wild-type and mutated HMGS1 in Arabidopsis caused an upregulation of HMGR and genes in sterol biosynthesis such as sterol methyltransferase 2, delta-24 sterol reductase, and C-22 sterol desaturase, which led to an elevated sterol content in leaves and seedlings and increased stress tolerance (Gil et al. 2005). Similar responses to over-expression of HMGS in tobacco have been observed, resulting in improved sterol content, growth, pod size and seed yield (Liao et al. 2014). In contrast, the collapse of AACT2 expression resulted in lower levels and altered sterol profiles and reduced expression of downstream genes encoding FPP synthases and sterol methyltransferase (Jin et al. 2012). HMGR activity also shows a positive response to downstream metabolic changes, such as reduced levels of cycloartenol in transgenic tobacco expressing sterol methyltransferase type 1 (SMT1) and depletion of endogenous sterols due to squalene synthase inhibition (Holmberg et al. 2002). In mutants of the MEP pathway, the simultaneous response of several genes to pathway disturbances is further observed. For example, silencing CMK in Arabidopsis causes MCT, MDS and HDS expression to be increased (Ahn and Pai 2008). In addition, pathway genes of MEPs in rice have been found to be co-expressed with downstream genes in carotenoid and phytyl biosynthesis (Jung et al. 2008). In accordance with these observations, detailed analysis of the transcriptional co-expression network in Arabidopsis showed that gene modules in both MVA and MEP pathways are co-regulated with genes in downstream pathways, and these findings set the stage for the identification of regulatory elements in these gene modules (Wille et al. 2004). Consequently, cis elements were mapped showing that the promoters of the Arabidopsis genes DXS, DXR, CMK, HDR, and phytoene synthase share a cis-regulatory element interacting with RAP2.2, a member of the ethylene response factor B-2 subfamily (Vranova et al. 2013). MEPs and MVA pathways respond to sugar metabolism regulators in conjunction with their light regulation (see below). Arabidopsis mutants of pleiotropic regulatory locus 1 (PRL1), a global regulator for the response of sugar, stress and hormones, accumulate end products derived from the pathway of the European Parliament (Flores-Perez et al. 2010). Due to post-translation modification, the same mutants have reduced HMGR activity, but no change in HMGR transcript or protein. PRL1 inhibits the protein kinase 1 (SnRK1) associated with SNF1 (sucrose nonfermenting), which regulates HMGR1 negatively by phosphorylation and

catalytic inactivation (Dale et al. 1995). During normal development and in response to salt stress, HMGR1 is also negatively regulated by protein phosphatase 2A (PP2A), which dephosphorylates the HMGR protein (most likely at a site other than SnRK1 phosphorylation (Leivar et al. 2011). The modulation of HMGR transcripts at the start of translation (Yoshioka et al. 1996) and glycosylation of HMGR isoforms (Denbow et al. 1995) have previously been discussed as other mechanisms for post-transcription or post-translation regulation of HMGR genes caused by stress. The isoprenoid pathway can be linked to other metabolic pathways by delivery and competition for carbon precursors (e.g. amino acid degradation) (Hemmerlin 2013), which will require further attention in order to gain a more comprehensive understanding of the flow of terpenoid biosynthesis. Nieto et al. (2009) described a link between isoprenoid metabolism and lipid biosynthesis, which found that the inhibition of sphingolipid biosynthesis in Arabidopsis resulted in a post-translational reduction in HMGR activity decoupling with HMGR transcription and protein levels and a reduction in sterol content. Recently, in trichomes of tomato mutants of the flavonoid biosynthetic enzyme chalcone isomerase (CHI) (Kang et al. 2014a, b), an unexpected simultaneous downregulation of flavonoid and terpenoid metabolite levels has been observed and has led to several hypotheses about the regulatory links between pathways. Changes in flavonoid levels due to accumulation (upstream of CHI) or depletion (downstream of CHI) may alter the expression of terpenoid biosynthetic genes or directly inhibit biosynthetic and regulatory proteins (Pourcel et al. 2013). On the basis of previous findings, CHI itself may also interact with proteins involved in the production of terpenoids or their regulation (89). In addition, regulatory factors that coordinate metabolic flux through both pathways will be examined (Ben Zvi et al. 2012).

8.2.4.5 Regulation by Light and External Stimuli

Recent efforts to identify gene expression patterns of MVA and MEP pathways through transcriptome and hierarchical cluster analyze have shown that the genes of both pathways have opposite patterns of expression in light or dark (Vranova et al. 2013). While exposure to light leads to the reduction of MVA pathway genes and reduced levels of sterols (Ghassemian et al. 2006), it stimulates the accumulation of MEP pathway genes and genes in the biosynthetic pathways of carotenoids and chlorophylls such as PSY (phytoene synthase) and HEMA1 (glutamyl-tRNA reductase), which are essential for the differentiation of chloroplast (Cordoba et al. 2009). Light also regulates biosynthetic genes like VTE3 (vitamin E defective3) tocopherol and plastoquinone (Ghassemian et al. 2006). The results are supported by studies that observed an increased flow of carbon through the MEP pathway in enhanced light conditions by measuring the accumulation of MEcDP when the activity of 2-C-methyl-d-erythritol 2,4-cyclodiphosphate reductase was inhibited (Mongelard et al. 2011). The expression of MEP pathway genes with the exception of HDR (Hsieh and Goodman 2005) is reduced during the light-dark transition (6) in contrast to the upregulation by light Dark exposure can lead to HMGR activity, as demonstrated in ginseng, where HMGRs play a regulatory role in the biosynthesis of triterpene ginsenoside (Kim et al. 2014). Phytochrome B (PHYB) controls the

light-dependent response of Arabidopsis MEP and MVA pathway genes, since phyB mutants have increased levels of HMGR transcripts and enzyme activity, but reduced levels of pathway products for MEPs (Cordoba et al. 2009). The phytochrome interacting factors (PIFs) of the basic transcription factor family of helix-loop-helix (bHLH) have therefore been identified as regulators involved in the light control of MEP and carotenoid biosynthetic pathway genes (Mannen et al. 2014). The MEP pathway enzymes (DXS and DXR) were also found to be correlated with the activity of Clp, a major plastid stromal protease (Flores-Perez et al. 2008). Downregulation of MEP pathway enzymes in the dark provides a dilemma for the biosynthesis of carotenoids and gibberellins required for the development of etiolated seedlings. Supported by observations from treatments with the MEP pathway inhibitor fosmidomycin, Rodriguez-Concepcion and coworkers suggested that during seedling germination in the dark, prenyl diphosphates derived from the MVA pathway are transported into etioplasts for gibberellin and carotenoid synthesis prior to the induction of MEP pathway enzymes upon illumination (Rodriguez-Concepcion et al. 2004). In light and dark, the responses of MEP and MVA pathway genes are not surprising that the expression of several genes is under circadian control (Vranova et al. 2012). Co-expression analyzes in the photosynthetic tissue of Arabidopsis connect several MEP pathway genes with core circadian oscillators (LHY, CCA1, PRR9), while only AACT2 of the MVA pathway follows the expression of circadian regulators in the dark (Vranova et al. 2013). However, in roots, expression of several MVA pathway genes such as HMGR1 is correlated with that of circadian regulators (TOC1, TIC) showing clear differences in the circadian control of early pathway genes in above-and belowground tissues. Interestingly, in triple mutants of the TOC1 related pseudo-response regulator (PRR) proteins PRR9, PRR7, and PRR5, genes and metabolites of carotenoid, chlorophyll, and tocopherol pathways are upregulated, which suggests a function of these proteins as negative regulators of the MEP pathway-dependent metabolic routes (Fukushima et al. 2009). To what extent the oscillation of MVA and MEP pathway gene transcripts directly corresponds to changes in enzyme activity and downstream metabolites requires further attention. In snapdragon flowers, the rhythmic emission of volatile monoterpenes in plastids and sesquiterpenes in the cytosol depends on the MEP pathway that is controlled by the circadian clock (Dudareva et al. 2005).

MVA and MEP pathways respond to multiple other external stimuli at gene transcription and post-translation levels in addition to their variance response to light (Hemmerlin et al. 2012). Carbon flow through the MEP pathway increases at elevated temperatures in order to support the production of terpenoids; to protect against temperature stress (Mongelard et al. 2011). Not only HMGR, but also other enzymes such as AACT show induced responses to abiotic stress in the MVA pathway and appear to be involved in the adaptation of abiotic stress through the MVA pathway (Soto et al. 2011). Changes in the redox state also affect pathway enzymes of MVA and MEP directly.

For DXR (Balmer et al. 2003), both HDS and HDR, which function as iron-sulfur reductases, have been identified as targets for redox protein thioredoxin (Balmer et al. 2003). In addition, it has been shown that HDS can receive electrons

directly via the photosynthetic electron-transport chain via ferredoxin without any reduction cofactor, which is different from the reduction of HDS in bacteria dependent on flavodoxin/flavodoxin reductase and NADPH (Seemann et al. 2006). Biotic stress such as pathogen attack often upregulates individual genes of HMGR families to direct flow to the production of sesquiterpene phytoalexins under simultaneous downregulation of squalene synthase and sterol biosynthesis (Vogeli and Chappell 1988). Tobacco studies have shown that the regulation of the expression of HMGR by pathogen involves the cascade of kinase MEK2-SIPK/WIPK MAP (Jin et al. 2003). Another example illustrates the importance of HMGR in the development of root nodules. Medicago truncatula's HMGR1 protein interacts directly with NORK, a receptor-like kinase required to signal the Nod factor. Reduced HMGR1 expression in transgenic plants causes severe decrease of root nodulation (Kevei et al. 2007).

8.2.4.6 Regulation and Metabolite Exchange across Subcellular Compartments

The compartmentalization of MEP and MVA pathways and associated downstream pathways allows for the subcellular regulation and coordination of photosynthesis-dependent and independent terpenoid biosynthetic routes. Despite the general notion that the MVA pathway enzymes are located in the cytosol or associated with the ER, peroxisomes have been discussed as localization sites for AACT (particularly AACT1 in Arabidopsis), PMK, and MVD based on the prediction of peroxisomal PTS targeting peptides and transient protein peroxisome import studies in Catharanthus roseus cells (Guirimand et al. 2012). For MVD1 in Arabidopsis, however, mass spectrometry analysis suggests a cytosolic localization and MVD2 is predicted to reside in the cytosol (Vranova et al. 2012). Our current view of the subcellular organization of the MVA pathway remains incomplete in the absence of additional evidence for the partial location of the MVA pathway in peroxisomes and possible transporters of isoprenoid precursors between the compartments. The exchange of intermediates between cytosol and plastids is usually not sufficient to rescue biosynthetic enzyme mutants of Arabidopsis in MVA or MEP pathways (Suzuki et al. 2009). However, studies of dxs2 mutants in tomatoes have shown that both pathways can compensate for each other to some extent (Paetzold et al. 2010). In addition, some exchange of isoprenoid intermediates between plastids and cytosol has been demonstrated in many cases on the basis of the use of MEP and MVA pathway-specific inhibitors and the inclusion of stable isotope precursors in primary and specialized terpenoid metabolites (Hemmerlin et al. 2003a, b; Zhao et al. 2014). There is frequent evidence of isoprenoid intermediates trafficking in photosynthetic tissues from plastid to cytosol (e.g. Laule et al. 2003). However, in the absence of light, the contribution of the MVA pathway to the biosynthesis of plastidial isoprenoids can be significant, as Opitz et al. (2014) demonstrated in the roots of cotton seedlings or in dark-grown Arabidopsis seedlings (Rodriguez-Concepcion et al. 2004).

No specific isoprenoid precursor transporters have been identified in the plastid membrane to date. The export of IPP from plastids to cytosol was suggested by a

symport system of plastid protons (Bick and Lange 2003). Studies by Flügge and Gao (2005) showed that IPP is not transported by plastic translocators of phosphate, but depends on phosphorylated counter-substrates. In addition to the transport of IPP, there is evidence that longer prenyl diphosphates such as GPP and FPP are moved from plastids to cytosol in tomato (Gutensohn et al. 2013), grape berry exocarp (May et al. 2013), and stevia rebaudiana glandular trichomes (Woelwer-Rieck et al. 2014). Genomic and proteomic analyses of single cells such as trichomes could be a promising approach to identify the isoprenoid transporter machinery between both compartments.

Despite the exchange of isoprenoid intermediates between plastid and cytosol, the spatial separation of terpenoid biosynthetic pathways was beneficial for the development of terpenoid end products. The expression and targeting of FPP synthase and sesquiterpene synthase to tobacco plastids prevented the competition of carbon flux with cytosol sterol biosynthesis and promoted the yield of sesquiterpenoids by a thousand times (Wu et al. 2006). The same approach was successfully used to produce high levels of triterpene squalene in plastids and tobacco trichomes, although the latter occurred at the cost of significantly lower growth (Wu et al. 2012). Efforts have also been made to insert the entire MVA pathway into the genome of the tobacco chloroplast, which leads to higher levels of mevalonate and carotenoids, but also squalene and sterols (Kumar et al. 2012). At post-translation levels, other interdependent regulatory mechanisms have been detected between the pathways. Recent tobacco studies have shown that the blockage of MEP-dependent protein geranylgeranylation by treatment with monoterpene S-carvone suppresses signals to induce the formation of sesquiterpene phytoalexin capsidiol dependent on the MVA pathway have been addressed (Verbitskiy et al. 2010).

8.2.4.7 Isomerization and Condensation of the C5 Building Blocks

The construction of terpenoids with more than five carbons requires adequate IPP and its more reactive electrophilic isomer DMAPP. Therefore, the activity of an IPP isomerase must convert the IPP derived from the MVA pathway to DMAPP. Isoenzymes of type I IPP isomerase in plants were located in mitochondria and plastids and shorter isoforms were expected to remain in cytosol (Phillips et al. 2008). In analogy to mammalian cells, an alternative location of IPP isomerases in peroxisomes was discussed (Sapir-Mir et al. 2008), but additional evidence is needed for the role of peroxisomes in the metabolism of isoprenoid plants. Although the formation of DMAPP from IPP derived from the MVA pathway is essential for downstream reactions in cytosol and mitochondria, isomerization of IPP appears to be less important in plastids where the MEP pathway produces both C5 building blocks. However, the plastic activity of IPP isomerase may be necessary to produce an optimal ratio of IPP and DMAPP for downstream condensation reactions and to provide precursors for the possible cytosol transport. In the second major phase of terpenoid biosynthesis, the catalytic activity of prenyltransferases (isoprenyl diphosphate synthases) fuses the IPP and DMAPP units to form prenyl diphosphates as the linear central precursors of all terpenoids. The initial reaction catalyzed by prenyltransferase is a head-to-tail condensation of IPP with allylic cosubstrate DMAPP

based on an ionization-condensation-elimination mechanism for the production of C10-allylic diphosphate (1′-4). The formation of short-chain (C15-C25), medium-chain (C30-C35) and long-chain (C40-Cn) prenyl diphosphates results in additional rounds of head-to-tail condensation of the allylic product with more IPP units. The cis-or trans-stereochemistry of the double bonds of the prenyl diphosphate product determines whether the enzyme operates as a cis-prenyltransferase or trans-prenyltransferase, which belongs to families of structurally unrelated enzymes (Kharel and Koyama 2003). A great deal of knowledge has been gained on the biochemistry and development of short-chain trans-prenyltransferases, which synthesize C10-geranyl diphosphate (GPP), C15-trans, trans-farnesyl diphosphate (E, E)-FPP), or C20-all-trans-geranylgeranyl diphosphate (all-trans-GGPP) as the main precursors in terpenoid metabolism, although more recent work has found similar roles in previously undetected short-cut metabolism.

8.2.4.8 Geranyl Diphosphate Synthases

GPP is synthesized from IPP and DMAPP as a precursor to the biosynthesis of C10-monoterpenoids by the activity of GPP synthase enzymes (GPSs), which are usually targeted at plastids. In plants, various classes of homodimeric and heterodi/tetrameric GPS were identified (Rai et al. 2013). The first GPS to be found in plants (Burke et al. 1999) was a heterotetrameric GPS from peppermint, which has since been found in a variety of other species, such as Anthirrinum majus, Clarkia breweri, and Humulus lupulus (Wang and Dixon 2009). The enzymes consist of a large subunit (LSU), which has a significant homology (~50%) for GGPP synthases (GGPS, see below) and can exhibit GGPP synthase activity as a recombinant protein, and a small subunit (SSU I), which has a similarity of only ~20% to homomeric prenyltransferases and is functionally inactive. It is generally believed that SSU I binding changes the LSU's activity to produce GPP. Structural analysis of the heterotetrameric GPS from peppermint confirmed the importance of the physical interaction of both subunits to make GPP (Rai et al. 2013). Wang and Dixon (2009) identified a separate lineage of SSU genes (SSU II) encoding GGPS in Arabidopsis. Arabidopsis GGR modifies GGPS 11's in vitro activity to produce GPP and contains two preserved CxxxC motifs, which are essential for the interaction of both subunits (Wang and Dixon, 2009). In contrast to the role of GPSs containing SSU I in the formation of monoterpenes in peppermint or hops, the function of heterodimeric GPSs carrying SSU II subunits is less clear due to the lack of a tight correlation between the expression of proteins and the biosynthesis of monoterpenes in different tissues (Wang and Dixon 2009). GPS activity engineering was achieved by the expression of GPS.SSU I in tobacco and tomato fruits from snapdragon. The expressed subunit recruits plastic GGPS proteins to form heterodimeric GPS proteins (Orlova et al. 2009). The tomato study also revealed that plastid-produced GPP is exported to cytosol, where monoterpene biosynthesis can be used (Gutensohn et al. 2013). However, the exchange of GPP between the two compartments may be limited in the absence of engineered GPP pools, as shown for bifunctional Arabidopsismonoterpene/Sesquiterpene synthase (TPS02), which is located in the cytosol and produces sesquiterpenes, but no monoterpenes.

Homodimeric GPS enzymes from angiosperms and gymnosperms were described (Schmidt et al. 2010a, b). These proteins belong to different lines and are linked to GGPS evolutionarily (see below). There has been controversial discussion of the existence of a homodimeric GPS in Arabidopsis. In order to encode a functionally active GPS enzyme, a single GPS1 gene was originally identified (Bouvier et al. 2000), but more recently, the GPS1 protein was characterized as a multiproduct synthase of prenyl diphosphate medium/long chain. This latter activity was observed when IPP was supplied in excess of DMAPP, GPP and FPP ally substrates and was supported by an active site structural analysis of an active-site cavity with sufficient size to accommodate the medium−/long-chain products (Hsieh et al. 2011). The GPS1 protein (renamed by Hsieh et al. as polyprenyl di(pyro)phosphate synthase, PPS) is targeted to plastids (Bouvier et al. 2000) where IPP and DMAPP are produced at ratios of approximately 5:1 by the MEP pathway.

8.2.4.9 Farnesyl Diphosphate Synthases

Trans-FPP synthases (FPS) catalyze the formation of (E, E)-FPP as a central precursor in the biosynthesis of primary metabolites of terpene (phytosterols, brassinosteroids, dolichols, ubiquinones), protein prenylation, and in the production of specialized metabolites such as sesquiterpenoids and triterpenoids. As FPS type I (eukaryotic), the trans-FPS plant builds a superfamily of homodimeric enzymes, which are often encoded by small gene families (e.g., Hemmerlin et al. 2003a, b). FPS isozymes of different sizes produced as a result of differential gene transcription have been located in cytosol or mitochondria, where FPP pools are produced for the biosynthesis of cytosolic and mitochondrial products downstream (Cunillera et al. 1997). On the basis of YFP fusion experiments in catharantus roseus cells (Thabet et al. 2011), the targeting of FPSs for peroxisomes was discussed. In Arabidopsis, however, no peroxisomal targeting of fluorescent FPS fusion proteins has been demonstrated which is consistent with the results of cytosol and purified peroxisom proteomic studies (Ito et al. 2011). Like the isozymes of the MEP and MVA pathways, it was of primary interest to clarify the possible functional differences of isoforms of prenyltransferase. In Arabidopsis, the two FPSparalogues, FPS1 and FPS2, have overlapping patterns of expression and can rescue each other's loss, while double mutants are impaired in the transmission of male genes and arrested in the early development of embryos (Closa et al. 2010).

However, there is no complete functional redundancy between the two isozymes, since FPS2 is the predominantly expressed isozyme in mature seeds and the development of early seedlings, and FPS1 appears to be expressed only in the breast seed coat (Keim et al. 2012). Seeds of fps2 mutants therefore have reduced sterol content (Closa et al. 2010). Keim et al. suggests that the specific expression of FPS2 in mature seeds is linked to its higher enzyme activity and thermal stability. The authors further speculate that FPP may be imported from the seed tissue in which FPS1 is expressed during the early development of the embryo (in the absence of FPS2 expression) (Keim et al. 2012).

8.2.4.10 Geranylgeranyl Diphosphate Synthases

Similar to (E,E)-FPP, in primary and specialized metabolism, all-trans-GGPP synthesized by all-trans-GGPS is an important point of connection for several downstream terpenoid pathways. These include the biosynthesis of carotenoids and their breakdown products (abscisic acid, strigolactones), chlorophylls, tocopherols, gibberellins, plastoquinones, and diterpenoids (all synthesized in plastids), geranylgeranylated proteins and poly−/oligoprenols (synthesized in the cytosol), and poly−/oligoprenols synthesized in the plastids and mitochondria. Larger gene families in comparison with FPSs represent GPPS isozymes. For example, the genome of Arabidopsis contains 12 GGPS paralogues, of which 10 were identified to encode functional GGPS proteins of the most likely homodimeric architecture and GGPP as the primary or sole product (Beck et al. 2013). The various GGPS isozymes are located in the plastids, mitochondria and ER in accordance with the subcellular compartimentation of the various terpenoid pathways depending on GGPP. With the exception of two of the Arabidopsis isozymes (GGPS1-mitochondrial, GGPS11-plastidial), which are expressed in the whole plant, the remaining family members exhibit distinct spatiotemporal expression patterns (Beck et al. 2013). In ggps1 mutants, seedling-lethal albino and embryo-lethal phenotypes indicate that GGPS1 has essential functions in the development and biosynthetic chlorophyll pathway (Ruppel et al. 2013). Although the possible redundant or more specific functions of most GGPS isozymes are not well understood, it is clear that the divergence in the gene family of Arabidopsis GGPS is the result of functional specialization and fine-tuning of metabolic pathways in different cellular compartments and tissues at different stages of development or under different environmental conditions. Both FPPS and GGPPS proteins have been expressed in modules with sesquiterpene synthases and diterpene synthases, respectively, to engineer the biosynthesis of sesquiterpenoids and diterpenoids in microbial systems and in planta (Dai et al. 2012). Specifically, the buildup of FPP pools in plastids improved the precursor supply and allowed for a substantial increase in yield of the desired sesquiterpene products. Other strategies to improve pathway productivity include generating combinatorial mutations in prenyldiphosphate synthase and downstream terpene synthases. For example, prokaryotic expression of pathway variants of a GGPPS and a terpene synthase, which produces a levopimaradiene product, they're by stressing the importance of protein engineering in these approaches (Leonard et al. 2010).

8.2.4.11 Chain Length Regulation and Evolution of Prenyltransferases

The structural analysis of short-chain prenyltransferase products combined with random or site-driven mutagenesis provided substantial insight into the regulation of the chain length. On the basis of crystal structures of several homodimeric FPPs and GGPS from eukaryotes and prokaryotes (Gabelli et al. 2006), short-chain prenyltransferases share a common protein fold consisting of 13 α-helices with 10 helices surrounding the active site cavity. Two highly preserved aspartate-rich regions,

the first motif DDx2–4D (FARM) and the second motif DDxxD (SARM) bind the IPP and the allylic substratum, which are placed on opposite cavity walls. The length of the product chain is partly regulated by amino acid residues upstream of the FARM motif (position-4,-5), which change the size of the polyisoprenoid chain's hydrophobic substratum binding or elongation pocket (Ohnuma et al. 1996). Type I FPSs such as Arabidopsis FPS1 and FPS2 have a smaller binding pocket due to the presence of aromatic amino acid residues "bulkier" according to this mechanism. Smaller residues such as alanine, serine and methionine in type II GGPSs, which comprise eubacterial and plant GGPSs, replace these aromatic amino acids. The length of the product chain is partly regulated by amino acid residues upstream of the FARM motif (position-4,-5), which change the size of the polyisoprenoid chain's hydrophobic substratum binding or elongation pocket (Ohnuma et al. 1996). Type I FPSs such as Arabidopsis FPS1 and FPS2 have a smaller binding pocket due to the presence of aromatic amino acid residues "bulkier" according to this mechanism. Smaller residues such as alanine, serine and methionine in type II GGPSs, which comprise eubacterial and plant GGPSs, replace these aromatic amino acids. These aromatic amino acids are replaced by smaller residues such as alanine, serine and methionine in type II GGPSs, which comprise eubacterial and plant GGPSs. GGPS yeast studies have shown that the termination of the chain at C20 depends on residues in the catalytic cavity deeper (Chang et al. 2006). Poulter and colleagues recently used a large-scale bioinformatics approach in combination with experimental enzyme characterization, protein crystallization and computer modeling to predict the specificity of the chain length of a large number of supposed polyprenyl transfers (Wallrapp et al. 2013).

Phylogenetic analyzes of prokaryotic and eukaryotic prenyltransferases place plant FPSs in a clade with other eukaryotic FPSs distinct from a cluster of plant proteins GPS and GGPS (Vandermoten et al. 2009). A comprehensive phylogenetic study of GGPS and GPS homologues of terrestrial plants and green algae showed a lineage and species-specific expansion of GGPS families indicating gene duplication and functional divergence (Coman et al. 2014). The phylogeny shows a number of evolutionary transitions from GGPS to GPS. Gymnosperm homodimeric GGPSs, for example, which form a distinct clade between GGPS plants, can produce shorter prenyldiphosphates or synthesize exclusively GPP. Gymnosperm GGPSs have acquired a second CxxxS (bifunctional GGPS) or CxxxC (GPS) motif compared to GGPSs from green algae and mosses that have FARM and SARM motifs and a preserved CxxxC motif. The two CxxxC motifs are typical of most GPS activity-related proteins. They are therefore present in the subunits of heteromeric GPS proteins SSU II and I and are critical for binding the LSU. The binding of both subunits limits access to the elongation cavity and terminates the elongation of the chain when a C10 product is formed (Schmidt et al. 2010a, b). Proteins SSU SSU II and I have lost both motifs rich in aspartate or carry mutated SARM. It is interesting to note that a previous study reported a flower specific GPS from orchids similar to SSU II (Hsiao et al. 2008). This protein lacks SARM, but as a homodimeric enzyme it maintains GPS activities. Several Arabidopsis PPS homologous proteins (former GPS1) have been reported from other plants and have been designated as

homodimeric GPSs. These proteins do not carry CxxxC motifs and it remains to be determined whether they function as true GPS in vivo or may exhibit the activity of medium-chain or long-chain polyprenyl diphosphate, as shown for the enzyme Arabidopsis. GPS activity in tomatoes, for example, has been demonstrated, but tests have been performed at a low IPP/DMAPP ratio (van Schie et al. 2007). In addition, silencing or mutation of this enzyme and PPS in Arabidopsis resulted in dwarfed or embryo lethal phenotypes, which could be related to promiscuous GGPS activities to produce GGPP for gibberellin biosynthesis or the synthesis of longer precursors in plastoquinone biosynthesis. The absence of aromatic amino acids near the FARM also supports the formation of longer chain products by Arabidopsis PPS. Computational predictions such as those presented by Wallrapp et al. (2013) should make it easier to determine the specificity of the chain length of PPS homologues. In summary, the activity of GPS appears to be the result of the promiscuity and neo-functionalization of GGPS proteins (or PPS?) in conjunction with the evolutionary adaptation of individual plant lines to produce monoterpenes as components of floral fragrance or chemical defense.

8.2.4.12 *Cis*-Isoprenyl Diphosphate Synthases

One of the surprising findings of the past 5 years in the field of terpene biosynthesis was the identification of short-chain cis-prenyltransferases (CPTs) and the conversion of their cis-prenyl diphosphate products into terpenoids by terpene synthases (see below). Prior to this discovery, CPTs were generally thought to synthesize prenyl diphosphate products with a chain length of more than 50 carbons by using all-trans short-chain prenyl diphosphates as allylic primers (Takahashi and Koyama 2006). Such prenyltransferases in plants include enzymes producing C70-C120 dehydrodolichol diphosphates or natural rubber (> C10,000) from (E, E)-FPP by cis orientation head-to-tail condensations (Schmidt et al. 2010a, b). Functional genomics studies of terpene biosynthetic genes in wild tomato glandular trichomes revealed the presence of short-chain (Z, Z)-FPP synthase (Z, Z)-FPP (Sallaud et al. 2009). The characterization of a nine-member CPT family in cultivated tomatoes provided additional evidence of short-chain enzyme activity by identifying three genes encoding the synthase of neryldiphosphate (NPP) (NDPS1 or SlCPT1, expressed in trichomes), a (Z, Z)-FPP synthase (SlCPT6, expressed in root and fruit), and a nerylneryl diphosphate (NNPP) synthase (NNDPS or SlCPT2, expressed in the stem), respectively (Akhtar et al. 2013). All three proteins are plastid-based (Akhtar et al. 2013). In particular, plastic sesquiterpene terpene synthases (santalene/bergamotene sesquiterpene synthase (Sallaud et al. 2009) and 7-epizingiberene synthase (Bleeker et al. 2012)), which are related to diterpene synthases, use the Z, Z-FPP pool produced by (Z, Z)-FPP synthase in trichome-specific plastids in wild tomatoes. The engineering of (Z,Z)-FPP synthase and 7-epizingiberene synthase in cultivated tomato trichomes resulted in the production of 7-epizingiberene and increased herbivorous resistance (Bleeker et al. 2012). NPP is converted to β-phellandrene among other monoterpenes by monoterpene synthase (Schilmiller et al. 2009). Co-expression of the NDPS1 enzyme with phellandrene synthase 1 has therefore been successfully used in the metabolic engineering of monoterpene formation in

tomato fruits (Gutensohn et al. 2014). Intriguingly, the expression of NDPS1 alone led to a reduction in fruit carotenoid levels due to NPP inhibition of GGPS feedback. Based on these findings, it is likely that the production of NPP is primarily limited to trichomes in order to avoid inhibitory effects on carotenoid biosynthesis. Structural diversity of metabolites and their precursors specialized in terpenoid. Examples of monoterpenoids and sesquiterpenoids produced by various synthases of Arabidopsis terpene (AtTPS) (Tholl and Lee 2011).

The combination of tomato CPT genes with terpenoid biosynthetic gene clusters (Akhtar et al. 2013) clearly shows adaptive functional specialization in the tomato CPT gene family in order to provide short-chain prenyl diphosphates for various terpene biosynthetic pathways, including trichome-specific terpene biosynthesis. In accordance with these findings, a prenyltransferase cis-type was found in lavender, which catalyzes the head-to-middle condensation of two DMAPP molecules to synthesize lavandulol diphosphate, the precursor of lavendulol (Demissie et al. 2013). In addition, a multi-product prenyltransferase (AtCPT6) was identified in the nine-member CPT gene family of Arabidopsis a multiproduct prenyltransferase (AtCPT6) has been identified that makes polyisoprenoid diphosphates with six to eight isoprene units as precursors of polyisoprenoid alcohols in roots (Surmacz et al. 2014).

As with trans-prenyltransferases, efforts were made to determine residues of amino acids that control the specificity of the chain length of CPTs (Takahashi and Koyama 2006). CPT sequences share five conserved areas and use residues for binding substrate and catalytic activity, which are different from trans-prenyltransferases (Takahashi and Koyama 2006). Kang and others (Kang et al. 2014a, b) exploited accession-specific sequence differences of NDPS and (Z,Z)-FPP synthase in tomato coupled with homology modeling and site-directed mutagenesis to identify four residues in region II that are important for product specificity. These residues are part of helix II, which, together with helix III, lines a hydrophobic cleft that influences product chain length (Noike et al. 2008).

8.2.4.13 Conversion of Prenyl Diphosphates and Terpene Synthase Function and Regulation

Trans-and cis-prenyldiphosphates in plastids, mitochondria and cytosol are the entry points to different downstream primary and specialized terpenoid biosynthetic routes. All these pathways are beyond the scope of this chapter, and the reader is referred to other chapters in this series (e.g. carotenoid biosynthesis) or to more specialized field reviews. The huge variety of terpenoids in specialized metabolism can be attributed to the activity of terpene synthases (TPSs). Therefore, TPS enzymes have become a focus in the plant and heterologous metabolic engineering of terpenoid end products used as pharmaceuticals, flavors, biofuels or chemical defenses for plants (Bohlmann and Keeling 2008). The TPS superfamily, which is divided into eight subfamilies (TPSa-h), comprises a large and still growing number of enzymes in the plant kingdom from nearly all taxa (Chen et al. 2011). TPSs convert intermediates of acyclic C5 to C20 cis or trans-prenyl diphosphate into C5 hemiterpenes such as isoprene, C10 monoterpenoids, C15-sesquiterpenoids, or C20-diterpenoids. In most cases, the primary enzyme products are acyclic or cyclic

hydrocarbons, which are often modified by secondary enzyme reactions such as hydroxylation, peroxidation, methylation, acylation, glycosylation or cleavage in order to produce biologically active end products with even greater structural diversity (Degenhardt et al. 2009). TPS enzymes facilitate the adaptation of terpene metabolism to the changing environment, as their promiscuous activity often leads to more than one compound (e.g. Tholl et al. 2005), and TPS proteins easily acquire new catalytic properties through minor structural changes (Keeling et al. 2008).

TPS proteins are automatically divided into enzymes of class I and II. The enzyme reaction catalyzed by Class I TPSs begins with the ionization of the prenyl diphosphate substratum by a divalent subtraction of the diphosphate mood depending on the cation. The produced intermediate carbocation then enters into different reactions, which may include cyclizations, hydride shifts and rearrangements before the reaction ends with the loss of protons or the addition of nucleophils such as water (Davis and Croteau 2000). By contrast, class II TPSs, which include oxidosqualene cyclases and diterpene synthases, catalyze the ionization of their substrate by adding a proton to an epoxide ring or via protonation at the 14,15-double bond of GGPP, respectively. Class II diterpene synthases that fall into this category are ent-copalyl diphosphate (CPP) synthases (CPSs), which are involved in gibberellin and phytoalexin biosynthesis (Zi et al. 2014). In the gibberellin biosynthetic pathway, CPSs catalyze a protonation-induced bicyclization of the substrate GGPP to form ent-CPP, which is further ionized and converted to ent-kaur-16-ene by a class I ent-kaurene synthase (KS) activity. Detailed genomic studies of land plants revealed that the gibberellin biosynthetic pathway gave rise to the biosynthesis of an array of specialized labdane-related diterpenoids largely by gene duplication and divergence of CPS and KS homologues (Zi et al. 2014).

The ability to produce kaurene arose early in the development of land plants, as can be assumed from the identification of bifunctional classII/I CPS/KS in the moss patens Physcomitrella, which catalyzes the formation of ent-kaurene (and16-hydroxykaurene) in the biosynthesis of kaurenoic acid via a CPP intermediate (Anterola et al. 2009). Diterpene synthases such as abietadiene synthase occur in gymnosperms in similar class II / can and I be considered early synthases of diterpene. These enzymes produce (+)-CPP from GGPP before the cyclization of (+)-CPP to the diterpene product initiated by ionization. An interesting new perspective on the development of plant TPS genes comes from genomic study of a large TPS gene family in the fern Selaginella moellendorffii (Peters et al. 2003).

Two different types of TPS genes have been identified: A group of diterpene synthases representing a new plant subfamily TPS – h and, surprisingly, a group of monoterpene synthases and sesquiterpene synthases, which are closer to microbial TPSs and may be the first indication of horizontal gene transfer of TPS genes (Li et al. 2012). It should be noted that the iridoid monoterpene biosynthetic pathway has recently discovered a new mechanism for the enzymatic formation of cyclic terpenes (Geu-Flores et al. 2012). Iridoids are pharmaceutical and antibacterial and are also produced as pheromones by aphids (Dewhirst et al. 2010). The iridoid synthase from Catharantus roseus is a short-chain reductase that most likely generates a C5-iridoid ring in the 10-oxogeranial linear monoterpene substratum by coupling

a reduction step with a cyclization step via a cycloaddition of Diels-Alder or an addition of Michael (Geu-Flores et al. 2012).

The analysis of a growing number of crystal structures, including those from isoprene synthase (Koksal et al. 2010), monoterpene synthases (Hyatt et al. 2007), sesquiterpene synthases (Gennadios et al. 2009), class I diterpene synthase (Taxadiene synthase (Koksal et al. 2011), class II CPP synthase (Koksal et al. 2011), class II CPP synthase (Koksal et al. 2011), class II CPP synthase (Koksal et al. 2011), class II CPP synthase (Koksal et al. 2011). Comparisons of the assembly of Class I type α-domain and Class II type β and β domains led to the prediction of an evolutionary scenario according to which the ancestral bifunctional ClassII/ClassI diterpene synthase (consisting of all three domains with a functional α- and β-domain) similar to the CPS / KS enzyme of P. patens resulted in synthases of Class II type diterpene (consisting of all three domains with a functional α- and β-domain) (Gao et al. 2012). A functionally active class I α-domain carries the highly conserved aspartate-rich motif, DDxxD, and a less conserved NSE/DTE motif, which are located on opposite sides of the entrance of the catalytic side and help position the diphosphate substrate by binding of a trinuclear magnesium cluster (Christianson, 2006). By contrast, functional class II β-domains carry a conserved DxDD motif, which is required for protonation-initiated carbocation formation (Cao et al. 2010). Although TPS enzymes may convert more than one prenyl diphosphate substrate in vitro, the substrate pool that is available in the respective cellular compartment largely determines their function in vivo. In this regard, TPS enzymes localized in plastids generally produce monoterpenoids or diterpenoids from plastidial GPP and all-trans-GGPP, respectively, whereas TPSs in the cytosol primarily convert (E,E)-FPP to sesquiterpenes (or squalene in the biosynthesis of C30 terpenes). However, this general rule has recently been challenged by the discovery of plastidial (Z,Z)-FPSs and sesquiterpene synthases in tomato, the latter of which are more closely related to kaurene synthases in the TPS-e subfamily (Schilmiller et al. 2009).

The existence of medium to large TPS families in Arabidopsis and many other plant species strongly supports the idea that TPS genes develop through gene duplication and neo-functionalization (Aubourg et al. 2002). Such duplication events in combination with genome relocation may include other genes that encode enzymes such as cytochrome P450s, leading to the assembly of gene clusters. Since the first discovery of a biosynthetic gene cluster of thalianol triterpene in Arabidopsis (Field et al. 2011), several of these clusters were found in the biosynthetic pathways of arabidiol, marneral and avenacin triterpene in Arabidopsis and oat (Field et al. 2011) (Sohrabi et al. in preparation), and for the biosynthesis of labdane-related diterpenoids in rice (Wilderman et al. 2004) or monoterpenoids and sesquiterpenoids in tomato (Falara et al. 2011). The biosynthetic clusters of triterpene carry genes for oxidosqualene cyclases (OSCs), which catalyze the cyclization of oxidosqualene into one or more cyclic triterpene alcohols via carbocationic intermediate formation (Phillips et al. 2006). Co-expression with other cluster genes (e.g., P450s, desaturase, acyltransferase) in an operon-like manner allows the triterpene precursor to be derived consecutively (Field et al. 2011). The evolutionary forces driving this

coordinated gene cluster assembly are believed to be twofold. Clustering of genes for pathway building facilitates the regulation of multiple genes at the level of chromatin and/or prevents the accumulation of possible cytotoxic products (Wilderman et al. 2004). However, a strict coregulation of gene expression does not seem to be the case in all clusters as was shown for a diterpene biosynthetic cluster in rice containing P450s that are differentially regulated and function in two different pathways (Geu-Flores et al. 2012).

Clusters that exhibit a coordinated expression of their genes have enabled the identification of possible key regulators, such as the basic transcription factor of leucine zippers, OsTGAP1, which regulates the cluster of diterpenoid biosynthetic genes in rice (Yamane 2013). Another transcription factor previously identified for the positive regulation of terpene biosynthetic genes is a cotton transcription factor of WRKY, GaWRKY1, which regulates the transcription of a sesquiterpene synthase gene in the pathway of gossypol biosynthesis (Xu et al. 2004). More recent studies on Artemisia annua suggest that APETALA2/ethylene response factors (AP2/ERF) are positive regulators of biosynthetic genes in the formation of sesquiterpene artemisinin, an insect deterrent and antimalarial drug produced in leaf glandular trichomes (Lu et al. 2013). However, these studies have not yet placed the identified transcription factors in the development and cell specification regulatory networks. In the process of flower maturation in Arabidopsis, a better understanding of the regulatory networks controlling the volatile formation of terpene has been gained. Two R2R3 MYB transcription factors, MYB21 and MYB24, were identified that promote gynoecium growth and nectary development and positively affect expression of the major floral (E)-β-caryophyllene sesquiterpene synthase TPS21 (Reeves et al. 2012). Both MYB TFs respond positively to jasmonic acid (JA), the levels of which are induced by the auxin response factor 6 (ARF6) and ARF8, both master regulators of flower maturation. TPS21 and the second floral sesquiterpene synthase, TPS11 (Tholl et al. 2005), also respond more directly to JA by the direct binding of their promoters to the bHLH transcription factor MYC2 (Hong et al. 2012), which is a central JA signaling pathway regulator in response to development and stress (Dombrecht et al. 2007). In addition, gene expression of TPS21 and TPS11 is regulated indirectly by gibberellins by binding DELLA proteins (signaling repressors for gibberellin) (Hong et al. 2012). Similar to the tissue-specificity of terpene formation in flowers, terpene-specialized metabolism in roots appears to be a highly coordinated cell type-specific process. Genes of the thalianol and marneral triterpene biosynthetic gene clusters are coexpressed primarily in the root epidermis (Field et al. 2011). Likewise, 14 genes of the Arabidopsis TPS family are expressed in different root tissues. For instance, a recently identified rhizathalene diterpene synthase (TPS08) has been found to be primarily expressed in the root stele (Vaughan et al. 2013). In addition, in the stele of the root elongation zone and the differentiation / maturation zone and in the epidermis and cortex of more mature roots, two genes of 1,8-cineole monoterpene synthase are constitutively expressed; a similar pattern of expression has been observed for two closely related synthases of sesquiterpene (Z)-bisabolene sesquiterpene (Chen et al. 2004). However, for these root-specific genes, no networks of temporal and spatial regulation have yet been defined.

8.2.4.14 Role of Terpenes and Terpenoids

Although terpenoids serve important primary functions such as photosynthetic pigments (carotenoids), electron carriers (side chains of ubiquinone and plastoquinone), growth and development regulators (gibberellins, abscisic acid, strigolactones, brassinosteroids, cytokinins), protein glycosylation (dolichols), or membrane structure and function elements (phytosterols), specialized terpenoid. In the protection of plants against abiotic stress, volatile or semivolatile, low molecular weight terpenoids, including isoprene, monoterpenoids, sesquiterpenoids and diterpenoids, are involved in the protection of plants against abiotic stress and in various biotic interactions above- and below ground (Loreto et al. 2014). The substantial emissions of isoprene and monoterpenes from various vascular and nonvascular plants have been associated with the protection against thermal stress. This process is presumably based on an intercalation of the volatile compounds with the photosynthetic membranes and thereby enhances membrane functionality (Behnke et al. 2007). Moreover, transgenic approaches in tobacco and poplar support a role of isoprene in oxidative stress protection (Velikova et al. 2014) and are addressed in a separate chapter by Vickers et al. Volatile terpenoids as constituents of floral scent are implicated in mutualistic interactions with plant pollinators. For instance, choice tests with bumblebees have indicated a role of monoterpenoids emitted by monkeyflowers in pollinator attraction (Byers et al. 2014). Nevertheless, distinct evidence for a specific role of terpenoids in pollinator attraction by the use of biosynthetic mutants is still missing, but it can be assumed that attractive effects depend on mixtures of volatiles rather than individual compounds. The notion that floral volatile terpenoids serve multiple functions has been supported by their role in the defense of floral tissues against microbial pathogens. This interaction was demonstrated in flowers of Arabidopsis mutants, which lack the emission of (E)-β-caryophyllene from their stigmatic tissue. The mutant flowers were more susceptible to infection by *P. syringae*, which resulted in lighter and often misshaped seeds suggesting reduced plant fitness. Similar findings were made by Junker et al. (2011) demonstrating that floral volatiles play roles in the structuring of bacterial communities that colonize flower petals by providing compound-dependent niches. Volatile terpenoids also serve important functions as constitutive or pathogenand herbivore-induced compounds in the defense of photosynthetic tissues. For example, repellent activities have been reported for monoterpene volatiles that are emitted by leaves of Chrysanthemum morifolium and, notably, herbivore-deterrent effects have been observed for isoprene (Wang et al. 2008). Furthermore, volatile terpenoids that accumulate in glandular trichomes function as insect repellents as was, for example, found for the activity of sesquiterpenes in trichomes of wild tomato against white flies (Bleeker et al. 2011). In conifers, the production of terpenoid oleoresin and terpenoid volatile emissions constitutes an important chemical defense system. In a search for resistance factors, the monoterpene (+)-3-carene was found to be associated with resistance of Sitka spruce (*Picea sitchensis*) to white pine weevil (Pissodes strobi) (Zulak and Bohlmann 2010). Variation of the (+)-3-carene production in resistant and susceptible trees was demonstrated to depend on the copy number of a (+)-3-carene TPS gene, differences in gene transcript and protein levels, and

variation in catalytic efficiencies. Similarly, in Arabidopsis, ecotype-specific variation of the herbivoreinduced volatiles, (E)-beta-ocimene and (E,E)-alpha-farnesene, is controlled by allelic variation and differences in subcellular targeting of the two terpene synthases, TPS02 and TPS03 (Hall et al. 2011).

The role of volatile mixtures induced by herbivores in attracting natural enemies of herbivores and at higher trophic levels has been examined in numerous studies (Gols 2014). Transgenic Arabidopsis work has provided strong evidence of the role of volatile terpenes in these interactions (Kappers et al. 2005). However, as indicated in the case of floral fragrance, the effect of these compounds must be taken into account in the context of the entire volatile mixture induced by herbivores, and the actual fitness benefits of the plant host under natural conditions are still under discussion (Kessler and Heil 2011). Indirect defense responses to insect oviposition are also mediated by volatile compounds (Hilker and Meiners 2006). For example, the deposition of eggs on the foliage of the European field elm (Ulmus minor) by the elm leaf beetle (Xanthogaleruca luteola) leads to the emission of volatiles, including irregular homoterpene, (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT), which play a role in attracting the specialist egg parasitoid, Oomyzus gallerucae (Buchel et al. 2011). In addition to their function in the interaction with herbivores and their enemies, constitutive and induced volatile mixtures (including volatile terpenes such as homoterpenes) can serve as interspecific, intraspecific and intraplant "alarm" signals in neighboring plants or unattacked tissues of the same plant (Heil 2014). In these interactions, volatiles may not necessarily need to enter the leaf tissue of the neighboring plant but remain on the leaf surface. This effect was observed for sesquiterpenoids that are emitted by rhododendron leaves and adsorbed on the leaves of birch trees, where they exhibit direct herbivore-repellent activities (Himanen et al. 2010). Moreover, terpenoids were suggested to be involved in parasitic plant interactions, specifically, in the attraction of the parasitic plant *Cuscuta pentagona* (dodder) to establish contact with tomato as its host (Runyon et al. 2006). The molecular mechanism of host plant detection in this response as in other volatile-mediated plant–plant interactions is still poorly understood. The described functions of volatile terpenoids in aboveground plant defense are complemented by nonvolatile terpenoids. As an example, glycosides of geranyllinalool serve as potent antifeedants in the wild tobacco, Nicotiana obtusifolia (Jassbi et al. 2010), and recently detected ent-kaurane–related diterpenoids in maize named kauralexins as well as acidic sesquiterpenes called zealexins function as pathogen-inducible phytoalexins (Huffaker et al. 2011). Similarly, *Barbarea vulgaris* metabolomics studies have shown that triterpene saponins contribute to flea beetle attack resistance (Kuzina et al. 2009). An increased interest in the role of specialized metabolites under the surface has shown that terpenoids serve similar functions to those above the surface. Recent studies in the roots of Arabidopsis have found semi-volatile hydrocarbons of diterpene with an unusual tricyclic structure called rhizathalenes. These compounds are produced in the root stele, from which they diffuse through the surrounding cell layers to act as local antifeedants by reducing the damage caused by root herbivores to these cell layers. The role of volatile terpenes in underground indirect defense has been well established on the basis of maize studies

showing that sesquiterpene, (E)-β-caryophyllene, emitted from the roots of the western corn root worm Diabrotica virgifera, attracts entomopathogens (Turlings et al. 2012). These findings led to attempts to manufacture (E)-β-caryophyllene in non-emitting American maize cultivars, which resulted in an increased attraction of nematodes and increased resistance to the attack of maize rootworms. However, constitutive emissions of (E)-β-caryophyllene were found to have additional costs inasmuch as they compromise seed germination, plant growth, and yield (Robert et al. 2013). Therefore, it may be necessary to develop more sophisticated engineering strategies to avoid these cost effects, taking into account herbivore-induced emissions. Nonvolatile terpenoids can be exuded from roots into the rhizosphere and the surrounding soil environment where different defense responses are involved. Studies using rice mutants have shown convincingly that labdanerelated diterpenoids called momilactones have allelopathic effects on competitors in barnyard grass (Xu et al. 2012). In addition, avenacins, which are saponins of triterpene exuded by oat roots, are known as phytoalexins for their potent activity (Thimmappa et al. 2014). Excitingly, a recent study by Osbourn and colleagues revealed that common triterpene precursors have additional signaling functions in root development. Specifically, it was demonstrated that β-amyrin is involved with determining the patterns of epidermal root hair cells (Osbourn 2010). These findings indicate that the roles of specialized metabolites in biotic interactions and potential "primary" functions become increasingly blurred. Signaling functions have also been demonstrated for the abietane diterpenoid, dehydroabietinal, which is produced at picomolar concentrations in Arabidopsis leaf tissue and serves as a vascular signaling compound and potent activator of systemic, acquired resistance. This activity seems to depend on the association of dehydroabietinal with vascular sap proteins. Finally, it should be noted that strigolactones have become an exciting model for the multifunctionality of small molecules. As carotenoid-derived compounds strigolactones have important roles as exogenous signals by recruiting arbuscular mycorrhizal fungi in the rhizosphere (Akiyama et al. 2005). Parasitic plants such as *Striga lutea* (witchweed) eavesdrop on these compounds by using them as germination signals. As internal signals, strigolactones function as growth and developmental hormones that suppress shoot branching. Other processes that involve strigolactone-signaling functions include root growth and development, stem elongation, secondary growth, leaf expansion and senescence, and responses to drought and salinity. Rapid progress has been made in understanding the perception of strigolactones but many open questions remain about downstream targets and the role of strigolactone-related compounds (Waldie et al. 2014).

8.3 Sequencing Technology for Genes and Gene Products

The sequencing of plant genomes allows researchers to discover the genetic composition of plants. This is essential not only to answer basic research questions, but also to design crop plants for the next generation. Even for de novo sequencing projects to obtain the primary genetic sequence of your plant species of interest, the

sequencing of plant genomes becomes easier and more cost-effective. Life Technologies has a wide range of sequencing instruments, reagents and analytical software to get you there faster and more reliably and accurately.

8.3.1 Current Sequencing Technologies

The development of recent sequencing technologies has generated a remarkable increase, by orders of magnitude, in sequencing throughput with a corresponding drop in cost per base. A simple exercise to comprehend the scale of acceleration in sequencing is to look back at the state of the art of sequencing in 1980. At that time, earlier improvements in Sanger and Maxam-Gilbert methodologies had initiated the wide use of sequencing in research laboratories around the world. Then, typical sequencing throughputs per slab gel run were under 10,000 bp. During the period from 1980 to 2005 sequencing platforms based on Sanger chemistry had a 500 to 1000-fold increase, to more than 5 Mbp per run. The number of reads that could be processed, quality, read length and analysis all improved and were optimized, propelled by the development of the human genome project. While these technological advances were certainly impressive, they dwarf when compared to the acceleration in sequencing capacity after 2005. At that time, novel ultra-high throughput technologies started to become commercially available. From 2005 through the second half of 2011, the throughput per run had increased an additional 100,000-fold, or 5 orders of magnitude. This acceleration has been unprecedented in science and technology. It has outpaced Moore's law that famously predicted that the number of transistors in a computer processor would double every 2 years. This fast increase in sequencing capacity has had important consequences in analysis and logistics, and has changed expectations in all aspects of plant genetics, breeding and biotechnology.

8.3.2 Sanger Sequence Analyzers

Sequencing based on the chemistry of Sanger and Maxam-Gilbert has been the only practical method for routinely determining DNA sequences in plants and other biological systems for more than 30 years and until recently. During the 80s and 90s, Sanger-based platforms increased by magnitude orders and became the choice method, while the Maxam-Gilbert method remained a low-throughput process. Technical innovations such as thermal cycle sequencing and single-tube reactions in combination with fluorescence-tagged terminator chemistry greatly facilitated the development of automated Sanger systems. Additional improvements in parallelization, quality, read length, and cost-effectiveness were achieved by the development of automatic basecalling and capillary electrophoresis. In the current version of Sanger sequencing a mixture of primer, DNA polymerase, deoxinucleotides (dNTPs) and a proportion of dideoxynucleotide terminators (ddNTP), each labeled with a different fluorescent dye, are combined with the DNA template. During the thermal

cycling reaction, DNA molecules are extended from templates and randomly terminated by the occasional incorporation of a labeled ddNTP. DNA is cleaned and denatured. The detection is achieved by laser excitation of the fluorescent labels following the separation of the extension products by capillary electrophoresis. The dye excitation differences create a "four color" system that is easily translated by a computer to generate the sequence. Modern Sanger sequencers such as the Applied Biosystems ABI3730 have reached a high level of sophistication and can achieve routine read-lengths of nearly 900 bp and 99.99% or higher "raw" accuracies per base. The ABI 3730xl analyzer can run 96 or 384 samples every 2–10 h, generating approximately 100,000 bases of raw sequence at a cost of a few hundred dollars.

8.3.3 Roche 454

The 454 platform was the first standalone NGS platform. DNA templates must be prepared by PCR emulsion and attached to beads, with 1–two million beads deposited in a titanium-covered plate in wells. The Roche 454 technology is based on Pyrosequencing and additional beads that have sulphurylase and luciferase attached to them are also loaded into the same wells to generate the light production reaction. DNA polymerase reactions are performed in cycles but, unlike Sanger, there are no terminators. Instead, one single dNTP is alternated in every cycle in limiting amounts. Fluorescence after the reaction indicates the incorporation of the specific dNTP used in the cycle. Because the intensity of the light peaks is proportional to the number of bases of the same type together in the template, the fluorescence can be used to determine the length of homopolymers, although accuracy decreases considerably with homopolymer length. The current 454 chemistry is able to produce the longest reads of any NGS system, about 700 bp, approaching those generated by Sanger reads. However, 454 systems can sequence several megabases for less than 100 dollars.

8.3.4 Illumina

The Solexa platform (now owned by Illumina) has become the most widely used NGS system in Plant biotechnology and breeding. Illumina captures template DNA that has been ligated to specific adapters in a flow cell, a glass enclosure similar in size to a microscope slide, with a dense lawn of primers. The template is then amplified into clusters of identical molecules, or polonies, and sequenced in cycles using DNA polymerase. Terminator dNTPs in the reaction are labeled with different fluorescent labels and detection is by optical fluorescence. As only terminators are used, only one base can be incorporated in one cluster in every cycle. After the reaction is imaged in four different fluorescence levels, the dye and terminator group is cleaved off and another round of dye-labeled terminators is added. The total number of cycles determines the length of the read and is currently up to 101 or 151, for a total of 101 or 151 bases incorporated, respectively. At the time of writing this review, this technology was able to yield the highest throughput of any system, with one of

the highest raw accuracies. One major disadvantage is the short read it produces. However, paired-end protocols virtually double the read per template and facilitate some applications that were originally out of the reach of the technology. The Illumina HiSeq 2000 sequencer is currently able to sequence up to 540–600 Gbp in a single 2-flow cell, 8.5-day run at a cost of about 2 cents per Mbp (http://www.illumina.com/systems/hiseq_2000.ilmn).

8.3.5 Life Technologies SOLiD

ABI (now part of Life Technologies) has commercialized the SOLiD (Support Oligonucleotide Ligation Detection) platform. This platform is based on Sequencing by Ligation (SbL) chemistry. SbL is a cyclic method but differs fundamentally from othercyclic NGS chemistries in its use of DNA ligase instead of polymerase, and two-baseencoded probes instead of individual bases as units. In SbL, a fluorescently labeled 2-base probe hybridizes to its complementary sequence adjacent to the primed template and ligated. Non-ligated probes are then washed away, followed by fluorescent detection. In SOLiD, every cycle (probe hybridization, ligation, detection, and probe cleavage) is repeated ten times to yield ten color calls spaced in five-base intervals. The extension product is removed and additional ligation rounds are performed with an n–1 primer, which moves the calls by one position. Color calls from the ligation rounds are then ordered into a linear sequence to decode the DNA sequence. SOLiD has similar throughput and cost per base to Illumina. It also has the best raw accuracy among commercial NGS systems.

8.3.6 Other NGS Platforms, Helicos Heliscope, Polonator

There are other NGS systems that have been marketed in the last few years, however, they have had limited use in plant sciences. Helicos developed the first commercial singlemolecule sequencer, called HeliScope. However, very few units were sold due to the cost of the machine, on-site requirements and other considerations. Currently, Helicos provides sequencing as a service. One additional company, Azco-Biotech is marketing the Max-Seq Genome sequencer (http://www.azcobiotech.com/instruments/maxseq.php). This commercial version of the academic, open-source Polonator can run either sequencing by synthesis or sequencing by ligation protocols, similar to Illumina and SOLiD, respectively, although it generates shorter reads, 35- or 55-bp-long.

8.3.7 Pacific Biosciences and the Third Generation

Pacific Biosciences has launched the PacBio RS platform, considered the first commercially available third-Generation system. The first early-access instruments were deployed in late 2010 and the first commercial batch became available by

mid-2011. The PacBio system is based on SMRT, a single-molecule sequencing chemistry with real time detection. The sequencing cell has DNA polymerases attached to nanowells and exposed to single molecule templates and labeled NTPs. No terminators are used, although conditions are set to slow polymerization to a level that can be detected by a CCD camera. Each dNTP has a unique fluorescent label that is detected and then cleaved off during synthesis. This method can generate reads exceeding 10 kilobases in a few minutes due to this real-time detection and enzyme processivity. The technology's potential for sequencing single molecules and producing long readings is immense. However, the PacBio technology may need to overcome a number of technical challenges before it reaches a widespread use in plant sciences. Average read length in current outputs exceeds 1 Kbp although single-pass error rate has been reported to be 15%, considerably higher than other sequencing platforms. One major source of errors consists of deletions produced during detection.

8.3.8 Role of Next Generation Sequencing in Crop Improvement

The basis of plant breeding is the identification and exploitation of genetic variation. Traditional phenotype selection is tedious and time consuming. Molecular markers help to associate the phenotype with genotype. Many DNA based molecular markers have been developed for major crops during the past decades and used for detecting the genetic variation among the cultivars. Marker assisted selection has been carried out in the progeny, which allows the early selection of desired progeny. DNA markers such as restriction fragment length polymorphism (RFLP), random amplification of polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), single sequence sequence repeat (SSR), and single nucleotide polymorphisms (SNPs) have been identified and applied to improve breeding of several plants. However, most of the conventional markers (RFLP, RAPD, AFLP, SSR) are selectively neutral, as they are located in non-coding and non-regulatory regions. When such markers are used for marker-assisted selection, there will be chances of false positives, due to genetic recombination. Gene based functional nucleotide polymorphism, if identifiable within the gene of interest, is more powerful and reliable. It is more advantageous than conventional markers, as there is no recombination between the marker and the gene of interest. Markers that allow for the identification of allelic variation of a particular trait are more valuable in crop breeding. The recent advances in genome sequencing through next generation sequencing (NGS) technologies provide opportunities to develop millions of novel markers, as well as the identification of agronomically important genes (Edwards and Batley 2010). SNPs now dominate over other molecular marker applications, with the advancement in sequencing technology. Traditionally, PCR amplification is performed for the genomic region of interest from multiple individuals representing the diversity in a population, followed by sequencing. The sequences were then aligned to identify polymorphisms (Edwards and Batley 2010). This approach is expensive and time consuming. Now, large quantities of sequences generated through NGS

platforms, together with the development of in silico methods, enable cheaper and more efficient SNP discovery. This approach also allows for the identification of functional indels (insertions or deletions), including partial or complete deletions of genes and different numbers of repeat motifs within SSRs. These markers have been used for the development of molecular genetic and physical maps, and for identifying the genes or quantitative trait loci controlling economically important traits.

Advances in NGS have enabled the development of genetic maps of high density. Based on their co-segregation, genetic mapping places the markers in linkage groups. The genetic map predicts the linear arrangement of markers in a chromosome based on the recombination frequency between genetic loci in a population derived from crosses of genetically diverse parents (Edwards and Batley 2010). The enormous sequence data obtained through NGS technologies have enabled the improvement of genetic maps by increasing marker density. Thousands of markers may be located in different linkage groups. It helps to localise corresponding scaffolds on the map, thus enabling the possibility of complete genome mapping. It also helps to replace traditional quantitative trait locus (QTL) mapping with association mapping, because QTL provide a wide genome range within which the gene is located, whereas association maps mark traits with high resolution.

The sequence data obtained from genomes and transcriptomes and their expression profiles associated with various physiological conditions help to identify the genes that determine different characteristics. These data allow regulatory mechanisms to be unravelled behind different characteristics and help to elucidate the entire path. These data also enable the identification of allelic variations in candidate genes controlling important agronomic traits, which is crucial for successful breeding programmes. Identification of the key genes underlying a trait enables the transfer of the trait to another cultivar or species by genetic modification; alternatively, these traits may be incorporated into a cultivar by marker-assisted selection (Edwards and Batley 2010). Furthermore, the analysis of copy number variations among and between species will contribute to the understanding of the mechanism of heterosis. In addition to the sequence variation, epigenetic changes are also responsible for heritable traits. Advancement in sequencing technologies allows for the survey of genome-wide epigenetic variation at high resolution through techniques such as bisulfite sequencing (Bi-seq), methylated DNA immunoprecipitation sequencing (MeDIP-seq), and methylation-sensitive restriction enzyme sequencing (MRE-seq) (Bell and Spector 2011).

Low agricultural productivity in the tropics can be explained by problematic soil due to humidity, rainfall variability, limited irrigation potential, pest and disease loads, and net photosynthetic potential differences (Gallup and Sachs 2000). The lack of freezing temperatures in the tropics favours an increased number of agricultural pests. Although the tropics are warmer and sunnier, it is generally cloudy, thus sunlight is blocked, which is disadvantageous for photosynthesis (Gallup and Sachs 2000). Likewise, nighttime temperature is generally high, which causes high respiration and slows the rate of plant growth (Gallup and Sachs 2000). Identification of genes associated with disease resistance and other abiotic stress management would be particularly important for improving tropical agriculture. The knowledge

obtained from genomes, transcriptomes, gene expression studies, and epigenetic variation studies would help to develop crop varieties that are capable of overcoming the disadvantages of tropical climates. Finally, one possible impact of genomics on plant breeding could be the development of a systems breeding approach, which integrates gene function information and regulatory networks to predict and estimate the contributions of genetic and epigenetic variations to phenotypes and field performance.

8.3.9 Sequenced Crop Genomes

Following the genome sequencing of the model plant Arabidopsis, a number of crop species have been sequenced, many being important to tropical countries (Table 8.1). Most genome assemblies are in the draft phase and extensive work is underway to close the gaps and re-sequencing. In addition to the genome sequence, transcriptomes and expressions profiles are also available for many crops. The large genome size and polyploidy exhibited by many crop species impedes the sequencing and further analysis. A high percentage of repeat elements is also a major hurdle in genome assembly. However, a platform has been established for many important crops and further research could lead could lead to more information for application in crop breeding.

8.3.10 Sequencing Food Crops

The recent surge in plant genome sequencing is primarily aimed to reduce hunger. Among the sequenced plant genomes, most are food crops that are especially important for tropical countries. These crops include different cereals, pulses, tuber crops, vegetables, fruits, and oil plants. Functional markers have been developed for many of these crops and genes controlling agronomically important traits have been identified. However, re-sequencing and gene expression studies are continuing to be completed for a comprehensive understanding of genetic mechanism behind each trait and to identify allelic variations. In addition to the sequenced crops, many genome projects are underway or at the planning stage.

8.3.11 Rice Genomes

Rice (Oryza sativa) is the most important crop, as staple food for more than half of the world's population. It is the main food crop in most of the tropical countries. Rice cultivation occupies 11% of the world's total arable land and it is a source of income for more than 100 million people around the world (Guimaraes 2009). *O. sativa* has two major sub species, indica and japonica. Japonica varieties are usually cultivated in temperate regions, while indica varieties are important for the tropics. A third sub species, javanica is also cultivated in the tropics and is also

known as tropical japonica. Glaszmann (1987) classified *O. sativa* into six groups; indica, japonica, aromatic, aus, rayada, and ashina, based on isozymes. The genetic improvement of rice, which preceded the green revolution, was given considerable attention in the 1960s. The main breeding objectives were to increase yield, grain quality, blast disease resistance and tolerance to drought (Guimaraes 2009). In the following years, hybridization developed many high-yielding semi-dwarf varieties (e.g. IR8). In the development of new rice varieties, mutation breeding was also popular. In rice breeding, it has been shown that biotechnological tools such as anther culture and protoplast fusion are promising tools (Guimaraes 2009). In the 1990s, several transgenic rice species were also produced (e.g. golden rice). In addition, different types of molecular markers were developed for rice and marker assisted selection has been employed in breeding programmes. A high-density rice genetic map was constructed with 2275 markers (Harushima et al. 1998).

8.3.12 Economically Important Crop Genomes

A few other economically important crops have also been sequenced in addition to food crops. Some of these crops are very valuable for the tropical countries' economy. Systematic mining and utilisation of these data would help to develop varieties with higher yield and tolerance to biotic as well as abiotic stresses, and would boost up the economy of many tropical countries.

8.3.13 Rubber and Oil Palm Genomes: Promises to Malaysian Economy

Natural rubber (NR) is a unique biopolymer used in the worldwide production of more than 50,000 products. The main source of NR is Hevea brasiliensis (rubber tree). The rubber tree originated from the amazon basin and has been domesticated in other tropical countries. Today, rubber cultivation is mainly performed in the Asian countries, which account for 93% of the world's supply. Malaysia has fourth position in NR production, after Thailand, Indonesia, and Vietnam. NR production in Malaysia was in its peak during the early twentieth century; however, rubber plantation area has been gradually decreasing over the past 10 years. The rubber cultivation area reduced to 1.02 million ha in 2011 (Economic Transformation Programme (ETP) 2012). Decreased yield and susceptibility to diseases are the major challenges for rubber cultivation. Several high yielding clones were developed by the Malaysian rubber board and by rubber research centres in other countries. However, global demand for NR is increasing and to cope with this demand, genetically improved clones with more productivity have to be developed. In addition to NR, rubber wood is used as a source of timber with export value. On the basis of RFLP, AFLP, microsatellite and isozyme markers, several molecular markers were developed for rubber tree and a saturated genetic linkage map was published. The same group also published a link map for H. Brasiliensis cultivar MDF

180, which is resistant to leaf blight from South America, and the resistance QTL has been mapped. Expressed sequence tags (EST) were produced from rubber latex, which provided more information on rubber biosynthesis (Chow 2006). Several transcriptome sequencing projects were completed with the advent of next-generation sequencing technologies and there have been several transcriptome-sequencing projects completed and made available in the public domain. (Li et al. 2012; Tang et al. 2010). To obtain more insight into the noncoding regions and their regulatory roles, the draft genome of H. brasiliensis was published recently. The assembly comprises 1.1 Gb of scaffolds of the estimated 2.1 Gb of genome. It was estimated that about 78% of the genome was repetitive DNA. A total of 68,955 gene models were forecast, 12.7% of which are unique to H. Brazilensis. Most genes associated with rubber biosynthesis, formation of rubber wood, resistance to disease and allergenicity has been identified. The genomic information and transcriptomes are a good basis for genetic studies and crop improvement. The yield of rubber depends primarily on three factors -the number of laticifer rings, the loading rate of sucrose in the laticifer and the polymerization rate of isopentenyl diphosphate (IPP) on the rubber particle. The systematic extraction of genomic and transcriptomic information and other expression studies would help to identify the key genes associated with these aspects, which could be used in higher yield breeding clones. The susceptibility to various diseases is a major impairment of rubber cultivation. Genomic and transcriptomic studies have identified genes resistant to the disease and further studies will reveal more insight into the genetic interaction of the rubber tree's genetic interaction with specific pathogens, leading to the development of disease resistant clones. Moreover, rubber cultivation is geographically restricted to a few regions. Increasing the area of rubber cultivation is another important approach to increase rubber production to cope with the global demand. Systematic mining of genomic and transcriptomic data would lead to the identification of genes imparting resistance to various geographical ailments and would lead to the development of clones suitable for various agro-climatic regions.

The main source of palm oil is oil palm (*Elaeis guineensis*). Palm oil is a food ingredient and is also used to produce biodiesel and other important products for industry. In addition, renewable energy, fuels and biodegradable products are generated using palm biomass. Oil palm is a plant native to western and central Africa that was domesticated in nineteenth century South East Asia. Malaysia, after Indonesia, is the second largest palm oil producer. Indonesia and Malaysia make about 85% of the world's palm oil. The palm oil industry is one of these countries' key economic drivers. The oil palm planted area in Malaysia reached 5.23 million hectares by 2013.

8.4 Gene Clusters and their Significance for Plant Products

The discovery of new plant natural product pathways and chemistries is now being revolutionized by two key developments. First, breakthroughs in sequencing technology and reduced cost of sequencing are accelerating the ability to discover new

natural product pathways by identifying the underlying genes, as genome sequences of numerous different plant species are becoming available. Transcriptomics in combination with metabolite analysis of different plant tissues, developmental stages and/or elicitor-treated material is also being used to great effect to identify candidate genes for the synthesis of natural products of interest and to piece together pathways (Lau and Sattely 2015). Second, it has emerged that the genes encoding certain natural product pathways are organized in biosynthetic gene clusters within plant genomes (Schneider et al. 2016). These examples include pathways for diverse classes of chemicals from a wide variety of different plant species, including eudicots and monocots (both annuals and perennials). This clustering phenomenon is interesting because it challenges the assumption that gene control is more or less random on plant chromosomes. It is also beginning to allow for the straightforward prediction and subsequent characterization of biosynthetic pathways from genome sequences, an approach that has been highly successful in bacteria and fungi (Van der Lee and Medema 2016). Much of what is known about pathways for the synthesis of natural products in plants until recently stemmed from the examination of individual pathways. In order to discover a large number of predicted genes and enzymes of biosynthetic pathways, multiple plant genomes can now be mined (Boutanaev et al. 2015). The challenges now lie in developing refined search methods to streamline this discovery platform, and in making sense of and exploiting the outputs. Understanding the features of the metabolic clusters that have been reported so far will be instrumental in enabling such methods to be developed. The biosynthetic gene clusters of natural products so far reported from plants range from ~ 35 kb to several hundred kb and consist of three to 10 genes. They include genes for at least three different types of biosynthetic enzymes (King et al. 2014).

8.4.1 Salient Features

8.4.1.1 Scaffolds and Networks –First Step in a Clustered Pathway

Plant metabolic clusters typically (but not always) contain the gene encoding the first committed pathway step and two or more genes encoding other downstream pathway enzymes. Examples of enzymes that catalyse the first committed step include oxidosqualene cyclases (OSCs) (for the synthesis of triterpenes) (Thimmappa et al. 2014), class I and II diterpene synthases (DTSs) (which together make diterpene scaffolds) (Zi et al. 2014), and the tryptophan synthase α homologue BX1 (which diverts indole-3-glycerol phosphate from tryptophan biosynthesis into indole for the synthesis of cyclic hydroxamic acids) (Frey et al. 1997). These enzymes draw metabolites from primary metabolism into specialized metabolism. A type III chalcone synthase-like enzyme required for the synthesis of diketone wax aliphatics in barley has also been reported (Schneider et al. 2016). While in most cases the nature of the ‘signature’ enzyme, and hence the type of scaffold, is evident based on cursory examination of these clusters, this may not always be the case. For example, the first committed step in cyanogenic glycoside biosynthesis (the conversion of amino acids to oximes) is catalysed by cytochrome P450 (CYP) enzymes

belonging to the CYP79 family (Saito et al. 2012). At first glimpse the scaffold for the cyanogenic glycoside clusters may not be immediately obvious. However, CYP79 enzymes are unusual amongst the CYP superfamily in having amino acids as their substrates (Zi et al. 2014). These CYPs convert amino acids to oximes, thereby siphoning them into specialized metabolism. Of note, CYP79 enzymes also catalyse the first committed step in the biosynthesis of noncyanogenic plant defence compounds such as glucosinolates and camalexin (Hamberger and Bak 2013), pathways for which the genes are not clustered.

Opium poppy (Papaver somniferum) produces a variety of different types of benzylisoquinoline alkaloids (BIAs), including the potential anticancer drug noscapine (Chen et al. 2015). A gene cluster for the synthesis of noscapine has recently been discovered in a high noscapine-producing poppy variety (Winzer et al. 2012). This cluster contains 10 genes encoding five distinct enzyme classes and includes the gene for the first committed step in the noscapine pathway- the conversion of scoulerine to tetrahydrocolumbamine by the O-methyltransferase PSMT1. One of the steps in the pathway has previously been shown to be carried out by the enzyme tetrahydroprotoberberine cis-N-methyltransferase (TNMT) (Liscombe and Facchini 2007). The TNMT gene is not represented within the noscapine cluster, although open reading frames with TNMT homology are present in the flanking regions. Unlike the rest of the pathway genes, TNMT is also present and expressed in non-noscapine-synthesizing poppy varieties and is likely to have additional functions (Winzer et al. 2012).

Analysis of a draft genome sequence for Madagascan periwinkle (Catharanthus roseus) has provided evidence for partial clustering of genes for the biosynthesis of the monoterpene indole alkaloids (MIAs) vinblastine and vincristine (Kellner et al. 2015). MIA biosynthesis is highly complex, involving condensation of the indole tryptamine with the iridoid secaloganin to form strictosidine. Strictosidine is a common intermediate in the biosynthesis of thousands of MIAs, including those of vinblastine and vincristine (De Bernonville et al. 2015). Thus, as for noscapine, the vinblastine/vincristine pathway is part of a much larger and more complex biosynthetic network that gives rise to a wealth of other diverse products. The MIA pathway also has one of the most complex and elaborate forms of compartmentalization known for a plant specialized metabolic pathway (Verma et al. 2012). The N50 scaffold size for the draft C. roseus genome sequence was in the region of 26–27 kb. Although not optimal for discovery of linked genes in plant genomes because of the large intergenic distances, Kellner et al. (2015), aided by bacterial artificial chromosome (BAC) sequencing, were able to identify seven small clusters each of two to three genes that contained genes encoding enzymes for vinblastine/vincristine biosynthesis and also other genes that may encode missing steps in the pathway, along with a gene for a predicted multi-antimicrobial extrusion protein (MATE) transporter that could potentially be involved in the transport of pathway intermediates. The development of a higher quality genome sequence will establish whether these small clusters are dispersed throughout the genome or whether they form a larger cluster, and how these genes are distributed relative to those required for the synthesis of other types of MIAs produced by *C. roseus*. A simplistic assumption may be

that clustering is a feature of dedicated linear pathways that are insulated from the rest of metabolism and that take place in the same cell types. Analysis of complex networks such as those for alkaloid biosynthesis in *P. somniferum* and *C. roseus* should help to test this hypothesis and to understand what precisely constitutes a metabolic gene cluster in the context of wider metabolism.

Most of the plant metabolic gene clusters that have been reported so far contain the genes encoding the enzymes for the first committed steps in the respective pathways. These may be obvious scaffold-generating enzymes (e.g. oxidosqualene cyclases; class I and class II diterpene synthases); other enzymes that divert metabolites from primary metabolism into specialized metabolism (e.g. the tryptophan synthase α homologue BX1, which catalyses the first step in DIBOA/DIMBOA biosynthesis; CYP79 P450s, which convert amino acids into oximes); or enzymes that siphon intermediates from complex specialized metabolic networks into dedicated pathways for specific end products (e.g. the O-methyltransferase PSMT1, which converts scoulerine to tetrahydrocolumbamine – the first committed step in the noscapine pathway). As more metabolic gene clusters are characterized from plants, the inventory of 'signature' enzymes that are diagnostic of particular types of biosynthetic pathways will grow, so making pathway prediction easier. In some cases, such genes do not appear to be present. For example, the tomato steroidal glycoalkaloid α-tomatine is predicted to be synthesized from cholesterol (Eich 2008). The genes for α-tomatine biosynthesis are located in one major cluster of six genes on chromosome 7, with a further two pathway genes adjacent to each other on chromosome 12. The CYP72 gene GAME7, which is predicted to catalyse the first step in this pathway (the conversion of cholesterol to 22-hydroxycholesterol) (Itkin et al. 2013), is also located on chromosome 7 but is 7880 kb away from the nearest cluster gene (Sato et al. 2012).

8.4.2 Tailoring Enzymes, Modularity, Collinearity and Moonlighting

CYPs, sugar transfers, methyltransferases, acyltransferases, dioxygenases, carboxylesterases, dehydrogenases / reductases and transaminases may be encoded in metabolic clusters (King et al. 2014; Boutanaev et al. 2015; Schneider et al. 2016). This list is likely to continue to grow as more clusters of natural products are discovered. The arbitrary definition of a metabolic gene cluster requires at least three different types of enzymes to contain genes. In this regard, it is important to note that the CYP superfamily is divided into families based on the similarity of sequences (family members with >40% amino acid sequence) (Nelson and Werck-Reichhart 2011), and that for the purposes of defining clusters each CYP family should be regarded as a different type of biosynthetic enzyme. The smallest plant metabolic clusters characterized so far are the Arabidopsis thaliana thalianol and marneral clusters. These are 35–38 kb of compact clusters (Field et al. 2011). The largest clusters are several hundred kb (Nützmann and Osbourn 2015). In some cases, the genes in the pathway are contiguous, with no other obvious intervening genes (e.g.

the thalianol cluster in A. thaliana and the avenacin cluster in diploid oat) (Qi et al. 2004). In other cases, some of the pathway genes are less tightly linked and there may be intervening genes with no obvious function in specialized metabolism between these 'peripheral' genes and the core cluster. For example, the the maize Bx7 gene, which encodes an O-methyltransferase required for DIMBOA biosynthesis (Jonczyk et al. 2008), lies within 15 Mb of the core cluster. Another gene, Bx9, which encodes a sugar transferase that is active towards DIBOA/DIMBOA, is located on a different chromosome (von Rad et al. 2001). In diploid oat, the Sad3 and Sad4 loci have been shown by mutation to be required for avenacin glucosylation (Qi et al. 2004) but not yet cloned. Sad3 is essential for avenacin glucosylation but is only loosely linked to the core cluster (genetic linkage distance 3.6 cM) (Qi et al. 2004). Full characterization of the wider avenacin cluster region will reveal the nature of Sad3 and the physical distance between this locus and the core cluster. Sad4is unlinked to the cluster, and unlike Sad3 has only a partial effect on avenacin glucosylation when mutated and also glucosylates other oat metabolites. Sad4 is therefore not a dedicated pathway component and appears to be 'moonlighting' (Mylona et al. 2008).

While it is important to emphasize that plant metabolic gene clusters contain at least three non-homologous genes encoding different types of enzymes in order to distinguish them from genome regions containing only tandem arrays of highly homologous genes, some of the characterized clusters contain duplicated genes that have developed different functions in some cases. A noteworthy example is the maize DIMBOA cluster, which contains genes for four CYPs belonging to the CYP71C subfamily (Frey et al. 1997). These closely related CYP genes (Bx2–5) are almost certainly the result of a common ancestor of duplication of tandem genes (Dutartre et al. 2012). However, functional analysis following expression in yeast has revealed that each carries out a highly specific and dedicated step in the conversion of indole (the product of the signature enzyme, BX1) to DIBOA (Frey et al. 1997). This group of four genes therefore provides some intriguing insights into the evolution of sequential CYP-mediated steps in this pathway.

A further intriguing finding is the observation that the genes required for a particular modification can be grouped within a cluster as a module. This is exemplified by a set of three genes within the oat avenacin cluster that are required for triterpene acylation. The major avenacin, A-1, is acylated with an N-methyl anthranilate group. This acyl group is introduced by the acyltransferase SAD7, which is a member of the SCPL family of acyltransferases. Unlike the BAHD acyltransferases, which use CoA-thioesters as the acyl donor, SCPL-acyltransferases use acyl sugar donors (Milkowski and Strack 2004). The acyl glucose donor used by SAD7 is N-methyl anthranilate glucose, which is generated by methylation of the readily available precursor anthranilate (a product of primary metabolism) by the methyl transferase SAD9 followed by glucosylation by the glucosyl transferase SAD10 (Owatworakit et al. 2013). The genes encoding SAD7, SAD9 and SAD10 are adjacent in the avenacin cluster, so forming and 'acylation module'. Interestingly, the genes within the noscapine cluster in poppy appear to show collinearity in that they are organized in groups that roughly correspond to the early, middle and late steps

of the pathway (Winzer et al. 2012). Occasional examples of apparent collinearity of metabolic gene clusters have been noted in bacteria and filamentous fungi (Osbourn 2010). Collinearity appears to be the exception rather than the rule in plants. Nevertheless, the fact that it occurs at all is noteworthy and suggests that, at least for the noscapine pathway, gene order (and by extrapolation temporal control of gene expression) may be significant for pathway function and/or potentially also for pathway assembly.

8.4.3 Core Clusters and Satellite Subgroups

Several examples of pathways that have core metabolic clusters along with one or two peripheral pathway genes have been mentioned earlier (the O-methyltransferase gene Bx7 and the sugar transferase gene Bx9 in maize, and the Sad3 locus required for glucosylation of avenacins in oat). In some cases, the pathways are more fragmented. For example, as already mentioned, most of the genes for the synthesis of the steroidal glycoalkaloid α-tomatine in tomato are clustered on chromosome 7. These include two early pathway genes – the dioxygenase gene GAME11, and the CYP72 gene GAME6 – along with the four sugar transferases, GAME1, GAME17, GAME18 and GAME2. Two further pathway genes encoding the transaminase GAME12 and the CYP88D GAME4 are adjacent to each other on a different chromosome (Itkin et al. 2013). All these regions, only the first contains genes for at least three different types of biosynthetic enzyme, i.e. fulfils the requirements to be classified as a metabolic cluster. The genes for the synthesis of the related steroidal glycoalkaloids α-solanine and α-chaconine are similarly organized in potato on chromosomes 7 and 12 in regions that are syntenic to those in tomato (Itkin et al. 2013). Thus it appears that these pathways share a common evolutionary origin but have acquired different functions in tomatoes and potatoes. By the same perfunctory, in cucumber (Cucumis sativus) five genes encoding enzymes required for the biosynthesis of cucurbitacins (triterpenoids that confer bitterness) are clustered on chromosome 6 – the gene for the oxidosqualene cyclase that synthesizes the triterpenoid scaffold cucurbitadienol, along with three different types of CYP genes (CYP81, CYP87, CYP89) and an acyltransferase gene. Four other CYP genes that are also required for cucurbitacin biosynthesis are located elsewhere (a CYP71 gene and two clustered CYP88 genes on chromosome 3; and a CYP87 gene on chromosome 1) (Shang et al. 2014). By extending such analyses out across the wider Solanaceae (for the steroidal glycoalkaloids) and the Cucurbitaceae (for the cucurbitacins), it should be possible to unearth other related pathways and enzymes that represent further variations on these themes. Such investigations will also help to establish whether these pathways may represent evolutionary snapshots of clusters in the process of assembling/disassembling/diversifying.

8.4.3.1 Architectures of Plant Metabolic Clusters

The archetypal plant metabolic cluster is compact and contains contiguous pathway genes, including the gene encoding the enzyme for the first committed step in the

pathway. In other cases, the genes for most of the metabolic pathway steps (including the gene for the first committed step) are clustered and contiguous, but there may be one or more peripheral genes encoding other pathway steps. These peripheral genes may be loosely linked to the core cluster (in cis) or unlinked (in trans). The relative importance of in cis and trans peripheral genes should become clearer as more plant metabolic clusters are characterized. It will also be interesting to determine whether these peripheral genes tend to encode specific types of enzymes (the earlier examples suggest that genes for sugar transferases may tend to be peripheral), although it is too early to generalize definitively. Pathways are more fragmented in other cases. In these situations, the majority of the pathway genes, including the gene for the first committed step, are organized into a core cluster with other genes present elsewhere in the genome as satellite subgroups. These satellite subgroups contain genes for one or two different types of adjustment enzymes for the examples reported so far. In the fourth scenario, the gene may be peripheral in the first committed step, as is the case with the α-tomatin cluster. However, it would be expected that these peripheral genes would bestrongly co-expressed with the clustered genes that encode the other steps.

8.4.3.2 Cluster Regulation

Clustered pathways for natural product biosynthesis are common in bacteria and fungi, and often contain genes encoding pathway-specific transcription factors that regulate the biosynthetic genes within the cluster (Brakhage 2013). Loss-of-function mutations in these transcription factors result in loss of expression of pathway genes, while overexpression can lead to coordinate up-regulation and enhanced metabolite production (Bergmann et al. 2007). Such transcription factors are therefore very useful tools, both for pathway delineation and for metabolic engineering. By contrast, the clusters that have been reported so far in plants do not appear to contain genes for pathway-specific transcription factors. However, recent advances are beginning to yield insights into how plant metabolic gene clusters are regulated.

In some cases, the temporal and spatial patterns of expression of certain clusters are quite well known. For example, the main product of the avenacin pathway for the synthesis of antimicrobial triterpene glycosides in oats, avenacinA-1, has a strong autofluorescence under ultraviolet lighting and can be easily located in the epidermal cells of the root tips and lateral roots, in accordance with its role in the defense of soilborne pathogens (Osbourn 2010). In-situ hybridization of mRNA indicates that the characterized genes of the avenacin pathway, including the first and last steps of the pathway, are specifically expressed in these types of cells (Qi et al. 2004; Mugford et al. 2004). Regulation of the avenacin pathway is therefore under strict developmental control and does not appear to be altered in response to pathogen attack or abiotic stress treatment. The avenacin pathway has evolved relatively recently in evolutionary time (since the divergence of oats from other cereals some 25 million year ago) and the ability to synthesize avenacins is restricted to oat. Interestingly, however, the promoters of the avenacin pathway genes retain very similar expression patterns when introduced into A. thaliana, rice and other plant

species as reporter fusion constructs, i.e. they are expressed in the epidermal cells of the tips of the primary and lateral roots, suggesting that that this pathway has hooked into an ancient root development process that is conserved across monocots and eudicots (Kemen et al. 2014). The class IV homeodomain leucine zipper transcription factor AsHDZ1 has been implicated in regulation of the avenacin gene cluster, but its function has not yet been validated in oat (Kemen et al. 2014).

The momilactone and phytocassane/oryzalide clusters in rice make diterpenes that are implicated in plant defence. Momilactones, oryzalexins and phytocassanes are phytoalexins (i.e. are produced in the leaves in response to pathogen attack or elicitor treatment), while oryzalides are present in unchallenged plants and so are regarded as phytoanticipins (Schmelz et al. 2014). Importantly, momilactones are also produced constitutively in the roots as part of normal growth and development, where they have been implicated in allelopathy (Kato-Noguchi and Peters 2013). These rice diterpene clusters are therefore subject to different types of regulation, both developmental and inducible. A chitin oligosaccharide elicitor-inducible basic leucine zipper (bZIP) transcription factor (OsTGAP1) coordinately regulates the gene clusters for the synthesis of momilactone and phytocassane/oryzalide diterpenes in rice (Miyamoto et al. 2014). However, this effect is not direct, as the promoters of the diterpene cluster genes are not targets for OsTGAP1 binding (Miyamoto et al. 2014). A conserved APETALA2/ethylene response factor (AP2/ERF) transcription factor (GAME9) is involved in regulation of synthesis of the cholesterol-derived steroidal glycoalkaloids α-tomatine in tomato, and α-solanine and α-chaconine in potato (Cárdenas et al. 2016). GAME9 control of steroidal glycoalkaloid biosynthesis is not restricted to the clustered pathway genes and also encompasses upstream genes of the cholesterol pathway. Although concerted changes in cluster expression are observed in GAME9 knockdown and overexpression lines, it is not yet known whether GAME9 directly regulates each cluster gene. Initial data suggest that GAME9 binds to the promoter sequences of some of the cluster genes (GAME4 and GAME7) cooperatively with an MYC2 transcription factor (Cárdenas et al., 2016).

In cucumber, two transcription factors that regulate cucurbitacin C synthesis in leaves and fruit, respectively, have been identified – Bl (Bitter leaf) and Bt (Bitter fruit) (Shang et al., 2014). Bl encodes a basic helix–loop–helix (bHLH) transcription factor required for cluster regulation. Yeast one-hybrid analysis, tobacco transient reporter (luciferase) activation assays, chromatin immunoprecipitation analysis and electrophoretic mobility-shift assays (EMSAs) have collectively shown that Bl can selectively bind to cis elements within the promoter of the gene encoding the first step in the cucurbitacin C pathway Bi. Bt is also a bHLH transcription factor and lies within 8.5 kb of Bl. The bt allele confers the nonbitterness trait of cultivated cucumber and has been selected during domestication. Bl and Bt can specifically bind to the promoters of nine different cucurbitacin C pathway genes, including those of the core cluster and satellite subgroup. Bl and Bt are on a different chromosome to the cucurbitacin C core biosynthetic cluster and satellite pathway genes.

In some cases, genes do not showconsistent co-expression in reported clusters. For example, the cyanogenic glycoside cluster in Lotus japonicus and the tomato

terpene cluster differ from the general pattern of metabolic gene clusters for co-regulation. The former is characterized in young leaf tissues by the overlapping expression of all clustered genes. The abundance of CYP79D3 and CYP736 transcripts in older leaf tissue is reduced, but the third pathway gene UGT85K3 remains constant (Takos et al. 2011). Tomato's terpene cluster shows coregulation of gene subsets. Sl-CPT1, Sl-TPS19, Sl-TPS20 and Sl-TPS41 are primarily limited to trichomes of stem and leaves, while Sl-CPT2, Sl-CYP71BN1 and Sl-TPS21 show highest expression in the petiole and Sl-CPT18 is expressed in the roots. Other examples of inconsistencies in expression patterns of cluster genes have also been reported (von Rad et al. 2001).

There is good evidence in filamentous fungi that gene clusters are regulated by chromatin-based mechanisms for natural product pathways (Brakhage 2013). There is increasing evidence that this is also the case in plants. Analysis of high-resolution DNA fluorescence in situ hybridization (FISH) indicates that chromatin decondensation is associated with the expression of the oat avenacin cluster (Wegel et al. 2009). Clusters of metabolic genes in A. Thaliana, maize, oat and rice show pronounced trimethylation of histone H3 lysine 27 (H3K27me3) and levels of this chromatin mark correlate inversely with cluster expression (Yu et al. 2016). H3K27me3 is a preserved modification of histone in eukaryotes and is usually associated with gene silencing. The thalianol and marneral clusters in A are consistent with this. Thaliana show increased expression in Polycomb mutants that are compromised in H3K27 trimethylation, and reduced expression in mutants for PICKLE, a positive regulator that counteracts H3K27me3 silencing (Yu et al. 2016). These two A. thaliana clusters are also positively regulated (either directly or indirectly) by the SWR1 chromatin-remodelling complex, which is required for the deposition of the histone 2 variant H2A.Z into nucleosomes, suggesting that H2A.Z is required for normal cluster expression. A model is emerging based on analysis of chromatin modifications and mutants in which H3K27me3 is implicated in cluster silencing and H2A.Z in cluster activation (Nützmann and Osbourn 2015). Importantly, these features can be used in genome-wide analyses to mine for new metabolic gene clusters (Yu et al. 2016). Although mutations in chromatin regulators have been reported to affect the biosynthesis of phenylpropanoids and glucosinolates, compounds that are made by unclustered pathways (Walley et al. 2008), it is not clear whether these effects are direct or indirect. RNAseq analysis of H2A.Z mutant lines did not support a role for this chromatin mark in the regulation of nonclustered glucosinolate and flavonoid pathways in *A. thaliana* (Yu et al. 2016).

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Sustainable Agriculture: Future of Plant Biotechnology

9

Abstract

Agricultural sustainable development is a very complicated issue and substantive by other factors like water resource shortage, cultivated land decline, environmental pollution etc. Early civilization was strongly base on agriculture and as such a need for alteration of crop which has been a longstanding practice which is traceable to 2500–2000 BC as recorded that in Africa the ancient Egyptians. However the scientific and technological advances of the past century have greatly expanded the breadth and power of agricultural innovations. There now exist a remarkable array of technologies to improve crop production and is documented in this chapter besides the biotechnological approaches to improve crop sustainability are also mentioned in the last section of this chapter.

Keywords

Biotechnology · Sustainability · Traditional · Innovation network · Models

9.1 Challenges of Sustainable Agriculture

Although food self-sufficiency is predicted to be acceptable in the next 50 years, agricultural sustainable development is still challenged by water resource shortage, cultivated land decline, environmental pollution, faults in the mechanisms for protection of cultivated land and inefficiencies in the management of land tenure rights.

9.1.1 Agricultural Water Use

In agricultural production, the use of agricultural water for irrigation and animal husbandry plays an important role and is the main component in the total use of water. The total use of agricultural water in China in 2000 was reported to be

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378.3 billion m^3, representing 68.83% of the total national water use. In 2000–2050, quota estimation and scenario analysis were used to predict agricultural and other water demands in China. The results show that demand for agricultural water will increase from 384.4 million m^3 in 2010 (64.29% of all water use) to 402 million m^3 in 2050. At the same time, by 2040, the national total demand for water will reach 677 billion m^3, which is close to the maximum available water resource in China (China's exploitable water resource is approximately 850–1100 billion m^3; Li and He 2000). There will therefore be a potential conflict between water demand and supply, and in the future there will be more competition for water. Agricultural water use will undoubtedly remain less competitive because in agriculture, the value of water output per unit is lower than in industry. In addition to scarce water resources the efficiency of agricultural water use is relatively low. The average use coefficient of the irrigation pipe is below 0.6, which means that nearly half of agricultural water is wasted during transport and about 140 billion m^3 of water is lost annually. Currently, 0.96 m^3 of water is required in developing countries to yield 1 kg of grain, which is twice that of developed countries. Israel, for example, only needs 0.43 m^3 of water to produce 1 kg of grain (Jiang 2001). The efficiency of water use can be increased in two ways:

(i) To modify pipe systems in order to reduce unnecessary transport losses.
(ii) Water saving technology and equipment must be advanced. Current flood irrigation can, for example, be transformed by small border irrigation or long border irrigation, sprinkler irrigation technology and equipment, drip irrigation and micro-sprinkler irrigation technology (Wang and Jiang 1998).

9.1.2 Cultivated Land Resources

9.1.2.1 Driving Forces for Cultivated Land Loss in the Future

Land supply and demand and land use are determined primarily by population, economy, society and natural conditions (Li et al. 2001). From the point of view of complex socio – economic and natural systems, the driving forces for the loss of land in cultivation around the world in recent decades have included an increase in the population with a trend of continuous growth, socio – economic development and environmental pressure (Yan et al. 2005). The growing population is the biggest challenge for global sustainable development and the main reason for the shortage of resources and environmental degradation (Heilig 1999). Continuous population growth before 2030 will undoubtedly increase the demand for cultivated land, grassland, and forest and construction land. The economic benefit of land use is the decisive parameter in the distribution of land resources, and cultivated land is often regarded as a relatively low economic benefit (Brown 1995). The growing demand for cultivated land can therefore only be met by the use of marginal land. However, intensive expropriation of marginal land would cause environmental problems, such as soil erosion and land degradation, which would threaten land use sustainability (Tian et al. 2003).

Developed countries 'experience shows that industrialization is always accompanied by the loss of cultivated land. During the process of industrialization, the decrease in cultivated land in developing countries appears normal. China's cultivated land is reported to be declining at an alarming rate, threatening China's food security and sustainable agricultural development. During 1996–2003, China's GDP increased by CNY 4880 billion, based on statistical data from CSSB; during the same period, China lost 6.65 Mha of cultivated land. This means that an increase in GDP of CNY 1000 leads to a loss of cultivated land of 1.36 ha. The cultivated loss of land due to industrialization is mainly a result of building and urban expansion use. The loss of cultivated land in 1996–2003 accounted for approximately 1.33 Mha of cultivated land, accounting for 43.8% of the total loss of cultivated land (excluding the loss of the Grain-for-Green Programme). Although urbanization has both positive and negative effects on cultivated land (Li and Liu 2003), an average decrease in cultivated land productivity is observed. Some studies have shown that more than 0.2 ha of reclaimed land must compensate for the loss of productivity of only 0.06 ha of cultivated land used for urban expansion (Yu and Hu 2003). The quality difference between the lost cultivated land and the recovered land that replaces it has reduced the overall quality of the cultivated land in China. According to research by the Institute of Remote Sensing Applications, CAS, the center of cultivated land in China has moved north 28.34 km and from 1985 to 1996 the quality of cultivated land has decreased by 2.52% (Gao et al. 1998). The patterns of land use are clearly influenced by policy. Previous inappropriate policies, such as "grain production as the core of agriculture" (yi liang wei gang), have caused environmental problems in China, although agricultural land has been increased in order to implement the policy (Yan et al. 2006). In the case of India, although the urban population has increased significantly in the last two decades, from 217 million in 1991 to 377 million in 2011 (Indian Census 2011), most of the country's urban transition has not yet taken place. India's urban population will grow by nearly 500 million between 2010 and 2050 (United Nations 2014), according to the United Nations. The area of non-agricultural land has more than doubled over a 50-year period, from 9.36 million hectares in 1951 to 22.97 million hectares in 2001 (Chadchan and Shankar 2012). India continues to lose its agricultural land by: (a) urban conversion (Fazal 2001) (b) reduction in farming suitability (Prokop and Poreba 2012) and (c) abandonment of agricultural land (Dhanmanjiri 2011). The country is currently facing the dilemma of increasing agricultural productivity while converting highly productive agricultural lands to urban uses (Brahmanand et al. 2013). The world has recognized these problems and adapted its agricultural policies to include the protection of the environment and environmental restoration. Several programs have been successfully implemented since the 1990s (Natural Forest Protection Programme, the Desertification Combat Programme, the Grain-for-Green Programme). Although these programs, in particular the Grain for Green Programme, cause a great deal of land loss in cultivation, their environmental benefits are vital in the long term for sustainable agricultural development. More serious environmental degradation due to inappropriate human activity has led to

a reduction in cultivated land and a reduction in the quality of cultivated land, such as land degradation and soil erosion, desertification and salinisation.

9.1.2.2 Protection Mechanisms for Cultivated Land

In order to ensure global food security and sustainable agricultural development, effective protection of cultivated land is essential. The current protection mechanisms generally have two major problems: (i) programming for land use, in which the authority of the relevant administrations can not be effectively countered, and there is no joint programming for land use and urban construction, and (ii) the protection of basic agricultural land, which focuses solely on the area of agricultural land in a protected area, but does not take into account the productivity of agricultural land, which leads to the degradation of agricultural land.

In order to overcome these restrictions on sustainable agricultural development, it is suggested that the following measures should be taken: (i) harmonization of different patterns of land use in order to meet the demands of economic development and agricultural production, (ii) improvement in the protection of the environment and the environment when additional land is cultivated, (iii) development of appropriate land (iv) increase capital investment to consolidate land and recultivate abandoned land. In addition, improving the efficiency of land use will also reduce the pressure on agricultural production due to the lack of cultivated land. According to our estimate, accommodation capacity can be improved by approximately 40% if the plot ratio is increased from 0.3 to 0.5. About 3.7 Mha of land is reported to be saved if land for residential use in rural areas is reduced to 120 m^2 per capita and 6 Mha of land is saved if it is reduced to 100 m^2 per capita. In the next 30 years, it is therefore possible to meet the demand for land for construction for a high population.

9.1.3 Tenure Mechanism of Agricultural Land

The basis of land tenure rights in agriculture is the system of household responsibility (HRS). Agricultural land is collectively owned, but individual households are responsible for land use and management. As farmland owners, households can benefit directly and indirectly. Land offers potential employment opportunities for farmers who have fewer opportunities to work in cities (Brandt et al. 2004), but, more importantly, access to agricultural land can help farmers avoid uncertainty in the supply of food and income and provide the necessary food security and insurance (Tao and Xu 2005). In the 1980s, the HRS stimulated the enthusiasm of farmers for production and accelerated agricultural production (CSSB 2006). However, the positive effects on the long-term improvement of agricultural productivity have gradually disappeared and the negative effects are becoming increasingly apparent. The HRS is associated with at least three problems:

(i) Agricultural land is divided into small parts and one household should farm each piece. The average agricultural land area for each household was found to

be 9.3 mu (1 ha equals 15 mu) and in the mid-1980s was even subdivided into smaller parts. The average area for one household decreased to 8.47 mu in 1990 (Zhao 2001). These fragmented areas of land can only support small-scale farming and restrict the use of modern agricultural technology.

(ii) The identification of farmers is a decisive factor in obtaining access to agricultural land. If access is lost, there is no economic compensation. Peasants engaged in non-agricultural activities are therefore reluctant to give up their access rights, which leads to a lack of investment in the inefficient use of some agricultural land.

(iii) To some extent, the separation of ownership and access to agricultural land has made farmers focus primarily on short-term outputs from agricultural land, leading to a decrease in the quality of land and damaging the sustainability of land use (Yu et al. 2003).

There are shortcomings in the current policy on land expropriation. Farmland can be converted into building land by expropriation, but the expropriation process lacks careful planning and the price of compensation is quite low (Guo 2001). This encouraged the rent of government land and encouraged the sale of land (Zhang 2000). In addition, a reasonable price is not created by an effective land market (Dowall 1993). The lack of an economic compensation system is an important reason for inefficiency in the protection of cultivated land, which leads to breaches of land use regulations and unreasonable land use. The current land tenure rights system should be reformed and HRS improved in order to solve these problems. It is suggested that the following measures be taken: (i) to identify separate land ownership rights and access rights, and to define reasonable rights and responsibilities of owners (collectively) and managers (peasants), (ii) to strengthen the supervision of land use in cultivated land by collective and government action, (iii) to improve the transfer mechanisms for land use rights, and to establish multiple distributions of land use rights in accordance with market rules (iv) establish market distribution mechanisms for agricultural land (Zhang 2000), and accelerate the introduction of price mechanisms suitable for the land market, (v) establish and improve the compensation tax system for the use of land and the reclamation of new building land, and increase the effects of economic measures, such as land prices and land taxes, on the adjustment and control of land use, and (vi) establish economic compensation mechanisms for the protection of cultivated land and adapt mechanisms for the participation of benefits in order to solve external problems and unparalleled cost profits in the protection of cultivated land.

9.1.4 Fertilizers and Pesticides Usage

In 2005, 47.66 Mt. fertilizers were used in China, including 22.29 Mt. nitrogen fertilizers, 7.44 Mt. phosphate fertilizers and 4.90 Mt. potash fertilizers (CSSB 2006). Fertilizers 'contribution to yield in China, however, is still low. The average contribution of fertilizers to the yield of grain is only 46.43%, which is increased by

approximately 8.84 tons of fertilizer/tons used (Peng 2000). On the basis of this contribution to yield, fertilizer consumption in the twenty-first century will be increased by at least 10.22–11.08 Mt. in support of a 100 Mt. increase in yield. In Asian countries, the average consumption of fertilizers in cultivated land in China and India is 356.7 kg per hectare, which is twice the maximum consumption of fertilizers in developed countries; the consumption of nitrogen fertilizers in cultivated land is 170.9 kg haK, 2.5 times higher than the world average; phosphate fertilizers are 56.6 kg haK, 1.86 times higher than the world average. These numbers suggest that fertilizers in Asian countries are being overused. On the other hand, fertilizer efficiency is relatively low. The efficiency of fertilizer nutrient use is reported to be only 30−40% in developing countries, which is only half that in developed countries (60−70%) in developed countries. Over-consumption and low fertilizer efficiency cause a large number of unabsorbed fertilizers in the soil, leading to a number of environmental problems. The China Council for International Environment and Development Cooperation (2004) reported that approximately 1.23 Mt. of nitrogen is discharged into rivers and lakes annually, 494,000 tons into underground water and 2.99 Mt. into the atmosphere. About 60 per cent of the annual use of fertilizer N in the Yangtze River area is lost from non-point sources of gaseous and agricultural products (Shen et al. 2001). China has the highest consumption of pesticides in the world. The total consumption of pesticides increased from 86 in 2003 to 1.33 Mt. 2003 from 862,000 tonnes in 1983. Abuse and low efficiency of the use of pesticides are also common in conjunction with fertilizer problems. According to the Chinese Academy of Agricultural Sciences (Zhang et al. 2004), 40% of pesticides used in the production of rice and 50% in the production of cotton are not necessary. The high proportion of highly hazardous pesticides in agriculture is another problem with the use of pesticides. Methamidophos, dimethoate, parathion, methylparathion and dichlorophos were frequently used in 1990 and accounted for 90,800 tonnes (Zhong et al. 2000). In Asia, Africa, Latin America, the Middle East and Eastern Europe, the environmental pollution caused by pesticides is now serious. Even in earlier years, DDT, lindane and dieldrin residues in fish, eggs and vegetables were far beyond India's safe range (Wu 1986). In India, the human body's DDT content was the highest ever. In the 1990s, the global sale of pesticides remained relatively constant, ranging from $270 to $300 billion, of which 47% were herbicides, 79% were insecticides, 19% were fungicides/bactericides and 5% were insecticides (Table 9.1). Herbicides ranked first in three major pesticide categories (insecticides, fungicides) between 2007 and 2008.

Fungicides/bactericides rapidly increased and ranked second. Europe, supported by Asia, is now the world's largest consumer of pesticides. China, the United States, France, Brazil and Japan are the world's largest producer of pesticides, consumers or traders. Fruit and vegetable crops are used with most pesticides worldwide. Pesticides, mainly herbicides, are mainly used for maize in the developed world. In agricultural environmental systems, pollution caused by careless and over-use of pesticides and fertilizers has become more severe. It is reported that there were 891 agricultural pollution events in 23 provinces in China in 2000, 40000 ha of agricultural pollution In addition, the lack of use of organic fertilizers has resulted in a

Table 9.1 List of some ambiguities in innovation

	Type of uncertainty	Issues on which there is uncertainty
1.	Technological uncertainty	Characteristics of the innovation (such as costs or performance)
		Relation between the innovation and the infrastructure in which it is embedded
		Uncertainty to what extent adaptations to the infrastructure are needed
		Possibility of choosing alternative (future) options
2.	Resource uncertainty	The amount and availability of raw material, human and financial resources
		How to organize the innovation process (e.g. in house or external R&D?)
3.	Competitive uncertainty	Behaviour of (potential or actual) competitors and the effects of this behavior
4.	Supplier uncertainty	Actions of suppliers as regards timing, quality and price of the delivery
5.	Consumer uncertainty	Consumers preferences with respect to the innovation
		Consumers' characteristics
		Long-term development of the demand over time
6.	Political uncertainty	About current policy (e.g. regarding interpretation or effect of policy, or a lack of regulation) or about future changes in policy, as well as reliability of the government

Source: – (Meijer et al. 2007)

reduction in organic soil, unbalanced soil nutrition and a reduction in fertility (Ma and He 2002). The content of organic matter in cultivated land in Asia fell to 1.5%, much lower than in North America (2.5–4.0%) and Canada (3.0–4.5%); the content of organic matter in black soil in the north-west Himalayas fell from 8–10% to 1–5% (Liu 2002). In order to achieve more sustainable agricultural production and agroecosystems, effective measures must be taken to control the abuse of fertilizers and pesticides and to improve the efficiency of the use of fertilizers and pesticides. We propose these measures: (i) Standardize pesticides and train farmers to correctly use the relevant technology, (ii) Improve existing production techniques and strengthen research and development on new fertilizers and safe pesticides, and (iii) Improve farming technology to eliminate the overuse of fertilizers and pesticides.

9.1.5 Ecological Agriculture

A new opportunity, eco-agriculture, is the combination of modern scientific, technological and traditional agriculture in the world. This requires the use of ecological theory and system science methods. Eco-agriculture has been widely recognized throughout the world as an effective tool and a general approach to sustainable agricultural development. The development and application of eco-farming is not without problems, however:

(a) The lack of sufficient research on the theory and methods of eco-farming. Technological innovation, the introduction and application of high technologies in eco-farming are slow and do not support the development of eco-farming.
(b) Low industrialization in eco-farming. At present, eco-agriculture is only a production system that focuses on agricultural production and neglects the production-market relationship. In addition, the characteristics of global agricultural production prevent eco-farming from industrializing. These include: more population with less cultivated land; a shortage of agricultural input; and the HRS system.
(c) Inefficient measures to promote eco-farming. Although economic, social and ecological benefits have been achieved in eco-farming regions, effective technologies and eco-farming methods have not been more widely popularized and the benefits of eco-farming for environmental protection are limited.

In order to develop eco-agriculture globally, the following measures will be required:

(i) Strengthening research in the theory and technology of eco-agriculture. The research should focus on: (1) Theory of agricultural ecological systems, (2) The application of the theory of scarcity of resources, the theory of externality and methods for eco-agriculture, (3) The introduction and use of high technology, such as genomic technology, information technology and other technologies, (4) The assessment of the impact of modern techniques on ecosystems and measures to protect against negative effects (5) Advancement in the design of the ecological engineering model.
(ii) Establishing advanced eco-agricultural engineering models based on current models and application conditions. It is also convenient and effective to optimize traditional technology for ecological benefit.
(iii) Seeking a model for eco-agricultural industrialization that fits local conditions. Zhou et al. (2004) proposed three practical ways: the comprehensive development way through optimization grouping or regrouping, the economic way according to marketing direction and the protective way according to limit of resources.
(iv) Popularizing eco-agriculture through information service and financial support. More technology information service and financial support need to be provided to households to promote the conversion from the traditional production model to the eco-agricultural model.

9.2 Sustainable Agriculture Innovation Network

The thinking of agricultural innovation systems (AIS) has become an increasingly used framework for analyzing technological, economic and institutional changes in agriculture (e.g. Devaux et al. 2009; Spielman et al. 2009). In the AIS approach, innovation is considered to be the result of a networking process and interactive

learning between a heterogeneous set of actors, such as farmers, input industries, processors, traders, researchers, extensionists, government officials and civil society organizations (Röling 2009). The AIS approach emphasizes that agricultural innovation is not only about new technologies, but also institutional change. Assuming the interaction between heterogeneous actors related to various dimensions of agricultural innovation (e.g. development of technology, institutional change, reorganization of the supply chain, market development, creation of social acceptance), it has been noted that AIS can be regarded as a complex adaptive system (CAS) (Hall and Clark 2009; Spielman et al. 2009). These are defined as self-organizing systems "whose properties cannot be analyzed by studying their components separately formed by many different types of agents, in which each defines its strategy, reacts to the actions of other agents and changes in the environment and tries to modify the environment in a way that suits its needs. Assuming the interaction between heterogeneous actors related to various dimensions of agricultural innovation (e.g. development of technology, institutional change, reorganization of the supply chain, market development, creation of social acceptance), it has been noted that AIS can be considered as a complex adaptive system (CAS) (Hall and Clark 2009; Spielman et al. 2009). These are defined as self-organizing systems "whose properties cannot be analyzed by studying their components separately formed by many agents of different types, in which each defines its strategy, reacts to the actions of other agents and changes in the environment and tries to modify the environment in a way that is appropriate to them (Spielman et al. 2009). Elsewhere, this environment is indicated as the incumbent 'socio-technical regime' (Geels and Schot 2007), and efforts to change it in favor of the realization and durable embedding of an innovation have been called 'effective reformism' (Roep et al. 2003).

There are several studies on the self-organizing nature of agricultural innovation systems and how they are linked to effective reformism processes, but their analytical focus is often at a high level of aggregation, such as the macro-level of a whole country and a very long time horizon, such as a process of change that takes several decades. Examples include studies on innovation in zero-tillage by Ekboir (2003), developments in the Green Revolution in India (Biggs 2007), innovation in food systems in Uganda (Hall and Clark 2009), rice innovation in Nepal (Pant and Hambly Odame 2009), agricultural development in Kenya (Ochieng 2007), and innovation in irrigation systems in Morocco (Poncet et al. 2010). Despite the usefulness of such an analysis to understand the main forces of socio-technical change in agriculture, such a focus risks failing to fully understand the activities of innovating actors in support of such change. The question addressed in this article is how forces contribute to socio-technical change at the micro level of individual innovation networks. By providing detailed insights into how actors interact with their environment at various levels in agricultural production systems and agri-food chains in their innovation efforts, we hope to contribute to building blocks for adaptive agricultural innovation policies that can address the unpredictability of agricultural innovation policies that can deal with the unpredictability of innovation processes. The article continues by drawing a conceptual framework of agency and innovation networks.

9.2.1 Innovation Agency and Structure

However, the CAS perspective on AIS interaction comprises several types of interaction between actors and their environment (e.g. between human actors and artifacts, i.e. technologies), the emphasis in this article is on social interaction in innovation processes. This focuses on the relationship between the agency of actors and the social structure described in the structuring theory of Giddens (1984). The Agency is capable of taking action and making a difference during events (Giddens 1984). Resources and competencies in the context of innovation determine the "innovation agency" available to an actor or organization for innovation (i.e. knowledge, skills, material, and financial resources). It also includes institutional features such as actors' norms and rules, a so-called 'innovation template' that orients and legitimizes action (Edwards 2000). No single actor can pursue its innovation objectives in self-organizing innovation systems without taking other actors into account due to a lack of sufficient power and resources (Aarts et al. 2007). This perspective allows actors to interactively shape a support network in order to achieve individual and collective goals (Engel 1995) and to obtain resources whose nature and source is unknown (Edwards 2007). The idea of a support network presupposes an innovation network with voluntary membership, known as "innovation configurations" (Engel 1995) and "coalitions" (Biggs and Smith 1998). This does not mean that the interests of partners in the innovation network are automatically aligned, since innovation networks are the negotiating scenes (Wiskerke and Roep 2007). In addition, an innovation network is not stable in the sense of a support network: It can change in composition over time. These innovation networks depend on many other peripheral players in their institutional environment, whose involvement may not be voluntary, but based on mutual interdependence. In Giddens' theory of structuring, actors and structures (i.e. their institutional environments) have a dual relationship, because the "structural properties of social systems of social systems are both medium and outcome of the practices they recursively organize" (Giddens 1984). This means that their environment conditions actors, but they actively or passively change their environment by their actions, so that another form of conditioning is exercised in turn. This reflexive relationship between actors and their institutional environment, which actors can adapt, change or complement, was called mutual embedding in the study of innovation systems (Markard and Truffer 2008). Actors carefully monitor the actions and aspects of the environments in which they operate, taking into account past, present and future events (Edwards 2007), in order to achieve their objectives and reduce the uncertainty in the process of achieving them (Geels and Schot 2007). Innovative actors' objectives are often embodied by more or less articulated visions that have an influential function to mitigate guidance, convincing, binding and uncertainty (Berkhout 2006). This is especially important, because innovation exposes many uncertainties to innovative players. These include, for example, complementary acquisition of resources, consumer demand development, adversity or instability in policy and legislation, and the behavior of network partners and competitors (Meijer et al. 2007). Although actors can deliberately try to reduce these uncertainties in their institutional environment, they are always

limited in their influence. In limiting or conditioning further activities, unintended consequences of the agency as well as external events outside the sphere of influence of agents themselves play an important role. These are therefore an important source of structural variation (Alexiou and Zamenopoulos 2008). For example, consumer preferences, government policies and market factors at regional, national and global levels influence innovation (Blay-Palmer 2005).

It is now clear that shaping innovation involves "selling a good story" (e.g. visions, speech), told by the right people (with conviction, credibility, power), at the right time, at the right place, and to the right people (acquiring complementary resources, building and capitalizing on momentum and using windows of opportunity). Since innovation actors must constantly react to their environment, which they actively try to change in their favor, it has been argued that this calls for adaptive innovation management (Smart et al. 2007), based on ideas from generic literature on CAS management (Westley 2002). This means that agricultural innovation policies should not seek to fully plan, control and manage the agricultural innovation system, but should be based on the likelihood of events, increase the likelihood of desired results and reduce the likelihood of unwanted results (Poncet et al. 2010). Although the AIS approach has proven its value as an analytical framework, it must still be transformed into an operational concept with policy options and targeted interventions to improve the ability to innovate everyday (Spielman 2006). The present study hopes to contribute to the understanding of innovation's microfoundations and thus optimally formulate adaptive innovation policies. Spielman et al. (2009) indicate that the analysis of the history of innovation focusing on important events is a useful method for mapping the dynamics and structured analysis of the interaction of innovation systems at the micro level, which was also applied in the analysis of "mainstream" innovation systems, where it is referred to as the analysis of innovation journeys (Van de Ven et al. 1999). Data were collected through semi-structured interviews with innovation networks and institutional environmental actors, who were both respondents telling their own experiences and informants giving a broader picture and observations. The interviews have been fully transcribed and analyzed using qualitative data analysis (Atlas 5.0). In order to reconstruct agency-structure interactions, the perspectives of both innovation network and institutional environmental actors were analyzed. This analysis was supplemented by an analysis of a range of internal network documents (minutes of meetings) and external documents (e.g. policy documents and journal articles). In addition, this multi-stranded approach allowed triangulation: A research methodology to prevent the risk of post-factual account distortions, increasing internal validity. Non-technological innovation is increasingly regarded as important for more socially sustainable farming systems, for example by reconfiguring the value chain (Devaux et al. 2009) and new arrangements for cooperation between different farms and non-farms (Veldkamp et al. 2009). One case relates to non-technological, organizational innovation in order to achieve social sustainability in arable farms that are too small to survive and have insufficient assets to sustain autonomous growth. This innovation can essentially be regarded as an innovation under the radar. Such innovations are not driven by politics or research, but are emerging from the bottom up

and increasingly considered as relevant sources of change (Hall and Clark 2009). These farmers sought to formally pool land, labor and other resources to increase scale in order to establish a joint venture (called Sjalon). This is an exception to the normal situation for individual family farms in the Netherlands. Other initiatives to increase the collective scale have emerged (Stevens 2007) in response to the need to create economies of scale in order to cope with low agricultural products prices (Röling 2009). The other case deals with innovation induced by research, which relates to the development of a concept of poultry husbandry that is respectful of the environment and animal welfare (Rondeel). This concept is the result of an interactive policy design process involving policy, business and societal stakeholders from the poultry sector (Groot Koerkamp and Bos 2008). This case forms part of in broader developments in animal welfare innovations in Dutch animal husbandry (Wiskerke and Roep 2007), in contrast to a production system characterized by industrialized animal production with low animal welfare. It combines both technological innovations (development of the Rondeel system) and non-technological innovations.

9.2.2 Event Analysis Sjalon

The starting point of Sjalon was a farmer's recognition that his farm was too small for his son to create a sustainable farming future. He decided to form a brainstorming group with non-agricultural actors (e.g. a machine vendor, an agricultural researcher) to develop the idea of increasing the scale by setting up a collective farm with a clear division of tasks. After a land measuring device symbolizing expansion, the project was called Sjalon. After a facilitator was hired to structure the process and help acquire resources such as financing and knowledge to concretize and materialize the plan, brainstorming gradually transformed into actual plan development. An important event was a research project by the Free University of Amsterdam, which calculated the effect of establishing a collective farm and thus made the vision more tangible. This enabled Sjalon to obtain funding from the province of Flevoland to draw up an initial business plan, assisted by an accounting firm and an applied research institute, which calculated the cost and return of collective farms. This business plan helped Sjalon obtain further support from influential parties such as the Ministry of Agriculture, Nature and Food Quality (Netherlands acronym: LNV). LNV referred TransForum (Veldkamp et al. 2009) to the Sjalon initiative, an innovation program to fund and facilitate the transition to sustainable agriculture. In addition, clear figures have been instrumental in recruiting potential participants into the plan and approaching banks for credit facilities for Sjalon. However, Sjalon never succeeded in attracting sufficient participants to set up the initially planned 600 ha farm (which is large according to Dutch standards), which also influenced banks 'willingness to provide loans, but eventually the remaining three participants managed to start a 100 ha farm. From 2004 to 2008, a legal problem was whether a collective farm would be legally permitted, given land tenure legislation in the Noordoostpolder (North-East Polder: NEP). Much land in this area is owned by the

government and allocated by the Treasury Department (TD) to farmers on long-term leasehold. Since this leasehold is personal, a situation that combined several leases (as would be the case in Sjalon) would be considered illegal sub-leasing. After long negotiations between Sjalon and the TD, an arrangement was concluded in 2008 that allowed individual farms to join a collective farm legally. Sjalon was therefore founded on 14 March 2008 as a limited liability company.

9.2.3 Agency in Particular Agency-Structure Interaction Loci in the Sjalon Case

The Sjalon innovation network dealt with the reduction of a number of uncertainties in the interaction with the institutional environment: (a) to obtain financing for their collective farm venture (financial uncertainty), (b) to obtain legal approval for a type of farm that did not fit leasehold legislation (political uncertainty), and (c) to ensure that farmers in the NEP are interested in participating in the NEP.

9.2.4 Sjalon Interaction Locus A: Finding Funding to Sustain the Development and Implementation of the Innovation

Sjalon's efforts to obtain financing can be divided into two separate but interconnected efforts: One to obtain funding to support the search and development process and the other to obtain funding for the eventual collective farm. As for the former, although the brainstorming group initially financed the facilitator, the Sjalon had to find additional funds for research and development and consultancy. The facilitator arranged for students from the Free University Amsterdam to carry out a feasibility study free of charge through his network of contacts. This study proved instrumental for Sjalon in obtaining funding from the deputy of the province of Flevoland, who was approached through informal facility contacts. The idea of Sjalon arrived at the right time for the provincial deputy and was in line with the ideas and sympathies of the deputy. Although there was no clear provincial development policy on initiatives to increase the scale, Sjalon was able to apply for an innovation subsidy and obtain it. This subsidy financed a legal-fiscal exploration of the legal form of Sjalon and drafted a business plan. This plan showed that a significant increase to more than 600 ha was needed to make the plan viable. Due to restrictions on state support, the province could not support this financially. They could only help to make spatial planning possible. The province therefore referred Sjalon to LNV, which would have more funding available (although LNV stated that it fell more within the remit of the provinces and municipalities). The Sjalon network met LNV's initial skepticism and felt that it was not taken seriously. Since the researcher in the brainstorming group was the then agriculture minister's nephew, however, he approached his uncle informally. Since the minister felt that Sjalon was a self-organized initiative that fitted well with the policy of agricultural innovation aimed at bottom-up changes, he ordered that a formal letter sent by Sjalon to LNV be taken

seriously. Then LNV officials entered into talks. As a result of these talks, LNV officials referred Sjalon to the TransForum innovation program financed by the government. TransForum has taken over the financing of the previously hired facilitator and has financed additional research and development and consultancy (to recalculate and adjust the previous business plan), as well as facilitation itself. The business plan was also important in dealing with the dual purpose of the bank. First, the then director of the local cooperative agricultural bank created a positive attitude towards the idea. This director began championing the idea and gave the Sjalon network the impression that the bank was prepared to take a risk and to support an innovative effort. It later served to convince the bank that there was a prospect of return on investment when the new bank director judged the plan on the basis of normal credit provision criteria and hesitated to provide a loan because there were no comparative cases for assessing risks and returns. It was unfortunate that the old director had left, according to the Sjalon network, because they had to face a less favorable financial environment that had become risk aversive. In addition, as the business plan now envisaged a 100-ha farm, it did not meet the bank's initial expectations.

9.2.5 Sjalon Interaction Locus B: Overcoming Legislative Barriers to Collective Land Tenure

The first phase of the relationship between Sjalon and the Treasury Department (TD) between August 2004 and March 2006 was initially troublesome, despite the fact that Sjalon was actively seeking contact because Sjalon was expected to attract many leaseholders. At an early stage, the Sjalon realized that their ideas would have legal implications for leasing arrangements. Sjalon's plans to amalgamate lands would be an illegal sub-lease, according to the interpretation of the TD. The TD saw this as an unwanted phenomenon that often occurred, but could not be tolerated. Illegality would arise because the land user should also be the lease for a certain period of time. Since the TD pursues a public good objective, they preferred to make these lands available for other purposes. The TD proposed a solution that would give individual leaseholders their individual leasehold rights to Sjalon, who would then become a collective leaseholder. Because some potential participants found this emotionally unacceptable-they would lose their individual tenure rights if they left Sjalon-the TD built into a special clause. This clause included that, if he could show that the new individual farm would be viable, leasehold rights would be returned to the individual farmer when he left Sjalon. This was TD's customized solution, which remained within the current law, although because of its unusual nature it had to be finetuned with the Ministry of Finance. This solution was communicated to Sjalon by the TD, but there was no favor. Since Sjalon thought there could be no further discussion, they did not enter into further discussions. Instead, Sjalon sought expert advice from a land tenure law professor in June 2007, who advised that the use (by Sjalon) and tenure (participants 'leasehold) could be legally separated. The TD thought Sjalon needed time to assimilate his proposal and waited for his answer. However, when the TD received a letter from professor on behalf of

Sjalon proposing the alternative, it was unpleasantly surprised. The TD thought Sjalon was too pushy to formulate the leasehold on them. The TD thought Sjalon was too pushy, wanting the leasehold formulated on its terms, which would lead to an unwanted sub-lease situation in one way or another. This conflict was mitigated by the facilitator of TransForum, who characterized it as follows: Through Sjalon's eyes, one gets the image of the TD as a bureaucratic obstacle, blocking participation in Sjalon, while the conversation with the director of the TD gave me the impression of an organization that is willing to go very far in supporting Sjalon, to the limit even a bit further, but that has to operate within the complex tenure situation in the NorthEastpolder. The facilitator organized an open discussion, in which once again it was explained what the TD proposal entailed; this resulted in Sjalon accepting the proposal.

9.2.6 Sjalon Interaction Locus C: "Selling the Story" to Recruit Potential Participating Farmers

Beginning with a vision of a collective farm of 600 ha, Sjalon had to attract many farmers who would be willing to amalgamate their land and conform to a less individual entrepreneurial orientation than they used to. The enthusiasm and active networking of the main champion farmer to engage potential participants was instrumental in recruitment, as many respondents indicated. In addition to this championing, the buzz was created by service providers supporting Sjalon and by articles in agricultural newspapers and magazines. The Sjalon network presented the plan and prospectus during a number of special meetings in 2006. This led to the signing of a letter of intention by six farmers. In the end, however, only three participants signed up, leading to a farm of 100 ha. One reason potential participants did not commit was that, despite the prospectus and other explanations, they could not obtain a clear picture of what Sjalon meant and were afraid that they would lose their independence from work and leasehold contract. Many respondents said that this difficulty in correctly interpreting the plan was due to the fact that it was developed in a relatively small circle over a long period of time. It was then sold as a ready-made package to potential participants who had no complete package to potential participants, who did not have full knowledge of the underlying ideas and assumptions. Another dilemma arose because the idea of Sjalon began with a planned size of more than 600 ha. When it became clear that this could not be achieved, the plan lost some of its attractiveness for many farmers. Farmers who were not willing to participate, on the other hand, indicated that they wanted to see the concept's "proof of principle," so it was better to start soon, albeit smaller. This was experienced as a major 394 L by the Sjalon network. Klerkx et al. / Agricultural Systems 103 (2010) 390–400 incentives to really launch the initiative in order to attract others by showing positive results. Another reason for non-interest was the strong association between other farmers very much associated the concept with the championing farmer. In the event that they had negative personal associations ("wants to play the boss"), or social associations with him (the NEP is a tightly knit

community in which reputations are well known), they also rejected the concept of Sjalon. On a higher level, there were a number of non-conducting factors in the socio-economic environment of potential participants outside the direct reach of the Sjalon network. One factor was that the Sjalon idea was not supported by the municipality. This was because it was a plan to strengthen agricultural activities, while the municipality favored other economic activities in order to reduce the agriculture of the municipality. In the municipality, an influential pastor who was both a farmer and an exofficial in the farmers' organization also criticized the plan. He considered the idea personally unviable. While he did not use his position to oppose the plan openly and publicly, it contributed to a non-conducting atmosphere. Sjalon's strategy was to bypass these people and not involve them except to arrange for formalities, but they nevertheless influenced the development of Sjalon. In addition, the banks and accountants of potential participants had a lack of a final business plan (the initial one had rough estimates) and despite the buzz created by the magazine, newspaper, articles banks and accountants of potential participants had not previously actively pointed to the Sjalon as a good opportunity for "weak" farmers to keep a viable business (which was how Sjalon wanted to sell itself). Their advice was conservative in the eyes of the Sjalon network and facilitators. Banks and accountants rebutted the argument that they could not act as advocates because of their position as neutral service providers (accountants) or had to adhere to certain criteria of judgment (banks). Other factors were that other cooperative farms had collapsed due to internal power struggles in the meantime, which influenced Sjalon's image among potential participants. Agricultural products prices have also risen significantly. Many respondents indicated that this reduced the need for less well-documented farmers to seek strategies for survival such as the one proposed by Sjalon. Consequently, in March 2008, only three farms finally joined a limited liability company, hoping that other farmers would find it attractive if it worked successfully.

9.2.7 Event Analysis Rondeel

Rondeel began with the interactive design project Caring for Hens (CfH) (Groot Koerkamp and Bos 2008), which resulted in visualizations and requirements (BoR) for new poultry farming systems (Plantage and Rondeel). The most obvious distinctive feature of the Rondeel concept is that it is a round hen housing system as opposed to normal rectangular systems. It also integrates standards of animal welfare comparable to free space and organic (open air) laying hen husbandry (e.g. natural shelter) with the advantages of closed hen housing systems producing cage eggs or barn eggs (e.g. protection from airborne diseases). Kwetters and Vencomatic (a manufacturer of poultry husbandry systems) formed a technical committee to develop a working prototype after a failed attempt to cooperate between Kwetters (an egg packing company participating in CfH) and the Animal Science Group (ASG-a research institute facilitating the CfH project). Vencomatic addressed technical problems such as nesting, collection of eggs and transportation of manure.

Kwetters dealt with the marketing of a segment between free range and organic eggs, with increased animal welfare as its main point of sale. The ASG researcher who managed and supported the CfH project as a technology champion, in addition to technical development activities (e.g. a manure drying carrousel based on the airflow effect of the chimney), the technical committee sought suitable locations for the construction of the new system. Initially, the search was concentrated in Barneveld, a municipality in which the poultry industry is clustered and which a global poultry center is. Members of the technical committee approached officials from Barneveld and several farmers from Barneveld interested in building a system from Rondeel. They also contacted various service providers (architects, construction contractors, feed suppliers, and researchers from ASG, environmental consultants, animal welfare consultants and business incubators). This network supported the fine-tuning of the design, facilitated the process of obtaining permits by checking compliance with construction and environmental standards and provided access to subsidies. At the same time, talks with the Dutch Animal Protection Society (APS) started with the technical committee. APS was a partner in CfH and piloted a certification system for poultry meat (Volwaard Chicken) indicating the value of animal welfare (by assigning welfare stars). They later hired a specialized animal welfare and corporate social responsibility (CSR) consultant to assist them in the talks. The aim was to negotiate one's assignment criteria, for the assignment of one, two, or the maximum of three welfare stars. Rondeel was temporarily assigned two stars by APS, and later even three. Kwetters and Vencomatic, together with the CSR consultant, successfully applied for TransForum financing and facilitation in their search for development funds. After Rondeel became a TransForum project, Kwetters 'internal strategic choices led to the project's withdrawal. Kwetters 'withdrawal opened new perspectives, in particular on alternative ways of distributing and marketing the egg. TransForum initiated workshops on the functioning, marketing and future development steps of the network to support the formation of new ideas and the consolidation of the network. This led to a greater sense of co-development between Vencomatic and farmers, who were first considered to be mere adopters of Rondeel. In addition to existing network partners, such as Vencomatic staff, CSR consultants, architects, officials and farmers from Barneveld, other consultants and champions from other welfare innovation networks (e.g. the Volwaard Chicken Network and the pork network described by Wiskerke and Roep (2007) participated. These workshops also resulted in cooperation between Rondeel Ltd. and the Southern Farmers 'Organization (ZLTO), which had experience with Volwaard Chicken marketing. Since the Rondeel housing system requires higher investment than normal hen housing systems, the production costs of the welfare value egg are higher. This makes it more expensive than usual eggs from the barn. For the Rondeel network, there was a great uncertainty as to whether consumers will see the difference and pay extra (a typical welfare innovation problem (Binnekamp and Ingenbleek 2006). Therefore, interested farmers, as well as their banks, hesitated to invest if the risks of non-return on additional investment were not covered (if the eggs had to be sold as eggs from the barn). This was a typical situation between the development and application of concepts between "valleys of

death" (Meijer 2008) or "chicken and egg" (Ansari and Garud 2009), which inhibited further development. The problem was that in order to have sufficient volume of the product, there must be sufficient Rondeels, but in order to have sufficient Rondeels, there must be sufficient guaranteed purchases of eggs from retailers (supermarkets); however, supermarkets were reluctant to market the egg because of the risk of their non-sale; And they also demanded a large volume immediately; but supermarkets still didn't think about marketing the egg, they first wanted the Rondeel to work, but no Rondeels would be built without a guaranteed purchase. In order to resolve this dilemma, Rondeel Ltd. engaged in talks with ZLTO and LNV in order to obtain a guarantee that would cover the additional investment in the event that the Rondeel eggs were unsuccessful and had to be sold at a lower price as normal barn eggs. This guarantee was given by LNV in July 2009, allowing the construction of the first hen housing systems. Generally number of agency-structure interaction loci in the Rondeel case is indicated that will be further analyzed. These reflect the way in which the Rondeel network dealt with: (a) obtaining building and environmental permits for the design of a hen housing system that did not fit neatly into an established category (political uncertainty), (b) obtaining a guarantee to cover additional investments in the event of a failure of Rondeel (financial uncertainty), and (c) obtaining certification from the Animal Protection Society as a proxy for Rondeel (financial uncertainty).

Rondeel Interaction Locus A

The adoption of a new design by current environmental and construction legislation. The design of Rondeel differs from other forms of hen housing systems because it is round. It is therefore not fully described in the legislation on the construction of the hen housing system and environmental regulations. This could lead to potential conflicts with regulatory bodies over issues such as construction dimensions, fire safety, landscape esthetic fit and gas emissions. However, construction and environmental permits were issued quickly in the case of Rondeel. There were a number of factors that caused this, as one factor was that the municipality of Barneveld adopted the innovative concept of Rondeel, since Barneveld could use it as an international poultry centre. In this aspiration, the marketing manager of Kwetters framed Rondeel (helped by vivid illustrations of the CfH project and a later model of scale). The CEO of Kwetters, who strategically pointed out that other municipalities were also interested, further enhanced the willingness of Barneveld; Barneveld was even more eager to have the first Rondeel built. As a result, economic development officials were champions of innovation in their organization. They did so by chasing their colleagues, the officials responsible for the permit procedures, by emphasizing that this was an innovative concept that would benefit the municipality. The system developers (i.e. Vencomatic R&D staff and ASG researchers who advised them) had a parallel purposeful strategy to equip the Rondeel with existing husbandry subsystems (feeding, extraction of manure, ventilation, etc.), which had already been approved for ammonia emission standards. Authoritative ASG calculations supported this approval, although researchers and environmental consultants recognized that in practice these figures could be different. Instead of a prolonged

admission and verification procedure for emissions (taking >5 years), the hen housing system nonetheless met existing standards. In addition, because Vencomatic hired a local environmental consulting firm and architect with good connections to the Barneveld civil servants, some of the remaining conflicting issues have been refined in interaction. For example, this concerned the Rondeel's fit with its round shape in the landscape. A more troubling problem was obtaining fire safety approval. This was because the Rondeel was regarded as a closed building with resulting security problems despite having outdoor spaces. Since the responsible fire department liked the project, however, he helped the architect in close interaction with the fire department to look for a solution. A specialized consulting agency later verified this solution, fireproof curtains that fall down in the event of a fire. Vencomatic has thus enhanced this strategy to frame Rondeel's component technology and appearance to comply with existing rules using specialized consultancies and researchers to verify compliance with existing standards. This made it possible to execute the integral concept as designed, with only minor adaptations.

Rondeel Interaction Locus B: Overcoming Risk Adversity Towards Non-proven Market Concepts

Rondeel Ltd. had to find ways to cover this risk in order to overcome the lock-in situation whereby the building of the hen housing system depended on the guaranteed sales volume and vice versa, therefore makes investment risks acceptable to Rondeel Ltd. and farmers. While innovation subsidies from innovation programs such as TransForum were available for the coverage of research and development costs, a major problem was to find risk financing (in the form of venture capital) or a guarantee. This was necessary to cover the risk of the surplus investment required compared to normal housing systems for barn hen. In this regard, Rondeel Ltd. and the support network around it pointed out that the public policy discourse was very supportive of innovations to improve animal welfare, such as Rondeel (e.g. Verburg 2008), but that they were not prepared to provide financial guarantees in practice. This was problematic because Vencomatic could only cover part of the financial risk as Rondeel Ltd's mother organization. Banks would only finance up to the amount of a normal housing system for barn hen, the returns of which are known. The initial search for risk financing with Oost NV, a business incubator, by the Rondeel Technical Committee (as was carried out before Rondeel Ltd. was established) proved unsuccessful. This was because Oost NV targets Barneveld's Gelderland Province. Since Vencomatic was from the Province of Brabant, Oost NV did not consider it to be its concern. In addition, the Economic Affairs Ministry funded Oost NV and the Rondeel system were considered to be an agricultural affair related to LNV. Later on, Rondeel Ltd. tried to convince ZLTO and LNV that they needed to provide risk financing or at least a guarantee. They did this by holding high-level talks between Vencomatic's CEO, ZLTO's president, and LNV officials. However, both ZLTO and LNV said they could not support specific companies: ZLTO because it is a political representative of all farmers, and LNV because of European state support rules. However, ZLTO provided in-kind support by providing the Volwaard Chicken with its experience and expertise, as they endorsed the Rondeel concept.

LNV stated that they had been responsible for financing the CfH project, a feasibility study, and now provided financing and facilitation for research and development through TransForum. LNV said companies should take some risk and should not rely on government alone. In order for the state to play a role, it would be necessary to develop generic instruments, since state support rules prevented individual companies from receiving support. Since the risk financing/guarantee problem could not be solved by formal means, Rondeel Ltd. to influence LNV first through informal probing by an ASG representative, but this was not appreciated. In addition, LNV said it was unrealistic for Vencomatic's CEO to expect that a guarantee could be provided solely on the basis of the Rondeel vision. Despite this setback, a new contact opening was forged thanks to the mediation of the CSR consultant hired as part of the TransForum project to facilitate processes and expertise in the marketing of welfare innovations. This was because of his good links to LNV. The people at Rondeel Ltd. highly appreciated this consultant's role, as the following quote shows:[the consultant], here we call him' the crowbar for opening closed doors.' Rondeel Ltd. sent a written formal support request and arranged a new formal meeting. A more detailed business plan with well-calculated financial figures has been drawn up on the basis of this meeting. The minister often referred to Rondeel as a promising concept, so the application and the business plan made explicit reference to this. Given the state support rules, LNV was willing to see what would be possible. Coincidentally, Rondeel was found to be able to use an existing guarantee regulation for large investments to individual farmers. The maximum amount of the guarantee had risen thanks to a recent change in this Regulation. This meant that this system could accommodate Rondeel. Furthermore, it facilitated the interaction with retailers. Retailers had earlier been hesitant to co-develop the Rondeel egg concept until there was a perspective of guaranteed supply, but now they became committed.

Rondeel Interaction Locus C: Getting Societal Support for Rondeel's Husbandry Concept

The third locus for interaction concerns Rondeel Ltd.'s dealings with what could be seen as a friendly actor in Rondeel's environment, the Animal Protection Society (APS). This is because APS supports innovations in animal welfare. This issue of welfare stars is linked to trade with retailers in order to convince retailers of the egg's' unique selling point.' In particular, the visualization/scale model of Rondeel and the BoR opened the doors to welfare star negotiations, as these showed that APS 'interests were clearly in line. However, hurdles must be overcome in the certification process. One hurdle was that the APS had to interpret the results of the pilot project it had carried out with its welfare certification system and has its members 'approval. This pilot project aimed to award the Volwaard Chicken welfare stars. APS perceived certain risks in attaching its reputation as a civic advocacy organization to a product (e.g. in the event of a food safety scandal involving a certified product), so it first had to evaluate the experience with Volwaard Chicken before assigning welfare stars to other products. These deliberations led to a delay in the awarding of stars, which Rondeel Ltd. used to convince others in the quest for

support, such as LNV in the guarantee issue. In addition, although the vision and the BoR attracted APS, the fact that the hen housing system could not yet be tested in practice meant that two stars were awarded provisionally. Rondeel sought three, and based his arguments on this number of stars. Another problem to be resolved was the interactional uncertainty between the CEO of Vencomatic and the APS representative. Conflicts over the critical opinion of APS on other Vencomatic products, coupled with the CEO's simplicity as an innovation champion, were bound to have a negative impact on the Rondeel trajectory. Naming the CSR consultant who also mediated the issue of the LNV guarantee as a neutral intermediary to take the sharp edges off the interaction and mediate an agreement mitigated.

9.2.8 China-UK Sustainable Agriculture Innovation Network (SAIN)

9.2.8.1 SAIN's Mission

To help achieve a resource-efficient, low-carbon economy and an environmentally friendly society SAIN will provide a coherent framework for the development and implementation of cooperation between China and the United Kingdom on environmentally sustainable agriculture, thus supporting the objectives of the current dialog on sustainable development as a basis for long-term cooperation between China and the United Kingdom (SAIN 2011a, b).

9.2.8.2 SAIN's Origins

The cooperation between China and the United Kingdom on sustainable agriculture dates back more than 20 years, but most of this has been inter-institutional, especially between universities. It has never been established in a long-term strategic framework designed to meet the objectives of national policy. The previous collaboration was of a technical nature and focused on soil erosion, management of crop nutrients, integrated pest management and biological control, waste management and plant breeding. More recent cooperation is mainly concerned with climate change, the impact of agriculture on the environment, ecosystem services and poverty in rural areas and the management of water resources. It is increasingly aimed at building capacity and supporting the development of policy, but much of it remains fragmentary and does not take into account possible synergies between different activities. However, events have provided the basis for a more ambitious approach in the last 3 years. First, the emergence of a scientific consensus on areas of mutual interest resulting from the UK-China Partners in Science Conference-Appropriate Science and Technology for Rural Sustainable Development held in November 2005 in Yangling, Shaanxi. Second, the Sustainable Development Dialog (SDD) was launched in November 2005, providing a new platform for cooperation between China and the United Kingdom, including a Sustainable Agriculture and Fisheries Work Programme. In November 2008, the signing of a Memorandum of Understanding (MOU) on Sustainable Agricultural Cooperation between the British Secretary of State for Environmental Food and Rural Affairs (DEFRA), Hilary

Benn, and the Chinese Minister for Agriculture, Sun Zhengcai, also indicated a strong commitment to the growing agricultural partnership. The ministers agreed to establish as an important vehicle the Sustainable Agriculture Innovation Network China-UK Sustainable Agriculture Innovation Network (SAIN) as an important vehicle for delivering on the MoU and the SDD Agriculture and Fisheries Work Programme (SAIN 2011a, b).

The main objectives of SAIN were: –.

(a) Support the implementation of the UK-China SDD and its theme for the management of natural resources by promoting innovation in three areas: Policy development; institutional mechanisms for collaborative research; and the implementation of policy and science on the ground.
(b) Stimulate innovative thinking and research on all aspects of sustainable agriculture and its relationship with the environment;
(c) Communicate information on issues relating to environmentally sustainable agriculture and opportunities for change and disseminate best practices to key audiences (farmers, policy makers, companies)
(d) Contribute to global sustainability through greater sharing of expertise between developed and emerging economies.

A high-level Board of Directors (GB), supported by a Secretariat Office in each country and four working groups (WGs), will supervise SAIN. The Secretariat Office in China is located at the North West University of Agriculture and Forestry (NWAFU), and the United Kingdom Secretariat Office is located at the University of East Anglia. The Governing Board of key government and academic stakeholders as well as independent experts will guide the program priorities and modalities. Each Working Group (WG) will lead a specific workflow and will be co-chairedby UK and Chinese experts with approximately five members from each WG from each country and supplemented by other national and international experts where appropriate. The SAIN Coordinators in the Secretariat Offices will be de facto members of the WGs. The WGs will function as a virtual network reaching out across both countries and with a rolling programme of well defined time-limited tasks. The membership and location of the WGs and the substance of their work may change in the future as new challenges, priorities and opportunities emerge (SAIN 2011a, b).

9.2.9 SAIN's Initial Work Focused on Four Inter-Related Themes

(a) Research and better communication tools are used to improve the management of soil and crop nutrients and reduce pollution from non-point sources.
(b) Increased use of agricultural biomass and manure for the production of biogas, liquid biofuels and organic fertilizers.
(c) Addressing the interface between agriculture and climate change, including the impact of climate change on agriculture and the way in which agriculture

contributes to greenhouse gas emissions, and therefore needs to adapt to it. This includes maximizing the potential contributions of I and II to adaptation and mitigation of climate change and helping to ensure that other agricultural policy issues are also addressed in support of climate change objectives.

(d) Providing policy advice on how the concept of a circular economy can be applied to agriculture by taking advantage of opportunities for greater recycling, minimization of waste and more efficient use of water and other critical resources.

However the main benefits of SAIN are:

(a) Improved focus on innovation in policy and increased relevance of research and development for SDD objectives and policy formulation
(b) Greater emphasis on cooperation in the development of integrated policy
(c) Better connections and more synergy between joint projects
(d) A more holistic approach to development of programmes. Enhanced complementarity with other bilateral and multilateral donors 'activities. Increased translation of research and development into action on ground
(e) Increased sharing of expertise and research
(f) Better implementation of core policies
(g) Improved learning (including expertise sharing between developed and emerging economies)

The Chinese Ministry of Agriculture supports SAIN and the United Kingdom Department for Environment, Food and Rural Affairs (Defra), providing financing to the United Kingdom and China Secretariat. Initial stakeholders include the NWAFU, China Agricultural University, the Chinese Academy of Agricultural Sciences, the Chinese Academy of Sciences, the Nanjing Agricultural University and the United Kingdom Research Council in China, and DFID, the FCO, BBSRC and CABI in the United Kingdom. As the WGs are appointed and the Work Program is developed, participation will be increased (SAIN 2011a, b).

9.3 Traditional Biotechnology and New Technological Approaches

9.3.1 Traditional Biotechnology

Early civilization was strongly based on agriculture and, as such, the need to alter the crop, which was a long-standing practice, which can be traced back to 2500–2000 BC, according to which ancient Egyptians in Africa produced wine using fermentation techniques with the help of microbiology, apart from the breeding of animals such as geese and cattle for dietary and nutritional use in their society. The fermentation technology was also used in bakery to cause an increase in dough, which resulted in 50 bread varieties more than 4000 years ago. The study and

understanding of advance plants in the seventeenth century was relatively poor. Nevertheless, plant breeding was a popular practice when Gregor Mendel, "the father of genetics," who was a plant breeder, studied the inheritable characteristics of peas and gave a better understanding of genetic inheritance, which led to a new terminology called crossbreeding, which is now known as hybridization. In addition, in 1865, at two meetings of the Natural History Society of Brünn in Moravia, he published a paper called Versuche über Pflanzenhybriden (Experiments on Plant Hybridization), which was criticized, but considered merely useful for hybridization rather than inheritance, and as such ignored. In this paper, it was shown clearly how dominant and recessive alleles produce their remarkable characteristics and possibly transfer them to offspring. A group of scientists who worked on breeding problems rediscovered the work in 1900 and made Mendel's findings more popular. Subsequently, Mendel and a new understanding of genetics found tangential points to traditional self-pollination and cross-pollination achieved significant advances in plant breeding with reference to the study. The identification and incorporation of characteristics in plant breeding is crucial. Breeders therefore traveled around foreign lands in search of plants with specific desirable characteristics.

These characteristics occasionally arise through the mutation process, which was considered too slow and unreliable for the desired production rate of various plants. This gesture of plant domestication also became the most important means of increasing yield, improving disease resistance and drought tolerance, improving nutrition and crop taste and facilitating crop harvesting for centuries. The Green Revolution has played an important role in popularizing the use of conventional hybridization to multiply yields in many folds through "high-yielding varieties" and has an enormous impact on crop production in the world. Nowadays, plant breeding is targeted at organic farming (OF), which can help to reduce the gaps in yield between both production systems: conventional farming (CF) and organic farming (OF). However, further analysis is needed to determine whether these systems should necessarily be considered as competing entities and whether they should necessarily be comparable in terms of productivity. Since it is not clear whether OF yields should be equal to CF yields or simply higher than they are at present. The breeding objectives for both conditions converge in order to achieve higher productivity, incorporation of resistance or tolerance to biotic and abiotic factors and higher resource-use efficiency (water, nutrients, light, etc.). Local adaptation may be more important for OF, as the recycling of resources and the quality of the inputs used may vary from region to region, even if OF practices are highly regulated. Organic plant breeding (OPB) also aims to fit cultivars into renewable organic resources farming systems. OF enthusiasts often noted that the cultivars grown for CF do not always perform well under OF conditions (van Bueren et al. 2011). However, there is no reason to believe that even in all CF environments, all cultivars produced by conventional breeding programs perform well. It is therefore unreasonable to believe that all the lines produced in an organic breeding program will perform well in all OF conditions. The interaction of genotype-by-environment (G + E) is a common situation that plant breeders have to deal with and significant progress in crop improvement can still be made if properly exploited. Even under CF, which

for some OF supporters consists simply of high-input standardized practices, G E is a very important aspect to be taken into account, because in reality there are also low-input and various CF systems, which are driven by resource-poor farmers in developing countries. From the point of view of pure plant breeding, OF can therefore be regarded as a separate environment with a strong component of local adaptation, in which the necessary characteristics and selection methods should be incorporated. Characteristics and sources of variation although the general breeding objectives for both OF and CF are similar, specific characteristics are required for OF, as the use of synthetic agrochemicals in this system is prohibited. The competitiveness of weeds and the ability to establish symbiont relationships with microorganisms in the soil are relevant to OF, since they can improve the use of resources and their efficiency of use (Zdravkovic et al. 2010). Research has shown that genetic variation in the competitiveness of weeds in cereals exists (Zystro et al. 2012), and that early vigor and allelopathy can be useful in improving weed suppression (Bertholdsson 2010). In potato (Tiemens-Hulscher et al. 2014) or wheat (Baresel et al. 2008), genetic variation for the efficiency of nitrogen use has been found, and genomic regions associated with this trait have been identified in barley (Kindu et al. 2014). In addition, studies have shown that agronomic practices can improve the efficiency of nitrogen use (Swain et al. 2014). In wheat, genetic variation and genomic regions associated with micronutrient intake have also been reported (White and Broadley 2009). However, Nelson et al. (2011) found that the percentage of arbuscular mycorrhizal fungi in winter wheat was negatively correlated with iron and zinc levels.

9.3.2 New Technological Approaches

The scientific and technological progress of the last century has greatly expanded the scope and power of agricultural innovation. There are now a remarkable range of technologies to improve crop production, many of which are now in practice, including:

9.3.3 Technologies for Natural Resources Management

Nigeria's future of sustainable agriculture depends largely on proper management of natural resources, in particular soil and water. It is considered that more than two thirds of agricultural land in northern Nigeria is degraded and the situation is equally alarming (FAO 1992). As a result of poor water management, irrigated and dry land salinity also poses serious threats to cropland in these lands. Technology includes:

9.3.3.1 Soil Management Techniques

One of the promising techniques for successful soil management practice is the application of organic manure and mulching with green plants, controlled overgrazing and bio – solid integration (Chrispeel and Sadava 1994).

9.3.3.2 Water Management Techniques

Effective and efficient irrigation methods include on – site storage tanks for the collection and storage of water in fields to minimize runoff water (IFAP 2005).

9.3.3.3 Technologies for Crop Improvements

Crop performance is affected by a combination of many factors, including the collection of genes that allow the plant to produce high yields in an agricultural environment (Abbott 1999). It takes years to develop high-performance plants and efforts in Nigeria have fallen behind, although new tools and techniques are emerging that can accelerate local crop improvement. The following are the techniques:

9.3.4 Annotated Crop Genome Sequencing

This technique helps to determine the genetic sequence and individual gene function of farmers 'local crops. Documenting the sequence and functions of these genes will help to accelerate crop breeding for sustainable productivity (Miflin 2000). Efforts to sequence the complete genome of Arabidopsis, rice and maize with the full sequence of chromosomes 2 and 4 have recently been published (Mayer et al. 1999). Cook (1998) outlined some of the methods for the collaborative project on crop plants. Usually the first approach is to look for homology with other known genes. Another way to understand the function of a particular sequence is to observe their expression under a range of defined conditions using micro array technology (Ruan et al. 1998). Gong et al. (2011) established the sequence of a small heat shock protein (sHSP) gene using a gene candidate method based on homology and a rapid amplification of cDNA ends (RACE). The cDNA sequence of this gene is 920 bp (GenBank: HM132040) and contains an open reading frame (ORF) of 636 bp, which is expected to encode a protein with 211 residues of amino acids. These studies and those conducted in other species show that this has considerable potential as a powerful tool for the discovery and functional analysis of plant genes and helps to understand genetic regulatory networks and gene interaction.

9.3.5 Germplasm Techniques

New genes can be used to improve a crop from plant, animal or bacterial species and molecular techniques to introduce them to a candidate crop. Once introduced into a plant, conventional approaches to plant breeding are used to incorporate them into the local elite germplasm (Chrispeel and Sadava 1994).

9.3.6 Biochemical Engineering

Biochemical plant metabolism studies have identified proteins that are essential to the functioning of most pathways. Many key genes were isolated by purifying and

(partially) sequencing proteins and then finding the appropriate cDNA and/or genomic sequences. Knowledge of changes in the specific function of a plant resulting from different treatments has led to the development of methods for isolating genes involved in metabolic pathways or their associated physiology (Miflin 2000). Osmolyte production in plants under abiotic stress conditions is frequently studied; OA results in a number of benefits that sustain the activity of cells and tissues under stress conditions (Serraj and Sinclair 2002).

9.3.7 Technologies to Overcome Biotic Constraint

Among the technologies emerging that can effectively overcome biotic constraints (weeds, disease pathogens and pest) so as to ensure sustainable yield are:

9.3.8 Plant Mediated Gene-Silencing Approach

This technique is used to induce plants to transfer genetic materials to other organisms and to target and interfere with the genetic interaction between plants and organisms. This approach benefits from the recently discovered powerful molecules known as small RNA that play a role in plant development and stress resistance. The plant-based gene silencing has shown promise to control viruses, nematodes and some insects (NAS 2009).

9.3.9 Bio-pesticides and Bio-control Approach

This approach focuses on natural means of fighting biotic constraints of crop rather than synthetic chemicals in order to avoid environmental degradation and pollution. Bio pesticides involve release of pest specific natural enemy to control its population and some allelopathic plants that can inhibit the growth of specific weeds while promoting that of agricultural crops (Bunza 2010).

9.3.10 Disease Suppressive Soils

The use of disease-suppressive soils involves management practices that encourage crop-associated microbial communities that naturally reduce plant diseases and pests. These practices might include manipulating carbon inputs, using crop rotation sequences that increase the presence of beneficial organisms, or inoculating soils with disease-suppressive microorganisms (NAS 2009).

9.3.11 Technologies for Energy Production and Storage

In view of the weather types and range of temperatures in these regions (Northern Nigeria), solar energy technology will provide reliable energy for crop production due to its potential scalability and cost. Due to the prevailing light and temperature of these locations, photosynthetic microbase fuel produced from algae and cyanobacteria will also excel well in these regions (Singh and Gu 2010).

9.3.12 Local Expertise and Participation

The new problems and technologies listed here are interdependent. A system-wide approach of all techniques must be implemented in local environmental conditions for successful crop production. Farmers need to be able to provide input and obtain information. These tasks require a committed, trained, local workforce of extension agents, scientists and engineers to be built with national efforts and international assistance (NAS 2009).

9.4 Physiological and Biotechnological Approaches to Improve Crop Sustainability

9.4.1 Physiological Approaches to Improve Crop Sustainability

In these sections, we focus on the contribution of crop physiology to plant breeding in relation to (1) the analysis of past achievements in increasing yield potential, yield stability and resource productivity, (2) the identification of characteristics that could improve the efficiency of genotypes with high yield potential and adaptation to the target environment, and (3) disentangling of complex interactions among traits and between traits and environment.

9.4.2 Analysis of Past Achievements of Plant Breeding

The concepts of crop physiology contribute to the analysis of past plant breeding achievements in increasing the yield potential; yield stability and resource productivity by identifying mechanisms indirectly affected by the selection process. The rationale is that realized changes in the phenotype resulting from yield breeding and agronomic adaptation could identify limits and opportunities for future improvements (Reynolds et al. 2009). Comparisons of cultivars released in different eras allow quantification of the contribution of genetic improvement to crop yield and the dissection of the characteristics involved, as shown in the example below.

In recent decades, breeding has consistently increased the yield potential of maize in Argentina. The higher yield of modern hybrids was linked to a higher production and harvest index of biomass (Echarte et al. 2004) and was mainly due to

more grains per unit of surface area, which increased the demand for reproductive sinks during the effective grain filling period. Compared to their older counterparts, modern hybrids generally produced a more stable yield and harvest index in response to increased plant density and environmental stress, mainly associated with a higher number of grains per unit of plant growth at a critical flowering period (Echarte et al. 2004). Modern hybrids, however, could show lower yield stability in response to source reductions during grain filling because of their high reproductive demand (Echarte et al. 2006; Cerrudo et al. 2013). Modern and old hybrids had similar use of water and absorption of nitrogen during vegetative stages, but after flowering, former hybrids absorbed more nitrogen (Robles et al. 2011). Finally, modern hybrids showed a higher yield of grain per unit of nitrogen absorption and evapotranspiration per unit than their older counterparts associated with an improvement in the harvest index (Nagore et al. 2010).

9.4.3 Identification of High Yield Traits and Adaptation to Environments

For the successful use of characteristics in crop breeding, five main conditions must be met: (1) relevance for crop growth and productivity; (2) genetic variability; (3) medium to high heritability; (4) easily monitored; and (5) absence of significant agricultural trade (Bruce et al. 2002). In this section, we emphasize the contributions of crop physiology to the identification of relevant characteristics related to crop performance in the field following top-down (gene phenotype; Chapman et al. 2002) or bottom-up (gene to phenotype; Ishitani et al. 2004).

(a) **The Top-down Approach**

The top-down approach begins by identifying consistent phenotypic variation in yield or other relevant features in the field. The search for mechanisms underlying these responses continues at decreasing levels of complexity (crop, plant, tissue, cell and molecule). Molecular mapping is then used to identify quantitative trait loci (QTL) associated with the trait of interest and, finally, methods based on genomics enable the selection and cloning of genes/QTL that control these traits of agronomic interest. The following two examples illustrate the top-down approach. The number of grains per unit of spike chaff weight (i.e. SF) explained a great deal of wheat yield variation among cultivars in a wide range of environments (Gonzalez et al. 2011). An appropriate method to determine this trait quickly and easily at maturity was developed using a small sample of individual spikes (Abbate et al. 2012). In order to investigate the genetic and molecular mechanisms underlying the trait, controlled crosses were made between wheat cultivars with contrasting SF under various mating designs. These studies have shown that a few genes control SF with low interaction between G and E and medium to high heritability (Pontaroli 2012). Two mapping populations are currently performing molecular marker analysis. Several markers were found to co-segregate with SF, which warrant further investigation (Deperi

et al. 2012). In comparison with 12 maize hybrids grown at 42 sites, Castro (2013) identified four maize hybrids of similarly high yield potential and contrasting yield in low yield environments leading to contrasting stability. For the two extreme hybrids in this set, he established the underlying physiological mechanisms responsible for the different yield stability in experiments in which plant density was used as an environmental stress surrogate (Andrade et al. 2002). These studies have been carried out on hybrids and their parental lines. The stable hybrid showed a higher yield of grain and a higher number of grains per unit of land area at high plant densities than the unstable hybrid. Only for the latter trait was the same response observed in their parental lines. In contrast, the partitioning of dry matter to the ear during the flowering period for the unstable hybrid and one of its inbred lines was greater. As in the example above, the next logical step would be to map the desired characteristics and develop quick, effective and easy phenotyping methods to facilitate the selection process. These top-down approaches allowed a complex characteristic assessed in a wide range of environments to be dissected into simpler components under less complex genetic control and with possible complementary effects.

(b) **The Bottom-up Approach**

Enzymes, metabolites and transcription factors that control key biochemical or physiological processes are identified and their genetic control is clarified in the bottom-up approach (Grillo et al. 2010). In order to develop genotypes with the desired feature, genetic engineering, mutagenesis and marker-assisted selection approaches can be used. The identification of relevant traits or genes associated with crop performance is a major challenge due to trade-offs, attenuation of traits and strong interactions resulting from scaling up across organizational levels and complex and redundant plant systems regulation (Sinclair and Purcell 2005). Therefore, scaling up across organizational levels because many factors interact and increase complexity generally attenuates the phenotypic effect of a given feature. This buffering response may reduce the relevance of many characteristics to the crop or farming system (Sinclair and Purcell 2005. Molecular genetics have provided a large amount of information on QTL and, to a much smaller extent, specific genes associated with yield-related characteristics (Mastrangelo et al. 2012). The clarification of gene function could be significantly improved by crop physiology, since it can help to close the phenotypic gap between the availability of DNA sequence and gene function related to crop performance in the field (Miflin 2000).

9.4.4 Disentangling Complex Interactions

Crop physiology also helps to disengage the complex interactions between features and environments and between features. Relevant examples of complex interactions at the level of the trait or quantitative trait locus are presented below to show that caution must be exercised when interpreting and analyzing the potential of new available information derived from crop physiology or molecular studies (Passioura 2012).

9.4.5 Interactions at the Trait Level

Water deficit tolerance is presented as an example of a complex feature. The effects of water deficit on crop productivity are not easy to predict, as they vary depending on the timing, duration and intensity of stress and factors, including previous crop history and other environmental aspects. The relevance of a particular trait varies depending on the type of drought. Features with putative advantages for adaptation to dry environments are not universal and interact strongly with other features and the environment (Tardieu 2012).

Deep roots would be a desirable feature in areas where substantial quantities of water are left in the soil at physiological maturity and the water stored in the soil can be replenished in the subsequent failure period (Sadras and Rodriguez 2007). The induction of a high resistance to water flow in the plant is also a way of saving water for later, more critical stages of growth in areas where drought is progressive and becomes more severe at the end of the growing season (Passioura 1983). Similarly, the most important component of tolerance in chickpea and pearl millet under terminal drought was the conservative use of water early in the crop cycle, partly due to a lower canopy conductance, which resulted in more water available in the soil profile during reproduction (Zaman-Allah et al. 2011). In addition, early flowering would be a positive feature in which crops are exposed to severe terminal water stress, so that plants can complete their growing cycle without severe stress at critical stages in determining grain yield (Debaeke and Aboudrare 2004). However, full-season cultivars would be preferred in environments or seasons with mild or no terminal water stress, as the yield potential is directly linked to the maturity group (Capristo et al. 2007). If the crop is based on rainfall during the season, early vigor would increase the efficiency of water use by reducing soil evaporation (Richards and Lukacs 2002). Expansion of the leaves and stomatal responses to dehydration, characteristics showing genetic variability (Sinclair et al. 2010), may have a variable value depending on the target environment. In environments where crops depend on stored soil water, reducing leaf area and stomatal conductivity would be desirable to save water for later, more critical reproductive stages. However, it would be appropriate to maintain high rates of leaf expansion and productivity under stress for short periods of drought during vegetative growth, since this strategy would allow the crop to use incoming radiation and resources more efficiently to relieve stress.

9.4.6 Interactions at the QTL or Gene Level

The following two case studies illustrate the physiological interpretation of complex molecular data. The first example refers to a genetic linkage analysis of two characteristics, namely growing degree-days in sunflower families derived from the cross between lines HA89 and ZENB8 (León et al. 2000, 2001). Two QTLs, A and B, were strongly associated with the response of the photoperiod that controls thermal flowering time. With the help of molecular markers, near-isogenic families for these

QTLs were developed through backcrosses. This genetic material has been used to study the effects of both QTL on the response of the sunflower photoperiod response, considering the moment of apex change, i.e. Stage 1.3 in the scale of Marc and Palmer (1981), and its subsequent rate of development (Fonts et al. 2008). When QTL A was selected homozygous as HA89 parental line and QTL B homozygous as ZENB8 parental line, the longest time for floral induction and floral differentiation was observed. This previously unrecognized gene interaction is compatible with the proposed web-cascade reaction system for time control to flowering and for the transition from vegetative to reproductive stages (Imaizumi and Key 2006). In addition, QTL B interacted significantly with both traits of the photoperiod (Fonts et al. 2008). The additive effect of QTL B on phase duration increased under extended photoperiod, indicating a short-day response. The presence ofZENB8 alleles in QTL B and HA89 alleles in QTL A resulted in a stronger developmental delay until the end of floral differentiation in long photoperiods than short photoperiods. For the development rate, a second-degree interaction was found between QTL A, QTL B and photoperiod. This higher order interaction is also compatible with the complex web of processes that control blooming responses to the photoperiod, in which light (long days) stabilizes a clock-regulated transcription factor and activates or suppresses key flowering genes (Imaizumi and Key 2006). The second example deals with the use of molecular markers to locate QTL in the same sunflower population, which is grown in a variety of latitudes from Fargo (46.8 ° N) to Venado Tuerto (33.2 ° S) (León et al. 2001, 2003), associated with oil concentration and growing degree days to flowering (DTF). Both the concentration of grain oil and DTF were associated with a QTL on the linkage group B. In accordance with the phenotypic correlations, ZENB8 was derived from additive effects for higher DTF and lower concentrations of grain oil in the linkage group B. These additive effects were also most significant at high latitudes. In combination with DTF, the highest LOD scores for this QTL were observed during long photoperiods in crops emergence. In Fargothe environment with the highest rate of decrease in temperature and radiation during grain filling, the highest LOD score for this QTL was also observed in combination with the percentage of grain oil. This environment also showed the most significant phenotypic correlation coefficients between the concentration of DTF and grain oil ($r = -0.29$; selection with P marker assisted (MAS) and transgenesis may not be completely straightforward and pose a continuous challenge (Edmeades 2013). Conventional breeding, MAS and genetic engineering for higher yield potential and adaptation of crops to the target environment can undoubtedly proceed more quickly if the physiological mechanisms of grain yield determination and their interaction with the environment are better understood.

Data from an analysis of genetic links in families derived from the cross between lines HA89 and ZENB8 (León et al. 2001, 2003). A negative sign of the additive effect means that the mean value of the trait due to HA89 alleles increases. A positive sign of the additive effect is an increase in the average value of the feature due to ZENB8 alleles. In this QTL, HA89 alleles reduce the growing degree of flowering and increase the percentage of grain-oil. This QTL's LOD scores and additive effects have a strong environmental impact. Crops were cultivated in two locations:

Fargo at high latitude (46.8 ° N) and Venado Tuerto at low latitude (33.2 ° S). Photoperiod measured in the emergence of crops In addition, some breeders used useful physiological concepts in their daily work above. In maize, breeding programs carried out at high plant densities and evaluation at many sites with different environments produced some hybrids with high yield stability and high yield potential (Castro 2013). This strong interaction between G and E for grain yields has a genetic basis and shows an opportunity to improve grain yields in medium and low yield environments. This relevant result probably reflects the understanding of breeders that high plant density is a substitute for environmental stress (Andrade et al. 2002). The weakening of the stem by removal of carbohydrates induced by a low source-sink ratio during grain filling (Uhart and Andrade 1991) was also used as a physiological concept by breeders to select the resistance to stalk lodging. In addition, another useful concept that helped breeders in their selection process was the need to optimize the physiological condition of the crop during critical periods for the determination of the grain number to obtain high yield (Egli and Bruening 2005). Physiological principles can also help to develop simple, precise and rapid phenotyping techniques that are essential for both top-down and bottom-up approaches (Pereyra-Irujo et al. 2012).

9.4.7 Biotechnological Approaches to Improve Crop Sustainability

The aim of plant breeding for stress environments is to accumulate favorable alleles that contribute to the tolerance of stress in a plant genome. Genes that confer stress resistance can be derived from germplasm collections, including wild relatives of crops in gene banks or organisms that currently live in water deficit or excess habitats, extreme temperature and salinity, which have developed to meet these conditions (Nevo and Chen 2010). Although conventional breeding (Blum 1985) has made some progress, breeding for abiotic stress tolerance is restricted: (I) by the complex nature of abiotic stress tolerance (timing, duration, intensity, frequency). Biotechnology is a viable option for the development of genotypes that can improve their performance under harsh environmental conditions, especially for (ii) and (iii). For example, advances in genomics and bioinformatics and stress biology can provide useful genes or alleles for stress tolerance. Superior genes or alleles identified in the same species can be transferred through molecular breeding (MB) to elite genotypes. In addition, there is no barrier to the transfer of useful genes or alleles from the animal or plant kingdoms to different species using an approach such as genetic engineering (GE). Biotechnology approaches therefore offer new strategies to produce appropriate crop genotypes that can withstand drought, high temperatures, submergence and salinity stresses. Zhu et al. (2010) outlines key strategies for genetic improvement in abiotic stress tolerance for crop improvement. A number of key approaches to improving crop productivity. Similarly, Ainsworth and colleagues critically analyzed biotechnological approaches that could be used to develop crops with potentially improved productivity in high temperature, high CO2 and high

ozone environments (Ainsworth et al. 2008). These included manipulation of leaf photosynthesis, partitioning of photosynthesis, total production of biomass and efficiency of nitrogen use (NUE) (see Glossary). Improved NUE in crops should reduce the use of fertilizers and thus reduce greenhouse gas emissions into the atmosphere. More than 50% of all US greenhouse gas emissions from agriculture are associated with the use of fertilizers and other cropping practices (EPA 2009). The biotechnology community should use biotechnological approaches to address multiple stresses directly under field conditions rather than focusing on individual stresses (Mittler and Blumwald 2010). Our increased understanding of the molecular and genetic basis of abiotic stress responses in plants should allow us to use crop-specific MB, GE and preferably integrated programs to introduce multiple stress resistance.

9.4.8 Fundamental Agricultural Biotechnology Approaches: Prospects and Progress

1. **Molecular Breeding**

In the field of genomics, significant progress has been made over the last 10 years. Many crop species, such as rice (Yu et al. 2002), poplar (Populus trichocarpa) (Tuskan et al. 2006), sorghum (Sorghum bicolor) (Paterson et al. 2009), maize (Schnable et al. 2009) and soybean (Glycine max) (Schmutz et al. 2010) are now available for genome sequences. In addition, the advent of so-called "sequencing of next generation" (NGS) technologies has enabled the sequencing of transcriptomes or genomes of any species (and of any number of individuals) relatively quickly and cheaply (Varshney et al. 2009). As a result, genome sequences have started to become available for less studied crops such as cucumber (Cucumis sativus) (Huang et al. 2009), pigeonpea (Cajanus cajan) (http://www.icrisat.org/gt-bt/IIPG/home.html) and large and complex genome species such as wheat (http://www.genomeweb.com/sequencing/wheat-genome-sequenced-roches-454) and barley (Hordeum vulgare) (http://barleygenome.org/).These genome or transcriptome sequences, combined with genetic approaches, can be used to identify appropriate genes that confer stress tolerance that can be used to improve crops using either MB or GE approaches. The use of genome sequences to identify genes associated with drought tolerance can be demonstrated in sorghum, a species that is well adapted to drought-prone regions. Analysis of the sorghum genome has shown that the characteristic adaptation of sorghum to drought may be linked in part to the expansion of one miRNA and several family genes. Rice miRNA 169 g, adjusted during drought (Xiao et al. 2009), has five sorghum homologs (sbi-MIR169c, sbi-MIR169d, sbi-MIR169.p2, sbiMIR169.p6 and sbi-MIR169.p7). The calculated target of the sbi-MIR169 subfamily consists of members of the transcription factor family of nuclear factor Y (NF-Y) B, which is linked to the improved performance of Arabidopsis (Arabidopsis thaliana) and maize during drought (Nelson et al. 2007). Domain-containing genes Cytochrome P450 have been found to be abundant in sorghum (326 cf. 228 in rice), often involved in scavenging toxins such as those accumulated in response to stress.

Expansins, enzymes that break hydrogen bonds and are responsible for a variety of growth responses that may be linked to the drought tolerance of sorghum, occurred in 82 copies in sorghum cf. 58 in rice and 40 in arabidopsis and poplar (Paterson et al. 2009). The MB approach first identifies quantitative trait loci (QTLs) for interest characteristics, such as abiotic stress tolerance. Until recently, linkage mapping has identified QTLs (Varshney and Tuberosa 2007), but now association genetics has begun to complement these efforts in several crops (Hall 2010). Nested association mapping is also used for the genome-wide dissection of complex traits in maize (Yu et al. 2008), which combines the advantages of linkage analysis and association mapping in a single unified mapping population. Association mapping is a high-resolution and relatively less expensive approach compared to linkage mapping (Gupta et al. 2005), particularly given the availability of high-throughput marker genotyping platforms (Varshney and Dubey 2009). The collaborative project between Cornell University and CIMMYT (http/www.maizegenetics.net/drought tolerance) is an example of the systematic use of association mapping for drought tolerance. The candidate QTLs or genes can be introgressed in elite lines via marker-assisted backcrossing (MABC) after identifying the markers associated with QTLs or genes for traits of interest. Although MABC has succeeded in developing superior genotypes for traits controlled by major effect genes or QTLs, such as bacterial blight and blast resistance in rice (Sundaram et al. 2009), there are few examples of complex traits such as drought and heat tolerance, which are the key traits that need to be targeted for developing crops that are adapted to low rainfall and high temperature conditions. However, MB has been used successfully in rice, with one major QTL effect for submergence tolerance (Septiningsih et al. 2009) and drought tolerance (Steele et al. 2006) identified and used in this approach. One of the difficulties in developing superior genotypes for abiotic stresses such as drought or heat is that these characteristics are usually controlled by QTLs or several epistatic QTLs (Messmer et al. 2009). MABC does not seem to be an effective approach to introgressing such QTLs, particularly in view of the large sizes of backcross populations required to pyramidize several QTLs in the same genetic context. Two new MB approaches, however-marker-assisted recurrent selection (MARS) and genomewide selection (GWS) or genomic selection (GS) – can be used to overcome this problem (Tester and Langridge 2010). The estimated genetic gain, which can be achieved using MARS or GWS, is greater than that obtained using MABC for the transfer or pyramidization of superior QTLs or gene alleles for complex traits such as drought or heat tolerance in one genetic context (Ribaut et al. 2010). Although the MARS approach is routinely used in breeding programs in the private sector (Ribaut and Ragot 2006), no reports on the use of MARS in public breeding programs have been published. GWS is another comprehensive approach to the improvement of complex characteristics. Although MABC and MARS require QTL information for complex characteristics, GWS does not necessarily need information on marker-trait associations (Heffner et al. 2009). In essence, GWS is concerned with the prediction of the progeny's genomically estimated breeding values (GEBVs). In this context, phenotyping data and the profiling of genome-wide markers on a "training population" are first necessary; GEBVs can then be calculated on the basis of

phenotyping and marker data. These GEBVs are then used to select the top line of progeny in the breeding cycle (Meuwissen et al. 2001). Several computational tools for calculating GEBVs are available or are being developed, such as the Best Linear Unbiased Prediction Method and the mixed geostatistical model (Schulz-Streeck and Piepho 2010) (http/genomics.cimmyt.org/#Software). However, at present there is little information available on the use of GWS in crop plants in public sector breeding programs, although some groups have started to explore this approach in crops such as maize (http://genomics.cimmyt.org/, http://www.synbreed.tum.de/index.php?id=31).

2. **Genetic Engineering**

The increase in genomic information and the use of related computer biology tools over the past decade has led to the identification of signaling pathways and regulatory genes and networks that control complex characteristics related to environmental stress. Crop GE with signaling components and transcription factors (TFs) leads to the expression of the target transcriptome consisting of several genes involved in the adaptation of stress. For example, the increased production of the signaling hormone abscisic acid (ABA) by over expression of the LOS5/ABA3 gene encoding the Molybdenum Cofactor Sulfurase, which is required for ABA synthesis, increased drought tolerance in transgenic rice plants under field conditions (Xiao et al. (2009). Similarly, the over expression of the rice AP37 (an APETALA2-type TF) gene resulted in the enhanced expression of several target genes and produced 16–57% higher grain yield under field drought stress conditions (oh et al. 2009). Hence, transcriptome engineering seems to be promising for the development of abiotic stress-tolerant crops. However, inducible expression rather than the constitutive over expression of TFs is preferable owing to the severe growth retardation and reduction in seed production that can occur even under normal environmental conditions in transgenic crops with constitutive expression of TFs (Liu et al. 1998). Nonetheless, several transgenic crops have been engineered using C-repeat binding factors (CBFs) and other TFs without a yield penalty (Yang et al. 2010). Transgenic rice plants overexpressing Arabidopsis CBF3/DREB1A or ABF3 TF showed improved tolerance to drought and salinity without growth retardation (Oh et al. 2005). However, only a few crops such as rice (Hu et al. 2006), maize (Castiglioni et al. 2008) and canola (Brassica napus) (Wang et al. 2005), expressing the desired TF and other genes, have been tested under real field stress conditions (Yang et al. 2010). RNA chaperones known for their active role, particularly in mediating transcription and translation both in bacteria and plants, have also been shown to increase yield under multiple stresses. For instance, Monsanto (http://www.monsanto.com/) researchers showed that bacterial cold shock proteins (Csps) can confer improved stress adaptation in multiple plant species. For instance, CspB codes for and is responsible for an RNA chaperone, which is a commonly occurring protein molecule that binds to RNAs and facilitates their function. The gene was first identified in bacteria subjected to cold stress conditions, and further research has demonstrated that CspB helps plants cope with drought stress. In maize and

rice, CspB works by helping the plant maintain growth and development during times of inadequate water supply [67]. Recently, a gene encoding aquaporin (NtAQP1) was identified in tobacco (Nicotiana tabacum) and shown to provide protection against salinity stress in transgenic tomatoes (Solanum lycopersicum) (Castiglioni et al. 2008). NtAQP1 plays a key role in preventing root/shoot hydraulic failure, enhancing water use efficiency and thereby improving salt tolerance. It simultaneously increased both water use and photosynthetic efficiency in plants. Moreover, the NtAQP1 gene, which increases stomatal conductance, might also lower canopy temperature and thereby reduce the level of heat stress experienced by plants. By contrast, decreased stomatal conductance and thereby transpiration by the suppression of farnesyltransferase genes (FTA or FTB) by RNAi in transgenic canola resulted in significantly higher yields compared with controls in a 3-year field trial (Wang et al. 2009). To make up for the water loss owing to higher stomatal conductance in the NtAQP1 transgenic plants, pyramiding genes for osmolyte biosynthesis expressed specifically in roots could lead to the growth of deeper roots, potentially enabling water uptake from deeper soil layers (Sinclair et al. 2004).

To offset the adverse effects of climate variability on plants, a combination of genes is required. In addition to genes that encode effector proteins, signaling proteins and/or TFs, a repertoire of promoters is required to conduct transgenic expression in specific tissues or plant organs in a precise and predetermined manner. In order to obtain desirable transgenic plants with high yield stability under stress conditions, appropriate promoters must be selected depending on the gene used. As discussed earlier, for example, the constitutive expression of ZmNF-YB2 in maize gave increased tolerance to drought (Nelson et al. 2007). Transgenic rice plants, by contrast, over express OsNAC10 under the rootspecific promoter RCc3, but not under the control of the constitutive GOS2 promoter, conferred a yield advantage under drought stress conditions in the field (Jeong et al. 2010). Other TFs, such as DREB2A with a variety of roles in biotic and abiotic stresses, may be used to design multiple stress tolerance and increased yields (Yang et al. 2010). The main concern about TF transgenics, however, is whether they perform consistently in the field in conditions of drought and/or heat stress.

9.4.9 Integrated Biotechnology Approach

Although the biotechnology community remains focused on either MB or GE approaches (Narayanan 2002), it is clear that the use of integrated biotechnology approaches must address complex problems caused by drought and heat. In this context, the maize community is an excellent example of several major projects, including Water Efficient Maize in Africa (WEMA, http///www.aatf-africa.org/wema/en/wema), Drought Tolerant Maize for Africa (DTMA, http/dtma.cimmyt.org/), and Improved Maize for African Soils (IMAS, http//www.cimmyt.org/en/projects/ improved maize for African soils). In collaboration with international partners, including multinationals such as Monsanto (www.monsanto.com/) and Pioneer (http//www.pioneer.com/), these projects use conventional breeding, MB and GE

approaches. CIMMYT provides high-yielding maize cultivars adapted to African conditions as well as expertise in conventional breeding and drought tolerance testing under the WEMA initiative. Monsanto provides proprietary germ plasm, advanced breeding tools and expertise and transgenes developed in cooperation with BASF tolerant of drought (http/www.basf.com/). The cultivars developed through this initiative will be distributed without royalties to African seed companies through the African Agricultural Technology Foundation (AATF) and made available to smallholder farmers as part of their seed companies. For example, under the DTMA initiative, more than 50 new maize hybrids and open-pollinated maize cultivars have been developed and distributed to seed companies and non-governmental organizations. These maize cultivars tolerant to drought produce approximately 20–50% higher yields under drought than other maize cultivars, and several of them have already reached farmers' fields. In contrast, the IMAS initiative is developing maize varieties that are better able to capture the small amount of fertilizer that African farmers can afford and that use nitrogen more efficiently to produce grain (i.e. increase NUE). In addition to MB and GE, there have recently been some new approaches to address complex stresses in a concerted way that should be integrated with MB and GE. (i) approaches to NGS or transcriptomics and proteomics to isolate new genes and promoters of multiple abiotic stress tolerance (Varshney et al. 2009); (ii) gene targeting for the genetic modification of crops (Osakabe et al. 2010); (iii) marker-free transgenic crop development (Parkhi et al. 2005); (iv) the development of cis-genics (Jacobsen and Schouten 2007); (v) allele mining for candidate genes in germplasm collections (Varshney et al. 2005); and (vi) the creation and use of mutations by deploying Targeted Induced Local Lesions in Genomes (TILLING) (Till et al. 2007).

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Advancement in Sustainable Agriculture: Computational and Bioinformatics Tools

10

Abstract

Sustainable agricultural production is an urgent issue in response to global climate change and population increase. Furthermore, recent increased demand for biofuel crops has created a new market for agricultural commodities. One potential solution is to increase plant yield by designing plants based on a molecular understanding of gene function and on the regulatory networks involved in stress tolerance, development and growth. Recent progress in plant genomics has allowed us to discover and isolate important genes and to analyze functions that regulate yields and tolerance to environmental stress.

Keywords

Transcriptomics · Proteomics · Informatics · Genomics · Integration

10.1 Bioinformatics and Its Applications in Plant Biology

Bioinformatics refers to the study of biological information using concepts and methods in computer science, statistics and engineering and can be divided into two broad categories: Biological information management and computer biology. The boundaries of these categories are becoming more diffuse and other categories will no doubt surface in the future as this field matures. In our society, our economy and our global environment, plant life plays important and diverse roles. For modern plant biotechnology, feeding the growing world population is a challenge. Crop yields have increased during the last century and will continue to improve as agronomy re-assorting the enhanced breeding and develop new biotechnological-engineered strategies. The onset of genomics is providing massive information to improve crop phenotypes. Accumulating sequence data enables detailed genome analysis through the use of friendly access to database and retrieval of information. Genetic and molecular genome co linearity allows efficient transfer of data

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revealing extensive conservation of genome organization between species. Genome research's goals are to identify sequenced genes and deduct their functions through metabolic analysis and reverse genetic screens from gene knockouts. More than 20% of the predicted genes occur as a cluster of related genes that generate a significant proportion of gene families. Multiple alignments provide a method for estimating gene numbers in gene families to identify previously described genes. This information allows for new strategies in plants to study patterns of gene expression. Available news technology information, as the DNA microarray expression data stored in the database, will assist functional genomics of plant biology. Expressed sequence tags also provide an opportunity to compare digital northern gene expression levels that provide initial clues to unknown regulatory phenomena. Collections of databases and bioinformatics resources for crop plant genomics were built on crop plant networks to harness the extensive genome mapping work. This resource facilitates the identification of ergonomically important genes through comparative analyzes between crop plants and model species, enabling genetic engineering of selected crop plants by the quality of the resulting products. Resources in bioinformatics have evolved beyond expectations, developing new biotechnologies in nutritional genomics biotechnology tools to genetically modify and improve food supply, for an ever-increasing world population. Bioinformatics can now be leveraged to speed up the translation into agriculture of basic discovery. Farming will be affected by predictive manipulation of plant growth at a time when food security, land reduction available for agricultural use, environmental stewardship, and climate change are all issues of growing public concern.

10.1.1 Sequence Analysis

Biological sequence such as DNA, RNA, and protein sequence is the most fundamental object for a biological system at the molecular level. Several genomes have been sequenced to a high quality in plants, including Arabidopsis thaliana (The Arabidopsis Genome Initiative 2000) and rice (Goff et al. 2002). Draft genome sequences are available for poplar (http://genome.jgi-psf.org/Poptr1/) and lotus (http://www.kazusa.or.jp/lotus/), and sequencing efforts are in progress for several others including tomato, maize, *Medicago truncatula*, sorghum (Bedell et al. 2005) and close relatives of *Arabidopsis thaliana*. Researchers also generated expressed sequence tags (ESTs) from many plants including lotus, beet, soybean, cotton, wheat, and sorghum (http://www.ncbi.nlm.nih.gov/dbEST/). Genome Sequencing Advances in sequencing technologies provide opportunities in bioinformatics for managing, processing, and analyzing the sequences. Shotgun sequencing is currently the most common method in genome sequencing: pieces of DNA are sheared randomly, cloned, and sequenced in parallel. Software has been developed to piece together the random, overlapping segments that are sequenced separately into a coherent and accurate contiguous sequence (Gibbs and Weinstock 2003). Numerous software packages exist for sequence assembly (Pop et al. 2004), including Phred/Phrap/Consed (http://www.phrap.org), Arachne (http://www.broad.mit.edu/wga/),

and GAP4 (http://staden.sourceforge.net/overview.html). TIGR developed a modular, open-source package called AMOS (http://www.tigr.org/software/AMOS/), which can be used for comparative genome assembly (Patil et al. 2001). Current limitations in shotgun sequencing and assembly software largely remain in assembling highly repetitive sequences, although the sequencing cost is another limitation. Recently developed methods continue to reduce sequencing costs, including sequencing using differential hybridization of oligonucleotide samples, polymorphism ratio sequencing (Blazej et al. 2003), four-color chip-based DNA sequencing and the "454 method" based on high-density micro-fabricated picoliter reactors (Margulies et al. 2005). In terms of experimental design, data interpretation and analysis, each of these sequencing technologies poses significant analytical challenges for bioinformatics in terms of experimental design, data interpretation, and analysis of the data in conjunction with other data (Di et al. 2005). Gene finding and Genome Annotation Gene finding refers to prediction of introns and exons in a segment of DNA sequence. Dozens of computer programs for identifying protein-coding genes are available (Zhang 2002). Some of the well-known ones include Genscan (http://genes.mit.edu/GENSCAN.html), GeneMarkHMM (http://opal.biology. gatech.edu/GeneMark/), GRAIL (http://compbio.ornl.gov/Grail-1.3/), Genie (http://www.fruitfly.org/seqtools/genie.html), and Glimmer (http://www.tigr.org/softlab/glimmer). Several new gene-finding tools are tailored for applications to plant genomic sequences (Schlueter et al. 2003). Ab initio gene prediction remains a challenging problem, especially for large-sized eukaryotic genomes. For a typical *Arabidopsis thaliana* gene with five exons, at least one exon is expected to have at least one of its borders predicted incorrectly by the ab initio approach (Brendel and Zhu 2002). Transcript evidence from full-length cDNA or EST sequences or similarity to potential protein homologs can significantly reduce uncertainty of gene identification (Zhu et al. 2003). Several software packages have been developed for structural annotation (Allen et al. 2004). In addition, one can use genome comparison tools such as SynBrowse (http://www.synbrowser.org/) and VISTA (http://genome.lbl.gov/vista/index.shtml) to enhance the accuracy of gene identification. Current structural annotation limitations include accurate transcript start sites prediction and identification of small genes encoding less than 100 amino acids, non-coding genes) and alternative splicing sites. The analysis of repetitive DNAs, which are copies of identical or almost identical sequences present in the genome (Lewin 2003), is an important aspect of genome annotation. There are repetitive sequences in nearly any genome and abundant in most plant genomes (Jiang et al. 2004). Identifying and characterizing repeats is essential for shedding light on the evolution, function and organization of genomes and for filtering many types. A small library of plant specific repeats can be found at ftp://ftp.tigr.org/pub/data/TIGRPlant Repeats/; this is likely to grow substantially as more genomes are sequenced. One can use Repeat Masker (http://www.repeatmasker.org/) to search repetitive sequences in a genome. Repeats with poorly conserved patterns or short sequences are hard to identify using RepeatMasker due to the limitations of BLAST. To identify novel repeats, various algorithms were developed. Some widely used tools include Repeat Finder (http://ser-loopp.tc.cornell.edu/cbsu/repeatfinder.htm) and

RECON (http://www.genetics.wustl.edu/eddy/recon/). However, due to the high computational complexity of the problem, none of the programs can guarantee finding all possible repeats as all the programs use some approximations in computation, which will miss some repeats with less distinctive patterns. Inevitably, a combination of repeat finding tools is required to obtain a satisfactory overview of repeats found in an organism. Comparing sequences provides a foundation for many bioinformatics tools and may allow inference of the function, structure, and evolution of genes and genomes. For example, sequence comparison provides a basis for building a consensus gene model like UniGene (Boguski and Schuler 1995). Also, many computational methods have been developed for homology identification (Wan and Xu 2005). Although sequence comparison is highly useful, it should be noted that it is based on sequence similarity between two strings of text, which may not correspond to homology, especially when the confidence level of a comparison result is low. Also, homology may not mean conservation in function. Methods in sequence comparison can be largely grouped into pair-wise, sequence profile, and profile-profile comparison. For pair-wise sequence comparison, FASTA (http://fasta.bioch.virginia.edu/) and BLAST (http://www.ncbi.nlm.nih.gov/blast/) are popular. To assess the confidence level for an alignment to represent homologous relationship, a statistical measure was integrated into pair-wise sequence alignments (Karlin and Altschul 1990). Remote homologous relationships are often missed by pair-wise sequence alignment due to its insensitivity. Sequence-profile alignment is more sensitive for detecting remote homologs. A protein sequence profile is generated by multiple sequence alignment of a group of closely related proteins. A multiple sequence alignment builds correspondence among residues across all of the sequences simultaneously, where aligned positions in different sequences probably show functional and/or structural relationship. A sequence profile is calculated using the probability of occurrence for each amino acid at each alignment position. PSI-BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) is a popular example of a sequence-profile alignment tool. Some other sequence-profile comparison methods are slower but even more accurate than PSI-BLAST, including HMMER (http://hmmer.wustl.edu/), SAM (http://www.cse.ucsc.edu/research/compbio/sam. html), and META-MEME (http://metameme.sdsc.edu/). In the detection of remote homologues, a profile-profile alignment is more sensitive than sequence-based search programs (Yona and Levitt 2002). Because of its high false positive rate, however, the comparison between profile and profile is not widely used. It is helpful to correlate the sequence comparison results with the relationship observed in functional genomic data, especially the widely available microarray data as discussed in the transcriptome analysis section below, given potential false positive predictions. For example, if a gene is predicted to have a particular function by sequence comparison, the prediction can be trusted if the gene has a strong correlation in gene expression profile with other genes known to have the same function. Proteins can be generally classified based on sequence, structure, or function. Several sequence-based methods were developed based on sizable protein sequence (typically longer than 100 amino acids), including Pfam (http://pfam.wustl.edu/), ProDom (http://protein.toulouse.inra.fr/prodom/current/html/home.php), and Clusters of Orthologous Group (COG) (http://www.ncbi. nlm.nih.gov/COG/new/).

Other methods are based on fingerprints of small conserved motifs in sequences, as with PROSITE (http://au.expasy.org/prosite/), PRINTS (http://umber.sbs.man.ac.uk/dbbrowser/PRINTS/), and BLOCKS (http://www.psc.edu/general/software/packages/blocks/blocks.html). The false positive rate of motif assignment is high due to high probability of matching short motifs in unrelated proteins by chance. Other sequence-based protein family databases are built from multiple sources. InterPro (http://www.ebi.ac.uk/interpro/) is a database that integrates domain information from multiple protein domain databases. Using protein family information to predict gene function is more reliable than using sequence comparison alone. On the other hand, very closely related proteins may not guarantee a functional relationship (Noel et al. 2005). One can use structure or function-based protein families (when available) to complement sequence-based family for additional function information. SCOP (http://scop.mrc-lmb.cam.ac.uk/scop/) and CATH (http://cath-www.bio chem.ucl.ac.uk/) are the two well-known structure-based family resources. ENZYME (http://us.expasy.org/enzyme/) is a typical example of a function family. A protein family can be represented in a phylogenetic tree that shows the evolutionary relationships among proteins. Phylogenetic analysis can be used in comparative genomics, gene function prediction, and inference of lateral gene transfer among other things (Doolittle 1999). The analysis typically starts from aligning the related proteins using tools like ClustalW (http://bips.u-strasbg.fr/fr/Documentation/ClustalX/). Among the popular methods to build phylogenetic trees are minimum distance, maximum parsimony, and maximum likelihood trees. Some programs provide options to use any of the three methods, e.g., the two widely used packages PAUP (http://paup.csit.fsu.edu), and PHYLIP (http://evolution.genetics.washington.edu/phylip.html). Although phylogenetic analysis is a research topic with a long history and many methods have been developed, various heuristics and approximations are used in constructing a phylogenetic tree, as the exact methods are too computationally intense.

10.1.2 Transcriptome Analysis

The primary goal of transcriptome analysis is to learn how an organism's growth and development and response to the environment changes in transcript abundance control. DNA microarrays have been shown to be a powerful technology for gene-wide gene transcription profile observation (Schlueter et al. 2003). Microarray data is also combined with other information to infer coregulated processes such as regulatory sequence analysis, gene ontology, and pathway information. Whole-genome tiled arrays are used to detect transcription without prejudice to known or predicted structures of genes and alternative variants of splices. Other types of analysis include the analysis of ChIP-chip (chromatin immune precipitation (ChIP) and microarray chip, combining microarrays with methods for detecting chromosomal locations where protein-DNA interactions occur across the genome (Buck and Lieb 2004). DNA immune precipitation (DIP-chip) is used by a related technique to predict DNA-binding sites (Liu et al. 2005; Brenner et al.

2000). Microarray analysis makes it possible to measure transcript simultaneously measurement of transcript abundance for thousands of genes (Zhu and Wang 2000). Two general types of microarrays are high-density oligonucleotide arrays containing a large number of relatively short (25–100-mer) samples synthesized directly on the surface of the arrays, or arrays of amplified polymerase chain reaction products or cloned DNA fragments mechanically located directly on the surface of the array. Many different technologies are being developed (Meyers et al. 2004). Competition between microarray platforms has resulted in lower costs and higher gene numbers per array. Unfortunately, the variety of array platforms makes it difficult to compare microarray results between microarray formats that use different probe sequences, RNA sample labeling, and data collection methods (Woo et al. 2004). Even for standardized arrays like those from Affymetrix, the optimal statistical treatment for the sets of samples designed for each gene still has arguments. The Affycomp software, for example, compares Affymetrix results using two spike-in experiments and a dilution experiment for different standardization methods under different evaluation criteria (Cope et al. 2004). You can use this information to select the appropriate methods for normalization. There are many tools available for conducting a variety of analysis on large data sets of microarrays. Examples include commercial software such as Gene Traffic, GeneSpring (http://www.agilent.com/chem/genespring), Affymetrix's GeneChip Operating Software (GCOS), and public software such as Cluster (Eisen et al. 1998), CaARRAY (http://caarray.nci.nih.gov/), and BASE (Saal et al. 2002). A notable example is Bioconductor (http://www.bioconductor.org), which is an open-source and open-development set of routines written for the open-source R statistical analysis package (http://www.r-project.org). Observing transcriptional activity patterns that occur under various conditions, such as genotypes or time courses, reveals genes that have highly correlated patterns of expression. The correlation, however, cannot distinguish between genes under common regulatory control and those whose patterns of expression merely correlate. Recent microarray analysis efforts have focused on experimental analysis of microarray data (Mockler and Ecker 2005). A Toxico genomics research consortium study indicates "microarray results can be comparable across multiple laboratories, particularly when using a common platform and set of procedures"(Bard and Rhee 2004). Meta-analysis can examine the effect of the same treatment on different studies in order to arrive at a single estimate of the true effect of treatment (Rhodes et al. 2004). Tiling arrays Known and predicted genes are typical microarray samples. Tiling arrays cover the genome at regular intervals to measure unbiased transcription to known or predicted gene structures, polymorphism discovery, alternative splicing analysis, and transcription factor-binding sites identification (Mockler and Ecker 2005). Whole-genome arrays (WGAs) cover the entire genome with regular gaps overlapping samples or samples. The WGA ensures that the experimental results are not dependent on the level of current genome annotation, and those new transcripts and unusual forms of transcription are discovered. Similar studies for the entire genome of Arabidopsis (Stolc et al. 2005) and parts of the rice genome have been performed

in plants (Toyoda and Shinozaki 2005). These studies identified thousands of novel transcription units including centromer genes, significant transcription of antisense genes, and transcription activity in intergenic regions. Tiling array data can also be used to validate the predicted boundaries intron/exon boundaries (Toyoda and Shinozaki 2005). Further work is needed to establish the best practices for determining when transcription has occurred and how to normalize array data across the different chips. Visualization of the output from tiling arrays requires viewing the probe sequences on the array together with the sequence assembly and the probe expression data. The Arabidopsis Tiling Array Transcriptome Express Tool (also known as ChipViewer) (http://signal.salk.edu/cgibin/atta) displays information about what type of transcription occurred along the Arabidopsis genome (Yamada et al. 2003). Another tool is Affymetrix's Integrated Genome Browser (IGB), a Java program that investigates genomes and combines annotations from multiple sources of data. Another option to view such data is collaborations such as those between Gramene (Ware et al. 2002) and PLEXdb (Shen et al. 2005), allowing users to overlay probe array information to a comparative sequence viewer. The major limitations of WGAs include a sequenced genome requirement, the large number of chips required for complete genome coverage, and recent duplicated (and thus highly homologous) gene analysis. Regulatory sequence analysis Discoverin includes the interpretation of the results of microarray experiments involves discovering why genes with similar expression profiles behave in a coordinated fashion. Regulatory sequence analysis approaches this question by extracting motifs that are shared between the upstream sequences of these genes (van Helden 2003). Comparative genomics studies of retained non-coding sequences (CNSs) may also help to identify key motives (Inada et al. 2003). There are several methods on the upstream of coregulated genes to search for over-represented motifs. Approximately two classes can be categorized: oligonucleotide-based frequency (van Helden 2003) and probabilistic sequence-based models (Roth et al. 1998). The frequency-based method of oligonucleotides calculates the statistical significance of a site based on the frequency tables of oligonucleotides observed in all non-coding regions of the genome of the specific organism. The oligonucleotide length usually varies from 4 to 9 bases. Hexanucleotide (6-length oligonucleotide) analysis is most widely used. It is then possible to group the significant oligonucleotides as longer consensus motifs. Frequency-based methods tend to be simple, effective and comprehensive. The main limitation is the problem of identifying complex patterns of motifs. Regulatory Sequence Analysis Tools (RSAT), the public web resource, performs sequence similarity searches and analyzes the genome non-coding sequences (van Helden 2003). The motif is represented as a position probability matrix for probabilistic-based methods, where the motifs are supposed to be hidden in the noisy background sequences. One of the strengths of probabilistic methods is the ability to identify motifs with complex patterns. It is possible to identify many potential motives; however, separating unique motives from this large pool of potential solutions can be difficult. Also, probabilistic-based methods tend to be computationally intense, as they must be run multiple times in order

to obtain an optimal solution. AlignACE, Aligns Nucleic Acid Conserved Elements (http://atlas.med.harvard.edu/), is a popular motif finding tool first developed for yeast but expanded to include other species (Roberts et al. 2000).

10.1.3 Computational Proteomics

Proteomics is a leading technology for protein qualitative and quantitative characterization and genome-scale interactions. The proteomics goals include large-scale identification and quantification of all protein types in a cell or tissue, post-translation modification analysis and association with other proteins, and characterization of protein activities and structures. Proteomics application in plants is still in its initial phase, mostly in the identification of proteins (Newton et al. 2010). Other proteomic aspects such as protein-protein interaction identification and prediction, protein activity profiling, local subcellular protein localization, and protein structure, have not been widely used in plant science. However, recent efforts such as the structural genomic initiative that includes Arabidopsis (http://www.uwstructuralgenomics.org/) are encouraging. Electrophoresis Analysis Electrophoresis analysis can qualitatively and quantitatively investigate expression of proteins under different conditions (Gorg et al. 2000). Several bioinformatics tools have been developed for two-dimensional (2D) electrophoresis analysis (Mao et al. 2005). SWISS-2DPAGE can locate the proteins on the 2D PAGE maps from SwissProt (http://au.expasy.org/ch2d/). Melanie (http://au.expasy.org/melanie/) can analyze, annotate, and query complex 2D gel samples. Flicker (http://open2dprot.sourceforge.net/Flicker/) is an open-source stand-alone program for visually comparing 2D gel images. PDQuest (http://www.proteomeworks.bio-rad.com) is a popular commercial software package for comparing 2D gel images. Some software platforms handle related data storage and management, including PEDRo (http://pedro.man.ac.uk/), a software package for modeling, capturing, and disseminating 2D gel data and other proteomics experimental data. Limited ability to identify proteins and low accuracy in detecting protein abundance are the main limitations of electrophoresis analysis. Protein Identification by Mass Spectrometry following protein separation using 2D electrophoresis or liquid chromatography and protein digestion using an enzyme (trypsin, pepsin, glu-C, etc.), proteins are typically identified using mass spectrometry (MS). MS provides a high-throughput approach for large-scale protein identification, unlike other protein identification techniques, such as Edman degradation microsequencing. The data generated from mass spectrometers are often complicated and the interpretation of computational analysis is critical in interpreting the data for protein identification (Gras and Muller 2001). The lack of open-source software is a major limitation in MS protein identification. Expensive commercial packages are the most widely used tools. Furthermore, current statistical models are generally oversimplified for matches between MS spectra and protein sequences. Consequently, confidence assessments are often unreliable for the results of computational protein identification. Two types of protein identification methods are available for MS: peptide mass fingerprinting (PMF) and tandem mass spectrometry (MS/MS).

10.1.4 Peptide Mass Fingerprinting

Identification of PMF peptides/proteins compares the masses of peptides derived from experimental spectral peaks with each of the possible protein computationally digested peptides in the sequence database. The proteins in the sequence database are considered candidates for the proteins in the experimental sample, with a significant number of peptide matches. MOWSE (Pappin et al. 1993) was a previous PMF protein identification software package, and Emowse (http://emboss.sourceforge.net/) is the latest MOWSE algorithm implementation. Several other computational tools for PMF protein identification have also been developed. MS-Fit in the Protein Prospector (http://prospector.ucsf.edu/) uses a variant of the MOWSE scoring scheme that incorporates new features, including restrictions on the minimum number of peptides to match for a possible hit, the number of missed cleavages and the molecular weight range of the target protein. The MOWSE algorithm extension is Mascot (http://www.matrixscience.com/). It incorporates the same scoring scheme with a probability-based score being added. A limitation of the identification of PMF protein is that it can sometimes not identify proteins because multiple proteins in the database can fit the spectra of PMF. In this case, further experiments with MS/MS are necessary to identify the proteins.

10.1.5 Tandem Mass Spectrometry

MS/MS further breaks each digested peptide into smaller fragments, whose spectra provide effective signatures of individual amino acids in the peptide for protein identification. Many tools have been developed for MS/MS-based peptide/protein identification, the most popular ones being SEQUEST (http://fields.scripps.edu/sequest/) and Mascot (http://www.matrixscience.com/). Both rely on the comparison between database-derived theoretical peptides and spectrometric tandem spectra of experimental mass. One of the earliest tools developed for this, SEQUEST produces a list of possible assignments of peptide / protein in a protein mixture based on a correlation scoring scheme (Yates et al. 1995). Mascot uses a similar algorithm to identify MS / MS peptide/protein together with its PMF protein identification capacity as SEQUEST. The limitations of these programs are that due to various factors, including sequencing and annotation errors in the search database, a significant portion of MS / MS spectra cannot be assigned. Furthermore, computational approaches are not currently used to handle post-translation modifications well. An active research area (Dancik et al. 1999) is the de novo sequencing approach based on MS / MS spectra. The algorithms typically match peak separations by the mass of one or more amino acids and infer the likely peptide sequences consistent with matched amino acids (Chen et al. 2001). Several popular peptide de novo sequencing software packages are available using MS/MS data, including Lutefisk (http://www.hairyfatguy.com/lutefisk/) and PEAKS (http://www.bioinformaticssolutions.com/products/peaks). One limitation of the current methods is that they are frequently used.

10.1.6 Metabolomics and Metabolic Flux

Metabolomics is the analysis at any given time of a cell's complete pool of small metabolites. Because of the proliferation of secondary metabolites, metabolomics may be particularly important in plants (van Helden et al. 2000). Metabolites are extracted from tissues, separated, and analyzed in a high-throughput manner in a metabolite profiling experiment (Dancik et al. 1999). Metabolic fingerprinting examines a few metabolites to help differentiate samples by phenotype or biological relevance (Shanks 2005). Technology has now advanced to quantify >1000 compounds from a single leaf extract semi-automatically (Ware et al. 2002). The key challenge in metabolite profiling is to identify metabolites from complex plant samples quickly, consistently, and unambiguously (Sriram et al. 2004). Identification is routinely carried out using time-consuming standard additional experiments using commercially available or purified preparations for metabolites. For gas chromatography-mass spectrometry (GC–MS) profiles from various biological sources, a publicly accessible database is needed that contains the evidence and underlying metabolite identification. Experimental metadata standards and metabolomi data quality standards are still in a very early stage and a large-scale public repository is not yet available. The ArMet (metabolomics architecture) proposal (Harris et al. 2005) provides a description and results of plant metabolomics experiments along with a database scheme. MIAMET (Minimum Information on a Metabolomics Experiment) (Gorg et al. 2000) provides reporting requirements with a view to standardizing descriptions of experiments, especially in publications. The Working Group on Standard Metabolic Reporting Structures (SMRS Working Group, 2005) developed standards to describe the biological sample origin, analytical technologies, and methods used in a metabolite profiling experiment. Metabolite data were used to build networks of metabolic correlation (Steuer et al. 2003). Such correlations may reflect the net partitioning of carbon and nitrogen through transcriptional or biochemical processes resulting from direct enzymatic conversions and indirect cell regulation. Metabolic correlation matrices, however, cannot infer that a change in one metabolite in a metabolic reaction network led to a change in another metabolite (Steuer et al. 2003). The steady-state flow between metabolites is measured by metabolic flux analysis. However, fluxes are even more difficult to measure than metabolite levels because of complications in intracellular metabolite transport modeling and incomplete knowledge of in vivo pathway topology and location (Shanks 2005). The most basic approach to metabolic flux analysis is stoichiometric analysis, which calculates the quantities of reactants and chemical reaction products to determine each metabolite's flux (Edwards and Palsson 2000). However, for large networks, this method is numerically difficult to solve and it has problems when there are parallel metabolic pathways, metabolic cycles, and reversible reactions (Wiechert et al. 2001). FluxAnalyzer is a MATLAB package that integrates metabolic network path and flux analysis (Klamt et al. 2003). Flux analysis using 13C carbon labeling data attempts to overcome some of the disadvantages of the abovementioned stoichiometric flux analysis (Sriram et al. 2004). In the 13C restricted flux analysis and the stoichiometric and isotopomer balances, more rigorous

analysis is needed to fully determine fluxes from all experimental data. Iterative methods were used to solve the resulting matrix of isotopomer balances, with the measurements of nuclear magnetic resonance or gas chromatography being used for consistency purposes. As more reliable data are collected, ordinary differential equations can be used for metabolic network dynamic simulations, combining information on connectivity, concentration balances, flux balances, metabolic control, and pathway optimization.

Ultimately, one may integrate all of the information and perform analysis and simulation in a cellular modeling environment like E-Cell (http://www.e-cell.org/) or CellDesigner (http://www.systems-biology.org).

10.1.7 Ontologies

Ontology is a set of vocabulary terms with explicit meanings and relationships with other terms used to annotate data (Ashburner et al. 2000). Bio-Ontology Types A growing number of common ontologies are being constructed and used in biology. Examples include ontologies to describe gene and protein function (Harris et al. 2004), cell types (Bard et al. 2005), anatomies and organism developmental phases (Garcia-Hernandez et al. 2002), microarray experiments (Stoeckert et al. 2002), and metabolic pathways (Mao et al. 2005). The Open Biological Ontologies Web site (http://obo.sourceforge.net/) provides a list of open-source ontologies used in biology. A lot of ontology is under development on this site and is subject to frequent changes. Gene Ontology (GO) (www.geneontology.org) is an example of bio-ontology, which has gained acceptance from the community. It is a set of more than 16,000 controlled vocabulary terms for the biological domains of molecular function, subcellular compartment, and biological process. GO is organized as a directed acyclic graph, a type of hierarchy tree that allows a term to exist as a specific concept belonging to more than one general term. Other examples of ontologies currently in development are the Sequence Ontology (SO) project (Eilbeck et al. 2005) and the Plant Ontology (PO) project (www.plantontology.org). The SO project aims to explicitly define all the terms needed to describe features on a nucleotide sequence, which can be used for genome sequence annotation for any organism. The PO project aims to develop shared vocabularies to describe anatomical structures for flowering plants to depict gene expression patterns and plant phenotypes. A few challenges in the development and use of ontologies remain to be addressed, including redundancies in the ontologies, minimal or lack of formal, computer comprehensive definitions of the terms in the ontologies, and general acceptance by the research and publishing community (Bard and Rhee 2004). An international repository of ontology standards is available to oversee the development and maintenance of ontologies. Ontology applications are mainly used to annotate data such as sequences, clusters of gene expression, experiments, and strains. Ontologies that have such annotations of data in databases can be used in numerous ways, including connecting different databases, refining search, providing a framework for interpreting the results of functional genomics experiments, and inferring knowledge (Bard

and Rhee 2004). For example, one can ask which functions and processes in an expression cluster of interest are statistically significantly over-represented in an expression cluster of interest compared to the functions and processes carried out by all of the genes from a gene expression array. Since GO is one of the more well-established ontologies, this section focuses on GO to illustrate ontology applications in biology. Many model organism databases (http://www.geneontology.org/GO.current.annotations.shtml, http://www.geneontology.org/GO.biblio.shtml#annots) used ontologies to annotate genes and gene products. Function annotations of genes using GO have been used primarily in two ways: predicting protein functions, processes, and patterns of localization from different data sources (http://www.geneontology.org/GO.biblio.shtml#predictions) and providing a biological framework or benchmark set for interpreting large-scale sampling results such as genes expression profiles and protein-protein interactions (http://www.geneontology.org/GO.biblio.shtml#geneexp). Furthermore, GO annotations were used to test the robustness of search methods for semantic similarity (Lord et al. 2003) and to study adaptive evolution. Using GO annotations to predict function and use them as a benchmark for large-scale data has several problems. One is the misuse or lack of use of evidence codes, providing the kind of evidence used to make the annotation (http://www.geneontology.org/GO.evidence.shtml). Only approximately half of the codes of evidence refer to direct experimental evidence. In addition, several codes of evidence are used for indirect evidence, indicating less certainty in annotation assertion than those made with direct evidence. Other codes are used for computationally derived annotations and do not have experimental support and are more likely to be incorrect. Researchers and computer programs using the annotations to provide knowledge or analyze functional genomics data should be familiar with these codes of evidence to minimize data misinterpretation. For example, methods for evaluating the relationship between sequence conservation and gene co-expression and using GO annotations to validate their results should ensure that no annotations are used to avoid circular arguments using ISS and IEA evidence codes. Similarly, studies seeking to define biological processes and functions from gene expression data using the GO annotations should ensure that no annotation with inferred from expression pattern (IEP) evidence code is used. The other caveat is that annotations to GO are not equivalently represented throughout GO. When looking for statistical over-representation of GO terms in genes of an expression cluster, there is low statistical power for detecting deviations from expectation for terms that are annotated with a small number of genes (Khatri and Draghici 2005).

10.1.8 Emerging Areas in Bioinformatics

In this section the main focus will be on text mining, biology of systems, and semantic web. Some other emerging areas, such as image analysis (Sinha et al. 2002), grid computing (Foster 2002), directed evolution (Dalby 2003), rational protein design (Looger et al. 2003), microRNA-related bioinformatics (Brown and Sanseau 2005),

and modeling in epigenomics (Fazzari and Greally 2004) are not covered due to the limitation of space. The Medline 2004 database had 12.5 million entries and is expanding at a rate of 500,000 new citations each year (Cohen and Hersh 2005). The goal of text mining is to allow researchers to identify needed information and shift the burden of searching from researchers to the computer. Without automated text mining, much of biomolecular interactions and biological research archived in the literature will remain accessible in principle but underutilized in practice. One key area of text mining is relationship extraction that finds relationships between entities such as genes and proteins. Examples include MedMiner at the National Library of Medicine (Tanabe et al. 1999), PreBIND (Donaldson et al. 2003.), the curated BIND system (Alfarano et al. 2005), PathBinderH (Ding et al. 2005), and iHOP (Hoffmann and Valencia 2004). Results on real-world tasks such as automatic extraction and assignment of GO annotations are promising, but they are far from achieving the required performance required by applications in the real world (Blaschke et al. 2005). A key challenge that needs to be addressed in this field is the complex nature of names and terminology such as the wide range of variants in free text for protein names and GO terms. The current system generation is beginning to combine statistical methods with machine learning to capture expert knowledge about how genes and proteins are referred to in scientific papers in order to create usable systems with high precision and to recall specialized tasks in the future. Computational Systems Biology Classical systems analysis in engineering treats a system as a black box whose internal structure and behavior can be analyzed and modeled by varying internal or external conditions and studying the effect of variation on external observables. The result is a comprehension of the system's internal makeup and working mechanisms (Kell et al. 2005). Biology of systems is applying this theory to biology. The observables are measurements of what the organism are doing, ranging from descriptions of phenotypes to detailed metabolic profiling. A critical issue is how different data types, such as sequence, gene expression, protein interac, can be effectively integrated and phenotypes to infer biological knowledge. Some areas that require more work include creating coherent validated data sets, developing common formats for pathway data (SBML) (Hucka et al. 2004) and BioPAX (http://www.biopax.org)), and creating ontologies to define complex interactions, curation, and linkages with textmining tools. The Systems Biology Workbench project (http://sbw.kgi.edu/) aims to develop an open-source software framework for sharing information between different types of pathway models. Other issues are that biological systems are underdefined (not enough measurements are available to characterize the system) and samples are not taken often enough to capture time changes in a system that may occur at vastly different time scales in different networks such as signaling and regulatory networks (Papin et al. 2004). The long-term goal of creating a cell's complete silico model is still a long way off; however, the tools being developed to integrate information from a wide variety of sources will be of short-term value. Semantic Web Semantic Web is a model for "creating a universal mechanism for exchanging information by giving meaning to the content of documents and data on the Web in a machine-interpretable manner" (Neumann 2005). This model will enable the development of search tools

that know what type of information can be obtained from which documents and understand how the information in each document relates to another, allowing the use of reasoning and logic by software agents to make decisions automatically based on the constraints provided in the query (e.g., automatic travel agents, phenotype prediction) (Berners-Lee et al. 2001). Bioinformatics could greatly benefit from the successful implementation of this model and should play a leading role in its implementation (Papin et al. 2004). Current efforts have focused on the development of standards and specifications for the identification and description of data such as Universal Resource Identifier (URI) and Resource Definition Framework (RDF) respectively (http://www.w3c.org/2001/sw). While implementation of web-based applications is scarce at this point, some useful examples are being developed, such as Haystack (a browser that retrieves data from multiple databases and allows users to annotate and manage the information to reflect their understanding) (http://www-db.cs.wisc.edu/cidr/cidr2005/papers/P02.pdf) and BioDash (a drug development user interface that associates diseases, drug progression stages, molecular biology, and pathway knowledge for users) (http://www.w3.org/2005/04/swls/BioDash/Demo/). Cellular Localization and Spatially Resolved Data Research in nanotechnology and electron microscopy enables researchers to select specific cell and tissue areas and to picture spatiotemporal distributions of signaling receptors, gene expression and proteins. Laser microdissection capture allows specific tissue types to be selected for detailed analysis (Emmert-Buck et al. 1996). In Arabidopsis, confocal imaging is used to model the patterns of auxin transport and gene expression (Heisler et al. 2005). Methods are applied in electron microscopy to image spatiotemporal signaling distribution of signaling receptors. Improved methods in laser scanning microscopes may allow measurements of fast diffusion and dynamic processes in the microsecond-to-millisecond time range in live cells (Digman et al. 2005). These emerging capabilities will lead to new understanding of cell dynamics.

Bioinformatics integration will influence plant science and will lead to crop improvements in the following areas:

(a) Identifying important genes through genomics, analysis of expression and functional genomics.
(b) The design of agrochemicals based on the analysis of signal perception and transduction pathways components to identify targets and chemin-formatics compounds that may be used as herbicides, pesticides or insecticides.
(c) Use of plant genetic resources to preserve genetic diversity in farming species.
(d) Efficient use of biological clone, cell, organism and seed repositories.

10.1.9 Software's for Microarray Data Analysis

Most statistical packages used in microarray experiment data analysis i.e. In gene expression studies, SAS, SPSS, MATLAB and R are not entirely unique. To analyze such experiments, many tools are available and much easier than the aforementioned pure statistical programs.

10.1.9.1 FlexArray

FlexArray is a Windows software package designed to simplify expression micro array data analysis. FlexArray's target audience is biological scientists. Currently the software supports Affymetrix Gene Chips, Nimble Gen, Illumina Bead Chips and various types of one-color and two-color arrays of expression. FlexArray is well suited to projects of small and medium size. FlexArray is on the programming language of R. FlexArray is a tool that generates gene lists that is not suitable for data mining. This tool is suitable for algorithms for standardization, statistical testing and other complex data processing tasks. It is also an exploration tool for methods and algorithms of analysis (Blazejczyk et al. 2007). This software can be found at http://genomequebec.mcgill.ca/FlexArray. As an example, Khojasteh et al. (2017) used the FlexArray software in order to identify responsive genes against two main pathovars of *Xanthomonas oryzae* in different rice varieties.

10.1.9.2 BioConductor

The Bioconductor package in R (http://www.r-project.org/) is an open source and open software project that is used to analyze and understand genomics data, particularly microarray data. Bio conductor is primarily based on the language of R programming although it is friendly with different programming languages. For different types of microarray analysis, a large number of different packages are available. Readers should follow http://www.bioconductor.org/ (Drăghici 2011) for more details on the Bioconductor. There are many studies in which various Bioconductor packages have been used to analyze microarray data. For instance, in the expression study of the Dof1 transcription factor in wheat and sorghum, the Affy package from Bio conductor was used for to normalize microarray data (Peña et al. 2017). Furthermore, for gene expression study of wheat leaves infected by Xanthomonas translucens, the DESeq2 package of Bioconductor was used to identify differentially expressed genes based on the Negative Binomial distribution. The Bioconductor q-value package was also used for p value correction (Garcia-Seco et al. 2017).

10.1.9.3 Gene ARMADA

For both cDNA and oligonucleotide (Affymetrix) microarray data, Gene ARMADA can provide a complete, open-source, flexible and handy platform. It was implemented in the program for MATLAB. Gene ARMADA is an independent platform that can be used either as a MATLAB tool or as an application on its own. This software specializes in the visualization, standardization and statistical testing of data. Gene ARMADA has been successfully used to process several datasets of microarrays (http://www.grissom.gr/armada) (Chatziioannou et al. 2009).

10.1.9.4 Babelomics

Babelomics is an integrative web-based platform that includes a complete suite of methods for the analysis of gene expression data i.e. normalization, pre-processing, gene expression analysis, predictors, clustering and large-scale genotyping assays. Currently, Babelomics has an average of more than 200 experiments analyzed per day,

(http://bioinfo.cipf.es/webstats/babelomics/awstats.babelomics.bioinfo.cipf.es.html), distributed among many different countries (http://bioinfo.cipf.es/toolsusage). The current version of Babelomics (Babelomics 5.0) is freely available at: http://www.babelomics.org (Alonso et al. 2015). In a comparison study of the transcriptomic and metabolomic profiles of six rice cultivars leaves under high night temperature conditions, the background correction of microarray signal intensities and differential expression investigation were performed by class comparison methods in Babelomics tool (Glaubitz et al. 2017).

10.1.9.5 Maanova

MAANOVA refers to the Micro Array Variance Analysis. MAANOVA is suitable for the analysis of microarray experiments on both small and large scale. MAANOVA, implemented in Matlab, is the statistical language R add-on package. Friendly, to run this software on any platform that supports these packages. This package provides a complete workflow for different aspects of microarray data analysis i.e. data quality checks, ANOVA model fitting (both fixed and mixed effect models), statistical testing (F and Fs statistics), p-value (using sampling and residual shuffling permutation approach) and summarizes the results in tables and graphics including. Volcano plot and bootstrapping-based tree cluster. Functions in MAANOVA have been developed and tested in Matlab Release 12 for Windows and Linux Redhat 7.0. Executable software and source code of mannova can be downloaded from the link http://churchill.jax.org/software/archive/maanova.shtml.R/maanovapackage is also available from http://churchill.jax.org/software/rmaanova.shtml (Wu et al. 2011). This software has been used in different plant species for microarray data analysis (Calla et al. 2009).

10.1.9.6 HD BStat

HD BStat (High-Dimensional Biology-Statistics) is a package for data analysis of microarrays. It was initially developed to analyze data on gene expression, but proteomics and other aspects of genomics can also be used. This software uses a variety of methods to analyze microarray data for standardization, transformation, statistical, and quality control analysis. HD BStat can also perform a test of hypothesis. The results of preprocessing methods of data, analysis of quality control and test methods of hypothesis can be displayed in the form of Excel CSV tables, graphs and Html. HDBStat, please! It is freely available platform-independent software. The link for more details http://www.ssg.uab.edu/hdbstat/documentation.html is addressed to readers. This software has been used in a study with respect to gene expression in tomato (Windram et al. 2012).

10.1.9.7 Expander

Another useful integrated software platform for analyzing data on gene expression is EXPANDER (Expression Analyzer and Displayer). It is intended to support data preprocessing and standardization and identification of differentially expressed genes, clustering; downstream enrichment analyzes of (GO functional categories, TF binding sites in promoter regions, 3'-UTR micro RNA sites and biological

pathways and chromosomal locations) and network-based expression data analysis. Expander operates on platforms such as Windows and Linux and provides analysis for various types of organisms such as humans, animals, pests, plants and microorganisms. This package is available free of charge for academic use at http://acgt.cs.tau.ac.il/expander/ (Ulitsky et al. 2010). In a case study, Expander software was used for the hierarchical clustering of transcriptomic data of *Lotus japonicus* (Regel) Larsen cv. Gifu (B-129-S9) (Pérez-Delgado et al. 2016).

10.2 Integration of Transcriptomics, Proteomics and Metabolomics

Proteomics, defined as a high-throughput protein study, has taken the lead in plant biological research and response to stress, particularly due to the growing number of plant genomes being sequenced and released (Jorrín-Novo et al. 2015). Additionally, advances in mass spectrometry (MS), quantitative methods and bioinformatics approaches have enabled a wide range of proteins from specific organ/tissue/cells to be identified, quantified, validated and characterized (Glinski and Weckwerth 2006). The information obtained through these advanced approaches is useful for the deciphering of protein structure and complex mechanisms such as enzymatic and regulatory mechanisms functions of proteins coded by specific genes (Abdallah et al. 2012). In addition, proteomics approaches provide valuable information such as levels of protein associated with stress tolerance, changes in stressed proteomes that link transcriptomics and metabolomics analyzes, as well as the role of expressed genes in functionally translated genome regions associated with traits of interest (Kosová et al. 2011). Many proteomics-based publications, particularly related to plant development and other biological phenomena such as leguminous symbiosis, can be found in model legumes and Arabidopsis thaliana, as well as in some plants such as rice (*Oryza sativa*), *Triticum aestivum*, *Zea mays*, *Solanum lycopersicum* and *Nicotiana tabacum* (Jorrín-Novo et al. 2015).

The evolution of the plant immune response has resulted in a highly effective defense system that can withstand microbial pathogens from potential attacks. The primary immune response is known as pathogen-related molecular pattern (PAMP) triggered immunity and has evolved to recognize common characteristics of microbial pathogens (Janeway and Medzhitov 2002). In response to pathogen effector protein delivery, plants acquired Resistance (R) proteins to combat pathogen attack. R-dependent defense responses are important in understanding biochemical and cellular mechanisms and underlying these interactions will allow increased molecular and transgenic approaches for crops. A new research area, i.e. the analysis of more complex microbial communities and their interaction with plants, has been initiated by recent developments in the field of proteome analysis. Such areas have great potential to elucidate not only the interactions between bacteria and their host plants, but also the interactions between bacteria and bacteria between various bacterial taxa, symbiotic, pathogenic and commensal bacteria. Plant hormonal signaling pathways give priority to defense over other cellular functions during biotic

stress. Some plant pathogens use the hormone-dependent regulatory system to mimic hormones that interfere with the immune response of the host to promote virulence (vir) (Woodward et al. 2010). The majority of bacteria are exposed to a constantly changing physical and chemical environment, unlike plant and animal cells. Phylogenetic diversity of plant-associated bacteria (PAB) can categorize them into commensals (acquire plant nutrients without harm), mutualists (influencing plant health positively) and pathogens (damaging plant). Notably pathogenic bacteria, commensals, or mutualists have developed strategies for interacting with overlapping plants, an exceptionally modified physiology that reflects individual needs (Ozinsky et al. 2000). Bacteria respond to changes in their environment by adapting structural protein patterns, transporting proteins, toxins and enzymes which adapt them to a particular habitat (Torres et al. 2004). Enzymes are either constitutive (always produced by cells independently of the medium's composition) or inducible (produced in cells in response to a pathway's end product). Regulation of enzyme activity, which is primarily used to regulate biosynthetic pathways and repression of catabolites is considered a form of positive control as it affects an increase in protein transcription rates. Plant immunity recognizing membrane protein pathogens is referred to as pattern recognition receptors (PRRs), which recognize pathogen-associated molecular pattern (PAMP) and is the basis of plant-inborn immunity(Witte et al. 2012). PAMP recognition also results in plant systemic acquired resistance and resistance (R) protein production leading to effector-triggered immunity (ETI), often accompanied by hypersensitive response (HR), and cell death programmed. Many R genes that confer resistance to various pathogens, including viruses, bacteria, fungi, or nematodes, have been isolated over the past 10 years. These R-gene products are divided into intracellular protein kinases (Pto), proteins with an extracellular leucine-rich repeat (LRR) domain and a cytoplasmic protein kinase region (e.g. Xa21), intracellular proteins containing a region of LRRs and a nucleotide bin, based on predicted protein sequences (e.g., Cf-4, Cf-9) (Zhang et al. 2012). Proteomic analyzes made it possible to analyze complex microbial communities that had great potential to elucidate not only the interactions between bacteria and their host plants, but also the interactions between bacteria and bacteria. For various PABs, proteomic reference data sets were established using two-dimensional polyacrylamide gel electrophoresis (2-DE) gels, resulting in a few hundred identified proteins or multi-dimensional liquid chromatography-tandom mass spectrometry (LC-MS/MS) techniques leading to the detection of over 1000 proteins (Anderson et al. 2006). Era is followed by gel-free proteomics, but before gel-based proteomics, quantitation procedures must be optimized before the gel-based proteomics can be replaced by gel free procedures. Complete genome sequence of a *Xylella fastidiosa* is available which can be very helpful in genomics and proteomic studies of plant-bacterium interactions (Bagnarol et al. 2007). In order to understand the molecular signaling pathways involved in plant-bacterial interactions, more genomic data are needed for pathogenic and symbiotic bacteria. Because of the agricultural importance and intensity of scientific research, P. syringae and Xanthomonas campestris are important PABs. On the model plant Arabidopsis, both are pathogenic (Andrade et al. 2008). There has been extensive study of pathogenic and mutualist

PAB (Jacobs et al. 2012). The mutualism process involved a significant change in the metabolism of the mutualists as well as the host, which involves a change in the metabolism of plant cells to support the mutualist's ATP synthesis and nitrogen fixation for nodule development (Delmotte et al. 2010). Transcriptomics data shows that pathogenic bacteria involve the hypersensitive reaction and pathogenicity (hrp) gene and different secretion systems (SS) for colonization and damaging host cells (Buttner and Bonas 2002). They typically exchange signals with their hosts and have a range of specific plant colonization adaptations. To understand the molecular mechanism by which bacteria adapt to live in association with plants for symbiosis and pathogenesis evolution is explored the importance of proteomics. This will open up new research areas on protein-based plant-microbe communication and provide important information on manipulating gene expression of specific proteins to modify plant behaviour associated with compatible or incompatible interactions. The use of proteomics for crop plant analyzes has increased rapidly over the last decade. While proteomic techniques are routinely used in plant laboratories around the world and are powerful study tools, considerable room for improvement still exists (Komatsu et al. 2013). The fraction of the plant proteome that can be detected using current approaches is significantly lower than that of other "Omics" techniques and therefore does not fully represent the cellular proteins. The predominant technique used for separating proteins is the two-dimensional electrophoresis (2-DE) gel. However, proteome analyzes based on liquid chromatography (LC) are increasing in many common laboratories. Both techniques of protein separation have specific benefits. Protein modification and degradation can be quickly visualized with a standard 2-DE approach, whereas LC-based methods require much lower starting material quantities. The limited availability of genomic information has hindered the application of crop proteomics. However, with the successful development of "next-generation" sequencing technologies, identification and annotation of proteins and their isoforms in a particular crop species is becoming much more straightforward (Komatsu et al. 2013). A specific advantage of proteomics over other "Omics" techniques is the capacity to reveal post-translational modifications (PTMs), which is a prerequisite to determine the functional impact of protein modification on crop plant productivity. To date, proteomic analyzes have identified approximately 300 PTMs. However, major efforts are needed to develop reliable tools and strategies to assess the impact of this growing number of different crop PTMs. Lastly, crop proteomics should become an essential part of integrated "Omics" approaches. A major challenge for crop proteomics, however, will be to keep pace with other "Omics" techniques' throughput capacity. Advances in plant phenotyping will benefit the application of proteomics for plant functional analysis. In particular, improved techniques for automated, non-invasive plant collection phenotyping will help in the selection of appropriate genotypes for proteomics-based functional analyses aimed at characterizing the relevant traits for future crop breeding (Wang et al. 2013).

Numerous proteins that play crucial roles in plant growth and development have been identified in proteomic studies. However, it is a major challenge to determine how this wealth of information can be applied to agriculture and artificial crop

regulation. As seed viability is related to crop yields, seed is one of the most important factors in crop production. He and Yang (2013) applied proteomics to the study of rice seed germination regulation and demonstrated that starch is degraded in endosperm and subsequently biosynthesized in the embryo during germination, a process that appears to promote the gradual use of nutritional reserves. Wide spread use of heterosis in crop production where sterile male line is critical for hybrid breeding. Identifying the proteins involved in the regulation of male sterility represents a major target in crop proteomic studies (Wang et al. 2013). Unlike traditional breeding methods, transgenic techniques are becoming increasingly popular to obtain crops with desired qualities quickly. It is essential to evaluate these GM crops using proteomic methods (Gong and Wang 2013). Maintaining food safety is a serious challenge worldwide due to impending changes in global climate and ongoing industrialization. Effective methods to increase the efficiency of sunlight conversion are needed to sustainably feed the world population (Driever and Kromdijk 2013). In light conversion, C4 plants are more efficient than C3 plants because they contain two different chloroplasts. Comparative proteomic analyses of C4 chloroplastsmight help to determine the key components that influence the efficiency of sunlight conversion (Manandhar-Shrestha et al. 2013). An important factor that influences crop growth and eventual crop yield is the interaction between crops and other organisms. For example, the pathogen *Fusarium graminearum* causes small grain cereal head blight and dramatically reduces grain yield and quality, which has a major economic impact on the cereal industry. Proteomic analysis is expected to complement traditional approaches to molecular genetics to study the mechanisms by which this pathogen attacks cereal crops(Yang et al. 2013). It has also been demonstrated the use of proteomics to analyze the interaction between crops and bacteria, especially the symbiotic interactions in legume root nodules. Most studies have been carried out on whole organs or tissues that do not allow spatial information to be collected. The use of MS imaging techniques, which have been successfully applied in the field of medicine, is therefore expected to help in obtaining information on the spatial distribution of metabolites and proteins (Matros and Mock 2013). In addition to proteomics, metabolomics is another important approach to functional genomics in which the identification and quantification of metabolomes (collection of metabolites or small molecules) within a cell, tissue or organism produced through cellular metabolism connects the cellular biochemical activity with the phenotype. Major approaches to plant metabolomics include metabolic fingerprints, metabolite profiling, and targeted analysis (Weckwerth 2003). Different metabolomic approaches or a combination of approaches are applied depending on the study objective. In addition, the use of MS, bioinformatics tools and software enables rapid measurement of metabolites at the same time, which are spatially localized within the biological material (Bhalla et al. 2005). As metabolites are closer to the phenotype, they more accurately reflect gene expressions and various regulatory processes, and metabolomics is a powerful tool for studying molecular phenotypes of plants in response to stress. For instance, the plant metabolism is affected under abiotic stress conditions due to factors such as metabolic enzyme inhibition, substrate shortage, extreme demand for specific compounds and a combination of these

factors. The plant is thus subjected to metabolic reprogramming to adapt to the predominant stress conditions by producing anti-stress components such as compatible solutes, antioxidants and stress-responsive proteins (Wienkoop et al. 2008). The use of metabolites as selection biomarkers has been of great interest in crop breeding programs, as metabolites integrate the complex interaction between genotype and environment (Wienkoop et al. 2008). With proteomics and metabolomics emerging as state-of – the-art functional biology disciplines for understanding plant adaptation mechanisms to stresses in different plant systems at cellular and developmental stages, there was great interest in applying knowledge to understand responses in different crop plants. These approaches, integrated with data obtained from genomics, enable accurate identification of candidate genes and pathways involved in important agronomic traits that can be applied in crop breeding programs (Langridge and Fleury 2011). PAMPs are the first layer of plant innate immunity and failure to recognize them may lead to increased susceptibility to disease. PAMPs are ideal elicitors for "non-self" surveillance systems such as chitin, ergosterol and fungal transglutaminase, and/or bacterial lipopolysaccharides and flagellin, which stimulate PAMP receptors encoded by plants (Chisholm et al. 2006). Intracellular responses related to PAMP-triggered immunity (PTI), including rapid ion fluxes across the plasma membrane, kinase activation of mitogen-activated protein (MAP), production of reactive oxygen species (ROS), and rapid changes in gene expression and reinforcement of the cell wall. Suppression of PTI can be achieved through the pathogens 'secretion of virulence (vir) effectors or plant signaling suppression. ETI is accompanied by R protein or HR production, which illustrates the dynamic co-evolution between plants and pathogens (Jones and Dangl 2006). Flagellin, elongation factor (EF) Tu, peptidoglycan, lipopolysaccharide, and bacterial cold shock proteins are important PAMPS and their induced plant responses are called "basal" defenses. Once the highly preserved amino terminus of flagellin (flg22) is recognized, flagellin sensing 2 (FLS2) induces a series of defense responses, including MAP kinase signaling, transcriptional activation and callose deposition a putative physical barrier at the site of infection (Gomez-Gomez et al. 1999). EF Tu potent bacterial PAMP in *Arabidopsis* and other members of the Brassicaceae family, serves as an adhesion factor at the bacterial surface, in addition to its primary role in translation. Asparate oxidase is required for PAMP-triggered RBOHD-dependent (responsible for stomatal closure) ROS burst and stomatal immunity against the *P. syringae* (Macho et al. 2012). The LRR receptor kinases, EF-Tu receptor and FLS2 are PRRs, contributing to disease resistance against the hemibiotrophic bacterium *P. syringae* (Roux et al. 2011). The plant hormones, salicylic acid (SA), jasmonic acid (JA) and ethylene, have emerged as key players in the signaling networks involved in plant immunity. Rhamnolipids are glycolipids produced by bacteria and are involved in surface motility and biofilm development and are considered as PAMPS. Ethylene is found to be involved in rhamnolipid-induced resistance to *H. arabidopsidis* and to *P. syringae* whereas JA is essential for the resistance to *B. cinerea*. SA participates in restriction of all bacterial and fungal pathogens, so involving in broadly rhamnolipid-mediated immunity (Sanchez et al. 2012). PAMPS are sometimes succeeded and sometimes fails to induce PTI

depending upon the type of compatible and non-compatible interactions. Flagellin is capable of suppressing HR via PTI induction during an incompatible interaction (Wei et al. 2012). Type III secretion system (T3SSs) were essential components of two complex bacterial machineries: the flagellum, which drives cell motility and the non-flagellar T3SS (NF-T3SS), which delivers effectors into eukaryotic cells. *P. syringae* use T3SS to deliver up to 40 effector proteins into host cells, inhibiting basal host defense responses, such as HR. PAMP induced PTI serves as a primary plant defense response against microbial pathogens, with MAP kinase cascade downstream of PAMP receptors. LRR-RLKs including PSKR1 act as PTI against pathogenic bacteria, and plants expressing this gene show enhanced PAMP responses and less lesion formation after infection with the bacterial pathogen *P. syringae* via jasmonate signaling pathway (McCann and Guttman 2008). Peptidoglycan, an important PAMP from *Staphylococcus aureus* results in PTI, such as medium alkalinization, elevation of cytoplasmic calcium concentrations, nitric oxide, and camalexin production, and the post-translational induction of MAP kinase activities. PAMP recognition also results in plant systemic acquired resistance and production of R proteins such as SUMM2 that becomes active when the MAP kinase cascade is disrupted by pathogens, leading to ETI (Zhang et al. 2012). In rice, the LRR-RK Xa21 confers resistance to *Xanthomonas oryzae* pv. oryzae strains carrying the Avr gene AvrXa21. AvrXa21 as a type I secreted sulfated peptide, is conserved among all *Xanthomonas* strains sequenced (P. Ronald, pers. communication), suggesting that *AvrXa21/Xa21* constitutes a PAMP/PRR perception system (Lee et al. 2008). Although many PAMPs recognized by plants have been described, number of known PRR and PTI is still in its infancy, constituting a highly active and competitive field of research. Protein analysis of asscoaited bacteria (PAB) either they are pathogenic or symbiotic bacteria adhere to plant surfaces, invade the intercellular space of the host tissue, counteract plant defense systems and acquire nutrients. However either there is establishment of a pathogenic interaction or mutualist relationship develops. Cell surface proteins such as adhesions, polysaccharides, lipopolysaccharides, and degradative enzymes enable the degradation of the plant cell wall and also result in basal plant defenses (Newton et al. 2010). Proteins of PAB are studied either in planta, by means of bacterial responses to selected biomolecule or plant extracts, synthetic media, or secretome analysis to study the vir factor of the bacterial pathogens (Gourion et al. 2006). Analysis of proteomas is very tricky when dealing with bacteria separation from infected plants and additional steps are needed to avoid changes in the map of proteomas. Bacterial separation protocols for density gradient centrifugation using percoll or saccharose gradients had been proposed(Gourion et al. 2006). The transcriptomics profile of R was discussed by Jacobs et al. (2012). Solanacearum *in vitro* and the importance of T3SS in the Ralsotonia Vir Cascade (45% HR and Hrp gene regulated). Pathogenic bacteria X proteome analysis. Pv campestris. Campestris in conjunction with *B. Oleracea* and savastanoipv Pseudomonas. Savastanoi led to a comprehensive analysis of expression analysis including stress and metabolic proteins (Andrade et al. 2008). Increased protein levels associated with xanthan biosynthesis, stress response, and induced metabolism in X. Unlike in vitro grown cells, campestris in

plant conditions Chaperonin is reported to be involved in stress responses, and EF, which acts in plants as an important PTI, is the key component of bacteria's translation machinery. Xanthan is an extracellular polysaccharide likely to cause disease symptoms in planta growth through the mucoid appearance of bacterial colonies and host plant wilting by blocking water flow in xylem vessels (Andrade et al. 2008).

Methylobacterium extorquens differential proteome analysis of 45 metabolic proteins and proteins involved in stress response such as extracellular protease, SOD, catalases, and DNA protein (Gourion et al. 2006) resulted in the identification of 45 metabolic proteins and proteins involved in stress response. The protein analysis of symbiosis-living cyanobacteria revealed several adjustments to a symbiotic lifestyle, including an increase in proteins involved in the production of energy and fixation of nitrogen. On the other hand, under symbiotic conditions, proteins involved in photosynthesis were reduced, pointing to a heterotrophic lifestyle. Bacteroids 'general proteome analysis is compared with *in vitro* grown cells in order to identify nodule specific adaptations, over time or when plants were exposed to drought stress (Nomura et al. 2010). ABC-type transporters was present in nodule bacteria for transport of amino acids and inorganic ions along with proteins involved in vitamin synthesis, fatty acid, nucleic acid, cell surface synthesis, and stress-related processes. Integrated proteomics and transcriptomics data for *B. japonicum* bacteroids resulted in 2315 proteins involved in carbon and nitrogen metabolism, including a complete set of tricarboxylic acid cycle enzymes, gluconeogenesis and pentose phosphate pathway enzymes, along with other proteins important in symbiosis. Amino acids (Asn, Gln, Pro), organic acids (threonic acid), sugars (Rib, maltose), and polyols (mannitol) were reported to be more abundant in symbiotic roots (Delmotte et al. 2010).

Plant metabolites are involved in many responses to resistance and stress and also contribute to fruit and flowers colour, taste, aroma, and scent. Since an organism's biochemical phenotype is the final result of genotype-environmental stimuli interactions, it is also modulated by intracellular physiological fluctuations that are part of homeostasis. Therefore, it is necessary to simultaneously identify and quantify metabolites to understand the metabolome dynamics analyze fluxes in metabolic pathways and decipher the role of each metabolite after different stimuli. Metabolomics 'challenge is to find changes in biochemical pathways and metabolic networks that may correlate with a cell, tissue, or organism's physiological and developmental phenotype (Bino et al. 2004). The completion of the entire genome sequences of model plants like Arabidopsis thaliana and rice is one of the greatest achievements of plant biology. In Arabidopsis ~27,000 genes were predicted based on information about nucleotide sequence; however, only half of these genes were functionally annotated based on sequence similarity to known genes, and only ~11% of these genes were confirmed with direct experimental evidence (Pichersky and Gang 2000). Therefore, elucidating the function of unknown genes is currently a major challenge in plant research. Because there is very little information about the number of genes in a specific gene family of a non-model plant, the profile of expression of these genes under different

conditions and stimuli becomes necessary. Integrating metabolomics with transcription profiles can provide clues for identifying the functions of the unknown genes, irrespective of whether they are from model or non-model plants (Fiehn 2001). Plants produce over 200,000 metabolites, many of which have specific roles in adapting to specific ecological niches (Fiehn 2001). Therefore, the main problems encountered when characterizing the metabolome of the plant are due to the fact that the metabolome is highly complex in nature compared to the proteome or transcriptome due to the huge chemical diversity of the compounds. Additionally, there is a wide range of concentrations of metabolites that can vary in magnitude over nine orders (pM to mM). These wide variations in the nature and concentration of analytes to be studied pose challenges to all the analytical technologies used in metabolomics strategies. Using metabolomics, it is possible to identify pathways that are responsible for producing important food metabolites that could be important to human health improvement. There are several examples where the modification of some metabolic pathways has resulted in plant production with an increased nutritional value. This is the case with Golden Rice (GR), a genetically modified rice accumulating β-carotene in endosperm (Ye et al. 2000). Production of this rice variety has helped alleviate vitamin A deficiency, a major global nutritional issue. GR's nutritional value was subsequently enhanced by the overexpression of a phytoene synthase gene leading to obtaining GR2 variety, which accumulates higher amounts of carotenoids (84% of the total is β-carotene) (Paine et al. 2005). Mehta et al.(2002) expressed a S-adenosylmethionine decarboxylase gene in tomato under the inducible E8 promoter. The transgenic variety shows higher levels of various polyamines during fruit maturation, including spermidine and spermine, leading to an increase in metabolite lycopene, which prolonged the life of the vine and increased fruit juice and nutrient quality(Mehta et al. 2002). Other examples include plant engineering to improve anthocyanin content. Anthocyanins are flavonoids, a pigment class that contributes to the plant's colors and antioxidant properties. In addition, these metabolites were linked to protection against several human diseases, but their natural levels in plants are inadequate to confer optimal benefits. It has also been reported that the expression of two transcription factors in tomatoes has resulted in the accumulation of higher anthocyanin concentrations at concentrations comparable to those founded in high antocyanin-containing plants such as blackberries and blueberries (Yeager 1927). The new variety has an intense purple coloring and an increased antioxidant capacity of 3 times. Plant metabolomics is increasingly being used to understand other processes like cellular responses to stress conditions. An example of this is the metabolic adjustment to sulfur deficiency. There was a close relationship between the metabolism of sulfur, nitrogen, lipids and purine metabolism and enhanced photorespiration. Metabolomics has also been applied to the study of the cold stress response (Blake-Kalff et al. 1998 and Miyagi et al. 2010). Other applications include metabolic engineering of biochemical pathways, gene function discovery, and engineering pathways for pharmaceuticals production.

10.3 Omic Plant Development Database Approaches

Technological advances in each research area of omics have become essential resources for gene function research in association with phenotypic changes. Some of these advances include the development of high-throughput methods to profile thousands of gene expressions, identify modification events and interactions in the plant proteome, and simultaneously measure the abundance of many metabolites. Furthermore, large-scale bioresource collections such as mass-produced mutant lines and full-length cDNA clones and their integrative relevant databases are now available (Brady and Provart 2009). Arabidopsis thaliana's entire genome sequencing was completed in 2000 (The Arabidopsis Genome Initiative 2000). Subsequently, the National Science Foundation (NSF) Arabidopsis 2010 project in the USA was launched with the stated goal of determining the functions of 25,000 genes of Arabidopsis by 2010. The genome sequencing project of *japonica* rice was completed in 2005, and the Rice Annotation Project (RAP), which was orchestrated via 'jamboree-style' annotation meetings, aimed to provide an accurate annotation of the rice genome (International Rice Genome Sequencing Project 2005, Itoh et al. 2007). In conjunction with the rice genome sequence and its related genomic resources, advanced development of mapping populations and molecular marker resources has allowed researchers to accelerate the isolation of agronomically important quantitative trait loci (QTLs) (Ma et al. 2007).

The aforementioned recent advances in high-throughput technology have provided opportunities for specific organisms to develop collections of sequence-based resources and related resource platforms. Each biological element comprehensively measured by a high-throughput method is depicted in a corresponding plane in a conceptual model with layers ranging from genome to phenome, a model called 'omic space'. Such comprehensive models often provide an excellent starting point for experiment design, hypothesis generation, or conceptualization based on the integrated knowledge found in a particular organism's omic space. Such comprehensive models often provide an excellent starting point for experiment design, hypothesis generation, or conceptualization based on the integrated knowledge found in a particular organism's omic space. In addition, the development of such omic resources and data sets for different species allows for the comparison of omic properties between species, which promises to be an effective way of finding collateral evidence for preserved gene functions that could be evolutionarily supported. Bioinformatics platforms have become essential tools for accessing omics data sets for efficient mining and biologically important knowledge integration (Table 10.1).

Examples of resources related to each omics instance are presented as the model plant in Arabidopsis, rice and soybean, as well as a monocotype model and a sequenced crop, and as an important crop recently sequenced. These resources can be accessed from the above-mentioned URLs or links (Mochida et al. 2011).

An overview of several representative resources available for use in omics plant research is given above, with particular emphasis on recent progress related to crop species in addition to sequence related resources such as whole genome, protein coding and non – coding transcripts, and updates of sequencing technology.

Table 10.1 Omic space and related resources in plants

1. http://www.arabidopsis.org/,
2. http://www.gramene.org/,
3. http://soybase.org/,
4. http://nazunafox.psc.database.riken.jp,
5. http://rarge.gsc.riken.jp/dsmutant/index.pl,
6. http://signal.salk.edu/tabout.html
7. http://tilling.fhcrc.org/,
8. http://www.postech.ac.kr/life/pfg/risd/,
9. http://tos.nias.affrc.go.jp/,
10. http://www.soybeantilling.org/psearch.jsp,
11. http://mulch.cropsoil.uga.edu/~parrottlab/Mutagenesis/acds/index.php,
12. http://arabidopsis.org.uk/home.html,
13. http://abrc.osu.edu/,
14. http://www.shigen.nig.ac.jp/rice/oryzabase/top/top.jsp,
15. http://www.irri.org/grc/GRChome/home.htm,
16. http://www.legumebase.agr.miyazaki-u.ac.jp/index.jsp,
17. http://www.plantcyc.org:1555/ARA/server.html,
18. http://pathway.gramene.org/gramene/ricecyc.shtml,
19. http://www.plantcyc.org/,
20. http://mediccyc.noble.org/,
21. http://prime.psc.riken.jp/,
22. http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/gmd.html,
23. http://ppdb.tc.cornell.edu/,
24. http://phosphat.mpimp-golm.mpg.de/,
25. http://cdna01.dna.affrc.go.jp/RPD/main_en.html,
26. http://proteome.dc.affrc.go.jp/Soybean/,
27. http://oilseedproteomics.missouri.edu/,
28. http://bioinfo.esalq.usp.br/cgi-bin/atpin.pl,
29. http://atpid.biosino.org/,
30. http://suba.plantenergy.uwa.edu.au/, 32. http://proteomics.arabidopsis.info/,
31. http://www.brc.riken.go.jp/lab/epd/catalog/cdnaclone.html,
32. http://rarge.gsc.riken.jp/,
33. http://cdna01.dna.affrc.go.jp/cDNA/,
34. http://rsoy.psc.riken.jp/,
35. http://www.arabidopsis.org/portals/expression/microarray/ATGenExpress.jsp,
36. https://www.genevestigator.com/gv/index.jsp,
37. http://bioinformatics.med.yale.edu/riceatlas/,
38. http://bioinformatics.towson.edu/SGMD/Default.htm,
39. http://soyxpress.agrenv.mcgill.ca/cgi-bin/soy/soybean.cgi,
40. http://mpss.udel.edu/at/,
41. http://mpss.udel.edu/rice/,
42. http://signal.salk.edu/,
43. http://rapdb.dna.affrc.go.jp/,
44. http://rice.plantbiology.msu.edu/,
45. http://www.phytozome.net/,

(continued)

Table 10.1 (continued)

46. http://walnut.usc.edu/,
47. http://www.oryzasnp.org/,
48. http://www.soymap.org/,
49. http://1001genomes.org/,
50. http://rarge.gsc.riken.jp/rartf/,
51. http://arabidopsis.med.ohio-state.edu/,
52. http://datf.cbi.pku.edu.cn/,
53. http://drtf.cbi.pku.edu.cn/,
54. http://grassius.org/,
55. http://soybeantfdb.psc.riken.jp,
56. http://legumetfdb.psc.riken.jp/

Comprehensively collected sequence data provide essential genomic resources to accelerate molecular understanding of biological properties and to promote the use of such knowledge. The recent accumulation of model plant nucleotide sequences, as well as applied species such as crops and domestic animals, has provided basic information for the design of functional genomics sequence-based research applications. Species-specific collections of nucleotide sequences also offer opportunities to identify the genomic aspects of phenotypic characters based on genome-wide comparative analysis and model organism knowledge (Tanaka et al. 2008).

10.3.1 Genome Sequencing Projects

The first genome sequence of a plant was completed for *A. thaliana*, which is now used as a model species in plant molecular biology due to its small size, short generation time and high efficiency of transformation. The Arabidopsis genome sequence project was performed as a cooperative project among scientists in Japan, Europe and the USA (Bevan 1997). The genome sequencing was completed and published in 2000 by the Arabidopsis Genome Initiative (AGI) (The Arabidopsis Genome Initiative 2000). The draft genome sequence of rice, *japonica* and *indica*, an important staple food as well as a model monocotyledon, was published in 2002 (Goff et al. 2002). Subsequently, the genome sequence of *japonica* rice was completed and published by the International Rice Genome Sequencing Project in 2005 (International Rice Genome Sequencing Project 2005).

There are a number of providers for sequences and annotations of plant genomes. Phytozome is a web-accessible resource of information that provides genome sequences and annotations of different plant species. This resource is a joint project of the Joint Genome Institute (DOE–JGI) of the Department of Energy and the Center for Integrative Genomics to facilitate comparative genomic studies among green plants (http://www.phytozome.net/Phytozomeinfo.php). Phytozome's current version (ver. 5.0, January 2010) consists of 18 plant species sequenced by JGI and

other sequencing projects. Gramene (http://www.gramene.org/) is a website-based information resource for grass species, and it provides various kinds of information related to grass genomics, including genome sequences (Liang et al. 2008). The current version of Gramene (#30, October 2009) provides genome sequence information for 15 plant species, including five wild rice genome assemblies. The release of sequenced genomes is expected to accelerate with ongoing innovations in sequencing technologies of the next generation (Ansorge 2009). Whole-genome sequence information allows us to derive sets of important genomic features including identification of protein coding or non-coding genes and constructs such as gene families, regulatory elements, repetitive sequences, simple sequence repeats (SSRs) and guanine–cytosine (GC) content. These data sets have become the primary sequence material for designing genome-based sequence platforms such as microarrays, tiling arrays or molecular markers, as well as reference data sets for integrating omics elements into a genome sequence. Chromosome-scale comparisons identifying conserved similarities of gene coordinates facilitate documentation of segmental and tandem duplications in related species (De Bodt et al. 2005). Whole-genome comparisons identifying chromosomal duplication and conserved synteny among related species provide evidence for hypotheses on comparative evolutionary histories with regard to the diversification of species in a related lineage (Paterson et al. 2009). ESTs are created through the partial one-pass sequencing of randomly selected gene transcripts converted into cDNA (Adams et al. 1993). Since cDNA and EST collections can be acquired irrespective of genomic complexity, due to polyploidy and/or their number of repetitive sequences this approach has been applied not only to model species but also to a number of applied species with large genome sizes. As of November 2009, dbEST, a public domain EST database (http:/www.ncbi.nlm.gov/dbEST/), which includes a number of plant species, has >63 million ESTs in the National Center for Biotechnology Information (NCBI) (Table 10.2) (Boguski et al. 1993).

Since EST data collected from a particular organism's cDNA libraries consist of redundant sequence data derived from the same gene locus or transcription unit, it is often necessary to perform EST grouping by transcription units and assemble these groups to create a consolidated alignment and representative sequence of each transcript prior to further analysis. Such steps are carried out in a computational manner: a typical workflow consists of 'base calling,' i.e. converting the output trace of a sequencer to identified nucleotide data, followed by a cleaning step involving identification and removal of contaminated sequences, masking of cloning vector sequences, clustering of contaminated sequences, the masking out of cloning vector sequences, clustering of identical sequences and alignment of clustered sequences (Masoudi-Nejad et al. 2006). Then, the obtained data sets of representative transcripts can be used as unified transcript data. There are several data resources that provide such unified data sets of plants, such as NCBI-UniGene, PlantGDB, TIGR Plant Gene Index and HarvEST (Duvick et al. 2008).

In addition to the mass volume data sets of their sequence tags, the comprehensive and rapid accumulation of cDNA clones has become significant resources for functional genomics. ESTs derived from various tissue types, including tissues from

Table 10.2 Numbers of ESTs and unified transcripts in plants

Species	No. of ESTs (dbEST)	No. of entries (UniGene)
Physcomitrella patens	*382,584*	*18,870*
Picea glauca (white spruce)	*299,455*	*22,472*
Picea sitchensis (Sitka spruce)	*175,662*	*18,838*
Pinus taeda (loblolly pine)	*328,628*	*18,921*
Aquilegia Formosa×Aquilegia pubescens	*85,039*	*8046*
Arabidopsis thaliana (Thale cress)	*1,527,298*	*30,579*
Artemisia annua (sweet wormwood)	*85,402*	*9462*
Brassica napus (rape)	*643,601*	*26,733*
Brassica oleracea	*59,946*	*5617*
Brassica rapa (field mustard)	*44,570*	*14,497*
Capsicum annuum	*116,541*	*8868*
Citrus Clementina	*118,365*	*9123*
Citrus sinensis (Valencia orange)	*208,909*	*15,808*
Glycine max (soybean)	*1,422,604*	*33,001*
Gossypium hirsutum (upland cotton)	*268,786*	*21,738*
Gossypium raimondii	*63,577*	*3297*
Helianthus annuus (sunflower)	*133,682*	*12,216*
Lactuca sativa (garden lettuce)	*80,781*	*7940*
Lotus japonicas	*195,385*	*14,493*
Malus × domestica (apple)	*324,308*	*23,731*
Medicago truncatula (barrel medic)	*269,237*	*18,098*
Nicotiana tabacum (tobacco)	*317,190*	*24,069*
Populus tremula × Populus tremuloides (hybrid aspen)	*76,160*	*9652*
Populus trichocarpa (western balsam poplar)	*89,943*	*14,965*
Prunus persica (peach)	*79,203*	*7620*
Raphanus raphanistrum (wild radish)	*164,119*	*18,788*
Raphanus sativus (radish)	*83,034*	*17,649*
Solanum lycopersicum (tomato)	*296,848*	*18,228*
Solanum tuberosum (potato)	*236,568*	*18,784*
Theobroma cacao	*159,320*	*24,958*
Vigna unguiculata (cowpea)	*187,443*	*15,740*
Vitis vinifera (wine grape)	*357,856*	*22,083*
Selaginella moellendorffii	*93,806*	*8810*
Hordeum vulgare (barley)	*501,614*	*23,595*
Oryza sativa (rice)	*1,249,110*	*40,978*
Panicum virgatum (switchgrass)	*436,535*	*20,973*
Saccharum officinarum (sugarcane)	*246,892*	*15,594*
Sorghum bicolor (sorghum)	*209,814*	*13,899*
Triticum aestivum (wheat)	*1,067,290*	*40,349*
Zea mays (maize)	*2,018,798*	*97,123*
Chlamydomonas reinhardtii	*204,076*	*11,310*
Volvox carteri	*132,038*	*5638*

Source: (Boguski et al. 1993)

organisms at various stages of development or under stress, could significantly facilitate gene discovery as well as structural gene annotation, large-scale analysis of expression, intra-specific and interspecific genome-scale comparative analysis of expressed genes and the design of expressed gene-oriented molecular markers and probing for microarrays.

10.3.2 Full-Length cDNA Projects

While partial cDNAs are useful in the rapid development of catalogs of expressed genes, they are not suitable for further gene function study. This is because the most popular method of preparing a cDNA library does not provide a complete cDNA that includes the sequence of the capped site. Hayashizaki's RIKEN group developed the biotinylated cap trapper method, which uses reverse transcriptase trehalose-thermostabilized and is an efficient method of building full-length cDNA-enriched libraries about 10 years ago. Full-scale cDNA libraries and large-scale clone data sets have become invaluable resources for life science projects that study different species (Tanaka et al. 2008). The sequence resources derived from full-length cDNAs can also help to identify transcribed regions in completed or draft genome sequences substantially. Full-length cDNA sequences were used in Arabidopsis and rice to identify genomic structural features such as transcription units, transcription starting sites (TSSs) and transcriptional variants (Yamamoto et al. 2009). Full-length cDNA clones were sequenced to help consolidate genomic infrastructure in species for which we have draft genomes, such as Physcomitrella, soybean and poplar; this should also contribute to gene discovery (Umezawa et al. 2008). Also full-length cDNAs are useful for the three-dimensional determination (3D) structures of proteins by X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy and for functional biochemical analyses of expressed proteins in the molecular interactions of protein–ligands, protein–proteins and protein–DNAs. In addition, recent advances in proteomics infrastructure require extensive data sets on the full-length amino acid sequences used to assign a protein to peptides. These advances also require functional annotations to support systematic knowledge extracted from proteins to identify peptides and residues modified by, for example, phosphorylation for use in combination with comparative analysis of modified events between species. By creating overexpressors used in reverse genetics, the full-length cDNA library also contributed significantly to functional analysis. Systems such as full-length cDNA overexpressor (FOX) gene hu have developed function-based gene discovery by systems such as full-length cDNA overexpressor (FOX) gene hunting, which use full-length cDNA transgenic plants as overexpressors, has provided an effective approach to high-throughput discovery of functional genes associated with phenotypic changes (Kondou et al. 2009). A full-length enriched cDNA libraries have been constructed for non-sequenced crops or forestry species, such as wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), cassava (*Manihot esculenta*), Japanese cedar (*Cryptomeria japonica*) and Sitka spruce (*Picea sitchensis*), as well as for plant species showing specific biological

characters such as salt tolerance in salt cress (*Thellungiella halophila*). These full-length cDNA libraries have been used to identify biological features through comparisons of target sequences with those of model organisms such as Arabidopsis, rice and poplar. These libraries also serve as primary sequence resources for designing microarray probes and as clone resources for genetic engineering to improve crop efficiency (Taji et al. 2008).

10.3.3 Emerging Layers in Plant Omics

10.3.3.1 Plant Epigenome Analysis

The epigenome, the genome-scale properties of epigenetic modifications, has attracted attention as a new area of omics that NGS technology-based solutions have advanced (Schmitz and Zhang 2011). Small RNAs (sRNAs) may direct and mediate epigenetic modifications in plants (Matzke et al. 2009). For the interpretation of genetic information, the epigenomic regulation of chromatin structure and genome stability is crucial (He et al. 2011). NGS-based cytosine methylome (methylC-seq), transcriptome (mRNA-seq) and small RNA transcriptome (small RNA-seq) sequencing in inflorescences of arabidopsis revealed patterns of genome-scale methylation and a direct relationship between the location of sRNAs and DNA methylation (Lister et al. 2008). At the entire plant level, bisulfite sequencing using NGS technologies, the so-called BS-seq, has generated a genome-scale map of methylated cytosine in Arabidopsis (Cokus et al. 2008). These analyzes based on NGS methylome enabled a holistic understanding of patterns of genome-scale methylation at a single base resolution. In addition to DNA methylation, histone N-terminal tail modifications such as acetylation are crucial in plant development (He et al. 2011) and mechanisms of defense (Kim et al. 2008). A genome-wide nucleosome positioning analysis combined with DNA methylation profiles revealed 10 basis periodicities in nucleosome-bound DNA methylation status of nucleosome-bound DNA (Chodavarapu et al. 2010). The Epigenomics of Plants International Consortium web site (https://www.plant-epigenome.org/) provides hyperlinks to plant epigenome data resources. In fact, numerous efforts have been made to acquire epigenome information from plant species (He et al. 2010).

10.3.3.2 Plant Interactome Analysis

Interactions between proteins are essential to nearly all cellular processes. The interactome, a comprehensive set of all protein-protein interactions within an organism, is crucial to our understanding of the cellular system's molecular networks (Morsy et al. 2008). Analysis of interactomes was used to characterize plant cellular functions such as cell cycle, Ca2+/calmodulin-mediated signaling, auxin signaling and membrane protein-signaling interactions in Arabidopsis (Vernoux et al. 2011). Recently, the Arabidopsis Interactome Mapping Consortium presented a proteome-wide binary protein–protein interaction map of Arabidopsis with around 6200 highly reliable interactions interactions between about 2700 proteins (Arabidopsis Interactome Mapping Consortium 2011).

To generate the large-scale Arabidopsis interactome map, the consortium prepared approximately 8000 open reading frames of Arabidopsis protein-coding genes and then analyzed all pairly protein combinations encoded by these constructs using an enhanced binary interactome-mapping pipeline based on the two-hybrid (Y2H) yeast system. A large-scale plant pathogen effector interactome network has been created using the Y2H pipeline method (Mukhtar et al. 2011). In rice, biotic and abiotic stress responses were addressed by a focused interactom analysis (Seo et al. 2011). A number of databases were available on the web for protein-protein interaction data sets (Aranda et al. 2010). In addition to the curated data sets, predicted protein–protein interaction data sets are a valuable complement to experimental approaches (Li et al. 2011).

10.3.3.3 Plant Hormonome Analysis

Plant hormones play a critical role in regulating plant development and environmental responses as signaling molecules. A number of low molecular weight plant hormones, including auxin, ABA, cytokinin, gibberellins, ethylene, brassinosteroids, jasmonates and salicylic acid, have been identified to date (Davies 2004). In addition, strigolactone, a novel plant hormone, has recently been identified as an inhibitor of shooting branching (Umehara et al. 2008). A special issue of strigolactone plant and cell physiology has been published to gather current knowledge on the topic (Yamaguchi and Kyozuka 2010). Small peptides (peptide hormones) also work in plan regulation as signaling molecules in the regulation of plant growth and development (Fukuda et al. 2007). A special issue on peptide hormones was also published to describe recent advances in the area of plant peptide research (Fukuda and Higashiyama 2011).

Many remarkable advances have been made in our understanding of the molecular basis of plant hormones over the past decade, including biosynthesis, transportation, perception and response (Santner and Estelle 2009). Umezawa et al. 2010 reported that ABA response of recent exciting advances in understanding the molecular basis of regulatory networks. A remarkable recent advance was the discovery of receptors for several important phytohormones, including auxin, gibberellins, ABA and jasmonates (Santner and Estelle 2009). Structural analysis of each complex revealed the structural basis of the interaction of each receptor with phytohormone and its signaling mechanisms (Sheard et al. 2010). According to a number of recent mutant analyses, it is almost certain that all plant hormones cross-talk with one or more other hormones depending on the tissue, developmental stage and environmental changes (Depuydt and Hardtke 2011). A comprehensive analysis known as hormonal analysis based on high-throughput, high-sensitivity and simultaneous profiling of plant hormones is a key approach to a holistic understanding of the plant hormone network and its association with biological phenomena because of the interplay between multiple plant hormones. A recently developed analytical platform for high-sensitivity, high-throughput plant hormone measurements enables 43 molecular species of cytokinin, auxin, ABA and gibberellin to be measured simultaneously (Kojima et al. 2009). The platform was used to acquire hormonal profiles of plant hormones in rice organ distribution patterns. The hormonomal analysis of

endogenous levels of cytokinin, gibberellin, ABA and auxin in wild type and gibberellin signaling mutants indicated that cytokinin, ABA and auxin metabolism cross talk with the gibberellin signaling system. In Arabidopsis, comprehensive hormone profiling was used to analyze the accumulation of ABA, gibberellins, IAA, cytokinins, jasmonates and salicylic acid in wild-type Arabidopsis seeds and an ABA-deficient mutant. The hormonal approach results suggested that ABA interacts with other hormones to regulate the development of seed (Kanno et al. 2010).

10.3.3.4 Plant Metabolome Analysis

In system approaches to plant functional analysis and applied plant biotechnology, plant metabolomics now plays a significant role. There are many applications for functional genomics, system biology and molecular breeding, driven by advances in related technologies including metabolite measurement instruments, analytical methodologies and information resources. A number of excellent metabolomics reviews describing emerging methodologies and attractive applications have been published (Sumner 2010). Here we will review recent developments in analytical platforms briefly and describe examples of practical applications for understanding plant metabolism systems. Analysis of metabolomes involves chemically diverse compounds. The metabolome of the plant consists of extremely large metabolite varieties with different dynamic ranges of concentration. Consequently, the integration of combined analytical techniques and data set from heterogeneous instruments was key to a comprehensive understanding of various metabolites. Simplified analytical platforms integrating analytical steps such as sample preparation, data acquisition and data analysis allow us to address the complex plant metabolome (Saito and Matsuda 2010). Improved coverage and performance to detect large numbers of metabolites simultaneously expanded the practical application significantly. Ultra-performance liquid chromatography–tandem quadrupole mass spectrometry is used by a widely targeted metabolomics platform that provides coverage and throughput (Sawada et al. 2009a). The approach allows us to simultaneously acquire hundreds or more metabolites of accumulation patterns for large numbers of samples. The platform allows us to address complex metabolic plant systems and develop practical genetic and breeding approaches. For metabolic profiling of the Arabidopsis knockout mutants for methionine chain elongation enzymes, the widely targeted metabolomics approach was used. The results suggest that the metabolism of these enzymes ranges from methionine to primary and related secondary metabolites (Sawada et al. 2009b).

Metabolome profiling provides a snapshot of metabolite accumulation patterns in response to different types of biological conditions such as treatments, tissues, and genotypes. For example, approaches to metabolome profiling were used to monitor changing accumulation of metabolites in response to stress conditions (Kusano et al. 2011). Metabolome profiling was also used to evaluate genetic resources, not only for Arabidopsis and rice model plants, but also for metabolic phenotyping in different crop species (Fujimura et al. 2011). Metabolome profiling was also used to evaluate natural and/or segregation populations ‘metabolic phenotypes. Several approaches in metabolite quantitative trait locus (mQTL) analysis have been performed in various plant species in recent years.

10.3.4 Omics Resources in Emerging Plant Species

In a number of plant species, the development of genomic resources has progressed. At Phytozome (ver. 7.0, http://www.phytozome.net/), genome sequence data sets of 25 plant species are available as a typical example. Recent sequenced plant species have typically been nominated for the development of genomic resources because: I they have specific systems not covered by' conventional' model plants; (ii) they are of evolutionary importance; or (iii) they provide a commodity resource such as food and energy.

10.3.4.1 Solanaceae Species

The Solanaceae family includes a number of important crops, including tomato, potato, pepper, paprika, petunia, and tobacco. Tomato (*Solanum lycopersicum*) is a representative crop species for which genomic resources have made significant progress. Tomatoes are an important crop sold fresh and used in processed foods. Because of its small genome size and shared conserved synteny with other Solanaceae genomes, the tomato is a model plant to study Solanaceae species in addition to its agricultural importance. The tomato has also become a model plant to study fruit development, maturation, maturation, and metabolic systems. The International Tomato Genome Sequencing Project was initiated in 2004. Following the initial BAC-by-BAC approach for the euchromatic regions, a whole-genome shotgun approach was initiated in 2009. The International Tomato Annotation Group provided the official annotation of the tomato genome assembly (http://sol-genomics.net/organism/Solanum_lycopersicum/genome). A full-length cDNA resource from the tomato cultivar Micro-Tom was recently launched (Aoki et al. 2010; http://www.pgb.kazusa.or.jp/kaftom/). Transcriptome data from 296 samples of 16 series using the Affymetrix GeneChip tomato genome array can be found in NCBI GEO (September 8, 2011). The tomato GeneChip data deposited in NCBI GEO includes, for example, data sets acquired for co-expression analysis using cultivar Micro-Tom (Ozaki et al. 2010), for comparative transcriptome analysis between salt-tolerant and salt-sensitive wild tomato species (Sun et al. 2010) and for examining the transcriptome of the ripening process of an orange ripening mutant (Nashilevitz et al. 2010).

Significant progress has been made with metabolome analyses such as metabolome profiling and mQTL analysis (Enfissi et al. 2010). Metabolome analysis information resources are available and updated, providing data archives for tomato metabolome data sets and analytical platforms such as Plant MetGenMAP, Metabolome Tomato Database (MotoDB) (Moco et al. 2006), KaPPA-View4 SOL (Sakurai et al. 2011) and KOMICS (Iijima et al. 2008). The Tomato Functional Genomics Database provides data on gene expression, metabolites and microRNAs (miRNAs) via a web interface as an integrative information resource. TOMATOMA was launched as a tomato-mutant resource as a web-based database for a phenotypically classified Micro-Tom EMS mutant collection and a Targeting Induced Local Lesions IN Genomes (TILLING) resource (Saito et al. 2011). The potato genome was recently sequenced using a homozygous doubled-monoploid potato clone and

86% assembly of the 844 Mb genome revealed 39,031 protein-coding genes predicted (Xu et al. 2011).

10.3.4.2 Poaceae (Gramineae) Species

The Poaceae family includes staple food crops such as rice, maize, wheat and barley, as well as grasses used for lignocellulose biomass production, such as switchgrass and *Miscanthus* (Lobell et al. 2011). Since the completion of the japonica rice (*Oryza sativa*) genome project (International Rice Genome Sequencing Project 2005), whole-genome sequences have been completed in sorghum (*Sorghum bicolor*), maize (*Zea mays*) and *Brachypodium* (*Brachypodium distachyon*) (International Brachypodium Initiative 2010). Rice is a model species of monocot plants as well as one of the three major staple cereals in the world. To date, the japonica rice genome sequence with high-quality gene annotations has played an important role in promoting the development of a number of genomic resources for the discovery and isolation of important genes for molecular breeding application. The sorghum genome has been sequenced as a representative Saccharinae species that includes starch, sugar and cellulose plants from the source of biomass. Maize is another of the major staple food and feed cereals and is a model organism for basic research into complex heritage and genomic properties such as domestication, epigenetics, evolution, chromosome structure and transposable elements (Walbot 2009). Accompanying the release of the maize B73 genome sequence, the '2009 Maize Genome Collection' was edited (Walbot 2009). Brachypodium is an emerging plant species of the Pooideae subfamily, a model plant for Triticeae crops such as wheat and barley, as well as for grass species understanding systems for the production of cellulose biomass. Brachypodium has attracted attention since the release of the Brachypodium Bd21 genome sequence, and a number of genomic resource projects have been initiated at different institutions (Brkljacic et al. 2011). Therefore, we have access to published reference genome sequences of four species from each of the three major subfamilies of Poaceae. Wheat and barley were the subjects of ongoing attempts to regenerate their highly complex genomes through genome sequencing. A tentative linear order of 32,000 barley genes was recently regenerated by incorporating chromosome sorting, NGS, array hybridization and preserved syntenia with the brachypodium genome (Mayer et al. 2011). To introduce genetic and genomic resources and their applications, a special issue on barley has recently been published (Saisho and Takeda 2011). For several species of Poaceae, data sets of transcriptome profiles are available. For example, Genevestigator's current version provides processed transcriptome data from data sets of GeneChip hybridization (1.626 for barley, 1275 for rice, 1000 for wheat and 458 for maize; https://www.genevestigator.com/gv/). The transcriptome from primordial development through pollination / fertilization to zygote formation throughout the reproductive process was analyzed in rice using an oligomicroarray as an atlas for rice expression (Fujita et al. 2010). Accumulated transcriptome data sets have been made for co-expression analysis of the transcriptome in Poaceae species. The Rice ArrayNet and Oryza Express databases provide web-accessible co-expression data for rice. The ATTED-II database also provides co-expression data sets for rice in addition to

those for Arabidopsis. A co-expressed barley gene network was recently generated and then applied to comparative analysis to discover potential Triticeae-specific gene expression networks (Mochida et al. 2011). To analyze transcriptomes of the male gametophyte and tapetum in rice, microarray analyzes coupled with laser microdissection were used (Hobo et al. 2008). Recently, transcriptome data from rice grown in field environments has been collected. For example, the plant proteome database (http://ppdb.tc.cornell.edu/) provides information about the proteomes of maize and arabidopsis as a proteomics resource in Poaceae. The RIKEN Plant Phosphoproteome Database (RIPP-DB, http://phosphoproteome.psc.database.riken.jp) has been updated with a large-scale rice phosphorylated protein identification data set. The OryzaPG-DB was launched as a shotgun-based rice proteome database on proteomics (Helmy et al. 2011).

10.3.4.3 Fabaceae (Leguminosae) Species

The large family of Fabaceae includes economically significant crops such as soybean, common bean and alfalfa. A biological phenomenon, especially in legume species, is the symbiotic nitrogen fixation produced by the communication between plants and nitrogen-fixing bacteria. For plant and microbial biology as well as for agriculture, understanding this symbiosis is important. Recent progress in plant-microbe symbiosis research, including nitrogen-fixing symbiosis in legume plants, was presented in a special issue on plant-microbe symbiosis by Ikeda et al. (2010) and Kouchi et al. (2010) and (Kawaguchi and Minamisawa 2010). *Lotus japonicus* and *Medicago truncatula* served as models for molecular genetics and functional genomics studies to investigate the symbiotic system and carry out gene discovery in legume species (Stacey et al. 2006). In 2008, the genome sequence of *L. japonicus* was released with a 315.1 Mb sequence corresponding to 67% of the genome, covering 91.3% of the gene space. The TILLING resource for *L. japonicus* is used to identify allelic series for symbiosis genes. Proteome analyses on pod and seed development were performed in *L. japonicus* (Dam et al. 2009,).In *M. truncatula*, a *number* of genome resources have become available in recent years (Young and Udvardi 2009). For example, a gene expression atlas provides an information resource for the transcriptome (Benedito et al. 2008). Insertional mutagenesis by the *Tnt1* transposon system and the flanking sequence data set has provided a reverse genetics resource (Tadege et al. 2008). The web site of the *Medicago truncatula* Genome Project in JCVI/TIGR (http://medicago.jcvi.org/cgi-bin/medicago/annotation.cgi) is an information resource that provides the current version of pseudomolecules (ver. 3.5) and an annotation of the *M. truncatula* genome. The web page of the *Medicago truncatula* HapMap Project (http://medicagohapmap.org/index.php) provides not only a reference genome sequence but also NGS re-sequencing data as a GWAS resource. sRNAs expressed in roots and nodules were analyzed using 454 pyrosequencing, and the MIRMED database (http://medicago.toulouse.inra.fr/Mt/RNA/MIRMED/LeARN/cgi-bin/learn.cgi) was constructed as an informative resource for *M. truncatula* miRNAs (Lelandais-Briere et al. 2009). Large-scale analysis of the phosphoproteome in *M. truncatula* roots was performed using immobilized metal affinity chromatography and MS/MS followed by launch

of the *Medicago* PhosphoProteinDatabase (http://www.phospho.medicago.wisc.edu/db/index.php) (Grimsrud et al. 2010). The world's largest legume crop, soybean (Glycine max), is widely grown for food and biofuel. A draft assembly of soybean genomes was released in 2010 and the first version of the Glyma1.0 genome annotation was based on homology and gene predictions based ab initio (Schmutz et al. 2010). Also in favor of homology-based gene prediction was applied a sequence data set of soybean full-length cDNAs (Umezawa et al. 2008). In order to improve productivity and stress tolerance, several genomic resources are available for soybean genomics as well as molecular breeding (Manavalan et al. 2009). Genome-scale gene exploration by using the soybean genome sequence and annotated gene models genome-scale exploration of gene families and those functional analyses have been performed to identify genes for molecular breeding (Mochida et al. 2010). A soybean transcriptome atlas (http://digbio.missouri.edu/soybean atlas/) was developed using a NGS platform to perform sample RNA-seq from 14 different conditions (Libault et al. 2010). A genome-scale survey of sRNAs also included NGS-based approaches (Song et al. 2011). Soybase (http://soybase.org/) has played a significant role as an information portal for soybean research in integrating various soybean research resources and analytical platforms (Grant et al. 2010).

10.3.5 Systems Analysis of Plant Functions

Analysis of systems based on a combination of multiple omics analyzes was an effective approach to determining the cellular system's global image. From the early stages of plant metabolomics research, we have made significant progress in our understanding of gene function in metabolic systems through the integration of metabolome analysis with genome and transcriptome resources (Okazaki et al. 2009). Multi-omics-based system analyzes have improved our understanding of cellular plant systems following these successes. For example, recently integrated metabolome and transcriptome analyzes were used to analyze rice developing caryopses under high temperature conditions (Yamakawa and Hakata 2010), molecular events underlying pollination-induced and pollination-independent fruit sets (Wang et al. 2009) and the effects of *DE-ETIOLATED1*down-regulation in tomato fruits (Enfissi et al. 2010). Integrated metabolome and transcriptome analysis has also been applied to investigate changing metabolic systems in plants growing in field conditions, such as the rice Os-*GIGANTEA* (Os-*GI*) mutant and transgenic barley (Izawa et al. 2011). In addition, a system approach combined hormonomal, metabolome and transcriptome analysis in transgenic lines of Arabidopsis, showing increased leaf growth to gain insight into the molecular mechanisms controlling leaf size (Gonzalez et al. 2010). To compare the differences in response to anoxia between rice and wheat coleoptiles, an integrated proteome and metabolome analysis was applied (Shingaki-Wells et al. 2011). To characterize the cascading changes in UV-B-mediated responses in maize, an integrated transcriptome, proteome and metabolome analysis was conducted (Casati

et al. 2011). These illustrative examples show the power to understand multi-omics-based systems analysis for understanding the key components of cellular systems underlying various plant functions. GWAS identified genetic loci associated with enzyme activity, metabolome profiles, and biomass using a large set of accessions to Arabidopsis and data sets of genome-scale variation (Sulpice et al. 2010). The hormonal responses of natural variations were addressed in order to find relationships between physiological hormonal response variations and other variations, such as in the genome and transcriptome (Delker et al. 2010). A combinatorial approach to population genomics using hormonome profiling would allow us to identify the link between genomic polymorphisms and plant hormone abundance as quantitative features that could be closely linked to environmental adaptation. Recently, in human population genomics, relational instances of epigenomic modification, gene transcription coding and non-coding RNAs were coupled with genome-scale nucleotide polymorphism data sets (Shoemaker et al. 2010). In a comparable manner, plant epigenome analysis can also be integrated with genome-scale variations to provide important clues to phenotypic diversity-related epigenetic and genetic regulation.

10.3.5.1 Ultrahigh-Throughput DNA Sequencing

The Sanger sequencing method has been used to complete microbial and higher eukaryote genomes sequencing over the past decade. A number of alternative technologies have become available in recent years, which are method adaptations such as pyrosequencing procedures, massively parallel DNA sequencing or single molecule sequencing (Ansorge 2009). In the fields of comparative genomics, metagenomics and evolutionary genomics, such new sequencing technologies have provided us with new opportunities to address the entire genome level (Varshney et al. 2009).

10.3.5.2 Whole-Genome Re-Sequencing

Next-generation sequencing technology coupled with reference genome sequence data enables us to detect variations between individuals, strains and/or populations. Effectively identifies nucleotide polymorphisms by mapping sequence fragments to a specific reference genome data set, a capability of immense importance in all genetic research. A full-genome resequencing project to detect all-genome sequence variations in 1001 Arabidopsis strains (accessions) will result in a data set that will become a fundamental resource for the promotion of future genetic studies to identify allles associated with phenotypic diversity throughout the genome and throughout the species (http://1001genomes.org/) (Weigel and Mott 2009). In rice, an Illumina Genome Analyzer generated high-throughput method for genotyping recombinant populations was performed (Huang et al. 2009). The application to whole-genome de novo sequencing is one of the most anticipated innovations for next-generation sequencers. Although this approach has only been realized in bacterial genomes to date (Moran et al. 2009), there are several attempts to realize this progress in higher species.

10.3.5.3 Comprehensive Discovery of Small RNAs (sRNAs)

SRNAs, including microRNAs (miRNAs), short-interfering RNAs (siRNAs) and trans-acting siRNAs (ta-siRNAs), also play roles in plants as key components of epigenetic processes and gene networks involved in development and homeostasis (Ruiz-Ferrer and Voinnet 2009). These RNA molecules are important targets to be fully identified and their expression should be analyzed using genomic technologies of the next generation (Chellappan and Jin 2009). In maize, deep-sequencing sRNAs in the wild type and isogenic mop1–1 loss-of-function mutant were analyzed using Illumina's sequencing-by-synthesis (SBS) technology to characterize maize complement sRNA (Nobuta et al. 2008). In poplar, expressed sRNAs from leaves and vegetative buds were also discovered using Roche 454 high-throughput pyrosequencing, then genes from miRNA families were identified, including the novel ones (Barakat et al. 2007). Deep sequencing of Brachypodium sRNAs was also performed at the global genome level, resulting in the identification of miRNAs involved in the response to cold stress. The miRNA plant database (PMRD) is a useful plant miRNA information resource available on the Web (http://bioinformatics.cau.edu.cn/PMRD/).

10.3.6 Resources for Variation Analysis

Recent innovations related to DNA sequencing technology and the rapid growth of genome and cDNA sequence resources allow us to design various types of molecular markers covering entire genomes (Feltus et al. 2004). For high-throughput genotyping, a number of platforms have been developed that have been applied to genetic map construction, marker-assisted selection and QTL cloning using multiple segregation populations (Hori et al. 2007). Such genotyping systems have also been used in post-genome sequencing projects such as genotyping of genetic resources, accessions to evaluate population structure and association studies to identify genetic loci involved in phenotypic changes of species.

10.3.6.1 Molecular Markers

The accumulation and saturation of available genetic markers contributes to progress in marker-assisted genetic studies and is an important resource with a wide range of uses. Genetic markers designed to cover a genome extensively enable not only the identification by QTL analyzes of individual genes associated with complex traits, but also the exploration of genetic diversity in natural variations (Caicedo et al. 2007). These sequence data sets have become quite efficient sequence resources for designing molecular markers with the advancement of genome sequencing and large-scale EST analysis in different species. A number of attempts to design polymorphic markers from accumulated sequence data sets have been made for various species. Several genome-wide rice (*Oryza sativa*) DNA polymorphism data sets have been constructed based on alignment between *japonica* and *indica* rice genomes (Shen et al. 2004). Large-scale EST data sets are also important resources for sequence polymorphism discovery, particularly for allocating expressed genes

to a genetic map. The computational discovery of ESTbase single-nucleotide polymorphisms (SNPs) and/or EST-SNP markers for the identification of sequence-tagged site (STS) markers has therefore progressed for many species, including barley, wheat, maize, melon, brassica, common bean and sunflower (Li et al. 2009). Several databases provide information about plant species molecular markers. PlantMarkers is a genetic marker database containing predicted molecular markers from different plant species, such as SNP, SSR and preserved orthology set (COS) markers (Heesacker et al. 2008). GrainGenes is a popular genomics site for Triticeae; it also provides genetic markers and mapping information on wheat, barley, rye and oat (Carollo et al. 2005). Gramene is a comparative plant genomics database that provides genetic maps of different species of plants (Liang et al. 2008). The Triticeae Mapped EST (TriMEDB) database provides information on mapped cDNA markers related to barley and their wheat homologs (Mochida et al. 2008).

Analysis of high-throughput polymorphism is a key tool for facilitating any approach based on genetic maps. To date, genome-wide genotyping using a hybridization-based SNP typing method has been used to analyze representative ecotypes of Arabidopsis and rice strains, and data sets have been released for each species containing the calculated genome-wide variation pattern. As typified by the project Arabidopsis 1001, the study of genome-wide variation is a key analysis that should be carried out for a particular reference strain after genome sequencing has been completed. Therefore, the demand for high-performance and cost-effective platforms for comprehensive analysis of variation or also known as variome analysis.

The whole-genome resequencing approaches are already being implemented in species whose reference genome sequence data are available as a direct solution for variable analysis. Diversity Array Technology (DArT) is a high-throughput genotyping system developed on the basis of a microarray (http://www.diversityarrays.com/index.html) platform (Wenzl et al. 2007). DArT markers were used together with conventional molecular markers in various crop species such as wheat, barley and sorghum to construct denser genetic maps and/or conduct association studies (Mace et al. 2009).

Affymetrix GeneChip Arrays has been used in barley and wheat to discover nucleotide polymorphisms as single-function polymorphisms based on the differential hybridization of GeneChip samples (Bernardo et al. 2009). The Illumina Golden Gate Assay allows simultaneous analysis of up to 1536 SNPs in 96 samples and was used to analyze genotypes of segregation populations to construct genetic maps allocating SNP markers in crops such as barley, wheat and soybean (Close et al. 2009).

10.3.7 Transcriptome Resources in Plants

Comprehensive and high-throughput gene expression analysis, called transcriptome analysis, is also a major approach to screening candidate genes, predicting gene function, and discovering cis-regulatory motives. The method of hybridization, such

as that used in microarrays and GeneChips, has been well established to acquire large-scale gene expression profiles for different species. The recent rapid accumulation of data sets containing profiles of large-scale gene expression and the ability of related databases to support the availability of such large data repositories has given us access to large amounts of public domain information. This public domain data are an efficient and valuable resource for many secondary uses, such as co-expression and comparative analyses. Furthermore, as a next-generation DNA sequencing application, deep sequencing of short fragments of expressed RNAs, including sRNAs, is quickly becoming an efficient tool for use with genome-sequenced species (de Hoon and Hayashizaki 2008).

10.3.7.1 Sequence Tag-Based Platforms in Transcriptomics

An early approach to the acquisition of transcriptome profiles was the large-scale sequencing of ESTs from cDNA libraries. In this approach, sequencing and/or assembly methods are used to classify randomly sequenced ESTs in an unbiased cDNA library into clusters of transcript sequences. The abundance of transcripts expressed in each tissue is then estimated by counting the number of ESTs for each cDNA library and/or sequence cluster with identifiers. Human and mouse have applied the same methodological principle in the form of a' body map' to derive the transcriptome in different organs (Ogasawara et al. 2006). In addition, this principle was also applied in the digital field of differential display (DDD), which is a component of NCBI's UniGene database and has been applied in large-scale cDNA projects for various species, including plants (Zhang et al. 2004). While this approach, coupled with clone resources from cDNA, has facilitated gene discovery and profiling of expression, its disadvantages include cost and limited resolution due to large-scale sequencing. Serial gene expression analysis (SAGE) is a method based on deep sequencing of short cDNA read tags. SAGE allows a large number of transcripts present in tissues to be identified and allows a quantitative comparison of transcriptomes (Velculescu et al. 1995). SAGE is designed to generate a short specific tag (13–15 bp) from the 3′ end of each sample mRNA, after which >10 tags are concatenated and cloned to generate a SAGE library. The sequencing of selected clones from the SAGE library allows efficient collection of transcript tag sequences. A data set of genome sequences or large-scale ESTs is required to identify genes corresponding to each SAGE tag. Some derivatives of the original protocol (MAGE, SADE, microSAGE, miniSAGE, longSAGE, superSAGE, deepSAGE, 5′ SAGE, etc.) have been developed to improve and expand the utility of SAGE (Anisimov 2008). For example, superSAGE is an improved version of SAGE that produces 26 bp fragment tags from cDNAs. This method has been applied to simultaneous and quantitative gene expression profiling of both host cells and their eukaryotic pathogens in rice (Matsumura et al. 2003). The 26 bp superSAGE tags have also been used to design probes directly for oligo microarrys (Matsumura et al. 2008).

Massively parallel signature sequencing (MPSS) is another technology based on sequencing. MPSS uses a unique method to quantify levels of gene expression; by sequencing 16–20 bp from the 3′ side of cDNA using a microbead array, it generates millions of short sequence tags per library (Brenner et al. 2000). Online (http://

mpss.udel.edu) databases containing MPSS data on plant species, including Arabidopsis, rice, grape and Magnaporthe grisea (the rice blast fungus). In addition, the genome-scale discovery and expression profiling of sRNAs in Arabidopsis and rice was also carried out using the MPSS method (Nobuta et al. 2007). The CT-MPSS was a recently developed method for quantitative transcript 5′end analysis coupled with cap-trapper method for full-length cDNA cloning. This method was used to carry out TSS high-density mapping in Arabidopsis to identify plant promoter genome-scale instances (Yamamoto et al. 2009). Arabidopsis CT-MPSS tags data set can be accessed from ppdb (http://www.ppdb.gene.nagoya-u.ac.jp), a plant promoter database providing promoter annotation of Arabidopsis and rice (Yamamoto and Obokata 2008).

10.3.7.2 Hybridization-Based Platforms in Transcriptomics

The DNA microarray history began with a paper from P O. Brown University Laboratory in 1995 (Schena et al. 1995). Since then, technologies related to microarray and DNA chips have advanced rapidly and their application has expanded to a wide range of disciplines in life sciences. The methodological principle of the DNA microarray or GeneChip analysis is to acquire in a given sample a comprehensive data set of the molecular abundance of each molecule based on its simultaneous hybridization with large numbers of molecular DNA species immobilized on a glass slide or on a silicon chip used as a sample set.

DNA microarrays can be classified into two main types: I the type of spotting developed at Stanford University; and (ii) the type of on-chip synthesis based on manufactured samples. During the early years of transcriptome research, spotting type was widely used. This method involved preparing microarrays of DNA by spotting a solution of cDNA on a glass slide. The on-chip (in situ) oligo synthesis method is a process of light-directed chemical synthesis combining solid-phase chemical synthesis with techniques of photolithographic manufacturing. This method was initially used only in conjunction with the GeneChip Array system manufactured by Affymetrix. In the Affymetrix GeneChip system, a known gene or potentially expressed sequence is represented on the chip by 11–20 unique oligomeric probes that are each 25 bases in length. Roche NimbleGen and Agilent Technology offer platforms to manufacture high-density DNA arrays based, respectively, on Roche's proprietary Maskless Array Synthesizer (MAS) technology and on a non-contact industrial inkjet printing process, both of which are also used for in situ oligo synthesis.

A number of DNA microarrays were also developed for transcriptome analysis in different plant species with the recent and rapid increase in the number of sequenced species in whole-genome and/or large-scale cDNA clones. For example, Seki and colleagues designed a custom DNA microarray that uses 7000 full-length Arabidopsis cDNA clones as samples and then screens genes successfully using a two-color method in response to abiotic stress (Seki et al. 2002). With the recent increase in commercially available DNA microarrays, laboratories can use a specific DNA microarray design to obtain transcriptome data from numerous experiments to accumulate more comprehensive data. AtGenExpress was a multinational

effort designed to uncover the transcriptome of *A. thaliana*. The data sets collected in AtGenExpress have been one of the most comprehensive resources for the Arabidopsis transcriptome to date (Goda et al. 2008). The Gene Expression Omnibus (GEO) of the NCBI and the ArrayExpress of the European Bioinformatics Institute (EBI) were the primary public domain transcriptome archives (Barrett et al. 2009). There are also several more focused databases that provide user-friendly interfaces and annotations on probes with calculated transcriptome data. ATTED II (http:/atted.jp/) is a database that provides data calculated from publicly available data on Arabidopsis ATH1 GeneChip for co-expression analysis (Obayashi et al. 2009). Co-expression analysis data sets generated from extensively collected transcriptome data sets have become an efficient resource that facilitates the discovery of transcriptome data sets have become an efficient resource capable of facilitating the discovery of genes closely correlated in their expression patterns. Genevestigator (https://www.genevestigator.com/gv/index.jsp), which is a reference expression database and meta-analysis system, also provides summary information from hundreds of microarray experiments on various organisms, including Arabidopsis, barley and soybean, with easily interpretable results (Zimmermann et al. 2004a, b). The electronic fluorescent pictograph (eFP) browser provides gene expression patterns collected from Arabidopsis, poplar, *Medicago*, rice and barley via a user-friendly interface on the Web (http://www.bar.utoronto.ca/) (Winter et al. 2007). The Arabidopsis Gene Expression Database AREX is a database that provides data sets of high-resolution gene expression patterns of root tissues in Arabidopsis (http://www.arexdb.org/index.jsp) (Brady et al. 2007). The RICEATLAS is a database housing rice transcriptome data covering various types of tissues (http://bioinformatics.med.yale.edu/riceatlas/). Tiling arrays, which are high-density oligonucleotide samples spanning the entire genome in a particular organism, are a platform for analyzing expressed regions across a whole genome; an effective method for discovering novel genes and clarifying their structure. Seki and colleagues performed transcriptome analysis in Arabidopsis using a whole-genome tiling array under abiotic stress conditions and discovered a number of transcripts of antisense induced by abiotic stress. The *A. thaliana* Tiling Array Express (At-TAX) is a whole-genome tiling array resource for developmental expression analysis and transcript identification in Arabidopsis (Zeller et al. 2009). Coupling this platform with the immune-precipitation method has recently extended the usefulness of tiling arrays. For example, the binding sites of AGAMOU-Like15, AGL15, a MADS domain transcriptional regulator promoting somatic embryogenesis, were identified using a chromatin immunoprecipitation (ChIP) approach coupled with the Affymetrix tiling array for Arabidopsis. This method found approximately 2000 sites (Zheng et al. 2009). Using the methylcytosine immunoprecipitation (mCIP) method in combination with the Arabidopsis tiling array, a comprehensive DNA methylation map of the genome was constructed as an Arabidopsis methylome data set (Zhang et al. 2006a, b). Sequencing of co-precipitated DNAs together with a protein using the next generation sequencer, 'ChIP-seq', has also become an alternative approach (Park 2009).

10.3.8 Combinatorial Approaches in Metabolomics and Other Omics Resources

Metabolome approaches also support understanding global relationships between cellular metabolic systems in combination with other instances of omics such as transcriptome and proteome profiles, as well as genetic variations. In the well-studied Arabidopsis, these combinatorial approaches have been successfully demonstrated by taking advantage of the many other omics resources that currently exist, including the full-genome sequence with mature annotations, large-scale transcriptome data sets and related co-expression data, and bioresources such as mutant collections and full-length cDNA clones. A conceptual scheme for the systematic clarification of molecules from gene to metabolites molecular networks through a combinatorial approach using transcriptome and metabolome resources has been demonstrated by Saito's group in the RIKEN Plant Science Center. A batch-learning, self-organizing map (BL-SOM) was used to analyze data sets containing transcriptome and metabolome changes of Arabidopsis under stress conditions induced by sulfur and nitrogen deficiency to identify genes involved in glucosinolate biosynthesis (Hirai et al. 2004). For the investigation of an activation-tagged mutant and overexpressors of a MYB TF, PAP1 gene, an integrated approach involving metabolome and transcriptome analysis was conducted to identify genes involved in anthocyanin biosynthesis in Arabidopsis (Tohge et al. 2005). The Arabidopsis transcriptome co-expression data provided by the ATTED-II database was applied to the investigation of key genes involved in specific metabolic pathways and then to the configuration of a metabolome analysis coupled with mutant lines of the targeted genes (Obayashi et al. 2009). The ATTED-II database was used to identify novel genes involved in lipid metabolism leading to the identification of a novel gene, UDP-glucose pyrophosphorylase3 (UGP3), which is required for the first step of sulfolipid biosynthesis (Okazaki et al. 2009). Co-expression analysis has also been used to identify all genes associated with flavonoid biosynthesis, leading to further detailed analysis of two UGT78D3 and RHM1 flavonoid pathway genes (Yonekura-Sakakibara et al. 2008). Approaches that have integrated metabolome and transcriptome data also have elucidated regulatory networks that respond to plant environmental stress. Metabolome analysis using different types of MS combined with microarray analysis of gene overexpressors encoding two TFs, DREB1A/CBF3 and DREB2A, investigated the metabolic pathways that act in response to cold and dehydration conditions in Arabidopsis (Maruyama et al. 2009). Metabolomic profiling was also used under conditions of dehydration stress to investigate chemical phenotypic changes between wild-type Arabidopsis and a NCED3 gene knockout mutant. The metabolic data was then integrated into the transcriptome data to reveal ABA-dependent regulatory networks (Urano et al. 2009).

Metabolome profiling was also used simultaneously to evaluate chemical phenotypes of natural variations and/or populations of segregation. A comprehensive exploration of the association between metabolic and genomic diversity will allow key genes involved in metabolic changes to be discovered and will also help to

identify genetic associations between metabolic and/or visible phenotypes (Fu et al. 2009). Analysis of metabolite QTL (mQTL) using segregated populations has been applied in a popular forward genetic approach to different plant species such as Arabidopsis, poplar and tomato (Schauer et al. 2008). In addition, together with the recent availability of genome wide variation acquired by high-throughput genotyping methods including resequencing, interest in the discovery of the genetic association between nucleotide variation and phenotypic changes has also increased, especially with regard to the identification of key genes that play significant roles in evolutionary histories. The attempts to mine correlative patterns between metabolic and genomic diversities have recently been applied to sesame and rice using seed stocks of natural variations (Mochida et al. 2009).

With the completion of genome sequencing in a number of plant species, comparative genome-scale analyzes can be used to produce and publish data sets that facilitate the identification of preserved and/or characteristic properties among plant species. Several efforts have been made to build comprehensive gene families using model proteome data sets deduced from sequenced genomes in establishing platforms to verify gene content and elucidating the process of gene duplication and functional diversification among species (Sterck et al. 2007). Comprehensive gene family data sets are usually produced through computational procedures, including a step that performs an all-against-all sequence similarity search and then a step for building protein family clusters by methods such as Markov Clustering (MCL) or consideration of protein domain structures (Hulsen et al. 2006). The results of such studies can produce databases that are useful for further phylogenetic studies (Wall et al. 2008). Correlated gene arrangements between taxa along with chromosome allocation, also known as synteny and collinearity, have become valuable frameworks for the inference of shared gene ancestry and the transfer of knowledge from species to another related species (Tang et al. 2008a). The plant genome duplication database (PGDD) provides a data set of intragenome or cross-genome syntenic relationships identified throughout genome-sequenced plant species (http://chibba.agtec.uga.edu/duplication/) (Tang et al. 2008b).

Databases that contain focused data sets together with rich annotations and well-related cross-references are also very useful for better understanding of focused issues in particular gene families and/or specific cellular processes. Sequence-specific DNA-binding TFs are key molecular switches that control or influence many biological processes, such as development or responses to environments. The genome-wide identification of gene repertories encoding Arabidopsis genome TFs was first reported in plants and comparisons with other organisms revealed the properties of plant-specific TFs (Riechmann et al. 2000). Over the past decade, we have been able to compile catalogs describing the function and organization of TF regulatory systems in a number of organisms with the availability of complete genome sequences. In many plant species, there are many databases that provide data sets of genes that putatively encode TFs; these are usually predictions based on computational methods such as sequence similarity search and/or hidden Markov model search for preserved DNA-binding domains. Recently, there has been further integration of data sets of TF-encoding genes, thereby creating an integrative,

knowledge-based resource of TFs across related plant species in terms of comparative transgenomics regulatory networks. GRASSIUS provides the first step toward building a comprehensive platform for integration of information, tools and resources for comparative regulatory genomics across the grass species. The Grass Transcription Factor Database (GrassTFDB) of GRASSIUS houses integrated information on MaizeTFDB, RiceTFDB, SorghumTFDB and CaneTFDB (http://grassius.org/grasstfdb.html). The LegumeTFDB provides predicted TF- encoding genes annotated in the genome sequences of three major legume species: soybean, *L. japonicus* and *M. truncatula* (http://legumetfdb.psc.riken.jp/). This database is an extended version of the SoybeanTFDB (http://soybeantfdb.psc.riken.jp/) and is aimed at integrating knowledge on legume TFs and providing a public resource for comparative genomics of the TFs of legumes, non-legume plants and other organisms (Mochida et al. 2010).

10.4 Integration of Interdisciplinary Approaches for Solving Biological Problem with Respect to Agriculture or Crop Improvements

The information and resources generated from various omic technologies offer prospects for the production of new biological knowledge. To describe and understand complex biological phenomena, it is essential to integrate various types of biological information and large-scale omics data sets through systematic analysis. We have developed a web-based system, Plant MetGenMAP, for this purpose, which can integrate and analyze large-scale gene expression and data sets of metabolite profiles along with various biological information. Under certain conditions, significantly altered biochemical pathways and biological processes can be retrieved quickly and efficiently using this system, and transcriptional events and/or metabolic changes in a pathway can easily be visualized. The system also provides a unique function that can identify candidate promoter motifs related to regulating specific biochemical pathways. Using data sets from Arabidopsis and tomato, we demonstrate the functions and application of the system. The results obtained by Plant MetGenMAP can contribute to a better understanding of the mechanisms underlying interesting biological phenomena and provide new insights into the biochemical changes associated with them at the gene and metabolite levels (Thijs et al. 2001). Extensive insight into molecular mechanisms and the coordination of biological networks has been obtained after the application of several different methods. Our knowledge of how the cell's different molecular entities interact with each other suggests that the integration of data from different techniques could nevertheless lead to a more comprehensive understanding of the data emanating from different techniques. The main focus on the pairwise integration of large-scale metabolite data with that of the transcriptomic, proteomics, whole-genome sequence, growth- and yield-associated phenotypes, and archival functional genomic data sets (Gonzalez et al. 2010).

The advances in high-throughput analytics have enabled us to gain insights into individual biomolecules using the various omics technologies. Any single omic approach, however, may not be sufficient to characterize the complexity and behavior of biological systems as a whole (Gygi et al. 1999). Therefore, molecular research gradually shifts towards the holistic perceptions of system biology, through the integration of the individual omics datasets, in order to obtain biologically meaningful interpretation of plant systems. The integration of multiple layers of biological information will therefore provide an accurate' picture' of the' whole' plant systems. Multiple omics datasets must be integrated after preprocessing (normalization, attribution of missing value and selection of features). Data integration is a key to successful system philosophy development through the development of comprehensive plant system models. Given the enormous promise to integrate multiple omics data, there is a growing interest in logical input to designing different experiments and analyzing heterogeneous data (Choi and Pavelka 2011). The successful integration of data will depend on appropriate experimental design, sound statistical analysis and correct interpretation of the results. The various aspects of successful integration of multiple heterogeneous omics datasets are to deposit individual 'omics' data to respective public repositories, to generate relationships among various kinds of datasets, visualization of the data and application of statistical and bioinformatics resources, where and when needed. These aspects have been elaborately discussed in Joyce and Palsson (2006).

The literature contains several instances of omics data integration. There are a number of reports on gene function elucidation through the combination of metabolomic analysis with genomic and transcriptomic data (Tohge et al. 2005; Okazaki et al. 2009). In maize hybrids, an integrated approach to transcriptomics and epigenomics has recently been used (He et al. 2013). Integrated use of transcriptomic and proteomic data has been reported in several recent studies involving whole plant nitrogen maize economy, growth to transition to dormancy in white spruce stems (Galindo González et al. 2012), phytohormone crosstalk (Proietti et al. 2013) and flour quality in wheat (Altenbach et al. 2010). Similarly, integrated metabolome and transcriptome analyses were recently applied in analysis of rice developing caryopses under high-temperature conditions (Yamakawa and Hakata 2010), molecular events underlying pollination-induced and pollination-independent fruit sets, the effects of DE-ETIOLATED1 down-regulation in tomato fruits (Enfissi et al. 2010) and changing metabolic systems in plants growing in field conditions, such as the rice mutant and transgenic barley (Kogel et al. 2010). An integrated metabolome and proteome analysis was applied in wheat and rice coleoptiles to illustrate the differences in response to anoxia (Shingaki-Wells et al. 2011) and characterization of starch and raffinose metabolisms to low and high temperatures in A. thaliana (Mostafavi et al. 2008). An integrated transcriptome, proteome and metabolome approach was adopted to describe the cascading changes to UV-B in maize (Casati et al. 2011). Moreover, an integrated hormonome, metabolome and transcriptome analyses in Arabidopsis transgenic lines, displayed increased leaf growth to gain insight into the molecular mechanisms that control leaf size (Gonzalez et al. 2010) have been reported. The literature mining is also a useful approach to knowledge

integration in plant biology (Winnenburg et al. 2008). Apart from single problems, more complex problems like photosynthesis have been addressed by Weston et al. (2011), where they characterized a network for heat transcriptome of three plant species (Arabidopsis, Populus and Soybean) where expression of one heat responsive module showed a negative correlation with leaf-level photosynthesis at a critical temperature. Later they proposed a conceptual model where traditional network analysis can be linked to whole-plant models (Weston et al. 2012). Also recently, Fouracre et al. (2014), threw light on the application of systems approaches in understanding the Kranz anatomy of the C4 plants. Several web-based resources like PLAN2L (Krallinger et al. 2009) and PosMed-plus (positional Medline for plant upgrading science) (Makita et al. 2009) are available to integrate literature-derived bioentities and associated information. The integration of multiple omics data has several challenges (De Keersmaecker et al. 2006; Steinfath et al. 2007). One of the problems with complex annotation and integration is the lack of agreed formats across various omics datasets due to the primary data sources 'heterogeneous repositories. The solutions to this problem include creating' data warehouses, using extensible markup language (XML), navigating hypertext, Unmediated MultiDB queries, creating a federated database and using controlled vocabulary. A Data Warehouse collects data from multiple resources, translates the formats and places them in a single database. The examples of data warehouses include: Atlas, BioMart, BioWarehouse, Columba, SYSTOMONAS, BioDWH, VINEdb, Booly, GNCPro (Turenne 2011). The XML is a general-purpose markup language that helps in sharing data across heterogeneous systems. The development of Systems Biology Markup Language (SBML) (Hucka et al. 2003) is probably the first and most successful efforts in this aspect. The Plant Ontology Consortium is a collaborative effort between plant genome model databases and plant researchers to create a controlled vocabulary (ontology) for plants to be maintained and facilitated (Avraham et al. 2008). The other problem includes statistical analysis, i.e. evaluation of integration complexity that differs from that of individual omics analysis and subsequently applying an appropriate method. Therefore, integrating omics data is far more than merely' joining the pieces;' it is actually a journey of exploring uncharted territories and transforming information into more useful biological knowledge.

10.4.1 Modeling and Simulation in Plant System Dynamics

The systems interest to biological sciences dates back to the days of von Bertalanffy (1933 1968), Wiener (1948) and Forrester (1958, 1961). In the context of biology, Biochemical Systems Theory (Voit 2000) and Metabolic Control Theory (Heinrich and Schuster 1996), proposed general mathematical models of biological systems at and around a steady state (equilibrium). Successful plant modeling is the ultimate goal of biology of plant systems. In a system, in mathematics, a model (Latin mode, meaning manner/measurement) usually represents the causal relationship. Cells or higher units of biological organization are understood as interacting element

systems in systems biology. The identity of the constituents, dynamic behavior and interactions between the constituents, of the biological system under study (Kitano 2002) must be known for an explanation of the system level. Ultimately this information can be combined into a model that is not only consistent with current knowledge but can also predict system behavior under new unexplored perturbations. Modeling and simulation are central to bridge the gaps between theory and experiment (Dhar et al. 2004). Experimental results usually require correct mathematical/statistical input, and model hypotheses require experimental evidence to provide meaningful biological interpretations. Modeling usually begins with building biological networks from the molecular data sets available. Network building and analysis are key components of biology of systems. In system biology, a network/graph has two basic parts: the system elements are represented as graph nodes and the interactions are represented as edges, i.e. lines connecting pairs of nodes. Edges can be directed (from a source (start node) to a sink (end node) and represent unidirectional flow of material or information) or non-directed (representing mutual interactions where the directional flow of information is not known). In biological networks, nodes represent the molecules present inside a cell (e.g., proteins, RNAs and/or metabolites) and links (or edges) between nodes represent their biological relationships (e.g., physical interaction, regulatory connections, metabolic reactions) (Blais and Dynlacht 2005). Activation or inhibition signs can be displayed on the edges to increase the network's information content. The important characteristics of biological networks are the scale-free structure (the number of nodes that connect with other nodes is much lower than the number of nodes with few connections) and the relative scarcity of hubs that connect directly with each other (Barabasi and Oltvai 2004). The interaction network nodes represent the biomolecular population whose abundance varies over time and in response to internal and environmental disturbances. The interaction network needs to visualize the changes and create a model which needs to be augmented by variables (expression, concentration, activity), thus indicate the state of each node and set of equations, signifying the how the state changes corresponding to the stimuli. Depending on their behavior in the system with time, models can be static or dynamic. The four common types of networks in plant biology systems include gene-to-metabolite networks, protein-protein interaction networks, transcriptional regulatory networks, and gene regulatory networks in which the first three types are often static, whereas the gene regulatory network is often dynamic (Yuan et al. 2008).

10.4.2 Gene-To-Metabolite Networks

Gene-to-metabolite networks are derived under a given set of conditions from the analysis of the correlation of genes and metabolites. The genes and metabolites act as nodes here, and the edges represent the interactions between regulators. Depending on the distance between genes and metabolites, interactions are interpreted. Due to the enormous diversity and number of metabolites produced in cells corresponding to their sessile lifestyle, these types of networks are highly complex

and difficult to study in plants. Different new research dimensions such as interrelation between biological processes, functional gene annotation, and discovery of new genes in biosynthesis, regulation and transportation of metabolites, have been added to plant science owing to the elucidation of gene-to metabolite networks (Yuan et al. 2008). The gene-to- metabolite networks have been worked out in various studies like in stress responses, discovery of novel candidate genes for terpenoid indole alkaloid biosynthesis in *Catharanthus roseus* (Rischer et al. 2006), in the response to nitrogen deficiency and during diurnal cycles (Scheible et al. 2004) an so on.

10.4.3 Protein–Protein Interaction Networks

In protein-protein interaction networks, nodes are proteins that are connected by direct edges if the information flow direction is known during their interaction, or non-directed edges if there is strong evidence of their physical interaction or association without evidence of interaction directionality (Assmann and Albert 2009). It may be possible to have two types of interactions: genetic or physical. A protein-protein genetic interaction is a network of genes characterized by genetic interactions in order to explain gene function in physiological processes (Boone et al. 2007). However, due to ploid levels and long life cycles, this approach is difficult to implement the ploidy levels and long life cycles of plants. On the contrary, physical interactions are easier to be characterized on the plant systems. In plants, interaction maps have been experimentally elucidated for homo and heterodimerization within two large classes of transcription factors: the MADS (MCM1, Agamous, Deficiens, SRF) box transcription factors (de Folter et al. 2005) and the MYB (myeloblastosis) transcription factor family (Zimmermann et al. 2004a, b). The further details regarding interactome are furnished in a preceding section in the current review namely ‘interactomics’.

10.4.4 Transcriptional Regulatory Networks

The transcription regulatory network explains the regulatory interactions between transcription factors and downstream genes. They have two types of nodes—transcription factors and regulatory genes and two types of directed edged viz. transcriptional regulation and translation (Babu et al. 2004). In addition, the regulatory edges can have two types of signs, corresponding to activation or repression. Despite the general organizational similarity of networks across the phylogenetic spectrum, there are interesting qualitative differences among the network components, such as the transcription factors (Babu et al. 2004). Transcription factors usually regulate multiple genes and hence transcriptional regulatory networks are unidirectional and do not have strongly connected components. The various approaches to deciphering transcriptional regulatory networks include genome-wide expression profiling,

genome-wide RNA interference (RNAi) screens (Baum and Craig 2004), transcription rate assessment by measuring mRNA decline rates, pair evaluation of promoter co-occupancyand cis-element computational prediction. A transcriptional regulatory map for cold signaling mediated by the transcription factor of ICE1 was created in Arabidopsis (Benedict et al. 2006). Recent transcriptional regulatory network reports include the role of oxidative signals in chilling stress in rice (Yun et al. 2010), those in response to abiotic stresses in Arabidopsis and grasses (Nakashima et al. 2009) as well as rice (Todaka et al. 2012), abiotic light-regulated transcriptional networks in higher plants (Jiao et al. 2007) and so on.

10.4.5 Gene Regulatory Networks

The nodes correspond to genes, messengers or proteins in a gene regulatory network and the edges represent the regulatory interactions (activation, inhibition, repression or other functional interactions) among the network components. Complex gene regulatory networks consist of genes, non-coding RNAs, proteins, metabolites and components of signaling (Long et al. 2008). This type of network includes all stages of gene expression regulation including DNA transcription regulation, RNA translation, post-transcription RNA processing, as well as post-translation changes such as protein targeting and covalent protein modification. Unlike other static networks in nature, these networks are often used to display the dynamics of plant systems (Yuan et al. 2008). The ABC model, one of the first modeled plant gene regulatory networks, explained the interactions between transcription factors that regulate plant species-wide floral pattern formation (Coen and Meyerowitz 1991). In several studies, gene regulatory networks were reported to study plant developmental and physiological processes. The studies include the attempt to model the essential components controlling stomatal closure of the cell size, determining the cell fate during flower development in A. thaliana, microRNA (miRNA)-mediated gene regulatory networks (Meng et al. 2011) and recently in explaining land plant evolution (Pires et al. 2013).

Biological network construction and analyzes were therefore an important approach to explaining the organism or a biological process as a whole in the biology of plant systems. In modern science, high-throughput technologies provide huge quantitative data. However, in systems where knowledge of mechanical details and kinetic parameters is scarce, the use of quantitative data is obstructed. In such cases, it may be helpful to model the system with a wealth of molecular data on individual constituents as well as interactions (Assmann and Albert 2009). The system biology's individual key components viz. Earl explained genomics, transcriptomics, proteomics, metabolomics, etc. have been explained earlier. The biological networks along with these components are chief aspects of plant systems biology. Although the models could not exactly mimic the system with pure accuracy, still are highly capable to explain the intrinsic complexity of the plant systems.

10.4.6 Softwares and Algorithms for Plant Systems Biology

Using software from bioinformatics is inevitable for the comprehensive study of biology of plant systems. In addition to the tools and resources used in the analysis of the individual omics platforms, it requires several resources to elucidate the' complete picture.' Joyce and Palsson (2006) and Turenne (2011) list detailed discussion of various algorithms and software used for system biology. These include network visualization tools, modeling environments, pathway building and visualization tools, modeling platforms for systems biology, and model repositories. Visualization is a means for analyzing research data and a key method for analyzing networks. The purpose of omics data visualization should be to create clear, meaningful and integrated resources without the inherent complexity of the data being undermined (Gehlenborg et al. 2010). There are several tools to help visualize' omics' data on a system scale such as Sungear (Poultney et al. 2007), MapMan (Thimm et al. 2004), Genevestigator (Zimmermann et al. 2004a, b), Cytoscape (Shannon et al. 2003), VirtualPlant (Katari et al. 2010), REACTOME (Joshi-Tope et al. 2005). Pathway databases are used for modeling systems as they provide a clear way to create network topologies by the annotated reaction systems. The various pathway databases for systems analyses include KEGG (Kanehisa et al. 2012), BioCyc (Caspi et al. 2010), Aracyc (Mueller et al. 2003), Pathway Interaction Database (PID) (Schaefer et al. 2009) and BioCarta (Nishimura 2001). Also, several comprehensive modeling environments are available, like Gepasi (Mendes 1997), Virtual Cell (Loew and Schaff 2001), Osprey (Breitkreutz et al. 2003), Arabidopsis eFP browser (Winter et al. 2007), COPASI (Hoops et al. 2006), R (http://www.R-project.org),MatLab and InfoBiotics workbench (Blakes et al. 2011), E-Cell (Tomita et al. 1999), Systems Biology WorkBench (Sauro et al. 2003). The Systems biology model repositories include BioModels database (Le Novere et al. 2006) or JWS (Olivier and Snoep 2004). Both are public, centralized databases of curated, published, quantitative kinetic models of biochemical and cellular systems. The core systems biology networks include SynBioWave (Staab et al. 2010), Cell Illustrator (Nagasaki et al. 2010), Moksiskaan (Laakso and Hautaniemi 2010), MEMOSys (Pabinger et al. 2011), Babelomics (Al-Shahrour et al. 2006), MetNet (Sucaet et al. 2012), etc.

10.4.7 Integrating Metabolite and Transcriptome Data

The initial integrative approaches with plant metabolism relevance included the combination of transcript data and metabolite profiling(Tohge et al. 2005). Such studies were initially limited to model species for which ESTs or oligonucleotides were available; early transcriptomic approaches in fact relied on differential hybridization of complementary DNA samples to known immobilized sequences on solid supports. However, this barrier has been removed by the advent of next-generation sequencing technologies and far more exotic species are beginning to be studied using this approach (Gechev et al. 2013). By combining transcript and metabolite, two basic questions are commonly addressed by combining transcript and

metabolite data. The first concerns whether a gene functions within a given metabolic pathway. When a better characterization of the pathway is achieved, it is also essential to examine the extent of transcriptional control (except in some cases, for example, by regulating post – transcription modifications of the enzyme and by regulating positive/negative feedback by substrates/products) under different physiological conditions and how it is distributed across the different enzymatic steps.

The initial focus of these investigations was specific pathways, such as hormone, glucosinolate, and flavonoid biosynthesis. For example, differential gene expression mechanisms helped clarify the involvement of two different genes of nitrilase in auxin synthesis in Arabidopsis. Similarly, the contributions of gene duplication and inducible gene expression (differential activation of biosynthetic gene subsets) have been shown to impact glucosinolates amount and composition. An additional early evidence of the role of specific transcript accumulation on a metabolic phenotype stemmed from the clarification of the role that various regulatory mechanisms affect Trp synthase α and β had on the amount of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one, a natural pesticide synthesized in maize (*Zea mays*) leaves. Another example of the coordination between transcripts and metabolite accumulation was the maize anther analysis, where a strong correlation was found between the expression of a structural gene (flavanone 3-hydroxylase) and the appearance of specific flavonols (mainly quercetin and kaempferol). In this case, the comparison of sweet and hot pepper varieties made it easier to identify certain placenta-specific, differentially expressed genes that were directly correlated with the accumulation of capsaicinoids. One of the first examples of this approach focused on the identification of transcripts strongly correlated with the abundance of given metabolites across tuber development, irrespective of whether the transcript was associated with the metabolic pathway under question or not (Urbanczyk-Wochniak et al. 2003).

Indeed, this approach was able to identify certain transcripts that exhibited very high correlations with the expression of certain genes and, as such, proved effective in identifying a number of biofortification candidate genes. The same approach can and has been used by corollary to elucidate the variation in gene-to-metabolite networks following short-and long-term nutritional stress in Arabidopsis or to identify gene expression metabolic regulators. For example, in Arabidopsis (Hannah et al. 2010), cryptoxanthin was found to be highly correlated with a large number of genes across diverse environmental conditions, and organic acid malate was putatively identified (Carrari et al. 2006) and subsequently confirmedto be important in mediating the ripening process in tomato (*Solanum lycopersicum*). Such current studies are all examples of the guilt-by-association approach, which in essence postulates biological entities as being functionally related if they exhibit strong correlation or coresponse across a wide range of cellular circumstances. The power of this approach is that it may have great use in identifying novel metabolic integration and/or new regulatory mechanisms, given that it does not rely on a priori pathway knowledge. The main drawback of the approach, however, is that, in the absence of subsequent rounds of experimentation, it is difficult to gain insight into the mechanistic links underlying the behavior observed, given that correlation between biological entities does not always imply causation or the existence of functional links (Tohge and Fernie 2010).

In this respect, it becomes imperative to validate the resultsof co-expression analyzes using follow-up approaches to prove the existence of putting functional links. Arguably, the greatest advances made to date following approaches to integrate transcript and metabolite data have been achieved in gene annotation and the structural elucidation of plant intermediary and secondary metabolism.

Two early studies of particular note are those from the laboratories of Saito and Dixon investigating the metabolism of Arabidopsis anthocyanin and Medicago truncatula triterpene. In the case of the anthocyanin pathway, no late biosynthetic genes involved in anthocyanin decoration steps were identified in Arabidopsis prior to the study of (Achnine et al. 2005; Tohge et al. 2005), although visible phenotype screening characterized all early biosynthetic genes. A combination of transcript and metabolite profiling on an activation-tagged line of anthocyanin pigment1 along with validation experiments involving both heterologically expressed enzymes and knockout mutations resulted in the identification of five genes and the identification of up to 11 anthocyanins. Such confirmatory experiments are essential to assign gene function unambiguously. Combining reverse genetic strategies with enzyme activity characterization when the gene is expressed in a heterologous system remains the gold standard for molecular identification of novel enzyme-catalyzed reactions. Subsequent follow-up studies have identified some six genes associated with the metabolism of flavonol, and some 24 compounds of this class (among 35 compounds found) have now been identified in Arabidopsis While the expansion of the characterized triterpenoid metabolism in M. Truncatula is not that impressive, Tohge et al. (2005)'s study enabled the functional annotation of 30 different saponins, and currently, over 70 metabolites of this compound class have been identified in *M. truncatula.* The usefulness of this approach is at its greatest for the relatively unchartered pathways of specialized metabolism; however, it should be noted that the gene encoding plant Thr aldolase(322–324) in Arabidopsis and 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one glucoside methyltransferase in maize was independently identified in this strategy(Meihls et al. 2013). The number of species and pathways for which this approach was adopted expanded massively to include several crops and medicinal plants a decade later. Strategies combining transcript and metabolite profiling have been effective in shedding light on the structure of several metabolic pathways involved in the synthesis of primary metabolites, flavonoids, terpenoids, and alkaloids (Lin et al. 2015). The combination of transcript and metabolite profiling has been commonly used on a broader level for multi-layered descriptions of plant responses, especially those to abiotic stress. This has resulted in a number of studies assessing the combined transcript and metabolite responses to water stress, temperature stress, light stress, and nutrient supply limitations (Urano et al. 2009). Though descriptive by nature, such studies can provide insight into global metabolic variations under certain conditions as well as identify which pathways are under tight control and which are under loose transcriptional control. Given the highly interconnected nature of the metabolic system and its nonlinearity of metabolic pathways in the global network structure, and even in the absence of flux profiling data, the integration of transcriptomics with wide metabolic profiling can, in any case, narrow down which metabolic steps could be active under specific conditions. Occasionally,

however, more mechanistic information can also be provided. A prominent example of this is the detailed analysis of several transgenic Arabidopsis lines with altered flavonoid levels through transcriptomic and metabolomic analysis, including hormone analysis, which revealed that flavonoid overaccumulation with strong oxidative capacity in vitro also gives oxidative stress and drought tolerance (Nakabayashi and Saito 2015). Moreover, a combination of transcript and metabolite profiles followed a range of developmental processes at high resolution. Such studies are dominated by fruit maturation and leaf development studies, but they are not limited to these processes, with studies also covering the development of various organs, lignin deposition, and the establishment of arbuscular mycorrhizal symbiosis (Nakamura et al. 2014). In this respect, these approaches prove informative in clarifying the relative importance of apparently redundant biosynthesis pathways and the degradation of specific metabolites or may also help to define the role of those primary metabolites (e.g., aminobutyrate) for which a signaling role was assumed. For example, ascorbate biosynthesis has been revealed as the dominant route of ascorbate biosynthesis during tomato maturation, which is one of the well-studied metabolisms in several higher plants, especially in Arabidopsis. Another example can be found in the elucidation of the arogenate pathway as an alternative route for Phe biosynthesis, a similar approach in Arabidopsis, based on feeding studies and analysis of co – expression, suggested an alternative pathway to degradation of Lys in dark – induced senescent leaves (Araújo et al. 2010). However, despite the fact that these examples illustrate that combined transcriptome/metabolome studies increase our understanding of metabolic network regulation, we argue that they remain at their most powerful in gene functional annotation and the elucidation of metabolic pathway structures specific to species and/or tissue.

10.4.8 Integrating Metabolite and Proteome or Enzyme Activity Data

The combination of proteome and metabolome analyzes is less commonly used to date than combined transcriptome and metabolome analyses. In addition, they are largely used in a manner similar to the more descriptive studies discussed above hence, this gained considerable insight into the structure of metabolic networks as well as general aspects of metabolic regulation. In the first of these examples, metabolite data were studied in parallel with enzyme data (and transcriptomics data) in different wild-type diurnal cycles and an Arabidopsis starchless mutant, revealing that rapid transcript changes are integrated over time to generate substantially stable changes in many sectors of metabolism. The same group went on to apply this approach to tomato fruit development and natural variance in Arabidopsis. In tomatoes, enzyme profiles were sufficiently characteristic to distinguish developmental stages and cultivars and wild species, but the comparison of enzyme activity and metabolites revealed remarkably little connectivity between enzyme developmental changes and metabolite levels, suggesting the operation of mechanisms for post-translation modification. They documented highly coordinated changes

between enzyme activities in Arabidopsis, especially within those of the Calvin-Benson cycle, as well as significant correlations between starch and growth in specific metabolite pairs. On the other hand, there were few correlations and therefore low overall connectivity. On the other hand, few correlations were observed between enzyme activity and metabolite levels(Sulpice et al. 2010), and thus low overall connectivity, but strong links were seen between starch levels and growth, which we describe below. In an alternative approach, proteomic and metabolic data were only used to extend the range of molecular entities to show that fascicular and extra fascicular phloems are isolated from each other and functionally divergent (Zhang et al. 2010).

10.4.9 Integrating Metabolite and Genome Data

Assuming that the advent of metabolomics more or less paralleled the release of the first plant genome, the integration of metabolomics and data on the entire genome sequence may be unsurprising(van der Werf et al. 2007). Suffice it to say that in such combinations there are considerable complexities; tellingly, early studies aimed at computational prediction of the size of the Escherichia coli metabolome estimated a complement of about 750 metabolites, while subsequent experimental approaches revealed many metabolites that were not computed from the genome. This discrepancy could be explained by several potential reasons (Tohge et al. 2014). The most likely reason for this is the lack of linear relationship between genes, their protein products, and metabolites, and the fact that most genomes remain incompletely annotated, including those of model organisms. Despite this serious drawback, in this section, we hope to illustrate that the integration of metabolomics and genomic data can be incredibly powerful in understanding natural metabolism variation and its regulation. Whole-Genome sequences for over 100 species of plants (including microalgae) are available. Metabolomics currently cannot match this massive acceleration provided by next-generation technologies, particularly when high-quality species are being adopted optimized approaches (Fukushima et al. 2014). The KNApSAcK database, which is one of the largest curated compendia of phytochemicals, contains over 700 compounds for early sequenced plants like Arabidopsis and rice (*Oryza sativa*) but no entries for recently sequenced species such as goatgrass (*Aegilops tauschii*) and wild tobacco (*Nicotiana tomentosiformis*). In this section, we will describe insight gained from combining metabolomic data with genome sequences in three different case studies: (1) a simple comparison of a reference genome with metabolomics data; (2) a comparison of natural allelic and metabolic variance; and (3) integrating genome sequence data into quantitative genetics approaches. The first of these has been covered in considerable detail recently (Tohge et al. 2014) so we will only briefly describe it here. The starting point is to perform genome-wide ortholog searches using functionally annotated genes; best practice is to use cross-species cluster-based BLAST searches such as those housed in the PLAZA database (Proost et al. 2009) or, in the case of photosynthetic microbes, pico-PLAZA (Vandepoele et al. 2013). Illustrations of

how such analyses have been performed for central, shikimate, phenylpropanoid, terpenoid, alkaloid, and glucosinolate metabolism have been presented. Important insights into pathway evolution can be gained from such approaches, as illustrated by the recent cross-kingdom comparison of ascorbate biosynthesis (Wheeler et al. 2015). The second case study, which is similar in scope but far more targeted than genome-wide association studies to evaluate alllic and metabolic variance across natural diversity, is described below. Most recent examples of its usefulness are derived from the analysis of wild tomato species; however, it is important to note that the approach itself is essentially a modification of that adopted over decades in the cloning of natural color mutants. In recent years, this approach has significantly enhanced understanding of both primary and secondary and cuticular cell wall metabolism been enhanced considerably via this approach (Koenig et al. 2013), Although the greatest insight into the latter was ultimately clarified, as described below, through the use of the introgression line population. In essence, this approach begins with the identification of metabolic variance within a population of ecotypes, cultivars, or similarly related species and attempts to link this with alllic diversity or gene duplication, as has been achieved with acyl-sugar metabolites (Schilmiller et al. 2015), terpenes, and isoprenoids (Kang et al. 2014), or even with the presence or absence of genes, as described recently for methylated flavonoids of glandular trichomes. The previous list documents the success of this approach; however, until recently, it has been constrained by the limits of our a priori knowledge needed to select the candidate genes in which we are searching for alllic variance. The development of RNA sequencing technologies means that we are no longer limited by the amount of sequence data; however, there may still be a potential hurdle to these integrative approaches when comparing highly genetically divergent individuals, as the number of genetic polymorphisms is too large to be evaluated one by one. The quantitative trait loci approach is therefore a powerful alternative method of associating phenotypes with their underlying genetic variance. The use of such approaches in plant metabolism has been the subject of several recent comprehensive reviews (Scossa et al. 2015), however, few examples of their usefulness to advance understanding of metabolite accumulation and metabolic regulation are as-. Tomato fruit, as the model species for fleshy fruit maturation, has been the subject of combined large-scale genomic, physiological, and metabolic investigations, often using specific biparental populations or large sets of unrelated individuals, in an attempt to understand the causal variants of metabolic variations (Sauvage et al. 2014). In particular, the use of introgression lines obtained from the cross between tomato and *Solanum pennellii* (a wild tomato species) has greatly helped to identify quantitative trait loci for a large number of physiological and metabolic traits. Profiling data of primary and secondary metabolites in this population was collected over several years (along with some classical yield-related traits), revealing more than 1500 metabolic quantitative trait loci affecting levels of multiple sugars, amino acids, organic acids, vitamins, phenylpropanoids, and glycoalkaloids. In some selected metabolic quantitative trait loci, the availability of sequences of both parental genomes (Bolger et al. 2014) reduced the origin of the metabolic variation to specific genetic polymorphisms (Alseekh et al. 2015). The

integration of genotypic and metabolic variance can and has been applied to large collections of unrelated individuals (metabolite-based genome-wide association studies): as in the case of biparental populations, also with this strategy, several cases of polymorphological variants of genomic sequences have been identified and related to metabolic variation. These two approaches, based either on biparental populations or on large collections of natural accessions, have been used in Arabidopsis and crop species (maize, rice, wheat (*Triticum aestivum*), and fruit trees (Luo 2015).

10.4.10 Integration of Transcriptomic and Metabolomics Level Genome-Scale Models

In the first of these studies, Arabidopsis microarray data exposed to eight different conditions of light and temperature were integrated into a model on a genome scale (Töpfer et al. 2014). We first digress to give a brief description of how genome-scale models are generated before discussing the outcome of this integration. Essentially, a model on a genome-scale match's metabolic gene with metabolic pathways in a way that generates a stoichiometrically balanced metabolic network that matches all gene functions annotated for that organism. In the turn of the century, these models were originally published for microbes, with many models for plant species subsequently available for Arabidopsis as well as crop species such as rice and maize (Simons et al. 2014). Returning to the superimposition of experimental data on the model, the addition of transcriptomic data has enabled flux capacities to be predicted and statistically assessed if these vary under the test conditions. In addition, this study introduced the concepts of metabolic sustainers and modulators, the former being metabolic functions that are differentially up-regulated with respect to the null model, while the latter are differentially down-regulated to control a certain flux and thus modulate the affected processes (Töpfer et al. 2013). In a follow-up study, predictions based on transcriptomics integration were complemented by metabolomics data from the same experiment. In doing so, the authors were able to bridge flux-centric and metabolomics-centric approaches and, in so doing, demonstrate that, under certain conditions, metabolites serving as pathway substrates in pathways defined as either modulators or sustainers display lower temporal variation with respect to all other metabolites (Töpfer et al. 2013). In addition, substantial evidence suggests that levels of specific metabolites such as Ala, pyruvate, 2-oxoglutarate, Gln, and spermidine are exceptionally stable across a wide range of cellular conditions. They also agree with observations that metabolite levels such as Ser coordinate the expression levels of genes encoding multiple steps of the pathways they themselves belong to (Timm et al. 2013). The high stability of these metabolites over a range of different stresses is in line with their requirements. It also emphasizes the fact that the most biologically relevant metabolites may be for metabolic regulation; this is an important point, since it is at odds with the manner in which the majority of the metabolomics community assesses their data. This observation additionally highlights the potential difficulties and challenges in

interpreting data from a single level of the cellular hierarchy and thus provides further grounds for integrated models.

The rapid proliferation of plant and other organism genome-scale data makes it possible to study various cellular processes systematically. Because heterogeneous high-throughput data sets have been acquired from various "omics" technologies such as genomics, transcriptomics, proteomics, and metabolomics, it has become necessary to develop computational tools that can effectively integrate and analyze them. Microarrays and recently developed RNA-Seq technology have proven to be crucial tools for generating transcription data sets by simultaneously detecting thousands of gene expression. These data sets contain useful information to study gene functions in various ways including stress responses and developmental programs. Meanwhile, metabolomics, which investigates the profiles of all metabolites in an organism under specific conditions using techniques such as gas chromatography-mass spectrometry (GC-MS), has been regarded as an important research field in the postgenomic area, especially for plants due to their significant chemical diversity. New functional gene annotations have been added to various biological networks in recent years, including regulatory networks, networks of protein-protein interaction, and metabolic pathways. Despite these advances, under specific conditions, dynamic gene behaviors are still largely unexplored in specific pathways. Thus, in addition to integrating heterogeneous data sources, their analysis in the context of pathways is considered an essential step for functional studies of a complex biological system. Transcriptomic data are normally mapped into specific metabolic pathways in this type of analysis to investigate a set of genes 'coordinated behavior. It is important to develop effective tools for this type of analysis to systematically characterize and understand the dynamics of biochemical pathways by using multi-level information.

As detailed information on biological pathways has been developed, more complete and accurate pathways have been mapped, both experimentally and computationally. MetaCyc (http://metacyc.org/) and the Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.ad.jp/kegg/) are currently representative biochemical pathway databases. MetaCyc contains experimentally verified metabolic pathways and information on enzymes curated from scientific literature as well as predicted computationally predicted metabolic networks for more than 1600 different organisms (Krieger et al. 2004). KEGG is a knowledge base in terms of the network of genes and molecules resulting from their activities (Kanehisa et al. 2006). These databases are the primary resources that can be utilized to understand how genes and molecules are connected in biochemical pathways. Moreover, they can be combined with new resources or technologies for genomic and functional analysis, making it possible to expand previous databases and obtain increased depth and range of functions. For example, the database EGENES was developed to place genomic information, including ESTs of many plant species, into metabolic pathways and was integrated into the KEGG suite of databases (Kanehisa et al. 2006). Several analytical tools were developed to identify gene expression patterns that are responsible for potent biological effects by integrating large-scale transcriptomic data with various biological information such as pathways and related

metabolites. Pathway Processor is a tool for visualizing metabolic pathway expression data and evaluating which transcriptional changes affect metabolic pathways. Specifically for plant species, several similar tools have been developed recently. Another plant species analysis system is KaPPA-View, a web-based tool used to display quantitative data for individual transcripts and metabolites on stored plant metabolic track maps stored in KEGG.

The Omics Viewer package in the Pathway Tools enables scientists to visualize for any organism of interest the large-scale gene expression and metabolomics data sets on metabolic pathways predicted by the Pathway Tools. KaPPA-View and Omics Viewer, however, provide very limited statistical analysis or project management functions. A web-based system, Plant MetGenMAP, has been developed that can identify significantly altered biochemical pathways and highly affected biological processes and predict functional roles of pathway genes from transcript and metabolite profile data sets and potential pathway-related regulatory motifs. Plant MetGenMAP is a user-friendly, powerful system of analysis that supports many functions of system biology analyzes in the context of biochemical pathways and terms of gene ontology (GO). It provides an analytical platform that allows for rapid and efficient exploration of highly altered pathways through intuitive visualization and robust statistical testing. Because it allows simultaneous analysis of transcriptional and metabolic changes for each pathway, the association between gene expression and biochemical changes in specific pathways can be easily inferred under specific conditions. Functional analysis of differentially controlled pathways can help define functional roles correctly of genes within pathways. Furthermore, the system embeds a function that can putatively identify major regulators related to changing transcripts and metabolites in specific pathways. Transcript and/or metabolite profiles of the model plant species Arabidopsis (Arabidopsis thaliana) and tomato (Solanum lycopersicum) have demonstrated the functions of Plant MetGenMAP. We present comprehensive results identified with Plant MetGenMAP, including differentially regulated metabolic pathways, pathway-related gene functions, putative regulators associated with these genes, and probabilistic associations between genes, metabolites, and phenotypes. MetaCyc contains experimentally determined biochemical pathways that can be used as a metabolism reference database. MetaCyc can be used together with the Pathway Tools software to predict the metabolic pathway complement of an annotated genome computationally. More than 60 plant-specific pathways have been added or updated in MetaCyc recently to increase the breadth of pathways and enzymes. Unlike MetaCyc, which contains metabolic data for a wide range of organisms, AraCyc is a species-specific database that contains only enzymes and pathways found in the Arabidopsis (*Arabidopsis thaliana*) model plant. The first computationally AraCyc (http://arabidopsis.org/tools/aracyc/) was the first computationally predicted plant metabolism database derived from MetaCyc. AraCyc has been under ongoing curation since its initial computational construction to improve data quality and increase the breadth of pathway coverage. Recently, twenty-eight pathways were curated manually from literature. Also recently, AraCyc's pathway predictions have been updated with the latest functional annotations of Arabidopsis genes using controlled vocabulary and

literature evidence. Currently, AraCyc has 1418 unique genes mapped with 1156 literature citations on 204 pathways. The Omics Viewer, a user data visualization and analysis tool, makes it possible to paint a list of genes, enzymes or metabolites with experimental values on a diagram of AraCyc full path map. Other recent improvements to both MetaCyc and AraCyc include the implementation of an ontology of evidence used to provide data quality information, the expansion of the secondary pathway ontology metabolism node to accommodate the cure of secondary metabolic pathways, and the enhancement of the ontology of the cellular component for the storage and display of enzymes and pathways within s The MetaCyc database's goal is to catalog every experimental biochemical pathway for small molecule metabolism (Krieger et al. 2004). In EcoCyc(Keseler et al. 2005) a model organism database for Escherichia coli, MetaCyc was initialized with all the manually curated pathways. MetaCyc has subsequently added pathways from more than 300 organisms, and more than 90% of its pathways are manually curated with literature quotations and species information. The other 10% of pathways originally imported from the WIT database (http://www.cme.msu.edu/WIT/) are manually curated. MetaCyc can be used as a reference database in conjunction with the Pathway Tools software to create new Pathway Genome Databases (PGDB) from annotated genomes or genes. The Pathway Tools software contains three components: (1) PathoLogic, which matches an annotated genome's gene product names against enzymes and reactions in a reference database such as MetaCyc, and predicts the organism's pathways using a scoring algorithm; (2) Pathway/Genome Editor, which allows manual updating of the derived database and supports data sharing between derived d AraCyc was PathoLogic's first plant metabolism database to predict computationally using MetaCyc as the reference database(Mueller et al. 2003). AraCyc will eventually describe a complete set of metabolic pathways for Arabidopsis (Arabidopsis thaliana) and display genes and enzymes within their metabolic context with continued manual curation. Although there are still many pathways and enzymes to be manually curated in AraCyc, AraCyc is currently the most comprehensive genome-wide metabolic database available for a single plant species. Both databases can be accessed easily through the World Wide Web (http://metacyc.org and https://www.arabidopsis.org/biocyc/). With the release of the fully sequenced plant genomes of Arabidopsis (The Arabidopsis Genome Initiative 2000) and rice (*Oryza sativa*; International Rice Genome Sequencing Project, http://rgp.dna.affrc.go.jp/IRGSP) and the initiation of sequencing projects for many other plant species, there is a fast growing desire to place the sequenced and annotated genomes in a metabolic context. Indeed, the benefits of a species-specific metabolic pathway database are substantial: (1) it depicts the biochemical components of an organism; (2) it assists comparative studies of pathways across species and facilitates metabolic engineering to improve crop metabolism and traits; (3) it can be used as a platform to integrate and analyze data from large-scale experiments, such as gene expression, protein expression, or metabolite profiling; and (4) by presenting pathway steps lacking assigned genes or having genes assigned but solely based on computational prediction, we can discern what remains to be identified and experimentally characterized. Despite these advantages, it may be labor intensive

and time consuming to create a pathway database manual de novo. SoyBase (http://soybase.agron.iastate.edu/), a soybean-specific metabolic pathway database (Glycine max), is the only other manually created species-specific plant pathway database. It is also possible to predict computationally species-specific plant pathway databases as a way to jump-start manual curation. A precise and comprehensive reference database is key to the quality of the derived databases for the predictions to be useful. Examples of comprehensive pathway databases include Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/kegg/; (Kanehisa and Goto 2000) and Enzymes and Metabolic Pathways (http://www.empproject.com/). As they stand, their usefulness as reference databases for plant genomes is somewhat limited for one or more of the following reasons: (1) pathways are not linked to literature quotations and therefore it is difficult to evaluate their accuracy; (2) individual path diagrams tend to be composites taken from several different species and are therefore not accurate for any single species; and (3) they are composites taken from several different species; The approaches taken, however, have been relatively straightforward to date and have generally not been carried out at a high level of spatial resolution. There are currently several methods for obtaining data from all the methods described here at the tissue, cellular, and even subcellular levels (Aharoni and Brandizzi 2012) while still technically challenging, it seems conceivable that such methods could provide data required to better understand the cell specialization of metabolism. In addition, methods to gain accurate metabolic flux estimates following $^{13}CO_2$ labeling have recently been established (Ma et al. 2014) but are not yet fully integrated with protein or transcript data. However, it is important to note that such experiments, albeit using (^{13}C)Glc as a precursor, have already been carried out in in vitro-cultivated *Brassica napus* embryos, providing considerable insight into the systems-level regulation of this organ (Schwender et al. 2015). Moreover, it seems highly likely that future research will draw more heavily on archived genomics data than it has up to now; thus, the continued availability and quality-control curation of such data sets is imperative if their value is to be fully exploited. To facilitate our understanding of transcriptome and metabolome data, there are several metabolic pathway databases available. The Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.ad.jp/kegg/) has a pathway database (PATHWAY) containing metabolite and gene information, as well as graphical representations of metabolic pathways and complexes derived from different biological processes. The metabolic pathways for 218 organisms, including Arabidopsis and rice, have been constructed to date. Organism-specific metabolic pathway maps can be generated according to assignment information on the KEGG/GENES database. The metabolic pathway reference database named MetaCyc (Krieger et al. 2004) pathways from 302 organisms (December 2004; http://metacyc.org/). The Arabidopsis pathway database AraCyc (http://www.arabidopsis.org/tools/aracyc/) was constructed by adding plant-specific pathways and reactions to basic pathway sets in the MetaCyc pathway collection (Mueller et al. 2003). While the comprehensive database contains metabolic pathway data that is representative of the plant kingdom, the pathways and reactions involved in alkaloid and isoflavonoid biosyntheses are not well represented, as these are not found

in Arabidopsis. A relatively common feature of plants is that a single enzymatic reaction is often attributed to several homologous gene products. Multigene families in plant genomes are considerably more prevalent than in animal genomes (The Arabidopsis Genome Initiative 2000). Recent research has shown that multiple genes are not simple repeats, but exhibit a variety of gene expression and therefore have a variety of functions. For example, in Arabidopsis and rice, 33 and 29 member genes are the XTH gene family, a group of genes encoding xyloglucan endotransglucosylase/hydrolase involved in xyloglucan metabolism. In dicot and monocot plants, the individual gene members exhibit tissue-specific and stage-dependent expression of growth. One of the tools on the AraCyc database is capable of painting transcript data values onto the metabolic overview diagram. However, only representative data are used for the painting when multigene families are thought to be involved in single reactions. For painting, only representative data is used. On individual metabolic pathway maps, individual transcript data is not displayed. Also, a recent AraCyc version can represent metabolite data but only on the overview diagram. A user-driven tool, MAPMAN, has recently been developed to represent transcript data on pictorial diagrams, categorizing all genes of Arabidopsis on the basis of biological function (Thimm et al. 2004). Metabolites were also categorized to represent quantitative values of each metabolite on pictorial diagrams. However, since MAPMAN provides only several metabolic pathways, users need to use the user-driven tool to prepare their own diagrams. We created a set of comprehensive metabolic pathway maps for Arabidopsis as a complementary approach to AraCyc and MAPMAN in which 1263 metabolic reactions were grouped together. We have also developed a web-based tool for analyzing plant metabolic pathways, KaPPA-View, to display quantitative data on the same set of metabolic pathway maps for individual transcripts and metabolites. We adapted Scalable Vector Graphics (SVG) to facilitate dynamic document generation with rich graphical features and pathway map editing by the user to represent data from the transcript/metabolite. We demonstrated the utility of the KaPPA – View tool by displaying the transgenic plant data set that overexpresses the PAP1gene encoding a MYB transcription factor on the metabolic pathway maps (Tohge et al. 2005).

10.5 Conclusion and Perspectives

Biological information management and computer biology are becoming more diffuse and other categories will no doubt surface in the future as this field matures. In our society, our economy and our global environment, plant life plays important and diverse roles. For modern plant biotechnology, feeding the growing world population is a challenge. Crop yields have increased during the last century and will continue to improve as agronomy re-assorting the enhanced breeding and develop new biotechnological-engineered strategies. The onset of genomics is providing massive information to improve crop phenotypes. Accumulating sequence data enables detailed genome analysis through the use of friendly access to

database and retrieval of information. Genetic and molecular genome co linearity allows efficient transfer of data revealing extensive conservation of genome organization between species. Genome research's goals are to identify sequenced genes and deduct their functions through metabolic analysis and reverse genetic screens from gene knockouts.

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