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We dedicate this book to the memory of Ara Kirakosyan' parents, Anna and Benik Kirakosyan, and to the memory of Peter B. Kaufman's wife, Hazel Kaufman.

Preface

Plant biotechnology applies to three major areas of plants and their uses: (1) control of plant growth and development; (2) protection of plants against biotic and abiotic stresses; and (3) expansion of ways by which specialty foods, biochemicals, and pharmaceuticals are produced. The topic of *recent advances in plant biotechnology* is ripe for consideration because of the rapid developments in this field that have revolutionized our concepts of sustainable food production, cost-effective alternative energy strategies, environmental bioremediation, and production of plant-derived medicines through plant cell biotechnology. Many of the more traditional approaches to plant biotechnology are woefully out of date and even obsolete. Fresh approaches are therefore required. To this end, we have brought together a group of contributors who address the most recent advances in plant biotechnology and what they mean for human progress, and hopefully, a more sustainable future.

Achievements today in plant biotechnology have already surpassed all previous expectations. These are based on promising accomplishments in the last several decades and the fact that plant biotechnology has emerged as an exciting area of research by creating unprecedented opportunities for the manipulation of biological systems. In connection with its recent advances, plant biotechnology now allows for the transfer of a greater variety of genetic information in a more precise, controlled manner. The potential for improving plant productivity and its proper use in agriculture relies largely on newly developed DNA biotechnology and molecular markers. A number of methods have been developed and validated in association with the use of genetically transferred cultures in order to understand the genetics of specific plant traits. Such relevant methods can be used to determine the markers that are retained in genetically manipulated organisms and to determine the elimination of marker genes. As a result, a number of transgenic plants have been developed with beneficial characteristics and significant long-term potential to contribute both to biotechnology and to fundamental studies. These techniques enable the selection of successful genotypes, better isolation and cloning of favorable traits, and the creation of transgenic organisms of importance to agriculture and industry.

We start the book by tracing the roots of plant biotechnology from the basic sciences to current applications in the biological and agricultural sciences, industry, and medicine. These widespread applications signal the fact that plant biotechnology is increasingly gaining in importance. This is because it impinges on so

many facets of our lives, particularly in connection with global warming, alternative energy initiatives, food production, and medicine. Our book would not be complete unless we also addressed the fact that some aspects of plant biotechnology may have some risks. These are covered in the last section.

The individual chapters of the book are organized according to the following format: chapter title and contributors, abstract, introduction to the chapter, chapter topics and text, and references cited for further reading. This format is designed in order to help the reader to grasp and understand the inherent complexity of plant biotechnology better.

The topics covered in this book will be of interest to plant biologists, biochemists, molecular biologists, pharmacologists, and pharmacists; agronomists, plant breeders, and geneticists; ethnobotanists, ecologists, and conservationists; medical practitioners and nutritionists; and research investigators in industry, federal labs, and universities.

Ann Arbor, MI — Peter B. Kaufman
Ann Arbor, MI — Ara Kirakosyan

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About the Authors

Ara Kirakosyan, Ph.D., D.Sc. is an associate professor of biology at Yerevan State University, Armenia, and is currently a research investigator at the University of Michigan Medical School and University of Michigan Integrative Medicine Program (MIM). He received a Ph.D. in molecular biology in 1993 and Doctor of Science degree in biochemistry and biotechnology in 2007, both from Yerevan State University, Armenia. Dr. Kirakosyan's research on natural products of medicinal value in plants focuses on the molecular mechanism of secondary metabolite biosynthesis in selected medicinal plant models. His primary research interests focus on the uses of plant cell biotechnology to produce enhanced levels of medicinally important, value-added secondary metabolites in intact plants, and plant cell cultures. These studies involve metabolic engineering coupled with integration of functional genomics, metabolomics, transcriptomics, and large-scale biochemistry. He carried out postdoctoral research in the Department of Pharmacognosy at Gifu Pharmaceutical University, Gifu, Japan, under the supervision of Prof. Kenichiro Inoue. The primary research topic was molecular biology of biosynthesis of several secondary metabolites in plants, particularly this was applied to the sweet triterpene glycyrrhizin in cell cultures of *Glycyrrhiza glabra* and dianthrones in *Hypericum perforatum.* In addition, he took part in several visiting research investigator positions in Germany. First, he was a visiting scientist under collaborative grant project DLR in Heinrich-Heine-University, Düsseldorf (project leader Prof. Dr. W.A. Alfermann). The research here concerned a lignan anticancer project, i.e., the production of cytotoxic lignans from Linum (flax). The second involved a carbohydrate-engineering project as a DAAD Fellow in the Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, under supervision of Prof. Dr. Uwe Sonnewald. His collaboration with US scientists started with the USDA-founded project on plant cell biotechnology for the production of dianthrones in cell/shoot cultures of *H. perforatum* (St. John's wort). This research has been carried out with Dr. Donna Gibson at USDA Agricultural Research Service, Plant Protection Research Unit, US Plant, Soil, and Nutrition Laboratory, Ithaca, New York, USA. In 2002, he was a Fulbright Visiting Research Fellow at the University of Michigan, Department of Molecular, Cellular, and Developmental Biology in the Laboratory of Prof. Peter B. Kaufman. Dr. Kirakosyan is principal author of over 50 peer-reviewed research papers in professional journals and several chapters in

books dealing with plant biotechnology and molecular biology. He is second author of the best-selling book, *Natural Products from Plants*, 2nd edition (2006). Ara Kirakosyan is a full member of the Phytochemical Society of Europe and European Federation of Biotechnology. He serves as an editorial board member in the *Open Bioactive Compounds Journal*, Bentham Science Publishers, and as an editor as part of the editorial board of 19 scientific domains journals, Global Science Books (GSB), Isleworth, UK. He has received several awards, fellowships, and research grants from the United States, Japan, and the European Union.

Peter B. Kaufman, Ph.D., is a professor of biology emeritus in the Department of Molecular, Cellular, and Developmental Biology (MCDB) at the University of Michigan and is currently senior scientist, University of Michigan Integrative Medicine Program (UMIM). He received his B.Sc. in plant science from Cornell University in Ithaca, New York, in 1949 and his Ph.D. in plant biology from the University of California, Davis, in 1954 under the direction of Prof. Katherine Esau. He did post-doctoral research as a Muelhaupt Fellow at Ohio State University, Columbus, Ohio. He has been a visiting research scholar at University of Calgary, Alberta, Canada; University of Saskatoon, Saskatoon, Canada; University of Colorado, Boulder, Colorado; Purdue University, West Lafayette, Indiana; USDA Plant Hormone Laboratory, BARC-West, Beltsville, Maryland; Nagoya University, Nagoya, Japan; Lund University, Lund, Sweden; International Rice Research Institute (IRRI) at Los Banos, Philippines; and Hawaiian Sugar Cane Planters' Association, Aiea Heights, Hawaii. Dr. Kaufman is a fellow of the American Association for the Advancement of Science and received the Distinguished Service Award from the American Society for Gravitational and Space Biology (ASGSB) in 1995. He served on the editorial board of *Plant Physiology* for 10 years and is the author of more than 220 research papers. He has published eight professional books to date and taught popular courses on plants, people, and the environment, plant biotechnology, and practical botany at the University of Michigan. He has received research grants from the National Science Foundation (NSF), the National Aeronautics and Space Administration (NASA), the US Department of Agriculture (USDA) BARD Program with Israel, National Institutes of Health (NIH), Xylomed Research, Inc, and Pfizer Pharmaceutical Research. He produced with help of Alfred Slote and Marcia Jablonski a 20-part TV series entitled, "House Botanist." He was past chairman of the Michigan Natural Areas Council (MNAC), past president of the Michigan Botanical Club (MBC), and former secretary-treasurer of the American Society for Gravitational and Space Biology (ASGSB). He is currently doing research on natural products of medicinal value in plants in the University of Michigan Medical School in the laboratory of Steven F. Bolling, M.D. and serves on the research staff of UMIM.

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Part I
Plant Biotechnology from Inception to the Present

Chapter 1
Overview of Plant Biotechnology from Its Early Roots to the Present

Ara Kirakosyan, Peter B. Kaufman, and Leland J. Cseke

Abstract In this chapter, we first define what is meant by plant biotechnology. We then trace the history from its earliest beginnings rooted in *traditional plant biotechnology*, followed by *classical plant biotechnology,* and, currently, *modern plant biotechnology*. Plant biotechnology is now center stage in the fields of alternative energy involving biogas production, bioremediation that cleans up polluted land sites, integrative medicine that involves the use of natural products for treatment of human diseases, sustainable agriculture that involves practices of organic farming, and genetic engineering of crop plants that are more productive and effective in dealing with biotic and abiotic stresses. The primary toolbox of biotechnology utilizes the latest methods of molecular biology, including genomics, proteomics, metabolomics, and systems biology. It aims to develop economically feasible production of specifically designed plants that are grown in a safe environment and brought forth for agricultural, medical, and industrial applications.

1.1 What Is Plant Biotechnology All About?

Today, when science and technology are progressing at ever increasing speeds and humankind is experiencing both positive and negative feedback from this progress, the presentation of an overview of modern plant biotechnology concepts is highly germane. Inherently, plant biotechnology, along with animal biotechnology, pharmaceutical biotechnology, and nanotechnology, constitutes a part of what we term *biotechnology*. An unprecedented series of successes in plant science, chemistry, and molecular biology has occurred and shifted plant biotechnology to new directions. This means that the newer aspects of plant biotechnology seen today are vastly different from our understanding of what constitutes the earlier, more traditional aspects of this field. The earlier ventures in biotechnology (*traditional biotechnology*) were concerned with all types of cell cultures, as they were sources of important products used by humans. These ventures included the making of beer

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and wine, the making of bread, cheese, yogurt, and other milk products, as well as the production of antibiotics, pharmaceuticals, and vaccines.

What has radically changed since these earlier discoveries in plant biotechnology? With the advent of recombinant DNA technology and new approaches that utilize genomics, metabolomics, proteomics, and systems biology strategies (Cseke et al., 2006), it may now be possible to re-examine plant cell cultures as a reasonable candidate for commercial production of high-value plant metabolites. This is especially true if natural resources are limited, *de novo* chemical synthesis is too complex or unfeasible, or agricultural production of the plant is not possible to carry out year-round. Indeed, a study of the biochemistry of plant natural products has many practical applications. Thus, specific processes have now been designed to meet the requirements of plant cell cultures in bioreactors. In addition, plant cells constitute an effective system for the biotransformation involving the addition of various substrates to the culture media in order to induce the formation of new products. The specific enzymes participating in such biotransformation processes can furthermore be isolated and characterized from cells immobilized on various solid support matrices, such as fiber-reinforced biocers (e.g., aqueous silica nanosols and commercial alumina fibers) that are now used in bioreactors.

Modern plant biotechnology research uses a number of different approaches that include high-throughput methodologies for functional analyses at the level of genes, proteins, and metabolites. Other methods are designed for genome modification through homologous and site-specific recombination. The potential for including plant productivity or agricultural trials is directly dependent upon the use of the new molecular markers or DNA construct technology. Therefore, plant biotechnology now allows for the transfer of an incredible amount of useful genetic information in a much more highly controlled and targeted manner. This is especially important for the use of *GM* (*genetically modified*) organisms, in spite of risks and limitations that have been voiced by individuals and organizations not in favor of this technology. It is noteworthy that a number of transgenic plants are being developed for long-term potential use in fundamental plant science studies (Sonnewald, 2003). Some of these transgenic plants also have significant and beneficial characteristics that allow for their safe use in industry and agriculture. Biotechnological approaches can selectively increase the amounts of naturally produced pesticides and defense compounds in crop plants and thus reduce the need for costly and highly toxic pesticides. This applies also to nutritionally important constituents in crops. The new techniques from the gene and metabolic engineering toolbox will bring forth many viable strategies to produce phytochemicals of medicinal and industrial uses.

Plant biotechnology research is, by nature, multidisciplinary. Systematic botany and organic chemistry, for example, aim to elucidate the systematic position and the evolutionary differentiation of many plant families. For instance, accurate and simple determination of chemotaxonomy can be attributed to the science of describing plants by their chemical nature. This interdisciplinary scientific field combines molecular phylogenetic analysis with metabolic profiling. Furthermore, it helps to investigate the molecular phylogeny and taxonomy of plants and to investigate the structural diversity of unique secondary metabolites found only in endemic species.

Besides the evaluation of some compounds as chemotaxonomic markers, one can also focus on the structural elucidation of these unique secondary metabolites, applying modern techniques of analysis.

We then come to the conclusion as to what plant biotechnology is all about: it aims to impart an understanding of plant metabolism and how plant metabo-

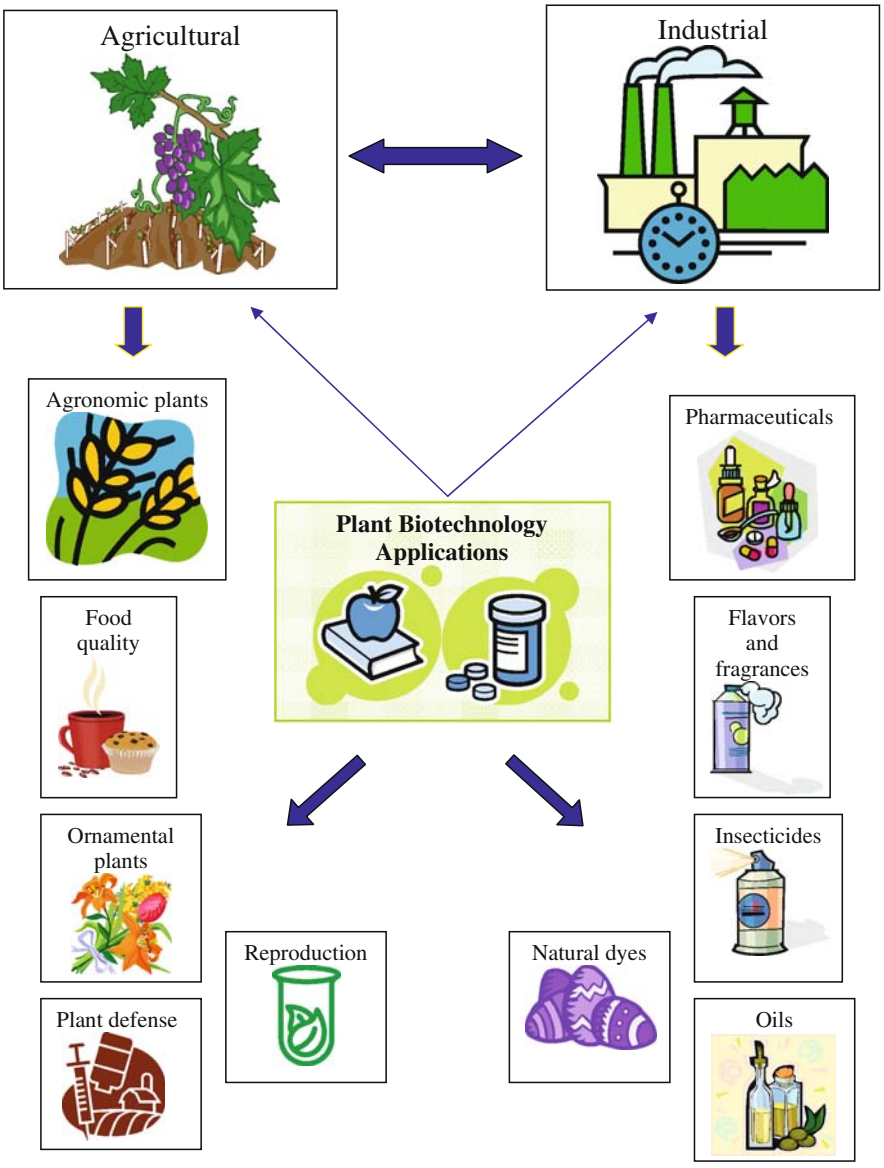

Fig. 1.1 A schematic representation of plant biotechnology applications

lite biosynthesis is regulated by particular enzymes, transcription factors, substrate availability and end-products and to apply this understanding to the economically feasible production of specifically designed plants that are grown in a safe environment and brought forth for agricultural, medical, and industrial applications (Fig. 1.1).

1.2 Tracing the Evolution of Classical Plant Biotechnology

Early in the twentieth century, plant cell culture was introduced (White, 1943, 1963). It had applications in plant pathology (Braun, 1974), plant morphogenesis, plant micropropagation, cytogenetics, and plant breeding. Then, protoplast culture was discovered (Cocking, 1960). It had applications in studies on cell wall biosynthesis, somatic cell hybridization, and genome manipulation (Power et al., 1970). Parallel studies led to the discovery that the ratio of auxin and cytokinin type hormones in tissue culture media largely determined whether one obtained shoots, roots, or undifferentiated callus tissue using tobacco (*Nicotiana tabacum*) as the model system (Miller and Skoog, 1953; Murashige and Skoog, 1962). These three discoveries in the plant sciences became the cornerstones of classical plant biotechnology.

The earliest roots of classical plant biotechnology emanate from studies by agronomists, horticulturists, plant breeders, plant physiologists, biochemists, entomologists, plant pathologists, botanists, and pharmacists. Their primary aim has been to solve practical problems associated with (1) the use of classical methods of plant breeding to develop new cultivars of plants that are resistant to plant pathogens, insect pests, and environmental stresses due to cold, drought, or flooding; (2) field-crop yield improvement, especially as related to the development of green revolution crop plants and of faster growing, higher yielding forest trees; (3) improvements in the postharvest storage and handling of crops; (4) the use of plant hormones to improve rooting responses of cuttings, enhancement of seed germination, breaking seed dormancy, prolongation of seed viability, and improvements in seed storage technology; (5) the employment of plant propagation (e.g., micropropagation via cell and tissue culture, grafting of new cultivars of plants); and (6) the use of plant natural products for human needs. These problems have been resolved successfully, primarily due to achievements in plant biology and crop science research. In connection with point (6) above, these earlier studies focused mainly on a description of the different kinds of natural products produced by plants. The pursuit of this direction became more popular in the past decades because many of the chemically synthesized constituents showed adverse effects on human health. Furthermore, for some constituents, chemical synthesis is either impossible or a very complicated and costly process.

Collectively, plants make a vast array of small-molecular-weight compounds. Most of these natural products are generally not essential for the basic metabolic processes of the plant but are often critical to the proper functioning of the plant in relation to its environment. With at least 100,000 so far identified, the total number of such compounds in the plant kingdom is estimated to be much higher. Plants are

capable of producing a variety of pharmaceuticals, adhesives, and compounds used for cosmetics and food preparation. Scientists working in this field have already discovered impressive amounts of potentially useful constituents with antibiotic, anti-inflammatory, antiviral, anticancer, cardiovascular, and other activities.

Natural products are believed to play vital roles in the physiology and ecology of plants that produce them, particularly as defense elements against pests and pathogens, or as attractants for beneficial organisms such as insect pollinators (Cseke et al., 2006). Most metabolites produced never leave the plant, but occasionally plant compounds, some of which attract and some of which repel, are the basis for a complex type of communication between plants and animals. Because of their biological activities, some plant natural products have long been exploited by human beings as pharmaceuticals, stimulants, and poisons. Therefore, there is an immense interest in isolating, characterizing, and utilizing these metabolites. While plant natural products hold a great deal of potential use for many human ailments, they are often made in only trace amounts within the specific plant species that produce them. Furthermore, the biosynthesis of the various metabolites proceeds along metabolic pathways that are highly complicated and located in one or more cell compartment(s) (e.g., cell walls, membrane systems, the cytosol, and various cellular organelles) within tissues that are often specialized for particular tasks. The specific enzymes that catalyze the respective steps in each metabolic pathway are encoded in nuclear, chloroplast, or mitochondrial genomes by specific genes.

Plant scientists enthusiastically endorsed the idea that plant cell and protoplast culture would eventually lead to the production of natural products using in vitro plant cell suspension cultures in bioreactors, similar to those produced by microbial and fungal cells cultivated in bioreactors. However, this expectation, in large part, failed to materialize, even in spite of ingenious strategies that were developed (Zenk et al., 1977). Only a few compounds were able to be successfully produced in plant cell cultures scaled-up in bioreactors for industrial applications (Verpoorte et al., 1994; Cseke et al., 2006). The main limitations were attributed to relatively slow growth rates of plant cells in shaker or bioreactor cultures, low rates of synthesis of desired products, and synthesis of compounds not present in intact plants. In fact, it was discovered in the course of these studies that biosynthesis of many types of plant metabolites occurs only in organized shoots or roots, but not in cell cultures *per se*. Thus, *in vitro* shoot or root cultures became an alternative strategy for the production of desired metabolites (Kirakosyan et al., 2004).

Many scientists have now combined extensive research experience using plant cell cultures in order to develop the best strategies for biotechnological application. This is enabling us to follow-up in greater detail points of interest, both theoretical and practical. Consequently, the development of an information base on a cellular and molecular level has been considered as a cornerstone of plant cell biotechnology. Using established cell cultures, it is now possible to define the rate-limiting step in biosynthesis by determining accumulation of presumed intermediates, characterizing the limiting enzyme activity, and probably relating it to the corresponding gene for eventual genetic manipulation. Generally, this approach works for known pathways. Therefore, step-by-step identification of all enzymatic activities that are

specifically involved in the pathway is more appropriate and has been carried out successfully. It is also quite common that blockage of one pathway leads to diversion of the substrate to alternative pathways. This would make it very difficult to identify the rate-limiting step in synthesis of a particular metabolite. It may also be that the pathway is subject to developmentally controlled flux at entry, as for example, through the activity of transcription factors. This kind of research must, therefore, focus on metabolic regulation by first establishing the pathways at the level of intermediates and enzymes that catalyze their formation. The subsequent step is the selection of targets for further studies at the level of the genes. This knowledge is also of interest in connection with studies on the role of secondary metabolism for plants and may contribute to a better understanding of resistance of plants to diseases and various herbivores. In addition, cell suspension cultures are used for biotransformation of added substrates, in order to search for new compounds not present in the intact plant, and finally to use plant cells for the isolation of enzymes that are responsible for the important metabolic pathways and to use them in chemical synthesis of natural products (reviewed by Alfermann and Petersen, 1995). Such complex studies that are based on molecular regulation of metabolite biosynthesis and on the creation of a systems biology type of information base may eventually lead to transgenic plants or plant cell cultures with improved productivity of the desired compounds (Fig. 1.2). Plant cell culture may therefore be a reasonable candidate for commercial realization if the natural resources are limited, *de novo* synthesis is complex, and the product has a high commercial value.

The biochemical capability of cultivated plant cells to transform exogenously supplied compounds offers a broad potential and can make an interesting contribution toward the modification of natural and synthetic chemicals as well. This attribute of plant cells is designated as *in vivo enzymatic bioconversion*. In many cases, the enzymes involved in this process can be identified, purified, and immobilized, and this accomplished by what is termed *in vitro bioconversion*. Then, the enzymatic potential of the plants can be employed for bioconversion purposes. The bioconversion process thus involves enzyme-catalyzed modification of added precursors into more desired or valuable products, using plant cells or specific enzymes isolated from plants. This type of metabolite modification is particularly accurate and is not so labor intensive. The biocatalyst may be free in solution, immobilized on a solid support, or entrapped in a matrix. Systems applied for bioconversion can consist of freely suspended cells, where precursors are supplied directly to cultures; immobilized plant cells, which are useful especially for secondary metabolite production but still need development to elicit an increase in the half-life of the cells; and finally enzyme preparation and further usage, which take into account problems connected with enzyme stability and sufficiency. In bioconversions elicited by whole cells or extracts, a single or several enzymes may be required for an action to occur.

In the same context, as described above, two biocatalytic systems can be employed in biotechnology. First, the catalysis of specific foreign substances, either chemically prepared or isolated from nature, can be carried out by enzymatic conversion outside the organisms. Second, bioconversion of a particular product uses

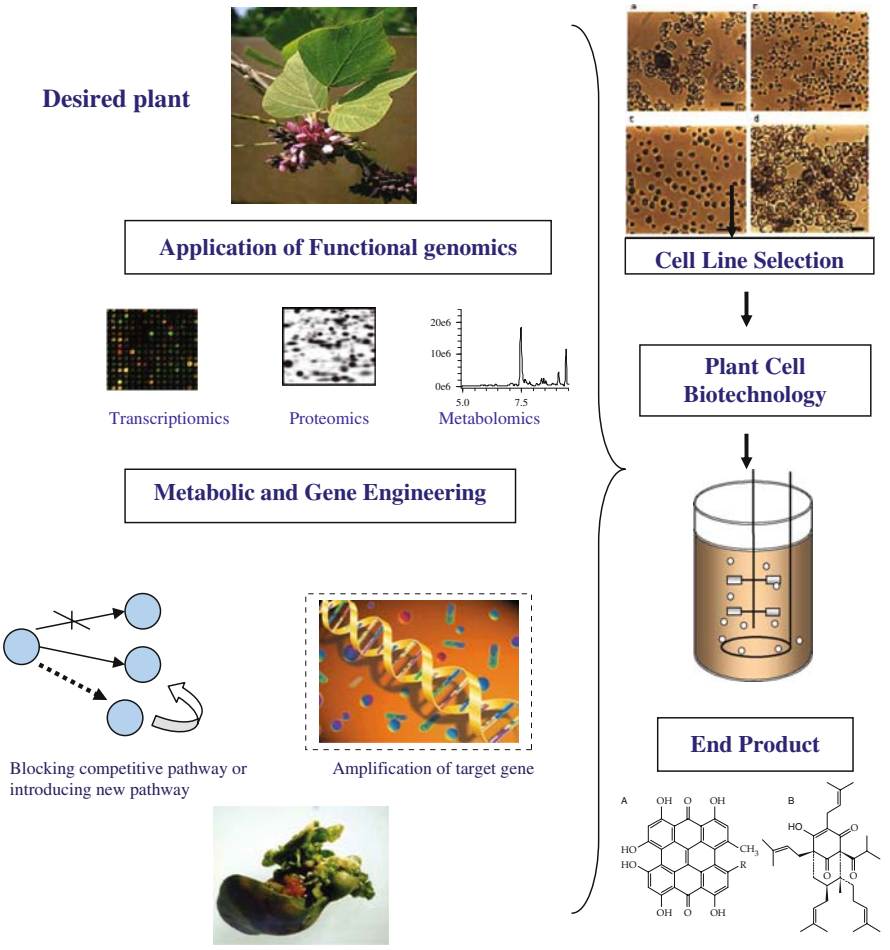

Fig. 1.2 Plant cell biotechnology for the production of high-value metabolites. The general steps presented involve the creation of an information base with the application of functional genomics, genetic and metabolic engineering of plant cells, and cultivation of modified plant cell lines in bioreactors for high-value secondary metabolite production

either plant cell cultures or whole plants. Improved metabolite production can be achieved by the addition of precursors to the culture medium. The advantages here are that the pharmaceutical, agricultural, and speciality chemical industries are increasingly requiring molecules that have distinct left- or right-handed forms, so-called chiral compounds. For example, the production of single left- or right-handed forms is not easy, and it is apparent that no single approach is likely to dominate. Scientists must continue to draw upon the entire range of chemical, enzymatic, and whole-organism tools that are available to produce chiral compounds. Despite some duplication in activity amongst enzymes, there is a need to characterize more of them in order to exploit their unique specificity and activity. In this

regard, plant enzymes are able to catalyze regio- and stereo-specific reactions and therefore can be used for the production of desired substances. *Stereospecificity* concerns high optical purity (100% of one stereoisomeric form) of biologically active molecules being catalyzed by plant enzymes. *Regiospecificity* allows for more precise conversion of one or more specific functional groups into others or, in the case of precursor molecules, selective introduction of functional groups on nonactivated positions.

In studies with the above-described plant cell cultures and their applications, we must, however, emphasize that not all aspects are clear and well-studied. Fundamental and practical researches are ongoing because problems related to monitoring the production of secondary metabolites in cell cultures still exist.

1.3 Modern Plant Biotechnology

Present-day studies are progressing in several different directions. It is noteworthy that each new plant gene, protein, or metabolite discovery may proffer several applications for agricultural, food, or pharmaceutical industries. These studies not only focus on the above topics but also utilize (1) genetically modified organisms (GMOs), (2) molecular farming techniques, (3) sustainable agriculture strategies, (4) production of green energy crops, (5) development of biological control strategies that can replace or reduce the use of toxic pesticides via integrated pest management schemes, (6) development of life-support systems in space, and (7) development of plant-derived products for use in medicine. These are topics that constitute the basis for recent advances in plant biotechnology. The current state of plant biotechnology research, using a number of different approaches, includes high-throughput methodologies for functional analysis at the levels of transcripts, proteins, and metabolites and methods for genome modification by both homologous and site-specific recombination.

Genetic and metabolic engineering are playing a substantial role in the development of agricultural biotechnology. This sector is therefore starting to move forward successfully, especially in the last several decades. The production and growth of improved cereals, vegetables, and fruits have been priority initiatives for agricultural biotechnology. Significant contributions have been made by plant biotechnologists to develop new crops involving the tools of gene and metabolic engineering. For example, scientists have been working on tomatoes that can be vine-ripened and shipped without bruising. Others have been trying to improve tomatoes that are processed for catsup, soups, pastes, or sauces by genetically engineering them to contain more solids, be thicker, and to contain more lycopene, β-carotene, and flavonoids, which provide the red color and medicinal value (Rein et al., 2006); see also Chapter 12 by Ilan Levin. The production of improved or "value-added" tomatoes, however, requires a long-term program involving multiple efforts. It is worth pointing out here that earlier, traditional plant breeding was also able to accomplish much of this improvement in tomato "germplasm." A good example is heirloom tomatoes, which have been passed down for generations.

The priorities are given for processing tomatoes with improved viscosity (thickness and texture, meaning fewer tomatoes for the same amount of catsup), higher soluble solids, better taste, improved color, and higher vitamin content. It also may include enhancing overall flavor, sweetness, color, and health attributes. Calgene was the first company to introduce a genetically improved tomato that ripens on the vine without softening and has improved taste and texture. Here, *antisense gene technology* was introduced to inhibit the polygalacturonase enzyme, which degrades pectin in the cell wall. The classical example here is the first genetically engineered slow-ripening tomato plant. It was commercially developed by Calgene Corp. in Davis, CA, and was called "FlavR Saver." This tomato has two distinct advantages over other tomato cultivars: first, it has a longer shelf life in storage, and second, the fruit of this tomato could be left on the plant until optimally ripe. Because of these attributes, FlavR Saver tomatoes are sold for premium prices.

Another successful marketing initiative was concerned with oilseed crops. Canola-producing laurate is the world's first oilseed crop that has been genetically engineered to modify oil composition. Similarly, Calgene isolated the gene responsible for laurate production from the California laurel (*Umbellularia californica*) tree. This gene was then engineered into canola (*Brassica napus* and *B. rapa*), resulting in the production of oil containing approximately 40% laurate – a fatty acid that is found in the seed oils of only a few plant species, mostly coconut and palm kernel oil from tropical regions. Laurate possesses unique properties, which make it desirable in edible and industrial products. Lauric oil is ideal for use in the soap and detergent industries, as it is responsible for the cleansing and sudsing properties of shampoos, soaps, and detergents.

Other examples of transgenic agricultural crops include many plants, such as potatoes with more starch and less water to prevent damage when they are mechanically harvested, crops with low saturated oils, sweet mini-peppers, modified lignin in paper pulp trees, pesticide-resistant plants, and frost-resistant fruits.

One of the important directions in plant biotechnology is the introduction of genetically engineered organisms (GMOs) to the market. This is based on a desire by consumers for more tasty and more healthy foods. It is also based on a preference for products grown without using pesticides or other soil additives. However, the choice of companies to keep the public ignorant of these genetic changes led to a great scare in the public once people found out what was going on. It would have been better if companies had informed the public prior to releasing any GMOs. As a consequence of these events, the regulatory requirements and safety assessment studies are far greater, not only in the United States but also worldwide.

An improvement in the quality or the composition of animal products has also been achieved through modern plant biotechnology. This has resulted in increased feed utilization and growth rate, improved carcass composition, improved milk production and/or composition, and increased disease resistance.

Modern plant biotechnology is also playing a role in "clean" manufacturing. Nevertheless, various types of chemical manufacturing, metal plating, wood preserving, and petroleum refining industries currently generate hazardous wastes, comprising volatile organics, chlorinated and petroleum hydrocarbons, solvents, and heavy met-

als. No one single plant species can handle all contaminants in any given environment. Rather, unique species have been found that can deal with a single or a few contaminants in a particular medium. For example, plants have been found that can break down or degrade organic contaminants (similar to microbes), while others are able to extract and stabilize toxic metal contaminants by acting as traps or filters. The ramifications of these phenomena for environmental cleanup (i.e., *phytoremediation*) were quickly realized. In theory, by simply growing a crop of the appropriate plant at a given polluted site, the contaminant concentration could be lowered to environmentally acceptable levels. This may involve several rotations of the plants, and indeed, it may even be possible to use a combination of plants (and microbes, too) to treat sites polluted with both heavy metals and organics. Chapter 7 discusses these several aspects of phytoremediation in detail.

These and other advances in plant biotechnology not only allow us to gain knowledge to answer fundamental questions in plant science but also make it possible for us to create new applications in response to threats of global warming, evolution of new resistant pests, development of new crop and forest species/cultivars and their products, and changes in market/consumer demands and needs.

For human health benefits, new technologies are required to introduce more naturally produced pharmaceuticals and vaccines. These may be possible if all aspects of plant natural product chemistry, including the biosynthetic pathways and possible biotransformation reactions, are included. This is true also for health issues where in-depth knowledge of molecular immunology, pharmacology, or related disciplines is required. Thus, plant biotechnology has a huge contribution to make for the world economy, largely through the introduction of DNA or RNA technologies to the production of biopharmaceuticals.

In summary, plant biotechnology concentrates much attention on the complexity and interrelatedness of plant biology, with such targets as agricultural and pharmaceutical biotechnology. Needless to say, and subject to clarification of certain ethical and public acceptance issues, plant biotechnology is also set to make an indelible contribution to human health and welfare well into the foreseeable future.

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Chapter 2
The Use of Plant Cell Biotechnology for the Production of Phytochemicals

Ara Kirakosyan, Leland J. Cseke, and Peter B. Kaufman

Abstract In this chapter, we bring together up-to-date information concerning plant cell biotechnology and its applications. Because plants contain many valuable secondary metabolites that are useful as drug sources (pharmaceuticals), natural fungicides and insecticides (agrochemicals), natural food flavorings and coloring agents (nutrition), and natural fragrances and oils (cosmetics), the production of these phytochemicals through plant cell factories is an alternative and concurrent approach to chemical synthesis. It also provides an alternative to extraction of these metabolites from overcollected plant species. While plant cell cultures provide a viable system for the production of these compounds in laboratories, its application in industry is still limited due to frequently low yields of the metabolites of interest or the feasibility of the bioprocess. A number of factors may contribute to the efficiency of plant cells to produce desired compounds. Genetic stability of cell lines, optimization of culture condition, tissue-diverse vs. tissue-specific site-specific localization and biosynthesis of metabolites, organelle targeting, and inducible vs. constitutive expression of specific genes should all be taken into consideration when designing a plant-based production system. The major aims for engineering secondary metabolism in plant cells are to increase the content of desired secondary compounds, to lower the levels of undesirable compounds, and to introduce novel compound production into specific plants. Recent achievements have also been made in altering various metabolic pathways by use of specific genes encoding biosynthetic enzymes or genes that encode regulatory proteins. Gene and metabolic engineering approaches are now being used to successfully achieve highest possible levels of value-added natural products in plant cell cultures. Applications through functional genomics and systems biology make plant cell biotechnology much more straightforward and more attractive than through previous, more traditional approaches.

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2.1 Plant Cell Factories as a Source of High-Value Metabolites

The presence of valuable metabolites in plants has stimulated interest on the part of industry in the fields of pharmaceuticals (as drug sources), agrochemicals (for the supply of natural fungicides and insecticides), nutrition (for the acquisition of natural substances used for flavoring and coloring foods), and cosmetics (natural fragrances and oils). The bulk of the market products, such as secondary metabolites from higher plants, are collected from plants growing in the wild or from field-cultivated sources. In using a plant strategy, major issues are that these plants need a seasonal period of growth before harvesting is possible. Other issues here include a relatively short growing seasons in temperate regions, disease and insect predation, and high costs for labor and machinery. On the other hand, total chemical synthesis of several compounds is possible, but economically not feasible. Therefore, an alternative, economically viable, and environmentally sustainable production source for desired secondary metabolites is of great interest. In this regard, plant cell cultures can be an attractive alternative as a production system, as well as a model system, to study the regulation of natural product biosynthesis in plants so as to ultimately increase yields.

The commercial-scale use of plant cell cultures is now progressing rapidly despite many drawbacks and limitations that scientists have acknowledged. Earlier, Verpoorte et al. (1994) had shown that biotechnological application of plant cell cultures on a large scale may become economically feasible. The limitation here, however, concerns the high price of the final product. This is mainly attributed to the slow growth of plant cell cultures, making the depreciation costs of the bioreactor a major cost-determining factor in future attempts (Verpoorte et al., 1994).

The detailed monitoring of functional status of cells is now routinely performed for plant cell cultures in order to permit accurate assessment of growth and metabolite production rates. The availability of plant cells for quantitative measurement parameters makes possible the accurate assessment of a culture's status and places the analysis of cell cultures on a par with the detailed monitoring that has been successfully applied for commercial microbial fermentations. The collected information may enable identification and clearer understanding of the biological and chemical constraints within the process, as well as optimization of cell culture production, planning, costs, and scheduling activities. All of these factors are now considered in relation to scale, geometry, and configuration of the bioreactor. In addition, in vitro plant cell cultures are currently carried out for a diverse range of bioreactor designs, ranging from batch, airlift, and stirred tank to perfusion and continuous flow systems. For a small scale of operation, both the conventional and the novel bioreactor designs are relatively easy to operate. In contrast, for a larger scale of operation, problems of maintaining bioreactor sterility and providing an adequate oxygen supply to the cells have yet to be resolved (Vogel and Tadaro, 1997).

While industrial applications of plant cell cultures are still in progress, recently, some promising advances have already been achieved for the production of several high-value secondary metabolites through cell cultivation in bioreactors. For example the valuable progress has been achieved for paclitaxel (Taxol), where yields have

improved more than 100-fold using multifactorial screening strategy (Roberts and Shuler, 1997). Such progress, however, is not universal and many trials with different cell cultures initially failed to produce high levels of the desired products. The failure to produce high levels of desired metabolites by cell factories is still due to our insufficient knowledge as to how plants regulate metabolite biosynthesis.

Earlier, Zenk and coworkers (1997) suggested a strategy to improve the production of secondary metabolites in cell cultures that is now being used by many researchers. This strategy includes the following general steps: (1) plant screening for secondary metabolite accumulation; (2) use of high producer plants for initiation of callus cultures; (3) biochemical analysis of derived cultures for their variability and productivity; (4) establishment of cell suspension cultures; (5) analysis of metabolite levels in cell suspension cultures; (6) selection of cell lines based on single cells; (7) analysis of culture stability; and (8) further improvement of product yields.

How does this strategy work and does it raise the bars of current modern plant biotechnology? Here, we will trace in detail the main points of such a strategy in order to show how these steps may work and what limitations may still occur when they are employed in modern plant biotechnology. As a part of such strategy, the primary effort has been devoted to the development of cultures from elite germplasm so as to take advantage of the wide range of biosynthetic capacities within cultures. This has been achieved either by selection or by screening germplasm for highly productive cell lines, as for example, in production of Taxol from *Taxus* cell cultures (Kim et al., 2005). On the other hand, several limiting factors can play crucial role for successful use of plant cell cultures in biotechnology. These limiting factors can include light intensity and quality; temperature; length of culture period, including kinetics of production; concentration and source of major limiting nutrients such as phosphate, carbon, and nitrogen; and concentration and source of micronutrients, vitamins, and plant growth regulators.

The other point concerns optimization of cell culture conditions. This has been carried out for a variety of media formulations and environmental conditions. The *Plackett and Burman* technique was particularly useful in these cases. It allows for testing of multiple variables within a single experiment (Plackett and Burman, 1946). This method relies on the following characteristics: (1) each variable is tested at a high level in half of the test cultures, or at a low level or not at all in the other half; (2) any two variables are tested in 25% of the test cultures; (3) both will be excluded in 25%; and (4) only one variable is tested in the remaining 50% of the test cultures. Since the production of secondary metabolites can be followed by HPLC or GC, a medium can be selected that supports good cell growth and production of secondary metabolites. The role of the cell cycle in plant secondary metabolite production must also be considered.

Screening of cell cultures for metabolite high productivity is carried out on several levels. In some cases, high-producing cell clones are obtained from single cells, and subsequently, these are used for screening high-producing strains. For rapid selection of high-producing cells, some simple techniques are applicable. A good example is flow cytometry, which may be useful. This technique is based on the fact

that cells contain fluorescent products (e.g., thiophenes), and therefore, it is possible to separate these (marked) cells from others. However, some problems may occur with cell line stability, especially as a result of cell differentiation or morphogenesis. Therefore, such stability problems of cell lines have probably made researchers reluctant to develop extensive screening programs, leaving this as the last step prior to an industrial application (Verpoorte, 1996). The fluorescent proteins from a wide variety of marine organisms have initiated a revolution in the study of cell behavior by providing convenient markers for gene expression and protein targeting in living cells and organisms. The most widely used of these fluorescent proteins, the *green fluorescent protein* (*GFP*), first isolated from the jellyfish *Aequorea victoria*, can be attached to virtually any protein of interest and still fold into a fluorescent molecule. Fluorescent proteins are increasingly being employed as noninvasive probes in living cells due to their ability to be genetically fused to proteins of interest for investigations of localization, transport, and dynamics. Martin Chalfie, Osamu Shimomura, and Roger Y. Tsien share the 2008 Nobel Prize in Chemistry for their discovery and development of molecular probe uses of the green fluorescent protein. To date, many plant cells, along with other organisms, have been selected using GFP as a marker for gene expression.

Alternatively, selection of high-producing cell lines by culturing cells on media containing certain additives, such as biosynthetic precursors or toxic analogues, also may be applied (Verpoorte, 1996). In this case, some instability of many precursors or toxic effects of some constituents on the cells is, however, possible. Therefore, it is not possible to use a universal screening platform for plant cell cultures. Instead, a specific screening for a particular plant cell culture must be employed in order to produce specifically desired metabolites.

Whether with plant cell cultures or with intact plants, the key to success in discovering naturally occurring phytochemicals rests on bioassay-guided fractionation and purification procedures. Generally, screening of both natural products and synthetic organic compounds has led to impressive advances in the identification of active agents. High-throughput screens and sensitive instrumentation for structural elucidation have greatly reduced the amount of time and the sample quantity that are required for analysis.

Still, the main criterion for future biotechnological success is connected to the biosynthetic capacity of cell factories. It is well known that the biosynthesis of plant secondary metabolites could be up- or downregulated by biotic and abiotic factors. In order to unravel the regulation of plant metabolism by such environmental stimuli, it is important to elucidate the factors that control the accumulation of secondary metabolites in plants. Therefore, nowadays, scientists are carrying out intensive research efforts to identify and apply limiting factors that can ultimately increase plant cells' biosynthetic capacities. With such research, attention has also been given to the accumulation and storage of desired secondary metabolites in plant cells. Secondary metabolites in plants, and perhaps in tissue cultures, are usually stored intracellularly, as for example, in vacuoles or multicellular cavities. Thus, transporters probably play an important role in the sequestration of secondary metabolites (Kunze et al., 2002).

Biotic factors are among the environmental factors that affect to a large extent the production of phytochemicals. Therefore, it is highly probable that there is a relationship with defensive responses that is manifested either in phytoalexin production or in the production of compounds produced along one of the signal transduction pathways. An approach to characterize the biotic parameters that may elicit the plant's defensive mechanisms may be revealed by an analysis of the expression of certain genes involved in the process and by correlation of gene induction with particular metabolite levels.

In addition to the strategy described above, new approaches based on genetic and metabolic engineering have been successfully introduced (Verpoorte and Alfermann, 2000). Consequently, the development of an information base on genetic, cellular, and molecular levels is now a prerequisite for the use of plants or plant cell cultures for biotechnological applications for the following reasons. First, a better understanding of the basic metabolic processes involved could provide key information needed to produce high-value metabolites. Second, many biosynthetic pathways in plants are extensive and complicated, requiring multiple enzymatic steps to produce the desired end-product. So, when engineering secondary metabolism in plant cells, the primary aim should be to increase the content of desired secondary compounds, to lower the levels of undesirable compounds, and finally to introduce novel compound production into specific plants. This kind of research must, therefore, focus on metabolic regulation by first establishing the pathways at the level of intermediates and enzymes that catalyze secondary metabolite formation (metabolic pathways profiling). The subsequent step is the selection of targets for further studies at the level of genes, enzymes, and compartments. Such studies on regulation of metabolite biosynthesis might eventually lead to the derivation of transgenic plants or plant cell cultures with an improved productivity of the desired compounds. Aside from practical applications with such organisms, the knowledge gained will be of interest in connection with studies on the adaptive/functional roles of secondary metabolism in plants. These are covered in the next section that deals with functional genomics.

2.2 Applications Through the Use of Functional Genomics

Interdisciplinary approaches that are based on molecular biology and biochemistry led to rapid advances in the identification of biosynthetic genes, the elucidation of specific biosynthetic enzymes, and the identification of end-products. The complete genetic makeup of an organism has been generated in the plant sciences as well. Because of the success of large-scale quantitative biology projects such as genome sequencing (*genomics*), the suffix "omics" has been extended to other directions. *Proteomics* is now well-established as a term that refers to a study of the proteome. More recently, *metabolomics* has been introduced, which is now leading to an incredible amount of research on all kinds of primary and secondary metabolites (Cseke et al., 2006). Thus, quantitative and qualitative measurements of all kinds of cellular metabolites, or metabolomics, yield a global view of the biochemical

phenotype or phytochemical database for a plant organism. This can be used to differentiate phenotypes and genotypes at a metabolite level that may or may not produce visible phenotypes. Due to the diversity of plant secondary metabolites, it is generally accepted that there is no single analytical method employed that can provide sufficient visualization of the entire metabolome. Multiple technologies are therefore needed to measure the entire metabolome of a given plant sample. Most metabolomic approaches seek to profile metabolites in a nontargeted way, i.e., to reliably separate and detect as many metabolites as possible in a single analysis. This is technically challenging due to the diverse chemical properties and large differences in the abundance of the metabolites. In contrast, selective profiling of a certain group of compounds, which is also called *targeted metabolic profiling*, is relatively easy to perform.

One of the major applications of genome sequencing of plants is functional genomics. In simple words, an understanding of the function of genes and other parts of the genome is known as functional genomics. It is a field of molecular biology that attempts to make use of the vast amount of data produced by genomic projects (such as genome sequencing projects) to describe gene (and protein) functions and their interactions. Unlike genomics and proteomics, functional genomics focuses on the dynamic aspects, such as gene transcription, translation, and protein–protein interactions, as opposed to the static aspects of the genomic information such as DNA sequences or structures (Cseke et al., 2006). It aims to determine the biological function of every gene within a given genome. Functional genomics, then, refers to the development and application of global (genome-wide or system-wide) experimental approaches to assess gene function by making use of the information and reagents provided by structural genomics. Functional genomics includes function-related aspects of the genome itself, such as mutation and polymorphism analysis, as well as measurement of molecular activities. Together, all measurement modalities quantify the various biological processes and powers in order to enhance our understanding of gene, protein, and metabolite functions and their interactions (Fig. 2.1).

Functional genomics uses mostly modern techniques to characterize the abundance of gene products such as mRNAs and proteins. It is characterized by high-throughput or large-scale experimental methodologies combined with statistical or computational analysis of the results. Some typical technology platforms are *DNA microarrays* and *SAGE* (serial analysis of gene expression) for mRNA analysis, two-dimensional gel electrophoresis and mass spectrometry (MS) for protein analysis, and targeting and nontargeting mass spectrometry and nuclear magnetic resonance (NMR) analysis in metabolomics. Because of the large quantity of data produced by these techniques and the desire to find biologically meaningful patterns, *bioinformatics* is used here for this type of analysis of complex systems. Bioinformatics refers to the extraction of biological information from genomic sequence and the reconciliation of multiple data sets based on DNA and RNA microarrays. In connection with the above, a *DNA microarray* (also called a DNA chip or gene chip) is a piece of glass or plastic on which pieces of DNA have been affixed in a microscopic array to screen a biological sample for the presence of many genetic

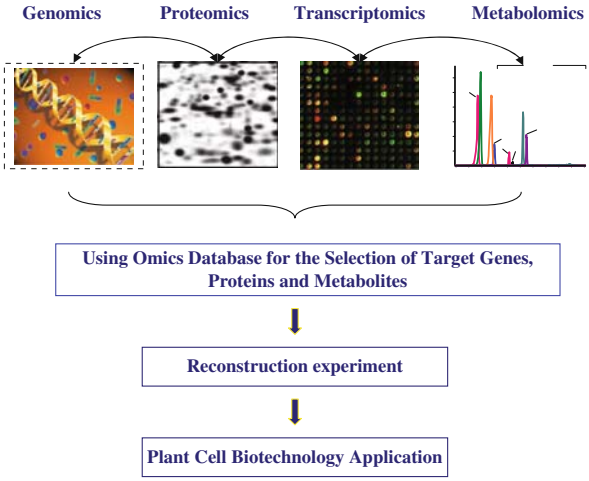

Fig. 2.1 Application of functional genomics tools in plant cell biotechnology

sequences simultaneously. The affixed DNA segments are known as *probes*. Thousands of identical probe molecules are affixed at each point in the array in order to make the chips effective detectors. Many microarrays use PCR products, genomic DNAs, BACs (bacterial chromosomes), plasmids, or longer oligos (35–70 bases) instead of short oligonucleotide probes of 25 bases or less. RNA microarrays are used to detect the presence of mRNAs that may have been transcribed from different genes and that encode different proteins. The RNA is converted to cDNA or cRNA. The copies may be amplified by RT-PCR (reverse transcriptase-polymerase chain reaction). Fluorescent tags are enzymatically incorporated into the newly synthesized strands or can be chemically attached to the new strands of DNA or RNA. A cDNA or cRNA molecule that contains a sequence complementary to one of the single-stranded probe sequences will hybridize, or stick, via base pairing (more so for DNA) to the spot at which the complementary probes are affixed. The spot will then fluoresce, or glow, when examined using a microarray scanner. The major components, then, of a functional genomics approach include *bioinformatics* (the global assessment of how the expression of all genes in the genome varies under changing conditions), *proteomics* (the study of the total protein complement expressed by a particular cell under particular conditions), and *reverse genetics* (deducing the function of novel genes by mutating them and studying mutant phenotypes).

Functional genomics, used as a means of assessing phenotypes, differs from more classical approaches, primarily with respect to the scale and automation of biological investigations. A classical investigation of gene expression might examine how the expression of a single gene varies with the development of an organism *in vivo*. Modern functional genomics approaches, however, can examine how 1,000–10,000 genes are expressed as a function of development.

The massive expansion of available genomic information in plants allows researchers to push the limits as to what can be produced by a chosen organism. Such technology continues to hold great promise for the future of plant

biotechnology. We now may simultaneously analyze the expression or silencing of thousands of genes in plants or in plant cell lines, screen for high- and low-producer lines of the desired phytochemical(s), or determine the full spectra of metabolites. With advances in proteomics, we should also be able to simultaneously quantify the levels of many individual proteins or to follow posttranslational alterations that occur. What are now needed are analogous analytical methods for cataloging the global effects of metabolic engineering on metabolites, enzyme activities, and metabolite fluxes.

So far as we are aware, many limitations or drawbacks occur when investigators try to engineer plant cells. The question here concerns: what cannot be genetically engineered? Our imagination creates thousands of possible applications for plant genetic engineering. It is easy to imagine, for example, that we will be able to derive coffee beans with less caffeine and with hazelnut aroma. Theoretically, that is possible. However, nothing can be successfully accomplished here without unraveling relevant gene expression phenomena, proteins with multifunctional tasks, or metabolic networks in particular plant organisms. Let us consider the fact that there are many identical genes in plants, animals, microorganisms, and even in humans. However, they all have so many differences in terms of their functions. For this reason, complex traits involving multiple functions are still impossible to genetically engineer without the use of a systems biology approach. The systems biology approach has four known steps in general. The first step consists of gathering various high-throughput data sets in addition to legacy data sets. All of these data are then used in the second step to reconstruct the biochemical reaction networks that underlie the cellular function of interest. When such data are put into the format of a biochemically, genetically, and genomically structured database, they have a mathematical format consistent with the underlying physicochemical processes. This mathematically structured database can then be mathematically interrogated (step 3). Constraint-based methods can be used to perform such interrogation at the genome- and network-scale levels. The mathematical computations are then used to perform new experiments. In plant cell biotechnology, extensive metabolic profiling must be more systematic and involve considerable analysis in this case. Due to the productivity issue we have mentioned previously, gene or metabolic engineering must be based on a systems biology approach involving integrated metabolomics, proteomics, and transcriptomics approaches (Carrari et al., 2003; Dixon, 2005). Likewise, metabolic engineering (see below) is a potentially very powerful tool in plant cell biotechnology for the regulation of secondary metabolism in transgenic plants or plant cell cultures, with potential to have wide applications in the phytochemical industry or in agriculture (Verpoorte and Alfermann, 2000).

2.3 Metabolic Engineering

Plant metabolic engineering treats the cell as a factory and adds or optimizes kinds and amounts of metabolites within the cell for some specific design purpose. In other words, metabolic engineering refers to a targeted metabolic pathway being

elucidated in plant or bacterial organisms with the purpose of unraveling and utilizing this pathway for future modification of a plant's end-products. It is generally defined as the redirection of enzymatic reactions so as to improve the production of high-value constituents, to produce new compounds in an organism, to mediate the degradation of environmentally toxic compounds, or to create plants that become resistant to environmental stress factors. In addition, metabolic engineering may include not only the manipulation of endogenous metabolic pathways but also the transfer of metabolic pathways into new host organisms.

The main goals of metabolic engineering in industry or agriculture are the stimulation of the production of secondary metabolite end-products, biosynthetic precursors, polymers that have plant origin, and the derivation of new plant organisms with high salt or drought resistance in agriculture. It is not surprising that metabolic engineering applications in plant biotechnology in recent years have had incredible achievements in agriculture, industry, and medicine.

This multidisciplinary field draws concepts and methodologies from molecular biology, biochemistry, and genetics, as well as biochemical engineering. In addition, the extension of metabolic engineering to produce desired compounds in plant organisms may answer many fundamental questions applied to plant development, physiology, and biochemistry. For example, plant metabolic flux analysis in the primary carbon-based metabolic pathways presents fundamental information on the application of plant metabolic engineering that is based on a thorough knowledge of plant biochemistry and plant physiology. Plant metabolism itself concerns thousands of interacting pathways and processes that are regulated by environmental and genetic stimuli. Therefore, engineering even known metabolic pathways will not always provide the expected results. Despite major advances in metabolic engineering, still only a few secondary metabolic pathways have been enzymatically characterized and the corresponding genes cloned. In this context, the biosynthetic pathways for alkaloids, flavonoids, and terpenoids are presently the best characterized ones at the enzyme and gene levels. More successful cases of gene discovery have also been considered for the lipid biosynthetic pathway, where most genes in plants encoding enzymes for fatty acid biosynthesis have been cloned. This information was applied for eventual manipulation through modification of many fatty acids in transgenic plants by means of metabolic engineering. As for targeting metabolite manipulation, DellaPenna advocated the conversion or chemical modification of an existing compound(s), rather than attempting to increase flux through a metabolic pathway. Another example, he cites, claims that modifications made in the end-products or secondary metabolic pathways have been generally more successful than in cases where manipulation of primary and/or intermediary metabolic pathways is attempted (DellaPenna, 2001). Recent achievements have been made in the altering of various pathways by use of specific genes encoding biosynthetic enzymes or genes encoding regulatory proteins (Verpoorte and Memelink, 2002; Maliga and Graham, 2004). Most current metabolic engineering studies have focused on manipulations of enzyme levels and the effect of amplification, addition, or blockage of a particular pathway. A new area is the manipulation of cofactors, which play a major role in plant biochemistry and physiology and in the fermentation process

of several end-products. Additionally cofactors are essential for many enzymatic reactions.

Metabolic engineering is becoming a powerful technology for the successful implementation of plant cell biotechnology in the future. This may be possible with the advances we already have mentioned above and some other important issues and key criteria that are cited as follows: (1) Metabolic flux analysis must be applied to well-documented and elucidated metabolic pathways; (2) extension of metabolic cross-talk between the desired metabolite pathway and other pathways for a possible direct impact on plant development and nutritional value must be considered; (3) identification of further elements in the complex regulatory network (such as transcription factors and their binding partners) needs to be examined; and (4) rigorous criteria must be developed for the assessment of the risk and benefit performance of engineered plants. Comprehensive studies in several directions may help to bring metabolic engineering out of the trial-and-error era and transform it into industrial applications.

Metabolic engineering approaches can be defined according to several different directions (Fig. 2.2). The first appropriate approach involves increases in the total carbon flux toward the desired secondary metabolite. In addition, decreasing the flux through competitive pathways is an alternative way to increase the biosynthesis of desired metabolite. Other possible directions involve the introduction of an *antisense gene* of the competing enzyme at the branch point, as well as overcoming *rate-limiting steps*, or blocking *competitive pathways*.

2.3.1 Increasing Total Carbon Flux Through Metabolic Pathways

Metabolic flux analysis determines the rate of carbon flow for each metabolic reaction in a biochemical pathway. A method to quantify flux through metabolite measurements is necessary for the analysis of original and modified pathways. Flux of carbon into a given metabolite pathway, diversion of metabolic flux at intermediate branches, and lack of final conversion at the end of a specific branch all may affect secondary metabolite production in plants. Therefore, it is important to identify points of possible flux limitation to be able to pursue pathway steps for genetic modification.

The biosynthesis of secondary metabolites in plants can be regulated by increasing the metabolic flux within cells through reconstruction experiments. In vivo, resource allocation is often accomplished by controlling the flux of branch point intermediates in metabolic networks. For example, Kleeb with coworkers used this approach to optimize an in vivo selection system for the conversion of prephenate to phenylpyruvate, a key step in the production of the essential aromatic amino acid phenylalanine (Kleeb et al., 2007). Careful control of prephenate concentration in a bacterial host lacking prephenate dehydratase, achieved through the provision of a regulable enzyme that diverts it down a parallel biosynthetic pathway, provides the means to systematically increase selection pressure on replacements of the missing catalyst. Successful differentiation of dehydratases, whose activities vary over

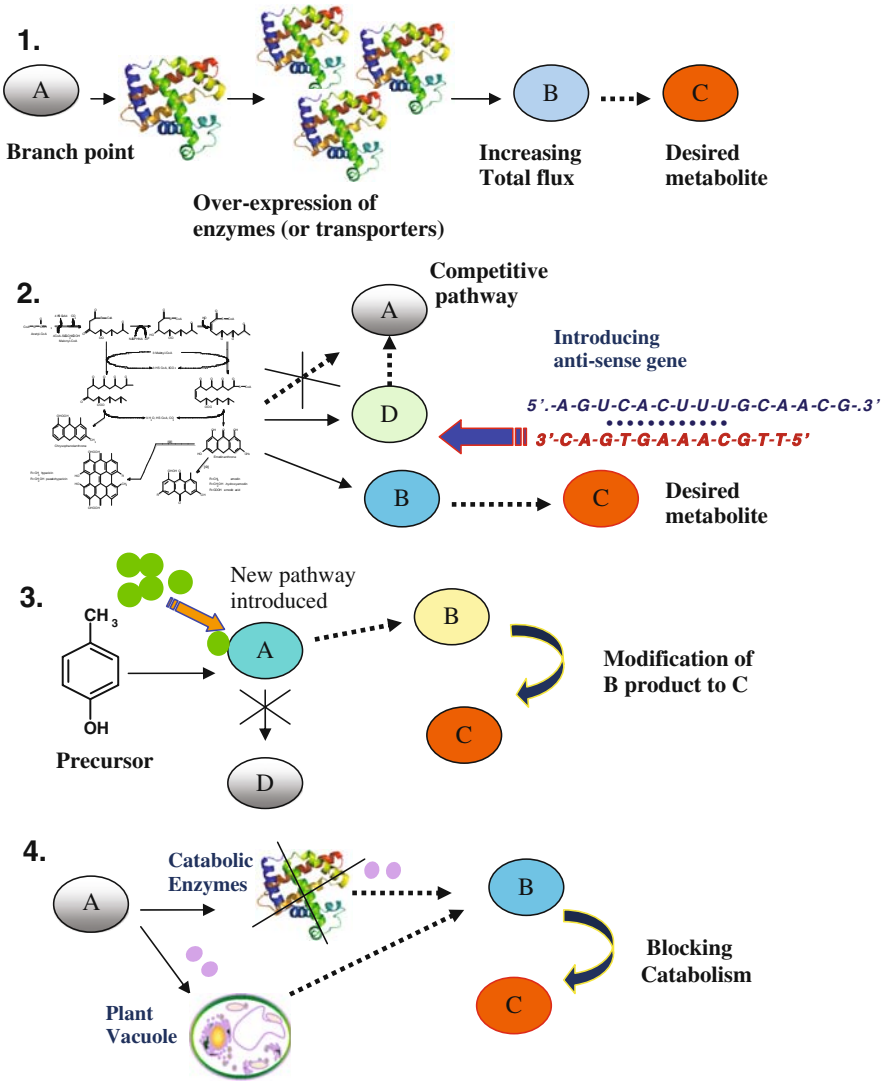

Fig. 2.2 Approaches in metabolic engineering: (1) increase in the total carbon flux at the branch point; (2) decrease in the flux through competitive pathways or introduction of an antisense gene of the competing enzyme; (3) regulation of desired metabolite yield either by competitive pathway determination and targeting of rate-limiting steps or by introduction of a new pathway; and (4) blocking catabolism either by increasing the transport of metabolites into the vacuole or by downregulation of catabolic enzymes

a >50,000-fold range, and the isolation of mechanistically informative prephenate dehydratase variants from large protein libraries illustrate the potential of the engineered selection strain for characterizing and evolving enzymes (Kleeb et al., 2007). This approach complements other common methods for adjusting selection pressure

and may be generally applicable to plant systems that are based on the conversion of an endogenous metabolite. There are several examples that have been reported for well-characterized rate-limiting enzymes of plants and their controversial role in the regulation of pathway flux (for review, see DellaPenna, 2001).

Analysis of a wide range of secondary metabolites has significant advantages as compared to a study of final product(s) accumulation. However, this approach may require fairly comprehensive study, because it is based on complex mathematical formulations for metabolite network analysis. The data are gathered from extracellular measurements of biomass composition, quantification of secreted metabolites, substrate utilization, and intracellular measurements of carbon partitioning. Such flux analysis may have some limitations due to the complexity of mathematical modeling.

A very interesting model that organizes the flux analysis by grouping metabolites of similar biosynthetic origin has been proposed by Morgan and Shanks (2002). They have quantified temporal profiles of metabolites from several branches of the *indole alkaloid pathway* in *Catharanthus roseus* (L.) G. Don (Madagascar pink) hairy root cultures and were able to examine the distribution of flux around key branch points. As a result, this analysis provides crucial information, such as an estimate of total flux for all the secondary metabolites produced in a multi-branched pathway. Another good example is the regulation of *metabolic flux* to *cellulose*, a major sink for carbon in plants, as reported by Delmer and Haigler (2002). As for many pathways, it is still unclear where carbon flux is rate-limited in the complex cellulose biosynthetic pathway. Cellulose is an important component of the cell walls of higher plants. As a major sink for carbon on the earth, possible means by which the quality or the quantity of cellulose deposited in various plant parts might be manipulated by metabolic engineering techniques is a worthwhile goal (Delmer and Haigler, 2002). Thus, putative mechanisms for regulation-altered flux through this pathway, as well as multiple genes for cellulose biosynthesis and their regulation, provide targets for metabolic manipulations. However, possible variation in flux control under environmental influences must also be considered.

2.3.2 *Introduction of an Antisense Gene of the Competing Enzyme at the Branch Point*

Metabolic engineering of a zeaxanthin-rich potato by antisense inactivation and cosuppression of carotenoid epoxidation is a classical example for this approach (Romer et al., 2002). In order to provide a better supply of zeaxanthin in a staple crop, two different potato (*Solanum tuberosum* L.) cultivars were genetically modified. Sense and antisense constructs encoding zeaxanthin epoxidase have been transformed into the potato plant. Subsequently, zeaxanthin conversion to violaxanthin was inhibited. In this study, both approaches (antisense and cosuppression) yielded potato tubers with higher levels of zeaxanthin, up to 40 $\mu g \cdot g^{-1}$

dry weight. As a consequence of this metabolic engineering manipulation, the amount of violaxanthin was diminished dramatically, and in some cases, the monoepoxy intermediate, antheraxanthin, accumulated (Romer et al., 2002). Most of the transformants with higher zeaxanthin levels showed simultaneous increases in total carotenoid content (up to 5.7-fold). The increase in total carotenoids suggests that the genetic modification affects the regulation of the whole carotenoid biosynthetic pathway in potato tubers, involving substantial higher phytoene synthase and a slight increase of the β-carotene hydroxylase transcripts levels in tubers.

Recent work has also led to the identification of a transcriptional regulator that is possibly involved in the control of carotenogenesis (Welsch et al., 2007). Another mechanism controlling carotenoid levels in plant tissues is their degradation (Ohmiya et al., 2006). The generality of such a mechanism remains to be tested, but it could provide an additional approach for biotechnological improvement of carotenoid synthesis. This is important because carotenoids are members of one of the most diverse classes of natural compounds. Plant carotenoids are composed of a C40 isoprenoid skeleton with or without epoxy, hydroxy, and keto groups. They are high-value compounds in human nutrition as antioxidants and vitamin A precursors. In previous years, several metabolic engineering efforts have been undertaken in edible plants, again with the aim to improve their nutritional value (for review, see Giuliano et al., 2008).

2.3.3 Overcoming Rate-Limiting Steps

The most important aspects in metabolic engineering are to identify enzymes that may be involved in intermediate biosynthesis and subsequently to determine if any of these may occur at regulatory steps, or as now named *rate-limiting steps*. Such determinations may play a key role in future manipulation of secondary metabolite biosynthesis, because rate-limiting steps can be considered as *docking targets*.

For known metabolic pathways, the single-gene approach is an excellent way to find out where a rate-limiting step occurs. However, if pathway architecture is quite complicated, it raises the bar from the linear to a complex network. The analysis should therefore start with a step-by-step identification of all enzymatic activities that are specifically involved in the pathway. As we have mentioned, blockage of one pathway may lead to diversion of the substrate to alternative pathways. In such a situation, the identification of the rate-limiting step for biosynthesis of a particular metabolite may be difficult and become a "fishing expedition." Therefore, pathway architecture is one of the important factors that will allow one to determine the most suitable approach for engineering plant cells. It may also be that the pathway is subject to developmentally controlled flux at entry, as for example, through the activity of transcription factors. Several other factors, such as regulatory mechanisms or compartmentation, can also play a significant role. Thus, regulatory mechanisms such as *feedback regulation* may affect secondary metabolite

yield in plants. This is especially relevant with the single-gene approach. In contrast, with *heterologous gene overexpression*, a heterologous enzyme is shown to be operative and, because of this, may have no feedback inhibition by downstream products. Such an enzyme may be introduced from another source (Chartrain et al., 2000). Compartmentation also plays a major role in the regulation of secondary metabolite pathways because some important pathways occur in compartments (Verpoorte et al., 1999). for example, the biosynthesis of terpenoid-type indole alkaloids requires at least three compartments: the plastids for the terpenoid moiety and tryptophan, the cytosol for decarboxylation of tryptophan, and the vacuole for the coupling of tryptamine with secologanin (Verpoorte et al., 1999). Similar rules are shown for plant folate biosynthesis pathway, where it is split among cytosol, mitochondria, and chloroplasts. For example, in pea leaves, folate is distributed among mitochondria (highest concentration), chloroplasts, and a fraction consisting of the cytosol, nucleus, and vacuole (Gambonnet et al., 2001). Folates and their biosynthetic intermediates must therefore move in and out of organelles, thus requiring unraveling of its transport mechanisms. Since nothing is known about folate or its precursor carrier, identifying and cloning some transporters have been considered to be a priority for metabolic engineering of plant folate biosynthesis (Basset et al., 2005). It may be based either on modification of folate transport or on compartmentation. The engineering of folate transport, as reported by the same authors, is also a potential strategy to prevent and stockpile folate within an "inert" compartment like the vacuole. As the folate biosynthetic enzymes are not present in the vacuole, it may be possible to accumulate folate without feedback inhibition of its synthesis by directing folate import into this organelle (Basset et al., 2005).

Another example concerns plant polyketides and their biosynthesis. The plant polyketide synthases, like most enzymes, display broad substrate specificity. Using alternative substrates is the most straightforward and powerful approach to generate new polyketides in vitro. Initial efforts here also focus on how active site variation among enzymes making various molecules leads to product specificity. For example, modification of the octaketide-producing polyketide synthase from *Aloe arborescens* Mill. leads to a variety of octaketide products, which were produced by certain bacteria polyketide synthases (for review, see Yu and Jez, 2008). Similarly, three substitutions in chromone synthase, which make a pentaketide, triple the volume of the active site and result in synthesis of the nonaketide naphthopyrone from nine malonyl CoA molecules (Yu and Jez, 2008).

Deletion of a key biosynthetic enzyme can severely affect metabolite flux within a pathway. For example, the flow of precursors into the disrupted pathway often results in the accumulation of one or more intermediates upstream of the blocked step. This is because elevated concentrations of the substrate for the missing enzyme boost nonenzymatic background reactions and favor the appearance of enzyme variants with low substrate affinity. Such problems can be minimized, or even eliminated, through metabolic engineering, where, for example, excess substrate can be efficiently removed from cellular metabolism by providing a second enzyme to channel it away from the blocked step (Kleeb et al., 2007).

2.3.4 Blocking Catabolism or Competitive Pathways

Generally, metabolic pathways contribute to *catabolism* – the oxidative degradation of molecules – and *anabolism* – the reductive synthesis of molecules. In this regard, the catabolic or anabolic nature of the pathways must be revealed prior to any reconstruction experiment. Since little is known about catabolism in secondary metabolite pathways, there is an important question as to whether catabolism is an important factor in secondary metabolite pathways for limiting product accumulation. Interesting questions are also raised concerning the possible toxicity of some compounds to plant cells and the role of catabolism in detoxification mechanisms. In this context, naturally occurring storage compartments (e.g., vacuole(s) and plastid(s) in plant cells) may play a key role in preventing secondary metabolites from being catabolized. Catabolism thus may be an important factor in metabolic engineering. A remarkable observation was made in plant cell cultures of *C. roseus*by Dos Santos et al. (1994) concerning equality of the rate of catabolism with the rate of de novo compound biosynthesis. The phenomenon of catabolism in secondary metabolites has not been studied very extensively, and still few enzymes have been characterized in catabolism of most secondary metabolites (Verpoorte et al., 2000). Catabolism can be blocked by antisense genes or even by using some antibodies.

Blocking competitive pathways is also powerful tool to increase desired metabolite yield. Isoflavone levels in *Glycine max* (L.) Merr. (soybean) have been increased via metabolic engineering of the complex phenylpropanoid biosynthetic pathway (Yu et al., 2003). Phenylpropanoid pathway genes were activated by the expression of the maize C1 and R transcription factors in soybean seeds, which decreased genistein and increased the daidzein levels, with a small overall increase in total isoflavone levels. Cosuppression of flavanone 3-hydroxylase to block the anthocyanin branch of the pathway, in conjunction with C1/R expression, resulted in higher levels of isoflavones (Yu et al., 2003). The combination of transcription factor-driven gene activation and suppression of a competing pathway provided a successful means of enhancing accumulation of isoflavones in soybean seeds.

2.3.5 Inverse Metabolic Engineering

In contrast to classical metabolic engineering, where manipulation of known genes affects metabolic pathways with possible systematic changes, *inverse metabolic engineering (IME)* aims to identify and construct desired cell phenotypes of interest so as to incorporate them into appropriate host organisms. The concept of inverse metabolic engineering was first introduced by Bailey et al. (1996). The key element of this concept is identification of the molecular basis of a desired phenotype and its subsequent transfer to an appropriate host organism. Generally the following approaches may be involved: (1) the identification of desired phenotype, (2) determination of the influence of environmental or genetic factors on phenotype sustainability, and (3) alteration of the phenotype of the selected host by genetic manipulation.

IME is a powerful framework for engineering cellular phenotypes (Bailey et al., 2002). Such cell phenotypes, for example, may be chosen based on the accumulation of a desired metabolite. In order to discover cells with the most desirable properties, the cells must be screened genetically to identify the genetic basis of the relevant phenotype. It allows determination of particular genetic modifications that could not be discovered with a more directed technique. Recent advances in functional genomics, described in Section 2.2, have dramatically improved our ability to relate changes in phenotype with associated changes in genotype. As a result, inverse metabolic engineering can be a method for discovering new genes to target with traditional metabolic engineering. Thus, the first step is to find the genes that underlie the relevant phenotypes. Genetic selection or screenings, together with conventional gene sequencing, may be used to identify such genes in mutations.

While IME was initially designed for prokaryotes, nowadays its application applies to plant or other eukaryotic organism's cells also. The best example described by Sauer and Schlattner (2004) concerns the ATP homeostasis exhibited by animal cells. A variable ATP turnover in these cells is achieved through temporal and spatial energy buffering, where phosphagen kinase systems (consisting of specific kinase and its cognate phosphagen) function as a large pool of high-energy phosphates that are used to replenish ATP during periods of high energetic demand. Thus, these authors suggest the use of recent advances and potentials of inverse metabolic engineering of cell types that do not normally contain such systems (bacteria, yeast, and plants) in conjunction with creatine or arginine kinase systems (Sauer and Schlattner, 2004). Beyond such applications in bioprocess engineering, engineering of phosphagen kinase systems is potentially important for medical and pharmaceutical applications. The advantage of inverse metabolic engineering may be more applicable if we can rationally modify a given phenotype to engineer cell behavior.

2.4 Development of Genetically Modified Plants That Express Resistance to Different Kinds of Abiotic and Biotic Stresses

Environmental stresses (e.g., high salt levels, low water availability that leads to drought, excess water that leads to flooding, or high- and low-temperature regimes) can adversely affect plant growth and productivity. The genetic or epigenetic responses of plants to these stresses are complex because they involve simultaneous expression of a number of genes or physiological reactions. Continuing efforts of scientists have resulted in engineering of plants resistant to high temperatures, low temperatures, and excess salinity. Some progress has also been achieved in generating plants resistant to water-deficit stress and to flooding. While such achievements are impressive, it is still a challenging task to understand complex functional genetic resistance responses to such stresses. Here, metabolic engineering can play an important role. The limiting factor in this aspect is the lack of information on what are the "useful genes", i.e., genes that would lead to better stress tolerance.

Metabolic engineering of rice leading to biosynthesis of *glycine betaine* and tolerance to salt and cold is one of the best examples in this field. Genetically engineered rice (*Oryza sativa* L.) with the ability to synthesize glycine betaine was established by introducing the *codA* gene for *choline oxidase* from the soil bacterium *Arthrobacter globiformis* (Sakamoto and Murata, 1998). This study indicates that the subcellular compartmentalization of the biosynthesis of glycine betaine was a critical element in the efficient enhancement of tolerance to salt and cold stresses in the engineered rice plants.

Metabolic engineering to accumulate osmoprotectants in plants may increase their drought and salinity tolerance. An effective mechanism to reduce damage from these stresses is brought about by the accumulation of high intracellular levels of *osmoprotectant compounds* including proline, ectoine, betaines, polyols, and trehalose. Engineering osmoprotectant biosynthesis pathways is a potential way to improve stress tolerance (Rontein et al., 2002). Several single genes for such osmoprotectant pathways have been successfully introduced into several plants, including rice (*O. sativa* L.), canola (*Brassica napus* L.), potato (*S. tuberosum* L.), tobacco (*Nicotiana tabacum* L.), and *Arabidopsis*, to improve their stress tolerance.

Another possible mechanism by which plants could survive salt stress is achieved by compartmentalization of sodium ions apart from the cytosol. Overexpression of a *vacuolar Na super*(+)/*H super*(+) *antiport* from *Arabidopsis thaliana* (L.) Heynh. in *Arabidopsis* plants promotes sustained growth and development in the soil/water environment. This salinity tolerance was correlated with higher-than-normal levels of *AtNHX1 transcripts*, protein, and vacuolar Na super(+)/H super(+) (sodium/proton) antiport activity, as reported by Apse et al. (1999). These results demonstrate the feasibility of metabolic engineering for salt tolerance in plants. Improving plant drought, salt, and freezing tolerance by gene transfer of a single *stress-inducible transcription* factor has also been reported to be successful (Kasuga et al., 1999). Overexpression of the cDNA encoding this transcriptional factor in transgenic plants activated the expression of many of these stress-tolerant genes under normal growing conditions and resulted in improved tolerance to drought, salt loading, and freezing.

Application of metabolic engineering to improve tolerance against UV radiation, intensive high light intensities, and high temperatures has been reported for *Arabidopsis* plants (Giuliano et al., 2008). Leaf carotenoids may have essential photoprotective roles, because they scavenge *reactive oxygen species (ROS)*, quench dangerous triplet states of chlorophyll, and participate in thermal dissipation of excess light energy. For example, zeaxanthin that is formed as a result of violaxanthin de-epoxidation under high irradiances enhances *nonphotochemical quenching (NPQ)* of chlorophyll fluorescence and protects membrane lipids from peroxidation. Thus, it is not surprising that genetically engineered *Arabidopsis* in which all leaf xanthophylls have been substituted with zeaxanthin shows more resistance to photooxidative stress and lipid peroxidation (Giuliano et al., 2008). However, due to genetic manipulations that may increase the levels of several β-xanthophylls simultaneously, each may have distinct photoprotective roles and therefore must be considered in future applications.

Improving resistance against pests or diseases also leads to improved yields. For resistance against pathogenic microorganisms, metabolic engineering can program for the expression of high levels of defense compounds, such as phytoalexins, or for pest resistance, improve the production of endogenous defense compounds (e.g., pathogenesis-related proteins), or introduce genes that produce new toxic compounds (e.g., the "B.T." gene from *Bacillus thuringiensis* that produces a toxic crystalline protein that interrupts digestion in many types of feeding insect pests) into plants to control insect predators.

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Chapter 3
Molecular Farming of Antibodies in Plants

Rainer Fischer, Stefan Schillberg, and Richard M. Twyman

Abstract Biopharmaceuticals are produced predominantly in microbial or mammalian bioreactor systems. Over the last few years, however, it has become clear that plants have great potential for economical, large-scale biopharmaceutical production. Following the commercial release of several maize-derived technical proteins, the first plant-derived veterinary vaccine was approved in 2006. Plants offer the prospect of inexpensive production without sacrificing product quality or safety. The first therapeutic products for use in humans – mostly antibodies and vaccine candidates – are now at the clinical trials stage. In this chapter, we discuss the different plant-based production systems that have been used to synthesize recombinant antibodies and to evaluate the merits of plants compared with other platforms. Despite the currently unclear regulatory framework, the benefits of plant-derived systems are now bringing the prospect of inexpensive recombinant antibodies closer than ever before.

3.1 Introduction

Antibodies are multisubunit glycoproteins produced by the vertebrate immune system. They recognize and bind to their target antigens with great affinity and specificity, which allows them to be used for many applications, including the diagnosis, prevention, and treatment of human and animal disease (Andersen and Krummen, 2003; Chadd and Chamow, 2001; Fischer and Emans, 2000). It is estimated that approximately 1,000 therapeutic recombinant antibodies are under development, up to one-quarter of which may already be undergoing clinical trials. A large proportion of these antibodies recognize cancer antigens, but others have been developed for the diagnosis and treatment of infectious diseases, acquired disorders,

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and even transplant rejection (Gavilondo and Larrick, 2000). As well as having biomedical applications, antibodies can also be exploited to prevent diseases in plants (Schillberg et al., 2001), to detect and remove environmental contaminants, and for various industrial processes such as affinity purification and molecular targeting (Stoger et al., 2005b).

With such a diverse spectrum of uses, the potential market for antibodies is extremely large and there is considerable interest in high-capacity production technologies that are robust, economical, and safe. Over the last 15 years, plants have emerged as convenient, economical, and scalable alternatives to the mainstream antibody production systems which are based on the large-scale culture of microbes or animal cells (Chu and Robinson, 2001; Wurm, 2004). In this chapter, we discuss the advantages and disadvantages of plants for antibody production, the diverse plant-based systems that are now available, and factors governing the success of antibody production in plants. We begin, however, with a brief overview of recombinant antibody technology.

3.2 Recombinant Antibody Technology

The typical antibody format is the mammalian serum antibody, which comprises two identical heavy chains and two identical light chains joined by disulfide bonds (Fig. 3.1). Each heavy chain is folded into four domains, two on either side of a flexible hinge region, which allows the multimeric protein to adopt its characteristic shape. Each light chain is folded into two domains. The N-terminal domain of each of the four chains is variable, i.e., it differs among individual B cells due to unique

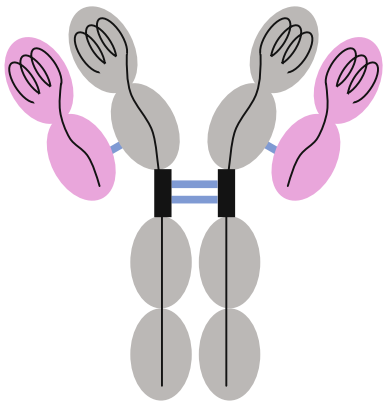

Fig. 3.1 Structure of a typical mammalian serum antibody, comprising two identical heavy chains (*gray*) and two identical light chains (*pink*). Solid *black lines* indicate continuation of the polypeptide backbone (*simple lines* indicate the constant parts of the antibody, *curly lines* indicate the variable regions, and *thick sections*represent the hinge region). Antibody domains are indicated by *colored circles*. Disulfide bonds are represented by *gray bars*

rearrangements of the germ-line immunoglobulin genes. This part of the molecule is responsible for antigen recognition and binding. The remainder of the antibody comprises a series of constant domains, which are involved in effector functions such as immune cell recognition and complement fixation. Below the hinge, in what is known as the Fc portion of the antibody, the constant domains are class-specific. Mammals produce five classes of immunoglobulins (IgG, IgM, IgA, IgD, and IgE) with different effector functions. The Fc region also contains a conserved asparagine residue at position 297 to which *N*-glycan chains are added. The glycan chains play an important role both in the folding of the protein and in the performance of effector functions (Jefferis, 2001).

Antibodies are also found in mucosal secretions, and these secretory antibodies have a more complex structure than serum antibodies. They are dimers of the serum-type antibody, the two monomers being attached by an additional component called the joining chain. There is also a further polypeptide called the secretory component, which protects the antibodies from proteases (Fig. 3.2).

Antibodies obtained from immunized animals are polyclonal, i.e., derived from many different B cells. The advantage of monoclonal antibodies, i.e., antibodies derived from a single clone of B cells, is that their binding specificity does not vary. The traditional source of monoclonal antibodies is murine B cells. To provide a constant source of the antibody, B cells of appropriate specificity are fused to immortal myeloma cells to produce a *hybridoma cell line*. However, the use of murine hybridoma-derived antibodies as therapeutics is limited because the murine components of the antibodies are immunogenic in humans, resulting in a so-called human antimouse antibody (HAMA) response. Therefore, numerous strategies

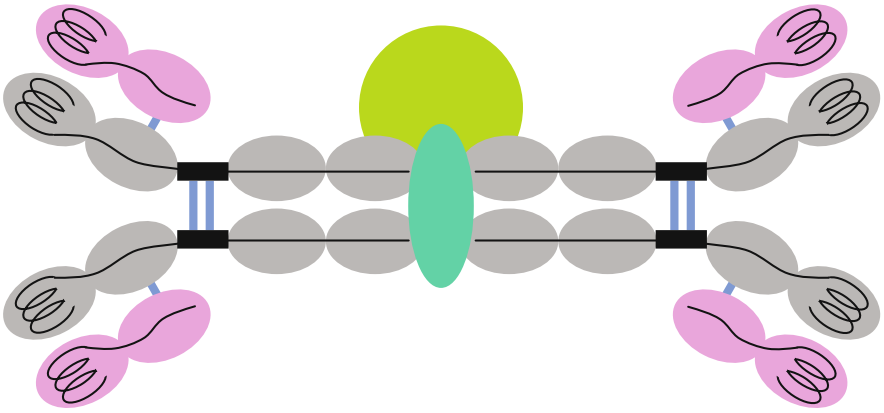

Fig. 3.2 Structure of a mammalian secretory antibody, comprising a dimer of the typical serum antibody and including two additional components, the joining chain (*blue disc*) and the secretory component (*green disc*). Heavy chains are shown in *gray* and light chains in *pink*. Solid *black lines* indicate continuation of the polypeptide backbone (*simple lines* indicate the constant parts of the antibody, *curly lines* indicate the variable regions, and *thick sections*represent the hinge region). Antibody domains are indicated by *colored circles*. Disulfide bonds are represented by *gray bars*

have been developed to humanize murine monoclonal antibodies (Kipriyanov and Little, 1999), culminating in the production of transgenic mice expressing the human immunoglobulin genes (Green, 1999). An alternative approach is to use phage display libraries based on the human immune repertoires. Phage display is advantageous because high-affinity antibodies can be identified rapidly, novel combinations of heavy and light chains can be tested, and the DNA sequence encoding the antibody is indirectly linked to the antibody itself (Griffiths and Duncan, 1998; Sidhu, 2000). This avoids the laborious isolation of cDNA or genomic immunoglobulin sequences from hybridoma cell lines.

The expression of serum-type or secretory-type antibodies as recombinant molecules requires the preparation and expression of two and four different transgenes, respectively. However, this is often an unnecessary complication, because in many cases, the effector functions conferred by the constant regions are neither required nor desired. The constant regions of native immunoglobulins are not required for antigen binding, and the variable regions of the heavy and light chains can interact perfectly well when joined on the same polypeptide molecule (Chadd and Chamow, 2001; Fischer and Emans, 2000). Smaller antibody derivatives, which still require two chains, include Fab and $F(ab')_2$ fragments (which contain only the sequences distal to the hinge region) and minibodies (which contain only part of the constant portion of the molecule). Other derivatives, such as large single chains, single-chain Fv fragments (scFvs), and diabodies, contain the variable regions of the heavy and light chains joined by a flexible peptide chain. Such derivatives are often more effective as drugs than full-length immunoglobulins because they show increased penetration of target tissues, reduced immunogenicity, and are cleared from tissues more rapidly. Another variant is the *camelid serum antibody*, which is unique in that it contains only heavy chains. A full-size camelid antibody can, therefore, be expressed from a single transgene. Further, more specialized derivatives include bispecific scFvs, which contain the antigen recognition elements of two different immunoglobulins and can bind to two different antigens, and scFv fusions, which are linked to proteins with additional functions. Examples of all these antibody derivatives are shown in Fig. 3.3.

3.2.1 Expression Systems for Recombinant Antibodies

Most of the recombinant full-length immunoglobulins being developed as pharmaceuticals are produced in mammalian cell cultures, a few in hybridoma lines, but most in immortalized lines that have been cleared by the FDA (Food and Drug Administration) and equivalent authorities in other countries. These lines include Chinese hamster ovary (CHO) cells, the murine myeloma cell lines NS0 and SP2/0, baby hamster kidney (BHK) and human embryonic kidney (HEK)-293 cells, and the human retinal line PER-C6 (Chu and Robinson, 2001). The main reason for this is the belief that mammalian cells yield authentic products, particularly in terms of glycosylation patterns. However, there are minor differences in glycan chain structure between rodent and human cells. For example, human antibodies contain only the

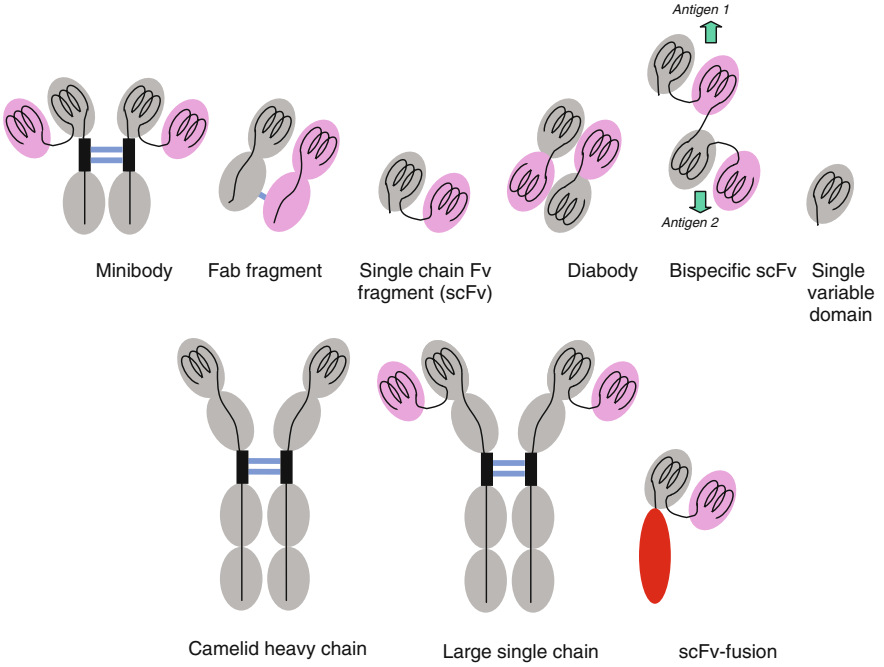

Fig. 3.3 Structure of recombinant antibody derivatives and atypical antibody formats, most of which have been expressed in plants. Heavy-chain derivatives are shown in *gray* and light-chain derivatives in *pink*. Solid *black lines* indicate continuation of the polypeptide backbone (*simple lines* indicate the constant parts of the antibody, *curly lines* indicate the variable regions, and *thick sections* represent the hinge region). Antibody domains are indicated by *colored circles*. Disulfide bonds are represented by *gray bars*. The *red disc* indicates a new functional protein domain in the scFv fusion protein

sialic acid residue *N*-acetylneuraminic acid (NANA), while rodents produce a mixture of NANA and *N*-glycosylneuraminic acid (NGNA) (Raju et al., 2000). There are many disadvantages to mammalian cell cultures, including the high setup and running costs, the limited opportunities for scale-up, and the potential contamination of purified recombinant antibodies with human pathogens. Bacterial fermentation systems are more cost-effective than mammalian cell cultures and are therefore preferred for the production of Fab fragments and scFvs, since these derivatives are not glycosylated. Even so, the yields of such products in bacteria are generally low because the proteins do not fold properly (Baneyx and Mujacic, 2004). The main reason for sticking to these systems is that they are well characterized and established and conform to the strict and extensive regulatory systems governing biopharmaceutical production.

Several alternative production systems have been explored, some of which are now well established while others are still experimental. In the former category, yeast and filamentous fungi have the advantages of bacteria (economy and robustness), but they do have the tendency to hyperglycosylate recombinant proteins

(Gerngross, 2004), while insect cells can be cultured in the same way as mammalian cells (although more cheaply) and also produce distinct glycan structures (Ikonomou et al., 2003). A more recent development is the production of antibodies in the milk of transgenic animals (Dyck et al., 2003). A disadvantage of animals, in common with cultured mammalian cells, is the existence of safety concerns about the transmission of pathogens or oncogenic DNA sequences. Finally, hen's eggs could also be used as a production system since they are protein-rich and already synthesize endogenous antibodies, but they remain a relatively unexplored potential expression system (Harvey et al., 2002). Plants offer a unique combination of advantages for the production of pharmaceutical antibodies (Twyman et al., 2003, 2005; Ma et al., 2003; Basaran and Rodriguez-Cerezo, 2008). Their main benefit is the low production costs, reflecting the fact that traditional agricultural practices and unskilled labor are sufficient for maintaining and harvesting antibody-expressing crops. Also, large-scale processing infrastructure is already in place for most crops. Scale-up is rapid and efficient, requiring only the cultivation of more land. There are minimal risks of contamination with human pathogens.

The general eukaryotic protein synthesis pathway is conserved between plants and animals. So plants can efficiently fold and assemble full-size serum immunoglobulins (as first demonstrated by Hiatt et al., 1989) and secretory IgAs (first shown by Ma et al., 1995). In the latter case, four different subunits need to assemble in the same plant cell to produce a functional product, even though two different cell types are required in mammals. The posttranslational modifications carried out by plants and animals are not identical to those in mammals, but they are very similar (certainly more so than in fungal and insect systems). There are minor differences in the structure of complex glycans, such as the presence in plants of the residues α-1,3-fucose and β-1,2 xylose, which are absent from mammals (Cabanes-Macheteau et al., 1999). These residues are immunogenic in several mammals, including humans, but curiously not in mice and only after multiple exposures in rats (Gomord et al., 2005; Faye et al., 2005). However, as discussed in more detail below, there are now many studies that show how the glycan profile of proteins produced in plants can be "humanized." As well as full-size antibodies, various functional antibody derivatives have also been produced successfully in plants, including Fab fragments, scFvs, bispecific scFvs, single-domain antibodies, and antibody fusion proteins (see Twyman et al., 2005).

3.2.2 Plant-Based Expression Platforms

The most widely used strategy for antibody production in plants is the nuclear transgenic system, in which the antibody transgenes are transferred to the plant nuclear genome. The advantages of this approach when used in our major terrestrial crop species include the following: (1) transformation is a fairly routine procedure in many plant species and can be achieved by a range of methods, the two most common of which are *Agrobacterium*-mediated transformation and the delivery of DNA-coated metal particles by microprojectile bombardment; (2) a stable transgenic line

can be used as a permanent genetic resource; (3) among the various plant systems, it is the simplest to maintain (once the producer line of transgenics is available) and is ultimately the most scalable; (4) it is possible to establish master seed banks. Disadvantages, compared to other plant systems, include the relatively long development time required for transformation, regeneration, analysis of transgenics, selection and bulking up of the producer line, the unpredictable impact of epigenetic events on transgene expression (e.g., posttranscriptional gene silencing and position effects), and the potential for transgene spread from some crops through outcrossing. A range of different crops have been explored for antibody production, and the main categories are described below.

Leafy crops have two major benefits: they have a large biomass, which translates to large product yields, and flowering can be prevented (e.g., genetically or by emasculation) to avoid the spread of transgenic pollen. On the other hand, leaf tissue is very watery such that proteins are expressed and accumulate in an aqueous environment in which they are subject to degradation. This means that antibody-containing leaves generally have to be processed soon after harvest or otherwise frozen or dried, which can add significantly to production costs. Tobacco (*Nicotiana tabacum* L.) has the longest history as a pharmaceutical production model crop system, having been used to express the very first plant-derived antibodies and many of the others since (Table 3.1). The major advantages of tobacco are the well-established technology for gene transfer and expression, the high biomass yield (over 100,000 kg/h for close cropped tobacco, since it can be harvested up to nine times a year), and the existence of large-scale infrastructure for processing that does not come into contact with the human or animal food chains. Particularly due to the yield potential and safety features, tobacco could be a major source of plant-derived recombinant antibodies in the future. Another leafy crop that has been evaluated for antibody expression is alfalfa (*Medicago sativa* L.). This has been developed as a production crop by the Canadian biotechnology company Medicago Inc., and they have secured a robust IP portfolio covering the use of expression cassettes for biopharmaceutical proteins in this species. Although not as prolific as tobacco, alfalfa nevertheless produces large amounts of leaf biomass and has a high leaf protein content. Alfalfa also lacks the toxic metabolites produced in many tobacco cultivars, which are often cited as a disadvantage, but instead it contains high levels of oxalic acid, which can affect protein stability. Alfalfa is particularly useful because it is a perennial plant that is easily propagated by stem cutting to yield clonal populations. Although alfalfa has been put on the biosafety "hit list" by the regulators because it outcrosses with wild relatives, this does not detract from the excellent properties of this species for antibody production under containment, as in greenhouses or programmed plant growth chambers. Alfalfa has been used for the production of a diagnostic IgG that recognizes epitopes specific to the constant regions of human IgG (Khoudi et al., 1999) and for several other antibodies in development by Medicago Inc.

The problem of protein instability in leafy tissue (see above) can be overcome by expressing antibodies in the dry seeds of cereals and grain legumes. Several different species have been investigated for antibody production including four major cereals (maize, rice, wheat, and barley) and two legumes (soybean and pea). The

Table 3.1 Recombinant therapeutic or diagnostic recombinant antibodies produced by molecular farming in plants and reported in the scientific literature (many antibodies in commercial development remain undisclosed until IP rights have been secured). Antibodies with alternative applications, such as phytomodulation or the prevention of plant disease, are not listed

Antigen	Antibody format	Production system	Comments	References
HIV gp120 (2F5)	IgG	Tobacco, maize, tobacco suspension cells	Maximum yield ~75 μg/g seeds	Floss et al. (2008), Sack et al. (2007)
HIV gp120 (2G12)	IgG	Tobacco, maize	Maximum yield ~100 μg/g seeds	Rademacher et al. (2008), Ramessar et al. (2008c)
B-cell lymphoma, murine 38C13	scFv	Virus vectors in tobacco leaves	Maximum yield 30.2 μg/g leaves	McCormick et al. (1999)
Carcinoembryonic antigen	scFv, IgG1	Tobacco agroinfiltration	Directed to apoplast or ER. Maximum yields 5 μg scFv/g leaves, 1 μg IgG/g leaves	Vaquero et al. (1999)
	dAb	Tobacco, agroinfiltration and transgenic plants		Vaquero et al. (2002)
	scFv	Rice, rice cell cultures	Directed to apoplast or ER. Maximum yields 3.8 μg/g callus, 29 μg/g leaves, 32 μg/g seed	Torres et al. (1999), Stoger et al. (2000)
	scFv	Wheat	Directed to apoplast or ER. Maximum yields 900 ng/g leaves, 1.5 μg/g seed	Stoger et al. (2000)
	scFv	Pea	Directed to rER. Maximum yield 9 μg/g seed	Perrin et al. (2000)
CD-40	scFv fusion	Tobacco suspension cells	Secreted into apoplast. Yield not reported	Francisco et al. (1997)
Colon cancer antigen	IgG	Virus vectors in tobacco leaves	Yield not reported	Verch et al. (1998)
Epidermal growth factor receptor (EGFR)	IgG	Tobacco	Aglycosylated antibody was directed to the ER and binds to EGFR expressed on the surface of human tumor cells	Rodriguez et al. (2005)
Human creatine kinase	IgG1, Fab	Tobacco and *Arabidopsis* leaves	Accumulated in nucleolus or apoplast. Maximum yield 1.3% TSP	De Neve et al. (1993), De Wilde et al. (1998)

Table 3.1 (continued)

Antigen	Antibody format	Production system	Comments	References
	scFv	Tobacco leaves	Directed to cytosol or apoplast. Maximum yield 0.01% TSP	Bruyns et al. (1996)
Rhesus D antigen	IgG1	*Arabidopsis* leaves	Reacted with RhD^+ cells in antiglobulin technique and elicited a respiratory burst in human peripheral blood mononuclear cells	Bouquin et al. (2002)
Ferritin	scFv	Tobacco leaves		Semenyuk et al. (2002)
Hepatitis B virus surface antigen	IgG	Tobacco leaves	Up to 25 mg antibody per kilogram biomass	Valdes et al. (2003[a],b)
	IgG	Tobacco suspension cells	Complement-dependent cytotoxicity demonstrated	Yano et al. (2004)
	scFv	Tobacco	Four different targeting constructs used, ER targeting achieved 0.22% TSP	Ramírez et al. (2002)
Herpes simplex virus 2	IgG1	Soybean	Secreted into apoplast. Yield not reported	Zeitlin et al. (1998)
HIV antibodies in blood	scFv fusion	Tobacco leaves, barley grains, potato tubers	Maximum yield 150 mg/g	Schunmann et al. (2002)
Human choriogonadotrophin	scFv, dAb, IgG	Tobacco leaves	Secreted into apoplast. Maximum yield 40 mg/kg fresh weight	Kathuria et al. (2002)
	IgG1, diabody	Tobacco and winter cherry leaves	Directed to apoplast or ER. Glycan patterns were analyzed	Sriraman et al. (2004)
Human IgG	IgG1	Alfalfa	Secreted into apoplast. Maximum yield 1% TSP	Khoudi et al. (1999)
Interleukin-4	scFv	Tobacco roots	Maximum yield 0.18% TSP	Ehsani et al. (2003)
Interleukin-6	scFv	Tobacco roots		Ehsani et al. (2003)
Protective antigen of *Bacillus anthracis*	IgG	*N. benthamiana*	Toxin activity was neutralized in vitro and in vivo	Hull et al. (2005)
Rabies virus	IgG	Tobacco	Directed to the ER. Activity of the rabies virus was neutralized. Glycan patterns were analyzed	Ko et al. (2003)
Salmonella enterica lipopolysaccharide	scFv	Tobacco	41.7 μg purified scFv per gram leaf tissue	Makvandi-Nejad et al. (2005)

Table 3.1 (continued)

Antigen	Antibody format	Production system	Comments	References
Streptococcal surface antigen (I/II)	sIgA	Tobacco leaves	Secreted into apoplast. Maximum yield 500 μg/g fresh weight	Ma et al. (1995)
	IgG1	Tobacco leaves	Directed to plasma membrane. Maximum yield 1.1% TSP in leaves	Vine et al. (2001)
	IgG1	Secretion from tobacco roots	Up to 11.7 μg per gram dry root weight per day	Drake et al. (2003)
Substance P	VH	Tobacco leaves	Secreted into apoplast. Maximum yield 1% TSP	Benvenuto et al. (1991)
Tetanus toxin C	IgG2a fused to tetanus toxin C	Tobacco	Animals immunized with recombinant immune complex without adjuvant were fully protected against lethal challenge	Chargelegue et al. (2005)
Tumor-associated antigen EpCAM	IgG	Tobacco	Secreted into the apoplast. Binding activity to colon cancer cells and tumor inhibition activity in nude mice	Ko et al. (2005)

idea is that such crops would be beneficial for production in developing countries, where on-site processing would not be possible and a cold chain could not be maintained. The accumulation of recombinant antibodies in seeds allows for long-term storage at ambient temperatures because the proteins accumulate in a stable form (Ramessar et al., 2008a). Seeds have the appropriate molecular environment to promote protein accumulation, and they achieve this through the creation of specialized storage compartments such as protein bodies and storage vacuoles that are derived from the secretory pathway. Seeds are also desiccated, which reduces the level of both nonenzymatic hydrolysis and protease degradation. It has been demonstrated that antibodies expressed in seeds remain stable for at least 3 years at ambient temperatures with no detectable loss of activity (Stoger et al., 2005a).

As well as their advantages in terms of product stability, seed expression might also be beneficial in terms of downstream processing. This is because seeds have a relatively simple proteome (therefore minimizing the likelihood that endogenous proteins would be copurified) and lack the phenolic compounds abundant in leaves that can interfere with affinity purification. The restriction of recombinant protein accumulation in seeds also helps to avoid any potentially negative effects on the growth and development of vegetative plant organs and on humans, animals, and microorganisms that interact with the plant or feed on its leaves.

Disadvantages of seed crops include the lower overall yields that have been obtained. The intrinsic yields are in a few cases higher than tobacco (e.g., on a kilogram-per-kilogram basis of harvested material, rice grains can accumulate more antibody than tobacco leaves; Stoger et al., 2002), but the vast abundance of harvested biomass per hectare from a tobacco crop far outweighs this. Also, seeds are regarded as viable genetically modified organisms in their own right. So while the transport of harvested transgenic tobacco leaves should not cause any problems, the transport of seeds could fall afoul of national and international regulations on the transport of GMOs (Sparrow et al., 2007; Spök et al., 2008); the seeds would have to be crushed to flour beforehand and this might offset the advantage of increased product longevity.

Maize (*Zea mays* L.) seeds have been investigated as an antibody production vehicle by Prodigene Inc. following successful demonstrations of the economical production of other valuable proteins using this system, including avidin and β-glucuronidase. Initial findings for the expression of a secretory IgA in maize showed that the four chains were expressed, directed to the cell wall matrix, and assembled correctly. The product accumulated to 0.3% of total soluble protein in the T1 seeds, and based on previous results, significant improvements were anticipated through selective breeding (Hood et al., 2002). An antibody derivative used for HIV diagnostics has been expressed in barley and has achieved a yield of 150 μg/g. More recently, the HIV-neutralizing antibody, 2G12, was produced in maize seeds with a yield >100 μg/g for use as a potential microbicide (Ramessar et al., 2008c).

Finally, antibodies have also been produced in soybean (*Glycine max* (L.) Merr.), although in this particular case, it was expressed constitutively and isolated from the leaves rather than from the seeds (Zeitlin et al., 1998). Soybean has been investigated

as a potential production crop by Prodigene and others because it is a self-fertilizing crop with a high biomass yield, but product yields have been low and the system has therefore been largely abandoned.

Recombinant antibodies have been produced in potato (*Solanum tuberosum* L.) and tomato (*Solanum lycopersicum* L.), which also offer certain advantages over other crops. Proteins accumulating in potato tubers are generally stable, because, like the cereal seed endosperm, these are storage organs that are adapted for high-level protein accumulation. The potential of potato tubers for antibody production was first demonstrated by Artsaenko et al. (1998), who produced an scFv fragment specific for the inflammatory agent oxazolone. Potatoes have since been developed as a general production host for antibodies (De Wilde et al., 2002) as well as other biopharmaceuticals based on antibodies (Schunmann et al., 2002). Fruit crops have another potential advantage, i.e., antibody expression in organs that are consumed raw allows the direct oral administration of recombinant antibodies designed for passive immunotherapy, such as protection of the oral cavity against pathogens. Stoger et al. (2002) describe preliminary experiments in which scFv84.66, recognizing the carinoembryonic antigen (CEA), is expressed in tomato fruits, although the accumulation levels are rather low (0.3 μg/g fresh weight). Other advantages of tomato include the high biomass yields (about 68,000 kg/ha, approaching the yields possible in tobacco) and the increased containment offered by growth in greenhouses.

Instead of introducing transgenes into the nuclear genome, they can be targeted to the chloroplast genome using particle bombardment or another physical DNA delivery technique and ensuring the transgene is embedded in a chloroplast DNA homology region (Maliga, 2003, 2004; Bock, 2007). The main benefits of the chloroplast system are that there are thousands of chloroplasts in a typical leaf cell, yet only one nucleus; therefore, the number of transgene copies in the cell following plastid transformation and the establishment of homoplasmy is much higher, promising greater product yields. This is enhanced by the absence of epigenetic phenomena such as transgene silencing in the chloroplast genome. Chloroplasts, derived from ancient bacteria, also support operon-based transgenes, allowing the expression of multiple proteins from a single transcript. Finally, and perhaps most importantly from the regulatory perspective, chloroplasts are absent from the pollen of most of our food crops, which limits the potential for outcrossing (Daniell et al., 2005a).

There are two disadvantages to the chloroplast system: first, chloroplast transformation is not a standard procedure and is thus far limited to a relatively small number of crops (e.g., tobacco, tomato, potato, cotton, soybean, lettuce, cauliflower, and sugar beet; Daniell et al., 2005b; Lelivelt et al., 2005; Nugent et al., 2006; De Marchis et al., 2009); second, since chloroplasts are derived from ancient bacteria, they lack much of the eukaryote machinery for posttranslational modification, i.e., they are unable to synthesize glycan chains. For this reason, they would be suitable for the production of scFvs but not full-size immunoglobulins.

In the only published report thus far dealing with antibody expression in terrestrial plant chloroplasts, a camelid antibody fragment was expressed in tobacco using an inducible T7-promoter system. Transcripts could be detected but no protein

(Magee et al., 2004). However, antibodies have been expressed successfully in algal chloroplasts (see below).

Transient expression assays are generally used to evaluate the activity of expression constructs or to test the functionality of a recombinant protein before committing to the long-term goal of generating transgenic plants. However, transient expression can also be used as a routine production method if enough protein can be produced to make the system economically viable. The advantages of this approach include the minimal setup costs and the rapid onset of protein expression, but scaling-up is expensive and impractical. So this type of system is particularly useful for the production of high-value proteins such as therapeutic antibodies, which have specialized markets and are required in small amounts.

An example of a transient expression system is the agroinfiltration method, where recombinant *Agrobacterium tumefaciens* is infiltrated into tobacco leaf tissue under vacuum and milligram amounts of protein can be produced within a few weeks (Kapila et al., 1997). This system has also been developed in alfalfa by Medicago researchers (D'Aoust et al., 2004) and is applicable in many other leafy species. Although stable transformation occurs at very low efficiency, many cells are initially transiently transformed, only for the exogenous DNA to get diluted and degraded. However, before this happens, most cells contain the T-DNA and can express any transgenes carried therein. As extrachromosomal constructs, these unintegrated T-DNAs are free from position effects and epigenetic silencing phenomena that often reduce or abolish the expression of integrated nuclear transgenes.

A number of different antibodies and their derivatives have been produced by agroinfiltration, including the full-size IgG T84.66 along with its scFv and diabody derivatives (Vaquero et al., 1999, 2002) and a chimeric full-size IgG known as PIPP along with its scFv and diabody derivatives, which recognizes human chorionic gonadotropin (Kathuria et al., 2002).

Plant viruses are advantageous for the production of antibodies because viral genomes are easier to manipulate than plant genomes, and the infection of plants with recombinant viruses is a very simple process compared to the regeneration of transgenic plants (Yusibov et al., 2006; Yusibov and Rabindran, 2008). Potentially, plants carrying recombinant viruses can be grown on the same scale as transgenic plants, but with a much shorter development time. Viral infections are generally systemic, so infected plants carry the virus in all cells and can produce the antibody systemically, resulting in potentially very high yields. A further advantage of viruses is that mixed infections are possible, making it a simple process to express, for example, the multiple chains of a full-size immunoglobulin. Although the transgene is carried on a viral genome rather than on the plant genome, the expressed protein is processed in the same manner as it would be in transgenic plants, meaning that appropriate folding, targeting, and modification of antibodies are possible. The viral system is therefore uniquely simple, flexible, and efficient, and it has the potential for protein manufacture in both contained and open facilities (Canizares et al., 2005; Yusibov et al., 2006).

There are two types of expression systems based on plant viruses, one for full polypeptides and one for peptide epitopes displayed on the virion surface. Both have

been used to express antibodies. In the polypeptide expression system, the antibody is encoded by a discrete transgene and accumulates as a soluble protein within the plant cell. In the epitope display system, a small antibody derivative such as an scFv is expressed as a fusion with the viral coat protein in such a way that the antibody is displayed on the surface of the virus particle.

Tobacco mosaic virus (*TMV*) has a monopartite RNA genome of 6.5 kb encoding four proteins all of which are essential for systemic infection. The normal strategy for polypeptide expression is to place the transgene under the control of an additional coat protein promoter, although not a perfect copy of the endogenous coat protein promoter, as this is an unstable configuration that leads to transgene elimination (Donson et al., 1991). Many antibodies have now been expressed in TMV-infected plants. McCormick et al. (1999) produced an scFv fragment based on the idiotype of malignant B cells of the murine 38C13 B-lymphoma cell line. When administered to mice, the scFv stimulated the production of anti-idiotype antibodies capable of recognizing 38C13 cells, providing immunity against lethal challenge with the lymphoma. This has been developed into a personalized therapy for diseases such as non-Hodgkin's lymphoma, where antibodies capable of recognizing unique markers on the surface of any malignant B cells could be produced for each patient. Up to 15 such antibodies were tested in phase I and phase II clinical trials by the US biotechnology company Large Scale Biology Inc., before they went into liquidation. Additionally, Verch et al. (1998) produced a full-length IgG in transgenic tobacco plants by infecting them with two TMV vectors, one expressing the heavy chain and one the light chain. This study showed that viral coexpression was compatible with the correct assembly and processing of multimeric recombinant proteins.

Potato virus X (*PVX*), the type member of the Potexvirus family, has a 6.5-kb monopartite RNA genome rather like that of TMV. Also, like TMV, PVX vectors contain extra subgenomic promoters to drive transgene expression, but in this case, the lack of a closely related alternative means that transgene elimination by homologous recombination is unavoidable. PVX vectors have been used for the expression of several different antibodies, but none of medical relevance. Single-chain Fv antibodies have been expressed, specific for proteins from potato virus V (Hendy et al., 1999), tomato spotted wilt virus (Franconi et al., 1999) and against granule-bound starch synthase I (Ziegler et al., 2000).

In addition to the use of complete viruses carrying additional foreign genes, another strategy uses deconstructed viruses that cannot spread systemically in the plant. The magnifection strategy, developed by Icon Genetics (now part of Bayer CropSciences), renders the systemic spread of the virus unnecessary through the use of *A. tumefaciens* as a delivery vehicle (Marillonnet et al., 2005; Gleba et al., 2005). The bacterium delivers the viral genome to so many cells that local spreading is sufficient for the entire plant to be infected. Like the infection stage, systemic spread is a limiting function, often one of the primary determinants of host range. Taking the systemic spreading function away from the virus and relying instead on the bacterium to deliver the viral genome to a large number of cells allow the same viral vector to be used in a wide range of plants. The system has been used to express antigens and antibodies at high levels in tobacco and other plants (Gleba et al., 2004).

The above systems all involve the use of whole plants as the expression platform. Even if antibody production is limited to specific tissues, such as seeds or leaves, these are harvested from the whole plant at the beginning of downstream processing. An alternative is to culture the specific organs or cells that produce the antibody and either isolate the antibody from these cells or tissues or collect it from the culture medium. A number of different culture systems have been developed, although most research has focused on cell suspension cultures. In most cases where nuclear transgenic plants have been used for the production of recombinant antibodies, the product has been extracted from plant tissues. An alternative is to attach a signal peptide to the recombinant protein, thus directing it to the secretory pathway. In this way, the protein can be recovered from the root exudates or leaf guttation fluid, processes known, respectively, as *rhizosecretion* and *phyllosecretion* (Borisjuk et al., 1999; Komarnytsky et al., 2000). Although not widely used, the secretion of recombinant antibodies into hydroponic culture medium is advantageous because no cropping or harvesting is necessary. The technology is being developed by the US biotechnology company Phytomedics Inc. A monoclonal antibody was shown to be secreted into hydroponic culture medium resulting in a yield of 11.7 μg antibody per gram of dry root mass per day (Drake et al., 2003).

Other systems are based on the culture of plant organs. Hairy roots are neoplastic structures that arise following transformation of a suitable plant host with *Agrobacterium rhizogenes*. If the plant is already transgenic, or if the transforming *A. rhizogenes* strain is transgenic, and transfers the foreign gene to the host plant during the process of transformation, then hairy root cultures can be initiated, which will produce recombinant antibodies and secrete them into the growth medium (Sharp and Doran, 2001b). Hairy roots grow rapidly and can be propagated indefinitely in liquid medium. Thus far, hairy root cultures have been used to produce a relatively small number of antibodies (Sharp and Doran, 2001a), mainly because of the relative ease with which multisubunit proteins can be produced. The cultures can be initiated from transgenic plants already carrying multiple transgenes, wild-type plants can be infected with multiple *A. rhizogenes* strains, or established hairy root cultures can be supertransformed with *A. tumefaciens*. A clonal root system based on a similar principle has been developed as a commercial platform by the Fraunhofer Center for Molecular Biotechnology in Newark, Delaware. In this case, the root system is combined with the use of viral-derived vectors for high-yield antibody expression in sealed vessels. A tissue culture system has been developed from *shooty teratomas*, which are differentiated cell cultures produced by transformation with certain strains of *A. tumefaciens* (Subroto et al., 1996). Thus far, there has been only one report of pharmaceutical protein production in teratoma cultures, and the levels of antibody were very low (Sharp and Doran, 2001a,b).

As stated above, most of the work in this area has focused on suspension cell cultures, which are individual plant cells and small aggregates thereof growing in liquid medium in a fermenter (Hellwig et al., 2004; Doran, 2006). Suspension cell cultures are usually derived from callus tissue by the disaggregation of friable callus pieces in shake bottles and are later scaled up for fermenter-based production. Recombinant

antibody production is achieved by using transgenic explants to derive the cultures, or transforming the cells after disaggregation, usually by cocultivation with *A. tumefaciens*. Suspension cultures have the same advantages as the simple plants, i.e., controlled growth conditions, batch-to-batch reproducibility, containment, and production under GMP procedures. Many foreign proteins have been expressed successfully in suspension cells, including antibodies, enzymes, cytokines, and hormones (reviewed by Hellwig et al., 2004; Fischer et al., 1999). Tobacco cultivar "Bright Yellow 2" (BY-2) is the most popular source of suspension cells for molecular farming, since these proliferate rapidly and are easy to transform. However, rice (*Oryza sativa* L.) suspension cells have also been used to produce several antibodies (e.g., Torres et al., 1999).

Recombinant antibodies expressed in plant cell suspension cultures may be secreted into the culture supernatant or retained within the cells. Localization depends on the expression construct design (see below) and the permeability of the plant cell wall to the antibody. Targeting signals included in the expression construct can be used to direct the protein to the apoplast or to retain it within intracellular compartments. The fate of antibodies targeted for secretion depends to a large extent on their size: molecules of 20–30 kDa (the size range of scFvs) will generally pass through the plant cell wall and be secreted into the culture medium, whereas larger proteins (such as IgGs) will be retarded in a size-proportional manner. The inclusion of a C-terminal KDEL sequence results in higher levels of antibody accumulation in cultured cells because the biochemical environment of the endoplasmic reticulum favors stable protein folding and assembly while reducing the level of proteolytic degradation (see below). However, this also makes it necessary to disrupt the cells in order to isolate the protein, which requires additional processing time and causes the release of phenolic molecules that interfere with purification and reduce production yield. Thus, the preferred approach is to secrete the target proteins and capture them from the culture supernatant or release them from the cells by mild enzymatic digestion.

Single-celled plants and aquatic plants can be maintained in bioreactors, offering two advantages over terrestrial plants. First, the growth conditions can be controlled precisely, which means that optimal growth conditions can be maintained, batch-to-batch product consistency improved, and the growth cycle can conform to GMP. Second, growth in bioreactors offers complete containment. Although more expensive than agricultural molecular farming, the use of simple plants in bioreactors is not as expensive as cultured animal cells because the media requirements are generally very simple. Added to this, the proteins can be secreted into the medium, which reduces the downstream processing costs and allows the product to be collected in a nondestructive manner. A final, major advantage is the speed of production. The time from transformation to first product recovery is on the scale of days to weeks because no regeneration is required, and stable producer lines can be established in weeks rather than months to years because there is no need for crossing, seed collection, and the testing of several filial generations to check transgene stability. Three major bioreactor-based systems are currently under commercial development: algae, moss, and duckweed (*Lemna* spp.).

Thus far, a single report discusses the production of monoclonal antibodies in the chloroplast of the alga *Chlamydomonas reinhardtii* (Mayfield et al., 2003). Production costs appear similar to those of recombinant proteins produced in terrestrial plants, mainly due to the inexpensive media requirements. The medium does not cost very much to start with and in any case can be recycled for algal cultures grown in continuous cycles. Aside from the economy of producing recombinant proteins in algae, there are further attributes that make algae ideal candidates for recombinant protein production. First, transgenic algae can be generated quickly, requiring only a few weeks between the generation of initial transformants and their scale-up to production volumes. Second, both the chloroplast and the nuclear genome of algae can be genetically transformed, providing scope for the production of several different proteins simultaneously. In addition, algae have the ability to be grown on various scales, ranging from a few milliliters to 500,000 l in a cost-effective manner. These attributes, and the fact that green algae fall into the GRAS (generally regarded as safe) category, make *C. reinhardtii* a particularly attractive alternative to other plants for the expression of recombinant proteins. The production technology has been reviewed recently (Franklin and Mayfield, 2005; Mayfield and Franklin, 2005).

The moss *Physcomitrella patens* is a haploid bryophyte, which can be grown in bioreactors in the same way as algae, suspension cells, and aquatic plants. Like these other systems, it has the advantages of controlled growth conditions, synthetic growth media, and the ability to secrete recombinant proteins into the medium (Decker and Reski, 2004). The unique feature of this organism, relative to all other plants, is that it is amenable to homologous recombination (Schaefer, 2002). This means that not only can it be transformed stably with new genetic information but also that endogenous genes can be disrupted by *gene targeting*. The major application of gene targeting in molecular farming is the modification of the glycosylation pathway (by knocking out enzymes that add nonhuman glycan chains to proteins), thus allowing for the production of humanized glycoproteins (Faye et al., 2005).

The *P. patens* system is being developed by the German biotechnology company Greenovation Biotech GmbH, which is based in Freiburg. The company has developed transient expression systems that allow feasibility studies and stable production strains that can be scaled up to several thousand liters. The Lemna System is based on duckweed (*Lemna minor*) and has been developed by the US biotechnology company Biolex Inc. Lemna has a number of significant advantages for the production of recombinant pharmaceutical proteins (Gasdaska et al., 2003). Unlike transgenic terrestrial plants, this aquatic plant is cultured in sealed, aseptic vessels under constant growth conditions (temperature, pH, and artificial light). Only very simple nutrients are required (water, air, and completely synthetic inorganic salts), and under these conditions, the plant proliferates vegetatively and doubles its biomass every 36 h. This provides the optimal production environment for batch-to-batch consistency. Duckweed constitutes about 30% dry weight of protein, and recombinant proteins can either be extracted from wet plant biomass or secreted into the growth medium. Biolex Inc. has reported the successful expression of tens of proteins in this system, including several recombinant antibodies and enzymes (Gasdaska et al., 2003).

3.3 Optimizing Antibody Production in Plants

The intrinsic production capacity of the chosen expression platform is a property that cannot be modified easily, because it is dependent on the overall biomass yield of the crop. However, the specific yield of recombinant protein per unit of plant biomass can be influenced by the optimization of transgene expression, which is achieved through expression construct design. Perhaps the most important component of the expression construct is the promoter used to control transcription of the transgene. For dicotyledonous species such as tobacco, potato, and tomato, the strong and constitutive *cauliflower mosaic virus 35S promoter* (*CaMV 35S*) is often chosen to drive transgene expression (Twyman et al., 2005). In cereals, the CaMV 35S promoter has a lower activity, and other promoters have been tested, such as the *maize ubiquitin-1* (*ubi-1*) *promoter* (Christensen and Quail, 1996). Some modified dicot promoters do work rather well in cereals, an example being the *pPLEX* series of constructs developed by Schunmann et al. (2003) adapted for use in monocots. The original pPLEX vectors were based on regulatory elements from subterranean clover stunt virus (SCSV). Modification was achieved by adding either the Ubi1 or Act1 introns, as well as GC-rich enhancer sequences from banana bunchy top virus (BBTV) or maize streak virus (MSV).

Regulated promoters can be used in preference to constitutive promoters to improve practicality and biosafety in addition to yields. For example, although constitutive promoters allow high-level accumulation of recombinant proteins in seeds, the proteins are also expressed in leaves, pollen, and roots. The use of seed-specific promoters largely restricts recombinant protein accumulation in the seeds, so the vegetative organs do not accumulate detectable levels of the recombinant protein. This increases the biosafety of the plants, since adventitious contact with nontarget organisms is unlikely (Commandeur et al., 2003). Among the many different seed-specific promoters that have been used (reviewed by Christou et al., 2004), the most impressive yields have been obtained with a novel seed-specific promoter from the common bean (*Phaseolus vulgaris* L.), which was used to express a single-chain antibody in *Arabidopsis thaliana* L. Heynh. In contrast to the CaMV 35S promoter, which resulted in antibody accumulation to 1% total soluble protein (TSP), the *bean arc5-I promoter* resulted in antibody levels in excess of 36% TSP in homozygous seeds, and the antibody retained its antigen-binding activity and affinity (De Jaeger et al., 2002).

The use of inducible promoters (Padidam, 2003) is also advantageous because recombinant protein synthesis can be delayed until just before harvest, or even after harvest, as is the case for the tomato hydroxy-3-methylglutaryl CoA reductase 2 (HMGR2) promoter developed by the now defunct CropTech Inc. as the MeGA promoter system (*mechanical gene activation*). The promoter used in this system is wound inducible, and gene expression is activated when the harvested tobacco leaves are shredded prior to protein extraction (Cramer et al., 1999). However, many endogenous inducible promoters show a degree of leakiness (background expression) and, in some cases, a low induction ratio. Recombinant systems such as those based on bacterial operons or animal hormones may be advantageous in these circumstances.

After promoter choice, the next most important aspect of construct design is the inclusion of sequences that control *subcellular targeting* of the protein. This is a general method to increase the yield of recombinant proteins because the compartment in which a recombinant protein accumulates influences its folding, assembly, and posttranslational modification (Ma et al., 2003; Schillberg et al., 2003). Comparative targeting experiments with full-size immunoglobulins and single-chain Fv fragments have shown that the secretory pathway is a more suitable compartment for folding and assembly than the cytosol and is therefore advantageous for high-level protein accumulation (Zimmermann et al., 1998; Schillberg et al., 1999). The endoplasmic reticulum (ER) provides an oxidizing environment and an abundance of molecular chaperones, while there are few proteases. Proteins are directed to the secretory pathway using either a heterologous or an endogenous signal peptide, located at the N-terminus of the native protein. Such proteins are cotranslationally imported into the ER and are eventually secreted into the *apoplast*, a supracellular network of interlinked compartments underlying the cell wall. Depending on its size, a protein can be retained in the cell wall matrix or can leach from the cell. Although the majority of recombinant proteins are generally more stable in the apoplast than the cytosol, they are even more stable in the ER lumen. Therefore, antibody expression levels can be increased even further if the protein is confined to the ER using an *H/KDEL C-terminal tetrapeptide tag* in addition to the signal peptide (Conrad and Fiedler, 1998). Accumulation levels are generally two- to ten-fold greater compared with an identical protein lacking the KDEL signal (Schillberg et al., 2002). As an added benefit, antibodies retrieved in this manner are not modified in the Golgi apparatus, which means they possess high-mannose glycans but not plant-specific xylose and fucose residues (Sriraman et al., 2003). Interestingly, recent experiments with antibodies expressed as fusion proteins with *elastin-like peptides* (*ELPs*) showed that the ELPs also had an impact on trafficking, albeit only in seeds. Two HIV-neutralizing antibodies expressed in tobacco seeds, namely 2F5 (Floss et al., 2008) and 2G12 (Floss et al., unpublished), were secreted into the apoplast when expressed as naked molecules bearing a KDEL tag, but initiated the formation of novel protein bodies that budded directly from the ER when expressed as ELP fusions. Aberrant antibody localization has also been reported in *Arabidopsis* seeds (Van Droogenbroeck et al., 2007).

Although the protein synthesis and folding pathways are highly conserved between plants and animals, there are some differences in the capacity for posttranslational modification. Plants do not, for example, hydroxylate proline residues in recombinant collagen. There are also various differences in glycan structure: plant-derived recombinant human glycoproteins tend to contain the carbohydrate groups $\beta(1\rightarrow2)$ xylose and $\alpha(1\rightarrow3)$ fucose, which are absent in mammals, but generally lack the terminal galactose and sialic acid residues that are found on many native human glycoproteins (Fig. 3.4). Since glycan structures can impact on the solubility, stability, immunogenicity, and biological activity of recombinant proteins, the "humanization" of glycan structures produced in plants has been an important topic of research and debate in the scientific community. There has been considerable interest in modifying the plant glycosylation pathway to humanize the glycan profile of recombinant proteins. Several changes in the pathway are required

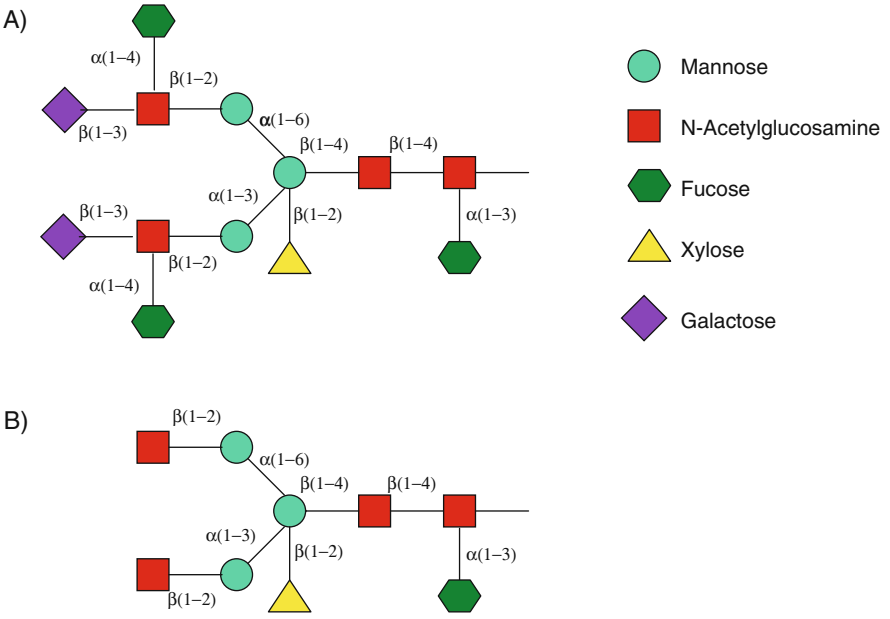

Fig. 3.4 Two glycan structures produced in plants. (**A**) Galactose-extended complex glycan. (**B**) Long-chain complex glycan. The xylose and α(1,3) fucose residues are not found in mammals

to produce proteins with typical human glycan structures (Warner, 2000; Gomord et al., 2005; Faye et al., 2005). Strategies used include the in vitro modification of plant-derived recombinant proteins by purified human β(1,4)-galactosyltransferase and sialyltansferase enzymes (Blixt et al., 2002) and the expression of human β(1,4)-galactosyltransferase in transgenic plants to produce recombinant antibodies with galactose-extended glycans (Bakker et al., 2001). In the latter case, 30% of the antibody was galactosylated, similar to the proportion found in hybridoma cells. In vivo sialylation will be more difficult to achieve because plants lack the precursors and metabolic capability to produce this carbohydrate group. A more recent report documenting sialylation in *A. thaliana* suspension cells has been challenged, although the subject remains a matter of controversy (Shah et al., 2003, 2004; Seveno et al., 2004). To remove the nonmammalian β(1→2) xylose and α(1→3) fucose residues, some researchers have explored the possibility of inhibiting the enzymes responsible for synthesizing these groups, while in one case, this goal has been achieved in whole *A. thaliana* plants by gene knockout techniques (Strasser et al., 2004). As discussed above, the moss *P. patens* can also be modified by gene targeting to eliminate these enzymes (Decker and Reski, 2004). Another approach is to prevent the glycoproteins passing through the Golgi so that only high-mannose glycans are added. This can be achieved simply by adding a KDEL C-terminal tag to the antibody, as demonstrated by Sriraman et al. (2004) and Triguero et al. (2005). This issue has been reviewed by Gomord et al. (2004).

3.3.1 Downstream Processing

Downstream processing, the isolation and purification of the recombinant product, is an integral part of every biomanufacturing process. Whichever production system is used, downstream processing represents up to 80% of overall production costs, although this depends on the required level of purity and is highest for clinical-grade materials (Drossard, 2003). In many cases, it is necessary to develop specific processing steps for each product, although certain classes of product can be isolated using a standardized approach (e.g., affinity chromatography to isolate recombinant antibodies; Stoger et al., 2005b). Several aspects of downstream processing have to be customized specifically for plant systems, including the removal of fibers, oils, and other by-products from certain crops and process optimization for the treatment of different plant species and tissues (Menkhaus et al., 2004; Nikolov and Woodard, 2004; Nikolov et al., 2009).

For the production of clinical-grade antibodies, downstream processing steps need to meet the standards that have been set for other biopharmaceutical production systems, including a strict regime of quality assurance and quality control to achieve approval by regulatory agencies (Fahrner et al., 2001). The initial stages of processing display the greatest variability and have to be optimized in a system-specific manner. Disruption of cell walls and membranes is the first postharvesting step, but different tissue types (leaves, seeds, fruits, etc.) require different forms of treatments (grinding, milling, etc.). After cell disruption, clarification of the extract is often carried out by dead-end or cross-flow filtration, sometimes preceded by bulk cell mass removal using a decanter, plate separator, or centrifuge.

Several liquid chromatography steps are required in a full purification protocol, and the initial chromatographic steps require the most specialization for plant-based production (Platis et al., 2008). In industrial processing, robust and inexpensive chromatography media are used in the initial steps, accepting that there will be some loss of selectivity and resolution (Bai and Glatz, 2003; Menkhaus and Glatz, 2005). However, important exceptions include the use of *Protein A* or *Protein G affinity chromatography* for antibody purification and the use of affinity tags and their respective capture agents (e.g., His_6 and Ni-NTA resin), which are highly selective initial capturing methods.

3.3.2 Regulatory Landscape

One of the greatest uncertainties surrounding the use of plants for the production of pharmaceuticals is the regulatory landscape. While plants are grown in glasshouses and in enclosed bioreactors, the production of pharmaceuticals is regulated in the same way as for other production systems and comes under the authority of the FDA and equivalent agencies in other parts of the world. The switch to open-field conditions adds another layer of regulatory complexity, because the transgenic plants then come under the authority of APHIS (Animal and Plant Health Inspection Service, a part of the USDA) or their counterparts in Europe and other regions. The

involvement of multiple regulatory agencies makes the production process more complex because the extent of each authority's jurisdiction is not always clear, and at the current time, only draft guidelines are available (FDA, 2002; CPMP, 2002, 2006). The impact of this is to suppress the market.

All recombinant pharmaceuticals, including those derived from plants, need to comply with the national and international GMP (good manufacturing processes) standards for product safety, quality, potency, and efficacy. However, it is not clear at which stage GMP requirements should come into effect when plants are used as the production system, since the strict rules governing defined growth conditions are difficult to implement in the field, where variables such as the weather, differences in soil quality, and the presence of other organisms need to be considered (Sparrow et al., 2007; Spök et al., 2008). This is increasingly important now that European regulatory requirements regarding GMP compliance for the manufacture of medicinal products have extended to the production of clinical trial material (Directive 2001/20/EC). Another important issue is the different regulatory structures for plant-derived pharmaceuticals and for genetically modified plants, in general, prompting calls for the harmonization of regulations internationally (Ramessar et al., 2008b).

3.4 Conclusions

The production of recombinant pharmaceuticals in plants is advantageous, theoretically offering unlimited production scales at unprecedented low manufacturing costs. We are beginning to overcome the technical limitations, such as low yields, instability, and nonauthentic glycan structures, that erect obstacles in the path toward commercialization. But more needs to be done to convince industry that plants represent a true alternative to CHO (Chinese hamster ovary) cells and bacteria. Despite the further limitations of a formative and, in some cases, restrictive regulatory framework, the potential of molecular farming can be seen in the rich IP (intellectual property) landscape and the multiple cross-licensing and collaborative ventures that are possible between companies developing production platforms, extraction, and separation technologies and those with experience in the latter stages of drug development and marketing. The welcome announcement of the first approved plant-derived veterinary vaccine may open the way for antibodies, and in particular antibodies with therapeutic and diagnostic potential, to follow.

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Chapter 4
Use of Cyanobacterial Proteins to Engineer New Crops

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Abstract Cyanobacteria, the closest living relatives of the ancient endosymbiont that gave rise to modern-day chloroplasts, offer a rich source of genes for plant genetic engineering, due to both similarities with and differences from the plant genetic systems. On the one hand, cyanobacteria share many metabolic pathways with plant cells, and especially with chloroplasts, which may be critical when the transgenic product needs to interact with endogenous systems or substrates to exert its function. On the other hand, most mechanisms involved in plant regulation of gene expression have arisen after endosymbiosis, permitting a more rational manipulation of the introduced trait, free from host regulatory networks. In addition, sequence divergence between plant genes and their cyanobacterial orthologues prevents, in most cases, the unwanted consequences of gene silencing and cosuppression. Finally, a few cyanobacterial genes involved in tolerance to environmental and/or nutritional stresses have disappeared from the plant genome during the evolutionary pathway from cyanobacteria to vascular plants, raising the possibility of recovering these adaptive advantages by introducing those lost genes into transgenic plants. In spite of their obvious potential, the use of cyanobacterial genes to engineer plants for increased productivity or stress tolerance has been relatively rare. In this chapter, we review several examples in which this approach has been applied to plant genetic engineering with considerable success. They include modification of central metabolic pathways to improve carbon assimilation and allocation by expressing unregulated cyanobacterial enzymes, development of chilling tolerance by increasing desaturation of membrane-bound fatty acids, pigment manipulation, shifts in light quality perception, production of biodegradable polymers, and synthesis of ketocarotenoids not present in crops. Tolerance to adverse environments could be achieved by the introduction of cyanobacterial genes lost from the plant genome during evolution, such as flavodoxin. The results obtained illustrate the power of gene and data mining in cyanobacterial genomes as a biotechnological tool for the

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design of transgenic plants with higher productivity, enhanced tolerance to environmental stress, and potential for biofarming.

4.1 Introduction

Development of crops with higher productivity, nutritional value, or potential for biofarming is a major goal of plant biotechnology. Direct transfer of plant genes has resulted in new varieties with improved properties. However, this approach is limited by the genetic stock of extant plant species. Therefore, the use of bacterial genes to engineer crop and model plants has become commonplace, with expression of the *Bt* toxin of *Bacillus thuringiensis* (milky spore bacterium) being the most conspicuous case of worldwide application to agriculture (for a recent review, see Jube and Borthakur, 2007). There are also limitations to the use of heterologous genes in transgenic plants, with important implications for the effectiveness of the desired manipulation. Several factors play a role in the success of this strategy, including the expression level of the transgene in the alien environment, successful interaction with suitable endogenous partners, availability of substrate if the transgene product is an enzyme, compartmentalization, and codon usage. In this sense, cyanobacteria offer special opportunities for crop improvement due to both important similarities with and differences from the plant genetic system. With respect to the former aspect, it is worth noting that many plant metabolic, regulatory, and dissipative pathways, especially those concerning chloroplast physiology, were evolved from cyanobacterial ancestors "enslaved" after the successful endosymbiosis that gave origin to photosynthetic eukaryotes. Many of these routes have not diverged much, thus allowing productive interactions of the transgenic products with the corresponding endogenous systems. At the same time, an unknown number of regulatory networks that complicate handling of transgene expression are newcomers in plant development and are not present in cyanobacteria, permitting a more customized manipulation of the engineered traits. The sequence divergence between plant proteins and their cyanobacterial counterparts also prevents in most cases the undesired consequences of gene silencing and cosuppression. Finally, a few genes of cyanobacterial origin have disappeared from the plant genome or have been profoundly modified. Their introduction into plants opens unpredictable possibilities to regain some of the adaptive advantages that allowed cyanobacteria to flourish and spread at the beginning of aerobic times on Earth.

The upcoming challenge for the scientists is to use specific genes from various sources to achieve a broad tolerance of plants to rapidly changing environmental conditions. In general, the final goal is to develop plants with higher yields or tolerant to unfavorable stress situations. It is also an important aim to generate plants with high-quality food properties or capable of producing renewable products (biofarming) (Fig. 4.1). Despite the many potential advantages of cyanobacterial genes, their use has still been relatively limited. We review herein the various existing

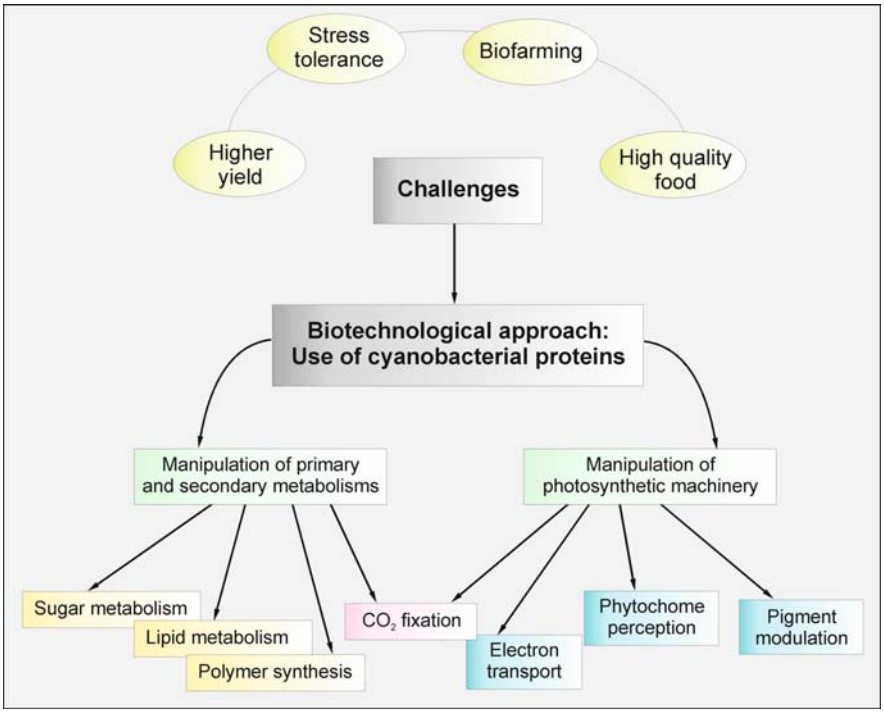

Fig. 4.1 Summary of current advances in plant biotechnology using cyanobacterial genes. Gene mining on cyanobacteria offers a promising opportunity as a tool for biotechnological approaches

examples, highlighting the cases in which their employment was particularly successful (Table 4.1).

4.2 Manipulation of CO_2 Fixation and Sugar Metabolism

Photosynthetic carbon metabolism is believed to be one major determinant for plant growth and final yield. To date, huge efforts have been made to use endogenous genes in order to modify photosynthetic carbon assimilation and partitioning with the aim to improve plant productivity (Morandini and Salamini, 2003; Geigenberger et al., 2004; Long et al., 2006). In most cases, these studies failed to provide evidence for improvement of plant biomass production, since endogenous genes derived from higher plants are likely to be prone to fine regulation *in vivo*. In this connection, the main reasons could be found in the modulation of enzyme activity by allosteric effects and covalent modification such as phosphorylation, or via intermediates and effectors such as glucose 6-phosphate (G6P) (Krause et al., 1998), suggesting that the use of cyanobacterial enzymes with different regulatory mechanisms could be a promising alternative. Attempts that have been made to manipulate photoassimilate production, sucrose metabolism, and sugar utilization will be discussed.

Table 4.1 A summary of recent publications related to the use of cyanobacterial proteins in plants to improve growth properties

Protein	Donor organism	Engineered plant	Pathway/function	Improvement/advantage	References
FBP/SBPase	*Synechococcus* PCC 7942	*Nicotiana tabacum,* Chloroplast-targeted	Carbon assimilation (Section 4.2.1) (Calvin cycle)	Avoidance of endogenous regulation Bifunctional enzyme Increased photosynthetic capacity	Miyagawa et al. (2001)
ictB	*Synechococcus* PCC 7942	*Arabidopsis thaliana Nicotiana tabacum*	Carbon assimilation (Section 4.2.1) (Calvin cycle)	Increased photosynthetic capacity and growth	Lieman-Hurwitz et al. (2003)
SPS	*Synechocystis* PCC6803	*Nicotiana tabacum, Solanum lycopersicum,* Oryza sativa	Sucrose metabolism (Section 4.2.2) (Sucrose synthesis)	Avoidance of endogenous regulation Increased sucrose synthesis	Curatti et al. (1998)
PEPC	*Corynebacterium glutamicum Synechococcus vulcanus*	*Vicia narbonensis Arabidopsis thaliana*	Sugar utilization (Section 4.2.3) (aminoacid biosynthesis)	Avoidance of endogenous regulation Increased protein content	Rolletschek et al. (2004) Chen et al. (2002)
Δ^9-desaturase	*Anacystis nidulans*	*Nicotiana tabacum* Chloroplast-Targeted	Lipid desaturation (Section 4.3)	Novel desaturase. Cold tolerance	Ishizaki-Nishizawa et al. (1996)
Acyl-lipid Δ^9-desaturase	*Synechococcus vulcanus*	*Nicotiana tabacum*	Lipid desaturation (Section 4.3)	Cold tolerance	Orlova et al. (2003)
Δ^6-desaturase	*Synechocystis*	*Nicotiana tabacum*	Lipid desaturation (Section 4.3) (γ-linolenic acid synthesis)	Polyunsaturated fatty acid synthesis	Reddy and Thomas (1996)

Table 4.1 (continued)

Protein	Donor organism	Engineered plant	Pathway/function	Improvement/advantage	References
CAO	*Prochlorothrix hollandica*	*Arabidopsis thaliana*	Pigment manipulation (Section 4.4)	Avoidance of endogenous regulation Change in pigment composition	Hirashima et al. (2006)
β-Carotene ketolase	*Synechocystis*	*Solanum tuberosum, Nicotiana glauca*	Pigment manipulation (Section 4.4) (ketocarotenoid synthesis)	Novel compounds (astaxanthin)	Gerjets and Sandmann (2006) Zhu et al. (2007)
Cyanophycin synthetase	*Thermosynechococcus elongatus*	*Nicotiana tabacum, Solanum tuberosum* (cytosol), *Nicotiana tabacum* (chloroplasts)	Production of biodegradable polymers (Section 4.5)	Biofarming. Production of cyanophycin	Neumann et al. (2005) Hühns et al. (2008)
Fd-PCB reductase	*Synechocystis* PCC6803	*Arabidopsis thaliana*	Phytochrome perception (Section 4.6) (synthesis of PCB)	Shift in phytochrome reaction spectra	Kami et al. (2004)
Fld	*Anabaena* PCC7119	*Nicotiana tabacum*	Electron transport (Section 4.7)	Multiple stress tolerance	Tognetti et al. (2006, 2007) Zurbriggen et al. (2008)
Cyt c_6	*Porphyra yezoensis*	*Arabidopsis thaliana*	Electron transport (Section 4.8)	Improved metabolism	Chida et al. (2007)
DnaE intein	*Synechocystis* PCC6803	*Arabidopsis thaliana* (cytosol and chloroplasts)	Protein splicing (Section 4.8)	Novel biotechnological tool	Yang et al. (2003) Chin et al. (2003)

4.2.1 Carbon Assimilation

The *Calvin cycle*, which is the primary route for carbon assimilation in the chloroplasts of C3 plants (Sharkey, 1985), can be divided into three phases. The first one involves carboxylation of the CO_2 acceptor molecule, ribulose-1,5-bisphosphate (RuBP) catalyzed by ribulose-1,5-bisphosphate-carboxylase/oxygenase (*Rubisco*), generating 3-phosphoglycerate (3PGA). In the second phase, the reduction step, 3PGA, is converted to triose phosphate, and finally, the regeneration phase produces the acceptor molecule RuBP for CO_2 assimilation (Fig. 4.2). The assimilates formed are used either to synthesize transitory starch in the plastids or to produce sucrose in the cytosol (Quick and Neuhaus, 1997). In order to maintain a balance between the photoassimilate export to the cytosol and the regeneration of the acceptor molecule, RuBP, an accurate regulation of the enzymes involved in the Calvin cycle is necessary. Transgenic approaches, mainly through downregulation of endogenous genes, have been performed to identify rate-limiting steps of the CO_2 fixation pathway. Decreases in the amounts of Rubisco activase, $NADP^+$-dependent glyceraldehyde-3-P dehydrogenase (GAPDH), plastidic fructose-1,6-bisphosphatase (FBPase), and aldolase have been reported (for details, see Frommer and Sonnewald, 1995). Based on the results obtained, the authors conclude that a successful modulation of metabolite distribution can only be achieved by using unregulated enzymes such as the plastidic aldolase. Following this rationale, Miyagawa et al. (2001) isolated a bifunctional unique enzyme, fructose-1,6-/sedoheptulose-1,7-bisphosphatase (FBP/SBPase), from *Synechococcus* PCC 7942 and demonstrated that it could hydrolyze both FBP and SBP with almost equal specific activities. The absence of homology between this enzyme and higher plants' FBPase and/or SBPase encouraged Miyagawa and coworkers to use it for genetic engineering. Overexpression of the cyanobacterial gene in tobacco plants (*Nicotiana tabacum*) under the control of the tomato *rbscS* promoter and chloroplast-targeting sequence led to an increased photosynthetic capacity in source leaves, carbohydrate accumulation, and accelerated growth rate (Miyagawa et al., 2001). Recently, Tamoi et al. (2006) generated transgenic tobacco plants expressing either *Synechococcus* PCC 7942 FBPase-II or *Chlamydomonas* SBPase in the chloroplasts to study the individual contribution of each enzyme. Interestingly, the same increase (1.6- to 1.7-fold) in the activities of either SBPase or FBPase resulted in different outcomes. While higher SBPase activity led to enhanced photosynthetic rates, FBPase overexpression failed to improve photosynthesis. Using antisense RNA technology, Kossmann et al. (1994) were able to show that photosynthesis and growth rate were drastically inhibited in potato (*Solanum tuberosum*) plants only when the FBPase activity was reduced below 14% of the wild-type (WT) levels. In contrast, antisense inhibition of SBPase activity strongly affected the photosynthetic pathway (Harrison et al., 1998, 2001). In plants with about 30% remaining SBPase activity, photosynthetic rates were diminished by 36%. Collected data suggest that SBPase is one of the primary limiting factors for RuBP regeneration in the Calvin cycle and that an increase in its activity causes a shift toward FBPase as a rate-limiting step of the photosynthetic carbon fixation. In conclusion, the use of

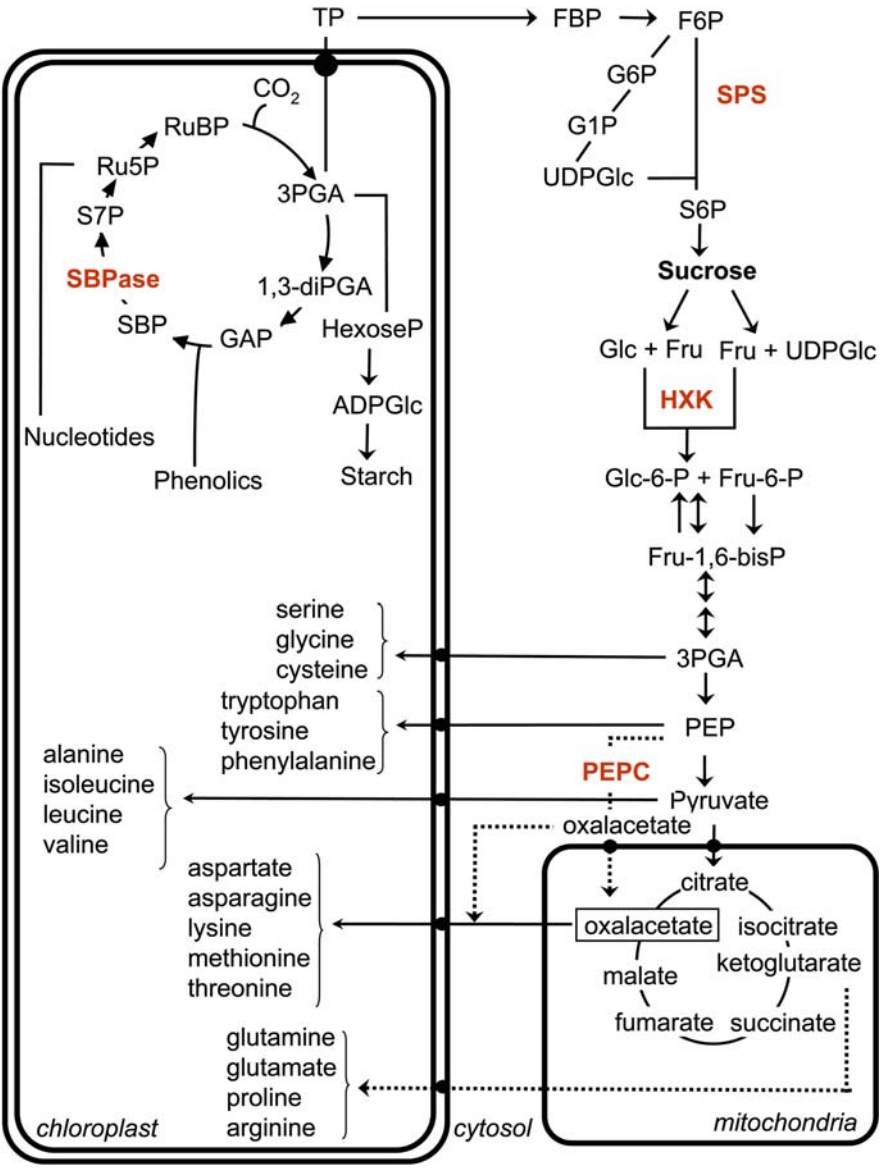

Fig. 4.2 Schematic presentation of the interaction between source and sink tissue in plants. Some crucial regulatory steps are indicated in *red*. Abbreviations: 1,3diPGA, 1,3-diphosphoglycerate; Fru, fructose; Glc, glucose; G1P, glucose 1-phosphate; GAP, glyceraldehyde 3-phosphate; Ru5P, ribulose 5-phosphate; S7P, sedoheptulose 7-phosphate. Other abbreviations are given in the text

either the unique enzyme FBP/SBPase or the single gene *SBPase* not only allows one to predict rate-limiting steps within the CO_2 fixation pathway but also leads to improvement of metabolic activity and thus to an increase in photosynthetic capacity and biomass production of plants.

In a further attempt to improve photosynthetic performance, Lieman-Hurwitz et al. (2003) expressed the *ictB* gene in *Arabidopsis* and tobacco plants. *ictB* is supposed to be involved in HCO_3^- accumulation within the cyanobacterium *Synechococcus* sp. PCC 7942. Characterization of a mutant of this strain with high CO_2 requirements revealed that the *ictB* gene is highly conserved among cyanobacteria and is probably involved in inorganic carbon accumulation (Lieman-Hurwitz et al., 2003). Transgenic *Arabidopsis thaliana* and tobacco plants expressing the *ictB* gene showed enhanced photosynthesis and growth at limiting CO_2 levels. The increased photosynthetic rate is thought to be due to a higher Rubisco activity. Similar results were also reported for the transgenic plants expressing the *Synechococcus* fructose-1,6-/sedoheptulose-1,7-bisphosphatase (FBP/SBPase) in order to increase the level of Ribulose-1,5bisP and thereby the photosynthetic rate (Miyagawa et al., 2001). Taking this possibility into account, *ictB* was shown to be a potential useful tool to enhance the yield of C3 plants, specially under specific conditions such as low humidity in which stomatal closure may lead to CO_2 limitation and thus to a retardation of growth (Lieman-Hurwitz et al., 2003).

4.2.2 Sucrose Metabolism

Photosynthetically produced assimilates are exported to the cytosol and distributed between various metabolic pathways such as glycolysis, amino acid metabolism, and sucrose biosynthesis (Fig. 4.2). Since sucrose is the preferred transport form of sugars toward sink organs in plants, its synthesis might be a rate-limiting step to establish a balanced partitioning between sucrose and starch biosynthesis. Sucrose is primarily formed from UDP-glucose (UDPGlc) and fructose-6-phosphate (F6P) followed by dephosphorylation of the resulting sucrose-6-phosphate (S6P) to yield sucrose. The first step of sucrose biosynthesis is catalyzed by sucrose-6-phosphate synthase (SPS), an enzyme modulated by several mechanisms. On the one hand, plant SPS is regulated by metabolites including G6P, which acts as an activator, and inorganic phosphate, which has an inhibitory effect. On the other hand, SPS is regulated by posttranslational modification via phosphorylation (Huber and Huber, 1992). Antisense RNA technology has been used to reduce the amount of SPS in potato plants to investigate whether it exerts a control step in sucrose synthesis (Krause et al., 1998). A 60–70% decrease in SPS activity led to a 40–50% inhibition of sucrose synthesis and to a 34–43% stimulation of starch and amino acid synthesis. Interestingly, the decrease in SPS amounts was partially compensated by an increase in the activation state of the residual protein, being about 1.4-fold higher in the best antisense plant as compared to nontransformed plants. Based on these results, Krause et al. (1998) concluded that SPS plays a crucial role in controlling

sucrose synthesis, but it is not the only step of regulation between sucrose and starch partitioning. All these observations led to the assumption that an increase of SPS might result in an enhanced rate of sucrose biosynthesis and thus to a higher final yield of plant productivity. Overexpression of maize SPS in transgenic tomato (*Solanum lycopersicum*) resulted in a three- to sevenfold increase in SPS activity (Galtier et al., 1993), while overexpression of spinach SPS led to a two- to threefold increase in SPS activity in transgenic tobacco and potato plants, respectively (Krause, 1994). A detailed investigation of the transgenic plants revealed that only a small stimulation of sucrose synthesis occurred in transgenic tobacco plants. Determination of the activation state showed that most of the excess SPS was deactivated, presumably due to posttranslational modifications. On the other hand, Curatti et al. (1998) identified and characterized a gene encoding SPS from *Synechocystis* PCC6803, whose product was insensitive to G6P and only weakly inhibited by phosphate. In addition, *Synechocystis* SPS lacks all the known phosphorylation sites found in plant SPS (Lunn et al., 1999). Expression of this nonregulated SPS in tobacco, tomato, and rice (*Oryza sativa*) under the control of the constitutive cauliflower mosaic virus 35S (CaMV 35S) promoter for tobacco and tomato, and the constitutive maize *Ubi1* promoter for rice, resulted in a two- to eightfold increase in transcript levels (Lunn et al., 1999). In spite of these high expression levels, no evidence could be found that the enzyme was active in leaf extracts (Lunn et al., 2003). Interestingly, purified *Synechocystis* SPS from transgenic tobacco and rice plants showed full catalytic activity. Based on these results, the authors proposed that *Synechocystis* SPS expressed in plants is inherently active, but it is inhibited in vivo by interacting with an endogenous plant protein. The nature of this protein, as well as the mechanism of its interaction with SPS, has not yet been elucidated.

4.2.3 Sugar Utilization

Among various attempts to engineer plants with enhanced sink capacity (see Frommer and Sonnewald, 1995), the use of genes playing a crucial role in assimilate utilization might be useful to introduce a C4-like pathway to C3 plants by genetic engineering. To this end, transgenic plants have been created using phosphoenolpyruvate carboxylase (PEPC) from *Corynebacterium glutamicum* (Rolletschek et al., 2004) or from the thermophilic cyanobacterium *Synechococcus vulcanus* (svPEPC, Chen et al., 2004). PEPC catalyzes the addition of CO_2 to PEP to produce oxalacetate, which is the direct precursor for the synthesis of amino acids such as aspartate, asparagine, threonine, methionine, and lysine (Fig. 4.2). The obvious advantage of the bacterial PEPC is that the enzyme is very stable, lacks a regulatory phosphorylation site, and does not require acetyl-coenzyme A (Ac-CoA), which usually acts as an allosteric activator (Chen et al., 2002). Furthermore, bacterial PEPC is, in contrast to plant PEPC, insensitive to feedback inhibition by malate (Chen et al., 2002; Chollet, 1996). When the *PEPC* gene from *C. glutamicum* was expressed in bean (*Vicia narbonensis*) plants, amino acid biosynthesis

was enhanced and an increase (ca. 20%) in protein content of dry seeds could be achieved (Rolletschek et al., 2004).

In a similar study using svPEPC, Chen et al. (2004) generated three different types of transgenic *Arabidopsis* plants: type-I was retarded in growth and leaf development; type-II displayed reduced leaf growth; and type-III was apparently normal. Biochemical analysis of the different plant types revealed that a switch in amino acid metabolism and growth recovery was observed by the addition of aromatic amino acids to the growth medium. Based on their results, the authors proposed that svPEPC is able to efficiently exert its activity in the plant cell environment (Chen et al., 2004).

Interestingly, cyanobacterial genes not only can be used to accelerate a specific metabolic route but could also be used to answer relevant biological questions. In this regard, Ryu et al. (2008) demonstrated that a cyanobacterial glucokinase, which has both a catalytic and a sugar-sensing activity in *Escherichia coli*, yeast, and mammals, can complement the glucose-sensing function of *Arabidopsis* hexokinase1 (HXK1). The gene encoding cyanobacterial glucokinase was overexpressed in the background of an *Arabidopsis* glucose-insensitive2 (*gin2*) mutant. This mutant lacks the normal specific physiological function of hexokinase (HXK1) in the plant glucose-signaling network. Noteworthy, the transgenic plants showed glucose-sensitive phenotypes with glucose-induced decreases of chlorophyll and transcript levels of the Rubisco small subunit (Ryu et al., 2008).

4.3 Lipid Desaturation and Cold Tolerance

Many plant species, including several important crops such as rice, maize (*Zea mays*), and soybean (*Glycine max*), are injured or killed by exposure to low non-freezing temperatures in the range of 0–15°C. Low-temperature photoinhibition is one of the major factors that limits plant productivity. It has been shown that low temperatures cause a decrease in the fluidity of biological membranes. The capability of cells to acclimate to cold is largely determined by their ability to synthesize the unsaturated fatty acids that fluidize the lipid bilayer and prevent lipids from undergoing cold-induced phase separation (Orlova et al., 2003). Polar lipids containing only saturated fatty acids display phase separations in the range of 30°C, but the presence of a single centrally positioned *cis*-double bond in the fatty acid decreases the transition temperature to about 0°C, providing the membrane lipids with enhanced molecular motions at low temperatures. Plant chloroplasts have a soluble desaturase that introduces double bonds at the Δ^9 position of saturated fatty acids linked to the acyl carrier protein (ACP) (Fukuchi-Mizutani et al., 1998; Orlova et al., 2003). It is believed that desaturation occurs largely in the chloroplast stroma by the acyl-ACP desaturase, limiting the cell's ability to respond to temperature shifts through desaturation of fatty acids already incorporated into membranes (Ishizaki-Nishizawa et al., 1996). Transformation of tobacco plants with a Δ^9-desaturase gene from *Anacystis nidulans* under the control of the CaMV 35S constitutive promoter

and a chloroplast-targeting sequence led to a significant increase in chilling tolerance (Ishizaki-Nishizawa et al., 1996). The cyanobacterial enzyme was nonspecific with respect to substrate and could use both acyl-lipids and acyl-ACP, resulting in higher levels of unsaturated fatty acids in most membrane lipids (Ishizaki-Nishizawa et al., 1996). Similar results have been obtained in tobacco plants transformed with an acyl-lipid desaturase gene from *S. vulcanus* (Orlova et al., 2003).

Lipid desaturation is also related to attempts to produce seed oils rich in essential fatty acids, making them nutritionally superior (Reddy and Thomas, 1996). Triunsaturated γ-linolenic acid (GLA), for instance, is important in human and animal diets, and consumption of vegetable oils containing GLA is thought to alleviate hypercholesterolemia and other related clinical disorders that correlate with susceptibility to coronary heart disease (Brenner, 1976). GLA does not accumulate in oilseed crops and can only be found in a few plant species such as evening primrose (*Oenothera biennis*), currant (*Ribes* spp.), and borage (*Borago officinalis*) (Reddy and Thomas, 1996). Cyanobacteria, instead, have a Δ^6-desaturase that catalyzes the synthesis of GLA from linoleic acid (Reddy et al., 1993). Transformation of tobacco seedlings with the Δ^6-desaturase gene from *Synechocystis* under the control of the CaMV 35S promoter generated transgenic plants with significant amounts of GLA in their leaves, irrespective of whether the foreign enzyme was targeted to chloroplasts, to the cytosol, or to the endoplasmic reticulum (Reddy and Thomas, 1996). Moreover, all lines produced even higher levels of octadecatetraenoic acid, a tetraunsaturated fatty acid not present in plants that has numerous industrial uses, including the production of oil films, special waxes, and plastics (Reddy and Thomas, 1996).

4.4 Pigment Manipulation

The organization of pigment molecules in photosystems is strictly determined. The peripheral antenna complexes may contain chlorophyll *a* and *b*, and even other types of pigments depending on the organism. But the core antennae of virtually all organisms displaying oxygenic photosynthesis admit only chlorophyll *a* and β-carotene. The diverse pigment composition of peripheral antennae is a beneficial feature that enables plants to absorb multiple wavelengths from the broad range of the light spectrum that is available for photosynthesis (Fromme et al., 2001). In contrast, the pigment and protein compositions of the core antennae do not change under any environmental conditions that have been tested. The reasons for this strict discrimination have been attributed to a regulatory domain of chlorophyllide *a* oxygenase (CAO), the enzyme responsible for chlorophyll *b* synthesis, which modulates the levels of this pigment. Cyanobacterial genes have been employed to evaluate this tenet by transforming *Arabidopsis* plants with a CAO gene from *Prochlorothrix hollandica*, which lacks the regulatory domain. About 40% of chlorophyll *a* in the core antenna complexes of the transformants could be replaced by chlorophyll *b* with concomitant changes in the photosynthetic action spectrum (Hirashima et al., 2006). Transgenic plants were able to grow like the wild type under low light intensity conditions

(80 μmol photons m^{-2} s^{-1}) but underwent severe damage at the level of photosystem II at higher irradiations ranging from 300 to 1,000 μmol photons m^{-2} s^{-1} (Hirashima et al., 2006).

Carotenoids constitute a vast group of lipophilic pigments synthesized by microorganisms and plants, in which they participate in light capture and photoprotection. Typical carotenoids contain 8 isoprenoid units (40 carbon atoms) and an extended conjugated polyene system, which may carry hydroxyl, epoxy, or keto groups. The ketocarotenoids, one type of carotenoids, are especially light stable and display high antioxidant capacities (Guerin et al., 2003; Higuera-Ciapara et al., 2006). They impart a distinct reddish color to tissues that accumulate them, such as the flesh of salmon and crustaceans, and their antioxidant effects are of particular interest in the food, nutraceutical, and aquaculture industries. Recent research has demonstrated their anticancer and antibacterial properties, as well as potential benefits in boosting the immune system and preventing cardiovascular disease, cataracts, and tissue damage from ultraviolet radiation (Guerin et al., 2003; Higuera-Ciapara et al., 2006).

Astaxanthin is one of the most important commercial ketocarotenoids derived from β-carotene by 3-hydroxylation and 4-ketolation at both ionone end-groups (Sandmann, 2001). Most of its demand is met by chemical synthesis; yet, natural sources are becoming more important (Guerin et al., 2003). The hydroxylation reaction is widespread in many organisms, but ketolation is restricted to a few bacteria (including cyanobacteria), fungi, and unicellular green algae. Plants are devoid of ketocarotenoids, but a cyanobacterial ketolase gene has been introduced in both potato tubers (Gerjets and Sandmann, 2006) and tobacco (*Nicotiana glauca*) flowers and leaves (Zhu et al., 2007). In the first case, plants were transformed with a *Synechocystis* β-carotene ketolase gene, *crtO*, and ketocarotenoids represented 10–12% of total carotenoids in leaves and tubers of the transformants (Gerjets and Sandmann, 2006). In the second case, the same gene was introduced in *N. glauca*, a species containing highly carotenogenic flowers, potentially representing new sources of ketocarotenoids. Upon transformation, high levels of ketocarotenoids were found in all flower parts and leaves, with no concomitant decrease in carotenoid contents accounting for an upregulation of total carotenoid quantities (Zhu et al., 2007).

4.5 Production of Biodegradable Polymers

Plants are being widely used as bioreactors for the industrial production of bioactive peptides, vaccines, hormones, antibodies, and other proteins (Fischer et al., 2004; Gomord et al., 2005; Hellwig et al., 2004; Twyman et al., 2003). *Biopharming* is also an environmentally acceptable and competitive way of producing several chemical compounds used as raw material for the pharmaceutical and chemical industries. An increasingly important challenge is the manufacture of biodegradable polymers in transgenic plants, such as polyamino acids, to replace petrochemical compounds,

which tend to become expensive and scarce (Neumann et al., 2005). Among them, polyaspartate is a soluble, nontoxic and biodegradable polycarboxylate widely used in many industrial, agricultural, and medical applications (Oppermann-Sanio and Steinbüchel, 2002). Polyaspartate is the backbone of the cyanobacterial carbon and nitrogen storage material cyanophycin, a zwitterionic copolymer of L-aspartic acid and L-arginine. It is produced via nonribosomal polypeptide biosynthesis by the enzyme cyanophycin synthetase, encoded by the *cphA* gene, which is present in many cyanobacterial and some noncyanobacterial eubacteria (Hühns et al., 2008; Krehenbrink et al., 2002; Ziegler et al., 2002). Cyanobacterial cyanophycin is polydisperse (25–125 kDa), water insoluble, and stored in granules without membranes.

No organism produces polyaspartate; consequently, its industrial production has relied either on chemical synthesis or on the hydrolysis of purified cyanophycin obtained from cyanobacteria, after expensive and resource-consuming growth and harvest of the microorganisms. Lately, a highly water-soluble polymer similar to cyanophycin has been produced in *E. coli* cells expressing a cyanophycin synthetase from *Desulfitobacterium hafniense* (Ziegler et al., 2002). Nevertheless, the need for cost-intensive bioreactors reduces the cost-effectiveness of this production procedure (Neumann et al., 2005).

Neumann et al. (2005) succeeded in producing cyanophycin in transgenic *N. tabacum* plants expressing the coding region of the *chpA* gene of *Thermosynechococcus elongatus* BP-1 in the cytosol under the control of the CaMV 35S promoter. The transgenic tobacco plants were found to produce up to 1.1% dry weight of both a water-soluble and a water-insoluble form of the polymer of size, composition, and structure very similar to those of the cyanobacterial cyanophycin. Afterward, they used the same technology in order to develop transgenic potato (*S. tuberosum*) plants with the aim of synthesizing cyanophycin in tubers. Harvesting of the polymer from the residues of starch isolation would conform to a high yield and a cost-effective method. However, the authors obtained a decreased content of cyanophycin in leaves (0.24% dry weight) in comparison to tobacco and could only demonstrate the presence of cyanophycin in tubers by electron microscopy. For both species, the resulting transgenic plants exhibited a decelerated growth rate, variegated leaves, and changes in chloroplast morphology. These undesired consequences could be related to exhaustion of the amino acid resources of the plant due to cyanophycin production or to the presence of cyanophycin aggregates in the cytoplasm, which could interfere with the normal metabolism of this compartment (Neumann et al., 2005). To overcome these limitations, and at the same time to increase polymer accumulation, Hühns et al. (2008) generated tobacco transgenic plants in which the gene of the cyanophycin synthetase was fused in-frame to a chloroplast-targeting sequence in order to direct the enzyme to this organelle. The resulting plants were able to produce 6.8% dry weight cyanophycin together with reduced stress symptoms. Achievement of higher polymer accumulation in chloroplasts than in cytoplasm could be due to the similitude of plastids with cyanobacteria, in which cyanophycin is synthesized naturally without causing any deleterious effects. What is more, the building blocks of cyanophycin, i.e., L-arginine and L-aspartate, are directly available in chloroplasts because

the synthesizing enzymes are located in this compartment (Hühns et al., 2008; Chen et al., 2006). Transgenic plants expressing specific cyanobacterial enzymes catalyzing new reactions could be utilized to produce renewable resources. In this example, plant-produced cyanophycin could provide for a nonexpensive and environment friendly production of polyaspartate, which could be a most likely biodegradable substitute for polycarboxylates and polyacrylates for the industry.

4.6 Phytochrome Perception and Plant Development

Light quality, quantity, and duration influence nearly every stage of plant growth and development. In vascular plants, red (R) and far-red (FR) lights are sensed primarily by the phytochrome family of photoreceptors (Casal et al., 2003). The covalently bound phytochromobilin (PΦB) prosthetic group is required for the diverse activities of all members of the family. Mutant lines that are unable to produce PΦB display aberrant photomorphogenesis with pleiotropic phenotypes that are most pronounced under R and FR illumination. Interestingly, green algal and cyanobacterial phytochromes employ the more reduced linear tetrapyrrole phycocyanobilin (PCB), which displays a slightly different action spectrum (Frankenberg et al., 2001). The difference is based on the existence of a distinct stock of enzymes in the two types of organisms: a PΦB synthase in plants that converts biliverdin into PΦB and a ferredoxin (Fd)-PCB reductase in algae and cyanobacteria that yields PCB as end-product.

To determine if PCB could be assembled in plant phytochromes and as a result to change the light quality responses of plants, Kami et al. (2004) introduced the Fd-PCB reductase gene of *Synechocystis* PCC6803 into an *Arabidopsis* mutant line that lacked PΦB synthase activity and was therefore unable to synthesize the normal phytochrome chromophore. The resulting transformants restored phytochrome activities to WT levels, albeit with blue-shifted absorption maxima. Expression of the cyanobacterial enzyme rescued phytochrome-mediated R and FR responses, and only the high-irradiance FR response was shifted to shorter wavelengths (Kami et al., 2004). This result indicates that PCB can function in vascular plants. It also allows dissection of functional features in the chromophore molecule.

4.7 The Case for the Lost Genes: Flavodoxin and Multiple Stress Tolerance

Environmental adversities such as drought and extreme temperatures, exposure to human-produced chemicals, and nutrient-poor soils usually affect plants growing in natural habitats (Vij and Tyagi, 2007). Among nutritional deficits, iron deprivation ranks at the top, as it is required for the function of a great number of metalloenzymes that are central to plant energetics and metabolism. Iron limitation is especially critical in the widespread alkaline calcareous soils where its bioavailability

is highly restricted (Guerinot, 2007; Kim and Guerinot, 2007). These factors place major limits on plant growth and yield, and they account for much of the extensive losses to agricultural production worldwide (Boyer, 1982). To overcome these limitations and to improve production efficiency in the face of a world with increasing food demands, more and better stress-tolerant crops must be developed.

Plant adaptation to environmental stresses is dependent upon the activation of cascades of molecular networks involved in stress perception, signal transduction, and the expression of specific stress-related genes and metabolites. Therefore, responses to abiotic stresses are multigenic and thus are difficult to control and engineer (Vinocur and Altman, 2005). Past efforts to improve plant stress tolerance through breeding and genetic engineering have had limited success precisely due to this genetic complexity (Cushman and Bohnert, 2000). In addition, many projects involving manipulation of endogenous plant genes faced intrinsic limitations such as cosuppression and misregulatory phenomena. One approach that has not yet been explored to any great extent is to take advantage of the tools available from plant ancestors, namely the cyanobacteria.

Ferredoxin (Fd) is an iron–sulfur protein present in all photosynthetic organisms ranging from cyanobacteria to plants. It is the final electron acceptor of the *photosynthetic electron transport chain* (*PETC*) and is essential for the distribution of low-potential reducing equivalents to central metabolisms like CO_2 fixation, nitrogen and sulfur assimilation, amino acid synthesis, fatty acid desaturation, as well as many regulatory (e.g., thioredoxin (Trx) redox regulation system) and dissipatory pathways (Fig. 4.3, see Hase et al., 2006). Fd levels experience a considerable decrease in response to environmental stresses and other sources of *reactive oxygen species* (*ROS*) production as a consequence of tight transcriptional and/or post-transcriptional regulatory systems operating under these conditions (Singh et al., 2004; Zimmermann et al., 2004). Likewise, iron deficiency also leads to diminished Fd levels. This affects central metabolisms as well as defense and regulatory mechanisms, thus compromising cell survival (Fig. 4.3, see Thimm et al., 2001; Erdner et al., 1999). Photosynthetic microorganisms like cyanobacteria and certain algae deploy an adaptive response meant to tackle Fd decrease upon stress by synthesizing an isofunctional electron carrier, flavodoxin (Fld). Fld contains flavin mononucleotide instead of iron as prosthetic group, is resistant to ROS inactivation, and is able to engage in most Fd reactions, albeit with somehow less efficiency. Fd substitution results in the restoration of electron delivery to productive pathways, therefore preventing misrouting of reducing equivalents to O_2 and the concomitant ROS production. The net outcome is augmented tolerance toward various sources of stress in algae and cyanobacteria (Erdner et al., 1999; Singh et al., 2004; Palenik et al., 2006). As a matter of fact, Fld induction has been used for many years as a reliable marker of iron deficiency in the oceans and constitutes a key selective advantage for colonization of iron-poor waters by phytoplankton (Erdner et al., 1999). Fld is absent in the plant genomes; it was lost somewhere in the evolutionary transition from green algae to vascular plants, rendering the latter unable to put into use such an efficient adaptive mechanism of defense (Zurbriggen et al., 2007). Nevertheless, some plant enzymes, whose cyanobacterial

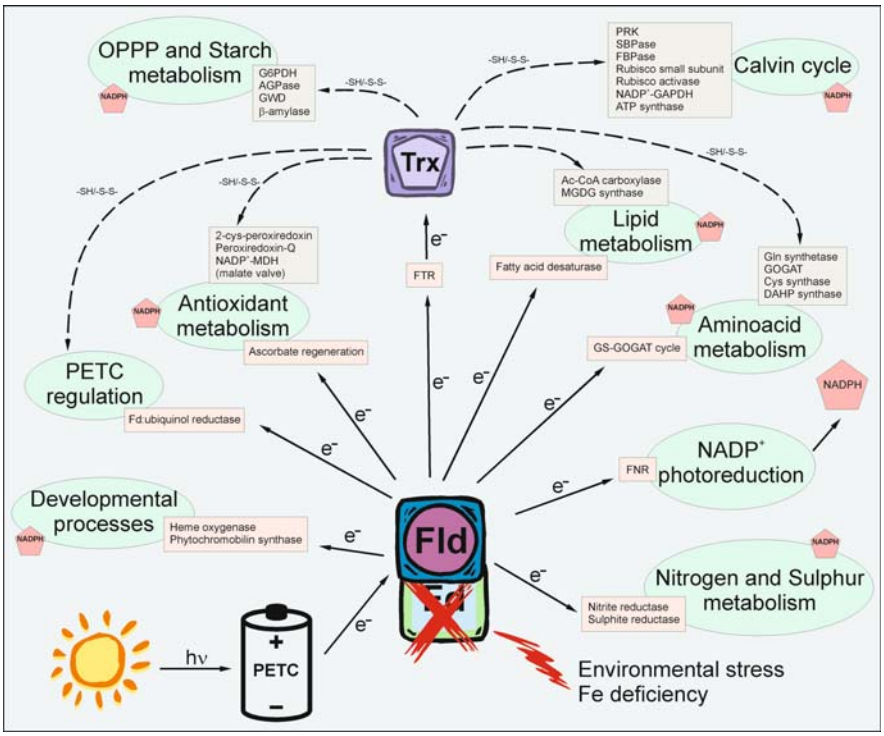

Fig. 4.3 Cyanobacterial Fld is able to substitute for chloroplast Fd functions. Chloroplast Fd plays a central role in the distribution of reducing equivalents generated during photosynthesis. Electrons originating in the PETC may be transferred via Fd to FNR for $NADP^+$ photoreduction, generating the NADPH necessary for the Calvin cycle and other biosynthetic and protective pathways. Reduced Fd is also the electron donor for nitrite and sulfite assimilation via nitrite and sulfite reductases, for fatty acid desaturation by fatty acid desaturase, and for glutamate synthesis mediated by glutamate–oxoglutarate aminotransferase (GS-GOGAT). Still other Fd molecules will participate in Trx reduction via Fd–Trx reductase (FTR). Reduced Trx will then activate key target enzymes through reduction of their critical cysteines (–SH/–S–S– exchange), resulting in the maintenance and/or stimulation of the Calvin cycle, the malate valve process, and other metabolic routes. Dissipative systems requiring Fd include regeneration of active peroxiredoxins, the most abundant peroxidase of chloroplasts, and of ascorbate. Fd also regulates the distribution of reducing equivalents between lineal and cyclic electron flow via Fd-ubiquinol reductase. Finally, it participates in developmental processes through the synthesis of phytochromobilin, the chromophore of the light sensor phytochrome, by donating electrons to two key enzymes of the pathway: heme oxygenase and phytochromobilin synthase. On exposure to iron-deficit or adverse environments, Fd levels are downregulated and the foreign Fld is proposed to take over electron distribution to Fd redox partners in chloroplasts. Abbreviations: Ac-CoA, acetyl-coenzyme A; AGPase, ADP-glucose pyrophosphorylase; DAHP, 3-deoxy-D-arabino-heptulosonate 7-phosphate; G6PDH, glucose 6-phosphate dehydrogenase; GWD, α-glucan water dikinase; MDH, malate dehydrogenase; MGDG, monogalactosyldiacylglycerol synthase; OPPP, oxidative pentose phosphate pathway; PRK, phosphoribulokinase. Other abbreviations are given in the text. Adapted from Zurbriggen et al. (2008)

counterparts used Fld as substrate (including Fd-$NADP^+$ reductase (FNR) and Fd-Trx reductase), are still able to engage with the flavoprotein in the electron transfer reactions normally performed by Fd (Fig. 4.3) (Tognetti et al., 2008; Zurbriggen et al., 2008). These observations triggered the obvious inquiry to evaluate if Fld introduction into plants could improve tolerance to abiotic stress and iron deficiency, as it occurs in microorganisms. Transgenic tobacco plants constitutively expressing the Fld gene from *Anabaena* under the control of the CaMV 35S promoter were thus engineered. A plastid-targeting sequence was fused in-frame to the Fld gene to direct the protein to chloroplasts (*pfld* lines). Several independent transformed lines were therefore obtained and selected for Fld expression levels. It is worth noting that transgenic plants from the different lines grown under normal conditions exhibited no significant phenotypic differences in relation to WT individuals with respect to growth rate, flowering time, and seed production (Tognetti et al., 2006).

Plants expressing 60 μM Fld (a level similar to endogenous Fd) were able to survive and even to increase in height, fresh weight, and dry weight when subjected to iron-deficiency protocols, whereas WT specimens exhibited the typical symptoms of this nutrient scarcity such as interveinal chlorosis, growth retardation, and high lethality rates. The transgenic plants failed to improve iron uptake or accretion and even developed a normal response to iron shortage, including induction of different genes involved in metal uptake, mobilization, and storage (Tognetti et al., 2007). Fld expression prevented the general decrease in CO_2 fixation capacity and downregulation of metabolic activities manifested by the nontransformed plants. Moreover, it preserved the activation state of key plastidic enzymes that depend on the Fd–Trx system like phosphoribulokinase (PRK) and FBPase. As a result, the levels of many central metabolites belonging to the Calvin cycle, energy storage, and anabolic routes, as well as the contents of most amino acids, were significantly higher in the transformants (Tognetti et al., 2007). Taken together, the results indicate that Fld expression could compensate for Fd decline occurring upon iron deficiency by successfully engaging in at least some of the Fd-dependent pathways of plant chloroplasts. It is tempting to consider that reallocation of available iron to other demanding routes probably contributes to the general welfare of the iron-starved *pfld* plants.

Plants expressing Fld in plastids were also able to withstand a remarkable range of environmental stresses, including high temperature, chilling, drought, high light intensities, UV radiation, and exposure to the redox-cycling herbicide methyl viologen (MV), all having as a common feature ROS buildup and the establishment of an oxidative stress condition (Tognetti et al., 2006). These stresses cause Fd downregulation independently of a general protein content breakdown, leading to potential malfunction of the electron transport pathways and systems dependent on the iron–sulfur protein. Tolerance was evidenced in the transformants by preservation of leaf turgor, cellular and membrane integrity, and plastid ultrastructure. Fld expression also preserved photosynthetic capacity, thus maintaining high levels of CO_2 fixation. Buildup of various ROS was impaired and there was little photooxidative damage to PETC components. These observations have direct implications for the

antioxidant protection provided by Fld. Its involvement in $NADP^+$ photoreduction and Trx reduction helps to relieve the electron pressure imposed onto the PETC by the stress condition. The latter function is crucial, as maintenance of high levels of reduced Trx in the transgenic plants favors dissipative and scavenging pathways, e.g., Prx-reductive regeneration to eliminate H_2O_2 and organic peroxides produced under stress, export of the excess of reducing power via the malate valve, and productive consumption of the surplus of NADPH by the Calvin cycle. Finally, as is the case with iron deficiency, the extent of protection conferred by the flavoprotein is strictly dose dependent, with low-expressing lines displaying WT levels of tolerance (Tognetti et al., 2006).

Fld expression thus restores electron transfer to productive routes, ameliorating the damage suffered by stressed (or iron-starved) plants caused by faulty electron distribution within plastids and cells, leading to a healthy physiological condition (Fig. 4.3, see Zurbriggen et al., 2008). It prevents an excessive reduction of the PETC, which would result in ROS accumulation and impairment of key metabolic, regulatory, and dissipatory pathways. Transfer of this technology to crops is still at a preliminary stage, but increased tolerance to MV, water deprivation, and/or UV irradiation has already been observed in tomato, potato, *Brassica*, barley, and maize lines, as reflected by lower damage to cells and tissues, higher chlorophyll levels and growth rates, and in some cases, higher seed yield (Zurbriggen et al., 2008). It is clear, then, that the expression of a single gene from a photosynthetic prokaryote can be used as a general technology to improve plant productivity and to utilize otherwise vast nonproductive agricultural areas.

4.8 The Case for the Lost Genes: Cytochrome c_6, Intein

Photosynthetic organisms possess two membrane-embedded multiprotein complexes, photosystems I and II, which mediate light energy conversion into electrochemical energy in the form of low potential electrons. They are connected by a series of electron carriers, namely plastoquinone, a cytochrome (Cyt) b_6f complex, and a soluble metalloprotein. The last-mentioned component can be either the heme-containing protein, Cyt c_6, and/or the copper protein, plastocyanin (PC), depending on the organism. For instance, some cyanobacteria only contain the Cyt c_6 gene, whereas others (as well as some algae) are able to synthesize both, depending on metal bioavailability. Plants, on the other hand, possess only PC (De la Rosa et al., 2002). At the times when the first oxygen-evolving photosynthetic organisms arose, the prevailing atmospheric conditions were highly reducing, favoring iron over copper bioavailability. Thus, in an evolutionary context, it seems clear why an iron-containing electron carrier, Cyt c_6, evolved initially. Later on, in the Precambrian, photosynthesis-derived oxygen started to accumulate slowly, turning the tables: now copper availability grew, whereas iron started to be scarce. PC appeared as a substitute for Cyt c_6, providing microorganisms

with an adaptive response toward nutrient deficiency analogous to the Fld/Fd system (see Section 4.7). The coexistence of both transporters in some cyanobacteria and algae conveys metabolic adaptability to the extremely changing environments they face living in seas, lakes, and rivers (De la Rosa et al., 2002). Plants lack this adaptive versatility, as Cyt c_6 was evolutionarily eliminated from their chloroplasts. However, the introduction in *A. thaliana* of a Cyt c_6 gene from *Porphyra yezoensis*, a red alga, fused to a luminal targeting sequence has led to the creation of transgenic lines exhibiting enhanced growth (height, root, and leaf length) and to an increase in the efficiency of photosynthetic electron transfer and CO_2 fixation rates. In this connection, the amounts of some energy-related metabolites such as NADPH, ATP, and storage sugars were higher in the transgenic plants than in WT siblings, suggesting an explanation for the improved growth of these plants (Chida et al., 2007).

Inteins are sequences within a protein that mediate posttranslational protein splicing. The intein element in a protein precursor catalyzes a series of reactions to remove itself from the precursor and ligate the flanking external protein fragments ("exteins") into a mature protein (Perler, 1998). The first and only naturally split intein identified so far is the DnaE intein of *Synechocystis* PCC6803. Many sequences potentially coding for inteins have been found in cyanobacteria, but none in plants. Inteins can be split into an N-terminal part and a C-terminal portion, which when fused to different polypeptides are able to perform a trans-splicing reaction assembly of the two separate precursors into a mature hybrid molecule, both in vivo (assayed on *E. coli* cells) and in vitro (Yang et al., 2003, and references therein). Yang et al. (2003) used the DnaE intein to reassemble the divided fragments (which would be the exteins) of β-glucuronidase (GUS) in transgenic *Arabidopsis* plants. The trans-splicing reaction resulted in a full-length GUS protein with catalytic activity, indicating accurate ligation and refolding of the enzyme throughout the entire plant without leaving any footprint. Chin et al. (2003) extended this approach to chloroplasts using the naturally split DnaE intein in which both intein fragments were incorporated into the chloroplast genome or separately in the chloroplast and nuclear genomes. As far as intein expression and excision in chloroplasts is concerned, the *aadA* gene and a soluble version of modified GFP (*gfp*) were ligated to sequences coding for the N- and C-terminal residues of the DnaE intein, respectively, and a chimeric polypeptide AAD-smGFP (soluble modified GFP) assembled. The strategy was further refined with respect to transgene containment by splitting the herbicide resistance gene 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) into two halves and integrating them separately into nuclear and chloroplasts genomes after ligating to coding sequences for split inteins. The functional, full-size EPSPS gene product was reconstituted either by crossing tobacco plants carrying split genes or by retransforming nuclear-transformed plants with a chloroplast transformation construct carrying C-terminal residues of both EPSPS gene and intein (Chin et al., 2003). These findings demonstrated intein usage in plant organelles and also explored the possibility of intein-mediated protein trans-splicing for limiting the environmental impact of herbicide resistance, by separating parts of the gene in plastid and nuclear

genomes while assembling the mature gene product in the stroma of chloroplasts (Khan et al., 2005).

This technology opens up important biotechnological applications. Thus, it might be possible to construct intein–extein fusions under the control of chemically inducible or tissue-specific promoters in order to perform the reconstitution of protein trans-splicing in plants. They could then be used as a *molecular switch* to turn on a gene expression mechanism or a metabolic pathway through reassembly of gene regulators or enzymes. In order to diminish the environmental impact of some transgenic products, different traits can be stacked in parental plants and brought together upon crossing. As explained before, it could also be possible to take advantage of the potential for trans-splicing of transgenes using inteins in conjunction with plastid engineering to provide for a more effective transgene containment strategy to yield transformed plants with greatly reduced risk of genetic outcrossing.

4.9 Concluding Remarks

Cyanobacterial genes display both important similarities with and differences from plant genes, and both could be exploited to improve plant productivity and stress tolerance by means of genetic engineering. Compared to other prokaryotes, many biochemical and physiological pathways from cyanobacteria, especially those related to chloroplast function, have been retained in plants. Therefore, transgenic cyanobacterial products could interact productively with plant routes and substrates, a condition that may be critical in an important number of cases. On the other hand, sequence divergence between cyanobacterial genes and their plant homologues precludes, in most situations, the unwanted consequences of silencing and cosuppression. Moreover, plants have evolved novel regulatory networks that are not present in cyanobacteria. Expression of a cyanobacterial gene product instead of overexpression of a plant counterpart can circumvent these endogenous regulatory constrains, thus allowing for a more customized manipulation of the introduced trait. Finally, a few cyanobacterial genes related to adaptive value for survival in hostile environments have been lost somewhere in the evolution of vascular plants. The case of Fld and Cyt c_6 shows that reintroduction of these genes in the proper subcellular compartment of model and crop plants restored some of the selective advantages that allowed photosynthetic microorganisms to thrive in hostile habitats. The examples described in this chapter illustrate the potential of gene and data mining in cyanobacterial genomes and physiology as a biotechnological tool for the generation of crops with increased yield and performance in the field, which are needed to feed an increasing world population. In addition, it shows the contribution of this strategy to the development of plants with biofarming potential in the frame of a high demand of natural and ecologically accepted sources of renewable compounds and materials.

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Chapter 5
Molecular Biology of Secondary Metabolism: Case Study for *Glycyrrhiza* Plants

Hiroaki Hayashi

Abstract Licorice (roots and stolons of *Glycyrrhiza* plants) is one of the most important crude drugs from ancient times, and its major constituent is an oleanane-type triterpene saponin, glycyrrhizin, which is a well-known sweetener as well as a pharmaceutical. We are using *Glycyrrhiza glabra* (common licorice) as a model plant to elucidate the regulation of triterpene biosynthesis in higher plants. Cultured cells of *G.glabra* do not produce glycyrrhizin but produce two structurally different triterpenoid constituents, namely betulinic acid and soyasaponins. Glycyrrhizin is localized exclusively in the woody parts of thickened roots, whereas soyasaponins are localized mainly in the seeds and rootlets. Betulinic acid, a lupane-type triterpene, is localized in the cork layer of the thickened roots. The cultured licorice cells converted exogenously administered glycyrrhetinic acid, the aglycone of glycyrrhizin, into seven biotransformation products, but formation of glycyrrhizin was not detected among the biotransformation products. To elucidate the regulation of the triterpene biosyntheses in *G.glabra*, cDNAs of squalene synthase and three oxidosqualene cyclaces were cloned and characterized. mRNA levels of these enzymes were differently regulated in the cultured cells and intact plants of *G.glabra*. Exogenously applied methyl jasmonate (MeJA) stimulated soyasaponin biosynthesis in cultured cells, and mRNA levels of squalene synthase and β-amyrin synthase were upregulated by MeJA.

5.1 Introduction

Roots and stolons of *Glycyrrhiza* plants (Licorice) are important crude drugs from ancient times, and the name *Glycyrrhiza* comes from Greek words meaning "sweet root" (Nieman, 1959; Gibson, 1978; Shibata, 2000). The sweet constituent in licorice is an oleanane-type triterpene saponin, glycyrrhizin, which is used in large quantities as a well-known natural sweetener as well as a pharmaceu-

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tical. Commercial licorice is derived from three *Glycyrrhiza* species, *G. glabra* L., *G. uralensis*, FISCH., and *G. inflata* BATAL. in the family Fabaceae, which are indigenous to Asia and the Mediterranean region (Shibata, 2000). *G. glabra* is found in South Europe, Turkey, Iran, Central Asia, and the northwestern part of China, and *G. uralensis* is found in Central Asia, Mongolia, and northwestern and northeastern parts of China. *G. inflata* is found only in the northeastern part of China.

Higher plants produce diverse triterpenes and triterpene saponins, which are of economical importance as drugs, detergents, and cosmetics. Although structural elucidation of triterpenes and triterpene saponins has been extensively studied (Mahato et al., 1988, 1992), our understanding about the regulation of their biosyntheses is quite limited (Chappell, 1995; Haralampidis et al., 2002; Jenner et al., 2005). We are using *G. glabra* (common licorice) as a model plant to elucidate the regulation of triterpene biosynthesis in higher plants. In this review, biosynthesis of various triterpene constituents in the intact plant and in cultured cells of *Glycyrrhiza* plants will be discussed.

5.2 Triterpene Saponins Isolated from *Glycyrrhiza* Plants

The major sweet-tasting triterpene saponin in roots and stolons of *Glycyrrhiza* plants is glycyrrhizin, which has the sweetness of about 200 times greater than that of sucrose. Glycyrrhizin is a conjugate of two molecules of glucuronic acid and glycyrrhetinic acid, an oleanane-type triterpene (Fig. 5.1). Glycyrrhizin is used in sweet foods to enrich the sweet taste. In addition, glycyrrhizin is used in salty foods to reduce the saline taste of foods, such as soy sauce and sausage, in Japan. Glycyrrhizin is also an active ingredient of *Glycyrrhiza* radix (licorice), which is the most frequently used component of the Chinese and Japanese traditional medicines (Shibata, 2000). Furthermore, glycyrrhizin is a pharmaceutical used in treatments of liver diseases and allergic diseases as an injectable preparation (Stronger Neo-Minophagen®) as well as a tablet (Glycyron®) in Japan, China, Korea, Indonesia, India, and Mongolia(http://www.minophagen.co.jp/en/index.html).

Chemical constituents of *Glycyrrhiza* plants have been extensively studied to isolate not only glycyrrhizin but also many triterpene saponins (Kitagawa et al., 1993a,b,c; Nomura and Fukai, 1998). Structures of these minor saponins, licorice-saponin B2 (11-deoxoglycyrrhizin), licorice-saponin C2, licorice-saponin E2, licorice-saponin G2, and licorice-saponin H2, isolated from licorice roots are shown in Fig. 5.1. Contents of these saponins in licorice roots vary among various *Glycyrrhiza*plants of different geographic origins (Kitagawa et al.,1998).

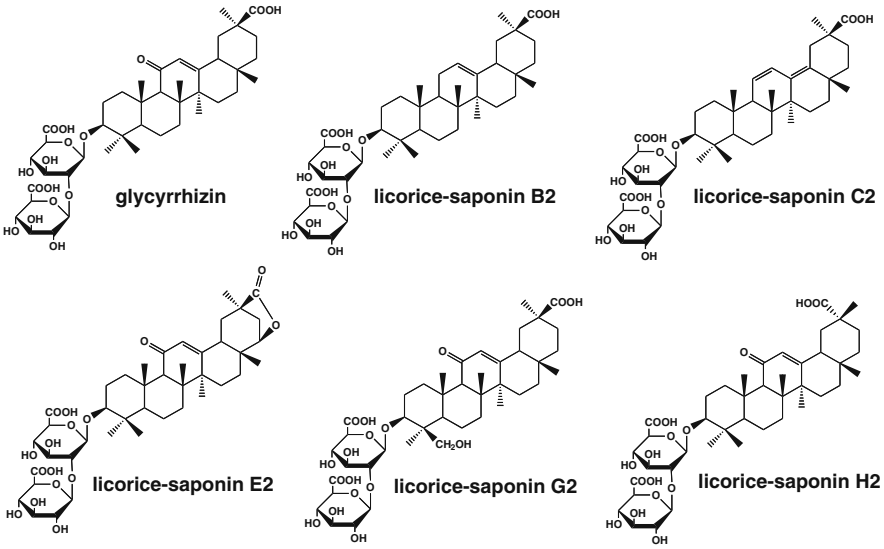

Fig. 5.1 Structure of glycyrrhizin, licorice-saponin B2, licorice-saponin C2, licorice-saponin E2, licorice-saponin G2, and licorice-saponin H2

5.2.1 Triterpenes and Triterpene Saponins Produced by Cultured Licorice Cells

Callus and cell suspension cultures were established from various organs of *G.glabra*, but they failed to produce detectable amounts of glycyrrhizin (Hayashi et al., 1988). However, the cultured licorice cells produced two structurally different triterpenoid constituents, namely soyasaponins (Hayashi et al., 1990b) and betulinic acid (Hayashi et al., 1988). Soyasaponins were isolated from various legumes, such as *Glycine max* (Kitagawa et al., 1974), *Pisum sativum* (Yokota et al., 1982), *Medicago sativa* (Kitagawa et al., 1988), and *Wisteria brachybotrys* (Konoshima et al., 1992), suggesting that soyasaponins are common saponins in leguminous plants. Soyasaponins are reported to possess antivirus (Nakashima et al., 1989; Hayashi et al., 1997; Kinjo et al., 2000), hepatoprotective (Ohminami et al., 1984: Kinjo et al., 1998), and antitumor-promoting (Konoshima et al., 1992) activities. On the other hand, betulinic acid, a lupane-type triterpene, is distributed widely in plant kingdom and was also isolated from licorice roots (Saitoh and Shibata, 1969; Hattori et al., 1986). Derivatives of betulinic acid were reported to be potent anti-HIV agents (Mayaux et al., 1994; Kashiwada et al., 1996), and one of them, 3-O-(3′,3′-dimethylsuccinyl)-betulinic acid (Bevirimat), is now undergoing clinical trials in the United States(http://www.panacos.com/index.htm).

Biosynthetic pathways of these triterpenoid constituents are shown in Fig. 5.2. Both glycyrrhizin and soyasaponins are oleanane-type triterpene saponins and share β-amyrin as a common biosynthetic intermediate. Specific oxidations of β-amyrin at C-11 and C-30 positions leading to glycyrrhizin are blocked in the cultured licorice cells, and the cultured cells convert β-amyrin into soyasapogenol B, the aglycone of soyasaponins I and II, via oxidations at the C-22 and C-24 positions. Betulinic acid is biosynthesized via oxidations at the C-28 position of lupeol, a common biosynthetic intermediate for lupane-type triterpenes. Gas chromatography (GC) analysis showed that the contents of soyasaponins and betulinic acid widely vary with the different culture strains (Hayashi et al., 1990b), and there was no significant correlation

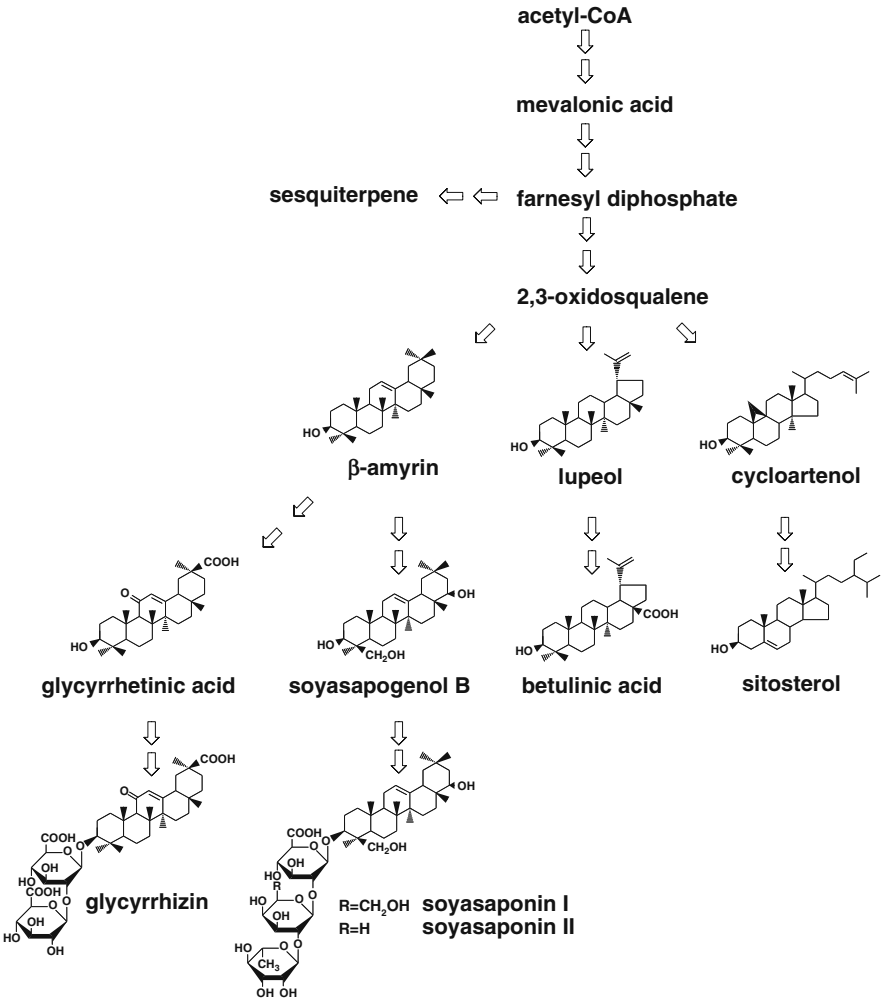

Fig. 5.2 Biosynthetic pathways of triterpenes and triterpene saponins in *G. glabra*

between the contents of soyasaponins and betulinic acid. It is also noteworthy that yeast extract promoted betulinic acid accumulation, whereas soyasaponin accumulation was suppressed, indicating that the regulation of soyasaponin biosynthesis was different from that of betulinic acid biosynthesis (Hayashi et al., 2005a).

5.2.2 *Distribution of Triterpenoids in Different Organs of* G. glabra

The contents of glycyrrhizin, soyasaponins, and betulinic acid in various organs of *G. glabra* were determined by HPLC (glycyrrhizin) and GC (soyasaponins and betulinic acid) as shown in Table 5.1 (Hayashi et al., 1988, 1993a, 2004). Glycyrrhizin was localized exclusively in the woody parts of thickened roots but not in the aerial parts, rootlets, or root nodules. Soyasaponins were detected in all parts of the plants examined, and the contents were higher in the seeds, rootlets, and root nodules than in other parts. It is also noteworthy that an inverse relationship was observed between the contents of soyasaponins and glycyrrhizin (Hayashi et al., 1993a). The contents of soyasaponins were higher in younger parts of a growing stolon, whereas those of glycyrrhizin tended to be higher in the older parts. Such an inverse relationship between soyasaponins and glycyrrhizin was also observed during the development of primary roots after germination. As the primary roots grew and became thicker, the soyasaponin content tended to decrease, while the glycyrrhizin content increased. On the other hand, betulinic acid was localized to the rootlets, root nodules, and the cork layer of thickening roots (Hayashi et al., 1988, 2004). Since both soyasaponins and betulinic acid were produced in the rootlets, root nodules, and cultured cells, the triterpenoid metabolism of the cultured licorice cells was similar to that of the rootlets and root nodules.

Table 5.1 Contents of glycyrrhizin, soyasaponin, and betulinic acid in various organs of *G. glabra* (Hayashi et al., 1988, 1993a, 2004)

	Content (% of dry weight) of		
Organ	Glycyrrhizin	Soyasaponins	Betulinic acid
Leaf	n.d.	0.001	n.d.
Stem	n.d.	0.001	n.d.
Seed	n.d.	0.35	n.d.
Old thickened root (diameter: 32 mm)			
Woody part	2.2	0.004	n.d.
Cork layer	0.006	0.004	0.32
Young root (6 months old)			
Thickened root (ϕ 4 mm)	0.23	0.28	0.14
Rootlet ($\phi < 1$ mm)	0.002	0.82	0.1
Root nodule	n.d.	0.98	0.06

5.3 Biotransformation of Glycyrrhetinic Acid by Cultured Licorice Cells

The cultured licorice cells do not produce glycyrrhizin but do produce soyasaponins, which are also oleanane-type triterpene glucuronides like glycyrrhizin. Thus, we examined whether or not the cultured licorice cells can convert exogenously supplied glycyrrhetinic acid, the aglycone of glycyrrhizin, into its glucuronide, glycyrrhizin (Hayashi et al., 1990a, 1992). The structures of seven biotransformation products derived from exogenous glycyrrhetinic acid administered to the cultured licorice cells were determined and are shown in Fig. 5.3. However, formation of glycyrrhizin was not detected among the biotransformation products.

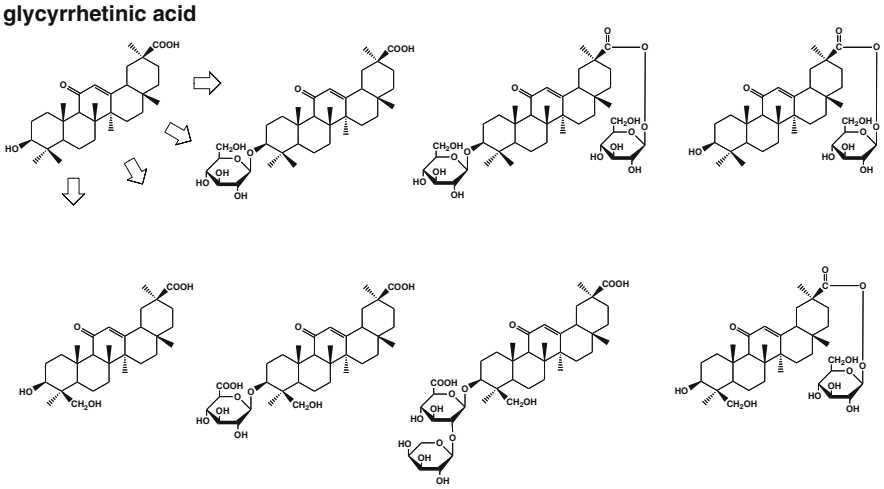

Fig. 5.3 Structures of biotransformation products derived from exogenous glycyrrhetinic acid administered to cultured licorice cells

Studies on time course for the production of metabolites as well as the pattern of chemical conversion of intermediate metabolites showed that hydroxylation of C-24 methyl group of glycyrrhetinic acid occurs at the first step of biotransformation, which is followed by glucuronylation (Fig. 5.4). Furthermore, glycyrrhetinic acid 24-hydroxylase activity was detected in the microsomal fraction prepared from the cultured licorice cells, and the participation of cytochrome P-450 in the reaction was shown by inhibitor experiments (Hayashi et al., 1993b). The 24-hydroxylation of glycyrrhetinic acid was prerequisite for glucuronylation in the cultured licorice cells. The glucuronosyltransferase activities for 24-hydroxyglycyrrhietinic acid and soyasapogenol B were also detected in the microsomal fraction of the cultured licorice cells, whereas the glucuronosyltransferase activity for glycyrrhetinic acid was not detected (Hayashi et al., 1993b, 1996). Since soyasapogenol B is the aglycone of soyasaponins I and II, the glucuronosyltransferase activity for soyasapogenol B would be responsible for biosynthesis of soyasaponins in the cultured

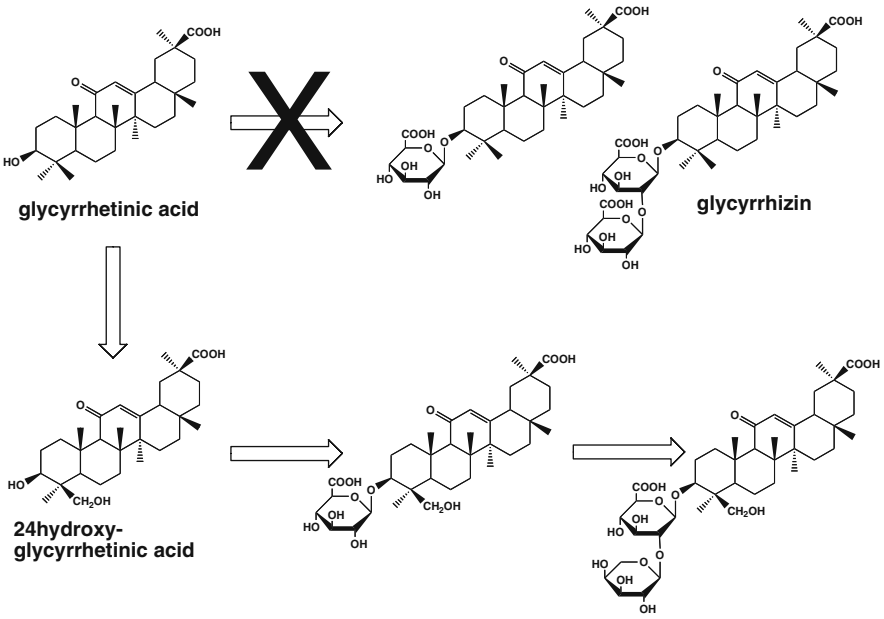

Fig. 5.4 Metabolic pathway of glycyrrhetinic acid in cultured licorice cells

licorice cells. Although the glucuronosyltransferase for glycyrrhetinic acid was not detected in the cultured cells, the glucuronosyltransferase for glycyrrhetinic acid should be involved in the biosynthesis of glycyrrhizin, which is localized to the thickened roots and stolons of *Glycyrrhiza* plants.

5.4 Saponin Variation in Seven *Glycyrrhiza* Species

It is of interest to elucidate the saponin variations among various *Glycyrrhiza* species from the viewpoint of the evolution of glycyrrhizin biosynthesis. Thus, the underground parts of seven *Glycyrrhiza* species, *G. glabra*, *G. uralensis*, *G. inflata*, *G. echinata* L., *G. macedonica* Boiss. et Orph., *G. pallidiflora* Maxim., and *G. lepidota* (Nutt.) Pursh, were analyzed by HPLC to examine the saponin variations in these species (Hayashi et al., 2000b, 2005b). Although the three *Glycyrrhiza* species, *G. glabra*, *G. uralensis*, and *G. inflata*, produce glycyrrhizin as a major saponin, the other three *Glycyrrhiza* species, *G. echinata*, *G. macedonica*, and *G. pallidiflora*, do not produce glycyrrhizin but instead produce macedonoside C as a major saponin (Shibano et al., 1999; Hayashi et al., 2000b). On the other hand, *G. lepidota*, American licorice, produces licorice-saponin H2 and macedonoside A as the two major saponins (Hayashi et al., 2005b). Licorice-saponin H2 is a minor saponin isolated from the glycyrrhizin-producing species (Kitagawa et al., 1993b), and macedonoside A is a minor saponin isolated from the macedonoside

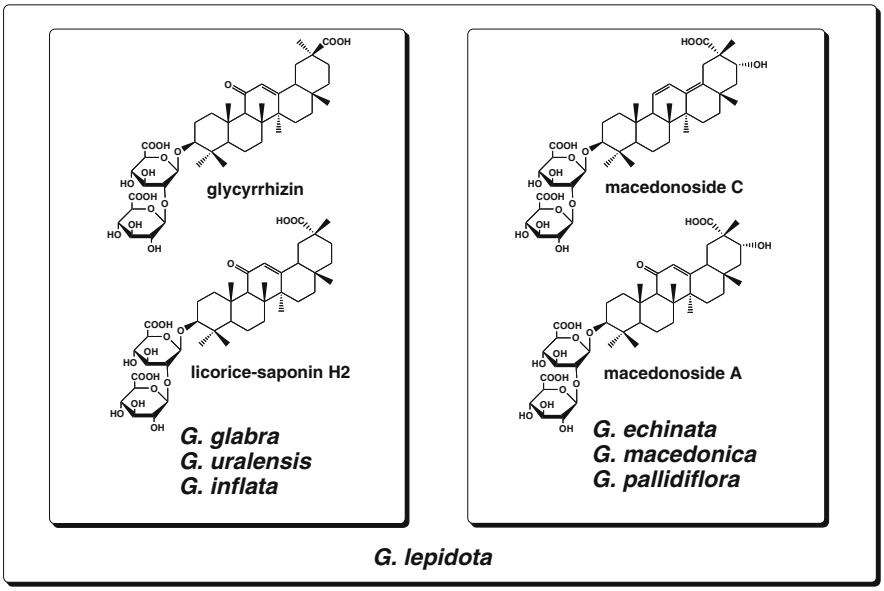

Fig. 5.5 Structures and distributions of glycyrrhizin, licorice-saponin H2, macedonoside C, and macedonoside A in seven *Glycyrrhiza* species

C-producing species (Shibano et al., 1999). In addition, *G. lepidota* produces trace amounts of glycyrrhizin and macednoside C. These results suggest that *G. lepidota* is a chemotaxonomical intermediate of the glycyrrhizin-producing and macedonoside C-producing species (Fig. 5.5).

To confirm the relationship of these *Glycyrrhiza* species, we determined the nucleotide sequences of a chloroplast gene for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbcL*). Based on the *rbcL* sequences, the seven *Glycyrrhiza* species were divided into three groups: the three glycyrrhizin-producing species (*G. glabra*, *G. uralensis*, and *G. inflata*), the three macedonoside C-producing species (*G. echinata*, *G. macedonica*, and *G. pallidiflora*), and *G. lepidota*(Hayashi et al., 2000b, 2005b). This phylogenetic relationship by their *rbcL* sequences is in accordance with their saponin compositions.

5.5 Molecular Cloning of Squalene Synthase and Oxidosqualene Cyclase cDNAs from *G. glabra*

Since *G. glabra* produces various triterpenes and triterpene saponins, this plant is an excellent model plant to study triterpenoid biosynthesis. Our next interest thus focuses on the gene expression of enzymes involved in triterpenoid biosynthesis (Fig. 5.6). Squalene synthase (SQS, EC 2.5.1.21) catalyzes the condensation of two molecules of farnesyl diphosphate into squalene, a common precursor of sterols and

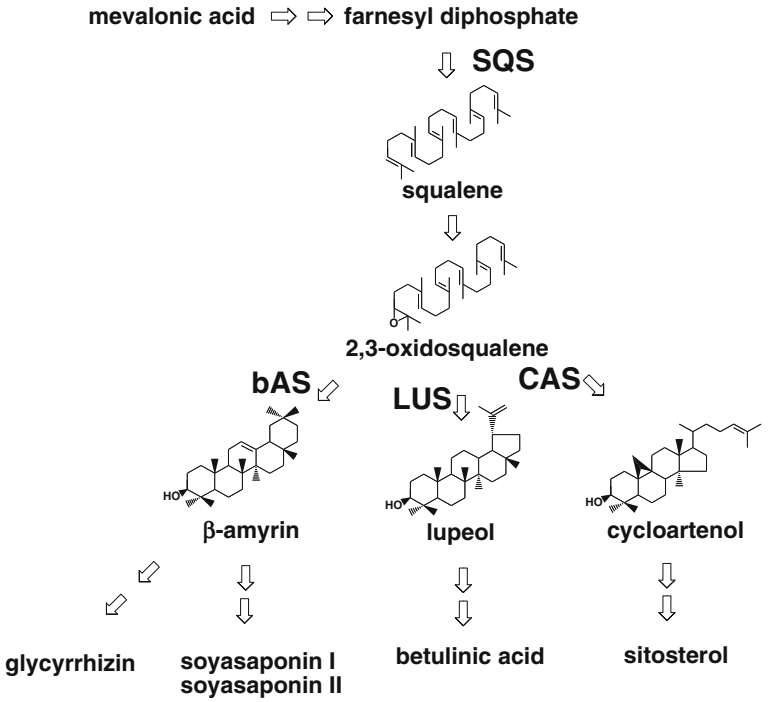

Fig. 5.6 Squalene synthase and oxidosqualene cyclases involved in the biosynthesis of triterpenoids in licorice. Squalene synthase (SQS), β-amyrin synthase (bAS), lupeol synthase (LUS), and cycloartenol synthase (CAS)

triterpenoids. Although the SQS genes of yeast and human was reported to be single-copy genes (Jennings et al., 1991; Robinson et al., 1993), two SQS genes are organized in a tandem array in the genome of *Arabidopsis thaliana*, a model plant used for genomic studies (Kribii et al., 1997). Since plants accumulate not only sterols but also various triterpenes and triterpene saponins, the regulation of the SQS gene might be different from that of mammals. Thus, cDNA for SQS was screened from a cDNA library of the cultured licorice cells to isolate two SQS cDNAs, designated GgSQS1 and GgSQS2 (Hayashi et al., 1999). The deduced amino acid sequence of GgSQS1 was 88% identical to that of GgSQS2. SQS activity was found in the cell-free extracts of *Escherichia coli* transformed with the expression plasmids for GgSQS1 and GgSQS2, indicating that the cultured cells of *G. glabra* produce two functional isozymes for SQS, which may play an important role in the regulation of biosynthesis of the different triterpenoids. Genomic Southern blot analysis suggested that there are three SQS genes in the licorice genome.

Oxidosqualene cyclases catalyze the cyclization of 2,3-oxidosqualene, a common intermediate of both sterols and triterpenes (Abe et al., 1993; Haralampidis et al., 2002). To elucidate the regulation of the triterpenoid biosyntheses in *G.glabra*, cDNAs of the three oxidosqualene cyclases, β-amyrin synthase

(bAS), lupeol synthase (LUS) and cycloartenol synthase (CAS), which are situated at the branching step for biosynthesis of oleanane-type triterpene saponins (glycyrrhizin and soyasaponins), lupane-type triterpene (betulinic acid) and phytosterols, respectively (Fig. 5.6), were cloned and characterized (Hayashi et al., 2000a, 2001, 2004). These three oxidosqualene cyclases cloned from *G.glabra* were monofunctional triterpene synthases, each of which produces a sole major triterpene product. However, a multifunctional triterpene synthase, producing multitriterpene products, has been cloned from other leguminous plants (Morita et al., 2000; Iturbe-Ormaetxe et al., 2003; Sawai et al., 2006).

5.6 Regulation of Triterpenoid Biosynthesis in the Cultured Licorice Cells and in the Intact Licorice Plants

Since the oxidosqualene cyclases are situated at a crucial branching step for biosynthesis of the respective triterpenes, molecular cloning of these cDNAs provides useful tools for studying regulation of triterpenoid biosyntheses in cultured licorice cells. Thus, the mRNA levels of two SQSs, bAS, LUS, and CAS were compared by Northern blot analysis to elucidate the effects of various plant hormones on the mRNA levels in cultured licorice cells (Hayashi et al., 2003, 2004). The mRNA levels of two SQS and bAS were upregulated by jasmonate (JA) and methyl jasmonate (MeJA), suggesting that jasmonates are positive regulators of soyasaponin biosynthesis in *G.glabra*. In contrast, the mRNA level of LUS was downregulated by JA and MeJA, whereas the mRNA level of CAS was relatively constant. On the other hand, gibberellin A_3 downregulated the mRNA level of bAS but not those of LUS and CAS, suggesting that gibberellin may be a negative regulator of soyasaponin biosynthesis in *G.glabra* (Hayashi et al., 2004).

Exogenously applied MeJA stimulated soyasaponin biosynthesis in cultured licorice cells (Hayashi et al., 2003), and accumulations of bAS and SQS mRNAs were not transient but lasted for 7 days after exposure to MeJA (Fig. 5.7), resulting in

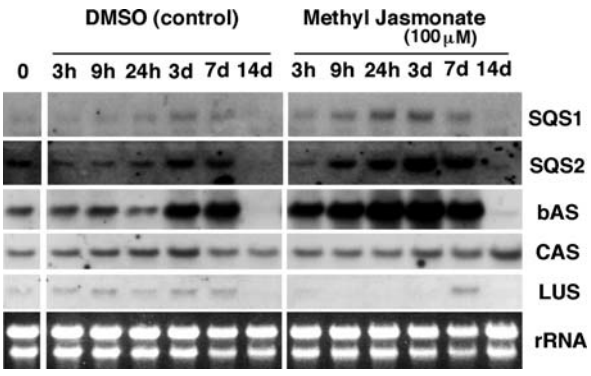

Fig. 5.7 Time course of accumulation of squalene synthase (SQS1, SQS2), β-amyrin synthase (bAS), cycloartenol synthase (CAS), and lupeol synthase (LUS) mRNAs in cultured licorice cells treated with MeJA (Hayashi et al., 2003)

the high-level accumulation (more than 2% of dry weight) of soyasaponins (Hayashi et al., 2003). Furthermore, enzyme activity of UDP-glucuronic acid: soyasapogenol β-glucuronyltransferase, an enzyme involved in a later step of soyasaponin biosynthesis, was also upregulated by MeJA, suggesting that the transcription of other enzymes involved in the late steps of soyasaponin biosynthesis might be upregulated by MeJA in cultured licorice cells.

Yeast extract was shown to induce isoflavone biosynthesis in cultured cells of *G. echinata* (Ayabe et al., 1986; Nakamura et al., 1999). Thus, the effect of yeast extract on the mRNA levels of cultured *G. glabra* cells was examined (Hayashi et al., 2003). The mRNA level of polyketide reductase, an enzyme involved in the biosynthesis of 5-deoxy-isoflavonoids in legumes, was transiently upregulated by both yeast extract and MeJA in cultured *G. glabra* cells, whereas the mRNA levels of two SQSs and bAS were coordinately downregulated by yeast extract in these cells (Fig. 5.8). The opposite effects of MeJA and yeast extract on the levels of SQS and bAS mRNA levels suggest that the signaling pathway leading to activation of soyasaponin biosynthesis is different from that of the flavonoid biosynthesis.

Although the mRNA levels of bAS and LUS were highly regulated in cultured cells of *G.glabra*, bAS and LUS also play an important role in the biosynthesis of glycyrrhizin and betulinic acid, respectively, in intact licorice plants. Thus, the mRNA levels of three OSCs in the intact plants were compared by Northern blot analysis (Hayashi et al., 2003, 2004). A high level of bAS mRNA was observed in the thickened main roots and root nodules. This finding was consistent with the high-level accumulation of soyasaponins in the root nodules and that of glycyrrhizin in the thickened main roots. The LUS mRNA was detected in the root nodules, in which a relatively high level of betulinic acid was detected. The mRNA level of CAS, responsible for sterol biosynthesis, was relatively constant in the various organs of *G.glabra*. These results indicate that there is independent regulation of three oxidosqualene cyclases in intact licorice plants. The levels of these mRNAs correlate well with the accumulation of respective end-products, suggesting that the transcription of oxidosqualene cyclases is an important regulatory step for triterpene biosynthesis.

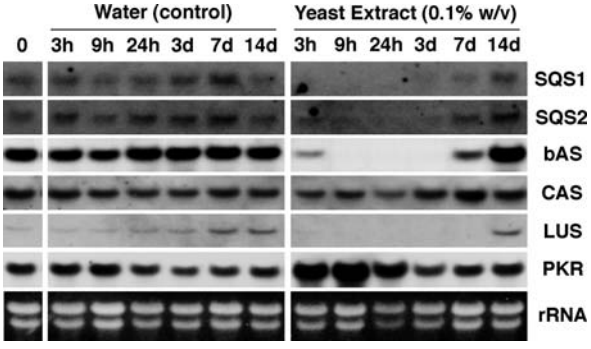

Fig. 5.8 Time course of accumulation of squalene synthase (SQS1, SQS2), β-amyrin synthase (bAS), cycloaretenol synthase (CAS), lupeol synthase (LUS), and polyketide reductase (PKR) mRNAs in cultured licorice cells treated with yeast extract (Hayashi et al., 2003)

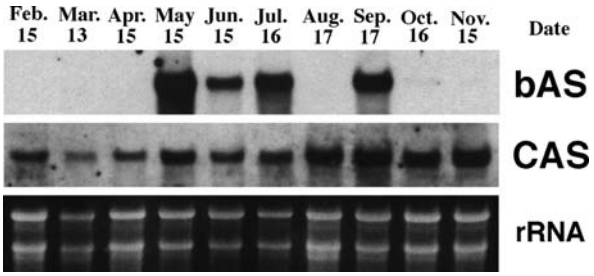

Fig. 5.9 Seasonal variation of accumulation of β-amyrin synthase (bAS) and cycloaretenol synthase (CAS) mRNAs in thickened main roots of licorice (Hayashi et al., 2005a). Reproduced with permission from Biol. Pharm. Bull. 27(7) Copyright (2004), Pharmaceutical Society of Japan

The site of glycyrrhizin biosynthesis is localized in the thickened roots and stolons of *G.glabra* (Fuggersberger-Heinz and Franz, 1984; Hayashi et al., 1998), and seasonal variation for the incorporation of ^{14}C-mevalonic acid into the glycyrrhizin fraction by the root segments was observed (Hayashi et al., 1998). The incorporation rate was high in May, June, and September and low in August and winter months. Seasonal variation of bAS mRNA level was also observed in the thickened main roots (Hayashi et al., 2004). The level of bAS mRNA in thickened main roots was high in May, June, July, and September when the aerial parts were growing (Fig. 5.9). The level of bAS mRNA was low in August, when many leaves had abscised due to the high temperature and high humidity in Japan, and in the winter months, when the aerial parts were dormant. These results indicate that the existence of the actively growing aerial parts is necessary for the expression of bAS mRNA and glycyrrhizin biosynthesis in the thickened main roots. The level of CAS mRNA was relatively constant, even during the winter months, suggesting that CAS is a housekeeping enzyme. The level of LUS mRNA was not detectable in the thickened roots.

5.7 Concluding Remarks

Glycyrrhizin, soyasaponins, and betulinic acid are localized in specific organs of intact licorice plants, and biosyntheses of these constituents are differentially regulated. Molecular cloning of SQS and oxidosqualene cyclase (bAS, LUS, and CAS) cDNAs revealed that the transcription of these genes is also differently regulated in the cultured cells as compared with intact plants of licorice. Upregulation of bAS and SQS mRNAs by MeJA in cultured licorice cells suggests that transcripts of other enzymes involved in the later steps of soyasaponin biosynthesis might be upregulated by MeJA. Induction of bAS mRNA and saponin accumulation by MeJA have also been reported in cultured cells of the model legume *Medicago truncatula* (Suzuki et al., 2002, 2005), and MeJA-inducible triterpene glycosyltransferase genes have been characterized from this plant (Achnine et al., 2005). Furthermore,

a cDNA of triterpene hydroxylase involved in soyasaponin biosynthesis was also identified from elicitor-inducible soybean P450s (Shibuya et al., 2006). Further experiments are underway to characterize the licorice genes involved in the later steps of triterpene saponin biosynthesis.

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Part II
Applications of Plant Biotechnology in Agriculture and Industry

Chapter 6
New Developments in Agricultural and Industrial Plant Biotechnology

Ara Kirakosyan, Peter B. Kaufman, and Leland J. Cseke

Abstract New developments in agricultural and industrial plant biotechnology are quite noteworthy and deserve special mention in this chapter.

In the *agricultural sector*, we have witnessed the advent of no tillage farming; significant increases in the use of organic farming practices, including a decrease in the use of toxic insecticides and herbicides; a quantum leap forward in the spread of farmers' markets and sale of locally grown food crops and products; an increase in the use of seeds of heirloom cultivars of crop plants; an increase in crop species diversity; an increase in the use of genetically modified food plants in America; a slowly emerging trend toward urban agriculture; and increasing use of hydroponic production systems to grow crops year-round in greenhouses.

In the *industrial sector*, we observe the advent of many new industrial-type products that are derived from plants. These include biodegradable plant-derived plastics, paints and varnishes, adhesives, auto biofuels, de-icers, cleaners, vegetable oils, essential oils, industrial solvents, pharmaceutical and industrial proteins; soy-based inks; soy-based spray foam insulation; soy-based carpet backing and padding; and soy-based wood-like composites used for floors, paneling, and table/countertops.

In this chapter, we present selected examples from each of these topics.

6.1 The Implementation of Organic Farming Practices: The Reasons, Benefits, and Disadvantages

Organic farming refers to the use of sustainable, environmentally safe practices in the growing of food crops for humans and domesticated animals. *Organic farming* is a form of agriculture which excludes the use of synthetic fertilizers and pesticides, plant growth regulators, livestock feed additives, and genetically modified (GM) organisms. As far as possible, organic farmers rely on crop rotation, green manure, compost, biological pest control, and mechanical cultivation to maintain

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soil productivity and control pests. Organic farming is often contrasted with conventional, or mainstream, farming (Adeyemi, 2000). *Advantages* include high consumer acceptance of organically grown products and willingness to pay a higher price for such food items; enhanced soil fertility and water-holding capacity, especially long term; gradual purging of the soil of toxic pesticides that may have been used previously after several years of instituting organic farming practices; organic certification of the grower as a certified organic farmer once organic standards have been met; enhanced value of the farmer's land once it has qualified for organic certification; fewer health-related problems for the organic grower because of the practices he/she uses; lower incidence of crop insect pests because of increased incidence of insect predators. *Disadvantages* include higher production costs and greater problems with weeds; hence lower yields over the short term. *Reasons for implementation of organic farming practices* include (1) consumer demand; (2) loss of soil fertility; (3) health problems for the conventional/corporate farmer who is exposed to a multitude of toxic chemical pesticides – herbicides, insecticides, fungicides, nematicides, and fumigants; (4) increased profits for the farmer/grower who grows certified organic crops; and (5) improved adherence to soil conservation practices.

Regarding GM crops (see Chapter 13), the absolutist position taken by organic farming adherents against any use of GM crops is currently meeting immense opposition worldwide from crop biotechnology proponents. The center of the controversy lies in European Union (EU) countries. The latest test comes in the following: The Public Research and Regulation Initiative (PRRI), a worldwide effort of public sector scientists involved in research and development of biotechnology for the public good, have sent an open letter to the members of the European Commission to aid them in their orientation discussion on biotechnology. PRRI has expressed deep concern about the effects of the political situation in Europe affecting genetically modified (GM) foods and crops.

The initiative notes, that despite clear EU rules and The European Food Safety Authority (EFSA) conclusions of GMOs not having adverse effect on human and animal health or the environment, EFSA opinions continue to be ignored. As a result of this situation, detrimental impacts have been felt both inside and outside the EU, particularly in developing countries.

Plant pathologist, Pamela Ronald, from the University of California at Davis, CA, believes that a combination of the two approaches – implementation of organic farming protocols and inclusion of GM crops – will be important for the future of global food production. Her view is that genetically modified seeds, when grown by the use of organic agricultural methods, can significantly increase yields, and at the same time, reduce the use of environmentally damaging chemicals. This hypothesis is not dissimilar from the conclusion of the recently published IAASTD (International Assessment of Agricultural Knowledge, Science and Technology for Development) report. This 2-year intergovernmental project is designed to investigate the role that agricultural science, knowledge, and technology can play in world poverty. The report concludes that a complete agricultural revolution is needed where agriculture is no longer thought of as production alone.

6.2 Recent Achievements in Improving Crop Diversity: What Are the Driving Forces in Play Here?

As of 2003, in the United States, there were only 20 major agricultural products (these are listed in a table along with amounts produced) in commercial production (Table 6.1).

The only other crops to ever appear in the top 20 in the last 40 years were, commonly, tobacco, barley, and oats, and, rarely peanuts, almonds, and sunflower seeds (in all, only 26 of the 188 crops the FAO tracks worldwide). Both alfalfa and hay would be in the top 10 in 2003 if they were tracked by FAO.

This has resulted in a major loss in agricultural product diversity as compared with that which existed in the 1800s. For example, concerning vegetable crop diversity, according to the Rural Advancement Foundation (now called ETC Group), 75 types of vegetables, or approximately 97% of the varieties available in 1900, are now extinct (Kimbrell, 2002). Accompanying this decrease in crop diversity of vegetable and most other types of crops is the trend for small farms to disappear and for existing farms to become much larger.

What are the attributes of this trend? Corporate agribusiness is the current model and has ended up controlling farming practices. Linked to this is the fact that monocultures have become most common. Furthermore, chemical agriculture is the primary system in use. As a result, we have increased production costs. Fewer people are engaged in farming. There is an increase in the incidence of crop pests. And, there is a reduction in quality of the crop products produced. As a consequence of these events, we might ask, is there any trend toward outsourcing crops from the

Table 6.1 The top 20 agricultural products of the United States by value as reported by the Food and Agricultural Organization of the United Nations (FAO) in 2003 (Products are ranked by their mass, multiplied by the 1999–2001 international prices. Mass is in metric tonnes.)

	Agricultural products	Mass (tonnes)
1.	Corn	256,904,992
2.	Cattle meat	11,736,300
3.	Cow's,milk,whole,fresh	78,155,000
4.	Chicken meat	15,006,000
5.	Soybeans	65,795,300
6.	Pig meat	8,574,290
7.	Wheat	63,589,820
8.	Cotton lint	3,967,810
9.	Hen eggs	5,141,000
10.	Turkey meat	2,584,200
11.	Tomatoes	12,275,000
12.	Potatoes	20,821,930
13.	Grapes	6,125,670
14.	Oranges	10,473,450
15.	Rice, paddy	9,033,610
16.	Apples	4,241,810
17.	Sorghum	10,445,900
18.	Lettuce	4,490,000
19.	Cottonseed	6,072,690
20.	Sugar beets	27,764,390

United States to other countries? No, this is really not the case because we are simply now using more of crops, such as banana, papaya, mango, star fruit, and kiwi, that are only grown in other countries.

With the advent of organic agriculture (see above), and because of consumer demand, we are now witnessing a steady increase in crop diversity on American farms. Consumers are demanding more nutritious products and foods that are healthier for them. This is driven, in part, by a parallel increase in the use of integrative medicine and preventive medicine in our health system.

What are some of the less common edible crops, not included in the top 20 major crop species that account for this increase in crop diversity? They include teff (*Eragrostis tef* (Zucc.) Trotter), quinoa (*Chenopodium quinoa* Willd.), grain amaranth (*Amaranthus* cruentus L.), wild rice (*Zizania aquatica* L.), canola/rapeseed (*Brassica napus* L.), sunflowers (*Helianthus annuus* L.), giant pumpkins (*Cucurbita pepo* L.), culinary herbs, heirloom vegetables (see Seeds of Change, www.seedsofchange.com), cassava, taro, kiwi, and many edible fruits that include new cultivars of grapes (*Vitis* spp. L.), blueberries, (*Vaccinium* spp. L.), sour cherries (*Prunus cerasus* L.), Goji berry/wolfberry (*Lycium barbatum* Thunb.), elderberry (*Sambucus canadensis* L.), thornless blackberries (*Rubus canadensis* L.), Cornelian cherry (*Cornus mas* L), chokeberry (*Aronia arbutifolia* (L.) Pers. and *A. melanocarpa* (Michx.) Elliott), and hawthorn (*Crataegus laevigata* (Poir.) DC. and *C. monogyna* Jacq.). Reasons why a wide spectrum of colored fruits and vegetables, like many of the above, are desirable for significantly improved health are described in "The Color Code: A Revolutionary Eating Plan for Optimum Health" by Joseph et al. (2002).

6.3 The Rise of Urban Agriculture

Grow gardens refer to collections of vegetables and flowers that are grown in relatively small plots in urban environments. The increasing presence of grow gardens in many cities in the United States, Europe, and Asia is one of the hallmarks of urban agriculture at work. The impetus for this activity is to satisfy the need to obtain our food locally rather than via world commerce, to save energy, to lower production costs, to improve human health, and to obtain fresher produce. Grow gardens allow urbanites to know where their food comes from, to be able to grow food crops without the use of toxic pesticides, to learn how it is grown and harvested, to get good exercise (and thus, to help fight a growing problem of obesity), to provide a greater diversity of foods in the diet, to promote human interactions, and to reduce urban crime. Grow gardens have also helped to restore the work ethic among urbanites. One other spin-off is that urban crops sequester carbon dioxide, and thus, help to reduce global warming. They also mitigate high summer temperatures via evaporative cooling from leaves of the crops grown (via the process of *transpiration* or *water evapo-transpiration* from the leaves).

Many grow gardens are now being developed as "rooftop gardens" where growing space is limited. They are also being located at ground level near churches and

schools, in parks, botanical gardens, and arboreta, and in lots where old houses and commercial buildings have been removed. It is essential that they should not be located in brown fields where the soil is contaminated with toxic residual chemicals and waste products.

6.4 The Use of Hydroponics Techniques for Commercial Food Production

Hydroponics refers to the cultivation of plants in complete nutrient solutions in the absence of soil. The first crops to be commercially grown with hydroponics included tomatoes and peppers, but the techniques were soon successfully extended to other crops such as lettuce, cucumbers, many kinds of culinary herbs, and cut flowers (Sustainable Living Articles at http://www.articlegarden.com; Mason, 2000). Valued at 2.4 billion dollars the hydroponic greenhouse vegetable industry has a growth rate of 10% per year and accounts for nearly 95% of the greenhouse vegetables produced in North America.

What are the advantages of hydroponics? Hydroponics, when used in greenhouses, allows for the cultivation of plants throughout the year. Other advantages include nutritious, healthy, and clean produce; improved and consistent quality of produce; and elimination of the use of toxic pesticides and herbicides. Notable, also, is the fact that specialty crops like culinary herbs, or even the above-mentioned vegetable crops or florist crops, can be grown hydroponically in gutter troughs in 42-day turn-around cycles (date of seed planting in biodegradable foam plugs to date of harvest) for most annual culinary herbs in greenhouses. The greenhouses can be heated from natural gas (methane) derived from garbage-filled landfills or from geothermal systems and illuminated continuously with full spectrum lamps with electricity generated by photovoltaic panels or wind generators.

Are there any disadvantages attributed to hydroponics? One entails high start-up costs. Another is that the whole process is highly labor-intensive as compared with field crop production, which, because of it being highly mechanized, involves much less hand labor.

What about yield comparisons? Commercial hydroponics systems have proven to be more productive than conventional systems of agriculture. Most commercial hydroponics greenhouse facilities are built sufficiently large to take advantage of economies of scale. Typically, these cover areas more than 10 acres, while smaller ones measure around 2 acres. In commercial practice, the yield of hydroponically grown tomatoes can be more than double that of soil-based systems due to the reduced turnover time between crops, better nutrition, and crop management. The dramatic increase in yields with hydroponics is best illustrated if we consider the actual production figures of soil-grown and hydroponically grown produce. Field grown tomatoes average yields ranging between 40,000 and 60,000 pounds per acre; on the other hand top growing hydroponics facilities in the United States and Canada report average yields of more than 650,000 pounds of tomatoes per acre.

Additionally, given the fact that only 10 years ago top hydroponics producers were producing around 400,000 pounds per acre, the increase in yields with improvements in growing practices has been truly phenomenal. Similar production figures can be quoted for other agricultural produce like cucumbers with 10,000 pounds per acre for field production and 200,000 pounds per acre for hydroponic greenhouse yields. Hydroponic lettuce and pepper yields too average around four times the corresponding yields of agricultural production.

In terms of global production, according to recent estimates, countries having substantial commercial hydroponics production include Israel – 30,000 acres, Netherlands – 10,000 acres, United Kingdom – 4,200 acres, and Australia and New Zealand – around 8,000 acres between them. The fastest growing area for commercial vegetable greenhouses is Mexico. There are several reasons for this. They include free trade and favorable winter conditions that attract vegetable growers in large numbers. Mexico has summers that are considered to be hot in the summer, but with greenhouses located at the right altitudes, vegetables can be grown in the hot summers as well as in the cold winters.

6.5 Examples of Food, Feed, and Industrial Products That Are Derived from Soybeans (*Glycine max* (L.) Merr.)

Soybeans are a versatile crop with many uses (see Indiancommodity.com). But before they can be used in food, feed, or industrial products, soybeans must be processed. More than 95% of the soybeans are processed by solvent-extraction industrial plants. When arriving at the processing plant, the soybeans are checked for quality. The soybeans then are processed to extract the *oil* and *meal*. From 100 pounds of soybeans, the soybean-crushing process produces 18 pounds of soybean oil and 80 pounds of soybean meal. There are several steps in the soybean-crushing process: *Dehulling* – First, the soybeans are cracked and the hulls are removed. *Soaking* – The soybeans then are flaked in special machines and moved to towers or tanks where they are soaked in a chemical solution. This solvent removes about 99% of the *pure, crude soybean oil* from the flake. *Refining*– The crude soybean oil may be refined further depending on how it is to be used. In the refining process, crude oil can be degummed, bleached, deodorized, or hydrogenated with hydrogen gas. In "degumming," the fatty acid content of the oil is neutralized with a caustic acid to produce some products like soap. The oil may also be "bleached" by treating it with an absorbent clay material before it is "deodorized" through a vacuum steam-distillation process. *Toasting and grinding* – After the oil is removed, the soybean flake then is cleaned, toasted, and ground to improve its nutritional value. This produces the *soybean meal*, which consists of 48% protein.

Soybeans are found in hundreds of human foods, animal feeds, and industrial products that are based on soybean oil and soybean meal. Some examples are as follows: *Soybean oil:* About 97% of soybean oil is used in a wide range of products for human use, such as cooking oils, salad dressings, sandwich spreads, margarine,

salad oils, coffee creamer, mayonnaise, shortenings, chocolate coatings, a flour ingredient, and medicines. Soybean oil also is used in such industrial products as printing inks, cosmetics, linoleum, vinyl plastics, paints, caulking compounds, pesticides, epoxy glue, protective coatings, yeast soaps, shampoos, and detergents. Other examples of industrial products that have been developed from soybeans include candles, cleaners, composite materials, crayons, diesel additives, fabric conditioners, flooring, paint removers, pens, polish, solvents, tables/furniture, and waxes (see information at soyworld.com). *Soybean meal:* About 98% of soybean meal is used as a feed ingredient in mixed rations for poultry, hogs, and beef and dairy cattle. The remainder is used for human food or industrial products. High-protein (48%) soybean meal is used as a starter ration and high-performance feed. A lower-protein soybean meal (44%) also may be produced by adding the high-fiber hulls for use in bulky feeds, or as a carrier for molasses and other ingredients.

6.6 Production of Biodegradable Plastics from Plant-Derived Starch

Biodegradable plastics derived from plant sources are now being developed in the United States, Europe, and Asia from renewable resources such as corn, wheat, and potato starches as substitutes for conventional and petroleum-based plastics. Examples are provided in Table 6.2.

The term *biodegradable* means that a substance is able to be broken down into simpler substances by the activities of living organisms, and therefore, is unlikely to persist in the environment (Gross and Kalra, 2002). There are many different standards used to measure biodegradability, with each country having its own standards. The requirements range from 90 to 60% decomposition of the product within 60–180 days of being placed in a standard composting environment.

The reason traditional plastics are not biodegradable is because their long polymer molecules are too large and too tightly bonded together to be broken apart and assimilated by natural decomposer organisms. However, plastics based on natural plant polymers derived from wheat or corn starch have molecules that are readily attacked and broken down by microorganisms (Fig. 6.1).

Table 6.2 Examples of products using plant-based plastics and the companies that produce them

Plant-based plastics	Producer companies
Plastic bags	BioBag
Water bottles	Biota Water
Disposable forks and knives	Cereplast
Wall carpets	Interface
Cups for smoothies	Mrs. Fields Brands
Electronics packaging and products	Sony
Car floor mats	Toyota
Produce packaging	Wal-Mart
Deli containers	Wild Oats

Plant-based plastics provide an alternative to conventional plastics, especially for *polyvinyl chloride* (*PVC*) that relies heavily on extremely toxic feedstocks and additives that have devastating impacts on our health and environment through their production, use, and disposal. Many of the chemicals used in PVC production are linked to cancer, birth defects, reproductive harm, and a host of other health problems. In contrast, bio-based plastics are generated from renewable materials, such as corn starch into plastic. The production of bioplastics uses fewer fossil fuels compared to petrochemical plastics, even after accounting for the fuel needed to plant and harvest the corn or other feedstocks. Plant-based plastics are also compostable. They can be effectively composted in a large-scale facility, where it will degrade

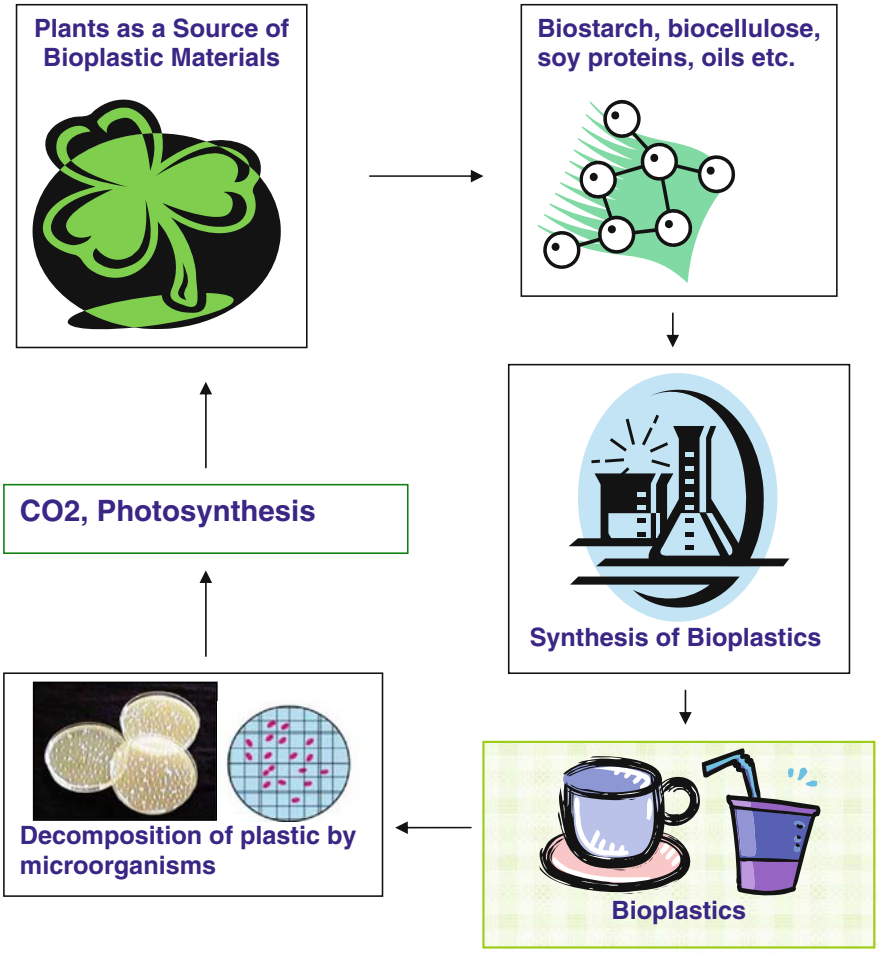

Fig. 6.1 Natural, plant-based plastics and their decomposition

within 45 days. Conventional plastics, in contrast, can take over 100 years just to begin the degradation process.

Starch is a natural storage polysaccharide-type polymer made up of α-(1,4)-linked glucose (amylose or straight-chain starch) or α-(1,4) + α-(1,6)-linked glucose (amylopectin or branch-chain starch) (see Cseke et al., 2006). It is a white, granular carbohydrate produced by plants during photosynthesis and it serves as one of the plant's energy stores. Grains of cereal plants and potato tubers normally contain starch in large proportions. Starch can be processed directly into a bioplastic, but because it is soluble in water, articles made from starch will swell and deform when exposed to moisture, thus limiting its use. This problem can be overcome by modifying the starch into a different polymer. First, starch is harvested from corn, wheat, or potatoes, then microorganisms transform it into lactic acid, a monomer. Finally, the lactic acid is chemically treated to cause the molecules of lactic acid to link up into long chains or polymers, which bond together to form a plastic called *polylactide* (*PLA*).

PLA has been commercially available since 1990. Certain blends have proved to be successful in medical implants, sutures, and drug delivery systems because of their capacity to dissolve away over time. However, because PLA is significantly more expensive than conventional plastics, it has failed to win widespread consumer acceptance.

Biodegradable plastic products currently available in the market are from 2 to 10 times more expensive than traditional petroleum-based plastics. Environmentalists argue that the cheaper price of traditional plastics does not reflect their true cost when their full impact is considered. A case in point is this: when we buy a plastic bag, we do not pay for its collection and waste disposal after we use it. If we included these kinds of associated costs, then traditional petroleum-based plastics would cost more and biodegradable plastics might be more competitive.

Another way of making biodegradable polymers involves getting bacteria to produce granules of a plastic called *polyhydroxyalkanoate* (*PHA*) inside their cells. Bacteria are simply grown in culture in bioreactors and the plastic is then harvested. Going one step further, scientists have taken genes from this kind of bacterium and transferred them into corn plants, which then manufacture the plastic in their own cells.

Unfortunately, as with PLA, PHA is significantly more expensive to produce. As yet, it is not having any success in replacing the widespread use of traditional petrochemical-based plastics. Perhaps as the price of oil increases and supply dwindles, biodegradable plastics will come into more favor benefit to our environment.

If cost is a major barrier to the acceptance of biodegradable starch-based plastics, then the solution lies in investigating low-cost options to produce them. In Australia, the *Cooperative Research Centre (CRC) for International Food Manufacture and Packaging Science* is examining ways of using basic starch, which is cheaper to produce, in a variety of blends, with other more expensive biodegradable polymers to produce a variety of flexible and rigid plastics. These are being made into film-molded and injection-molded products, such as plastic wrapping, shopping bags, bread bags, mulch films, and plant pots. Depending on the application,

scientists can alter polymer mixtures to enhance the degradative properties of the final product. For example, an almost pure starch product will dissolve upon contact with water and then biodegrade rapidly. But, by blending quantities of other biodegradable plastics into starch, scientists can now make a waterproof product that degrades within 4 weeks after it has been buried in the soil or composted (Fig. 6.1).

In the United States, the primary company manufacturing bioplastics is NatureWorks, owned by Cargill. They can produce 300 million pounds a year of a plastic called PLA or poly lactic acid that is made from corn grown in Nebraska and Iowa. Starch from the corn is extracted and converted into its basic monomer, D-glucose, and then into lactic acid by fermentation. The lactic acid is further refined into pellets that can be made into different end-products. This is actually a much better use of non-feed corn than the production of ethanol. It gives more and is much more efficient. Other companies manufacturing plant-based plastics include Dupont, BASF, Eastman, Proctor & Gamble, and Cereplast. The end plastic products, indistinguishable from those derived from petrochemicals, are used to create food packaging, disposable cups and forks, water bottles, auto parts, carpeting, compact disks, bedding materials, and other consumer products.

In Europe, bioplastics are even more popular. Consumption doubled between 2001 and 2003. An Italian company called Novamont manufactures a plant-based plastic called *Mater-Bi* that is used in many similar applications to PLA, including food packaging and disposable food service items. Production is expanding across the globe where capacity for bio-based plastics is around 800 million pounds and is expected to top 1.3 billion pounds in 2008.

Despite numerous environmental and health benefits of plant-based plastics, significant environmental challenges need to be addressed. These include the impacts of industrial agricultural production, the use of harmful additives, and the impact on recycling infrastructure and markets. Conventional corn production uses significant amounts of toxic pesticides that can adversely impact groundwater and surface water, leads to soil erosion, and impacts soil production and wildlife habitats. In addition, much of the corn made into NatureWorks' plastic is genetically modified. Many environmental organizations are working to address the use of genetically modified organisms (GMOs) in NatureWorks' feedstock.

One concern raised by recyclers is the impact that bioplastics have on the recycling of conventional plastics. Bio-based plastics, such as PLA, cannot be mixed with conventional plastic such as *PET/PETE* (*polyethylene terephthalate*) because these materials are not compatible for recycling purposes. PLA itself can be recycled, but at present, the infrastructure to separate and recycle this material does not exist in the United States. Until these problems are solved, the most sustainable disposal option for bio-based plastics is composting. Clear labeling of bio-based plastics is critical to ensuring that these materials are properly disposed of in composting facilities. The technology is a step in the right direction in terms of responsible use of plastics.

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Chapter 7
Phytoremediation: The Wave of the Future

Jerry S. Succuro, Steven S. McDonald, and Casey R. Lu

Abstract As the industrial age developed, societies have allowed large amounts of contaminants to enter the environment unchecked. As a result of this neglect, the incidence of heavy-metal contaminated sites has been on the rise. These sites are polluted with toxic hydrocarbons and radionuclides, as well as heavy metals, such as cadmium (Cd), chromium (Cr), copper (Cu), lead (Pb), and zinc (Zn). The result is unsightly areas left untreated, undeveloped and are accurately referred to as "Brown Fields." Heavy metals in the soil can create a contaminated and possibly toxic top layer ranging 2–5 cm deep in addition to the possibility of entering the food chain. The typical and most common method of removing contaminants is to excavate the soil by mechanical means and store it at off-site locations.

Phytoremediation is an innovative, emerging technology that utilizes plant species to remove contaminants from the environment using a distinct set of plant-based technologies. Four types of remediation technologies have been employed: (1) *phytostabilization* is the use of a plant's root system to stabilize the metal-contaminated soil thus preventing the spread of the contaminant; (2) *phytodegradation* is the process of using plants to convert toxic contaminants into less toxic forms; (3) *rhizofiltration* is the process of using plants to clean aquatic environments; and finally, (4) *phytoextraction* is the practice of using plants to take up metals from the soil and translocate them to the above-ground tissues which can then be harvested. By utilizing phytoremediation techniques, the environmental disruption is minimized, soil fertility is maintained, secondary air- and water-borne wastes are reduced, and these techniques are well received by the public as in situ methods. This chapter will discuss the use of multiple plant species in each of the listed remediation techniques for the goal of rejuvenating Earth's ecosystems.

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7.1 Introduction

During the industrial age, humans have allowed large amounts of contaminants to enter the environment unchecked. As a result of this neglect, many toxins have been permitted to accumulate in our soils and water systems. Since this neglect was allowed to continue for so long, the incidence of heavy-metal contaminated sites has been on the rise. The public is made aware of this issue through the media. In most cases, the media have a tendency to paint a very grim picture of how these contaminated sites will affect humans and animals alike (Environmental Protection Agency, 2007). For the most part, their interpretation of the situation is correct. Contaminated sites do exist and are polluted with toxic hydrocarbons and radionuclides, as well as heavy metals, such as cadmium (Cd), chromium (Cr), copper (Cu), lead (Pb), and zinc (Zn) (Amaya-Chavez et al., 2006). This heavy-metal contamination is a result of anthropogenic activities such as metal mining and smelting, agriculture, sewage sludge, fossil fuel combustion, and chemical manufacturing (Alloway, 1995). In addition to manufacturing types of activities, recreational sports such as the use of shooting ranges and improper storage techniques add to the list of sources (Alloway, 1995; Ebbs and Kochian, 1997). Often these sites of contamination become unsightly reminders of the lackadaisical attitude people take toward the environment.

These large, unsightly areas are often left untreated, undeveloped and are accurately referred to as "Brown Fields." Brown fields, or any other smaller toxic sites, are subject to wind-blown dispersion if the soil is disturbed and the heavy metal is set free. As the wind blows across the disturbed soil, soil particles and associated contaminants can be blown into the upper atmosphere and travel rather large distances, thus contaminating locations, such as playgrounds, parks, and yards thousands of miles from the original site (Xei et al., 1999). In addition to wind dispersal, when a heavy metal such as lead contacts the soil, it becomes tightly adsorbed to the soil particles. This can create a contaminated and possibly toxic top layer of soil that ranges 2–5 cm deep (Sharma and Dubey, 2005), where, in playgrounds or backyards, it becomes a threat to human health. Another concern for heavy-metal contaminated sites is the possibility of it entering the food chain. Although animals, plants, and microbes have no biological need for certain specific heavy metals, they can be taken up and sequestered in the cells of living organisms. Furthermore, these heavy metals can be moved from plants to animals as they graze on contaminated sites. Once the heavy metal enters the food chain, secondary and tertiary predators can be adversely affected by the quantities of metal present (Taylor and Crowder, 1983a). As these organisms in the higher trophic levels continue to consume heavy-metal contaminated foods, the toxic level within these organisms also increases (Taylor and Crowder, 1983a,b). This process is known as *bioaccumulation*.

In response to public outcry, the US Environmental Protection Agency (EPA) has spent billions of dollars on Superfund site cleanup projects across the nation (Bouchier and Lu, 2002; USEPA, 1993). As public awareness increases, so have the questions concerning how safe areas such as playgrounds, homes, and gardens, are for plants and animals (including humans). The largest concern regarding the

toxicity or accumulation of heavy metals, such as lead, is directed toward small children. Their bodies and central nervous systems are developing rapidly and any exposure to lead, even blood levels as low as 10 μg dL^{-1}, can cause long-term health problems within many organ systems. Examples of affected systems include, but are not limited to, gastrointestinal tract, cardiovascular system, nervous system, kidneys, immune, reproductive, and mental and physical impairment (www.epa.gov/iaq/lead.html, 2007; ATSDR, 2007; Pyatt and Gratten, 2001). Potential areas of concern for children are the ingestion of soil particles in playgrounds, and most importantly, the consumption of small lead-based paint chips in homes built before 1960, and the inhalation of lead dust from painted friction surfaces.

In an attempt to reduce exposure to heavy-metal contamination in soils, several methods of disposal have been developed. The typical and most common method is to excavate the soil by mechanical means using tractors and bulldozers, and then to transport the soil to another location where it is stored (Memon et al., 2001; Cunningham et al., 1995). Even though these storage facilities are lined with material to prevent the spread of contamination, the sites themselves become areas that are uninhabitable. In some cases, these sites become problems as heavy metals escape the confines of the protective barriers such as native claystone soils (ATSDR, 2005) and leach into the surrounding area and possibly nearby sources of groundwater. In addition to excavation of contaminated sites, some sites are remediated by the process of covering the soil with large quantities of concrete, asphalt, and/or clean, uncontaminated soil (Berti and Cunningham, 2000), which temporarily fixes the problem right where it lays by covering it to reduce contact. When these types of remediation techniques are used, they only satisfy an immediate and temporary fix to a long-term problem. In addition, these options can be very costly. Depending on the type of management strategy, remediation of a site by traditional means can cost between \$10 and \$3,000 m^{-3} per year (Cunningham et al., 1995). No one really knows what the long-term ramifications will be if these types of management techniques are allowed to continue. In light of this problem, a developing new technology has begun to focus on utilizing plants to decontaminate areas of high concentrations of heavy metals and organic contamination. This new technology is termed phytoremediation.

7.2 What Is Phytoremediation?

Phytoremediation is an innovative, emerging technology that utilizes plant species to remove contaminants from the environment (Tian et al., 2007; Amaya-Chavez et al., 2006). Much research has been conducted in this field and is gaining global acceptance because of the possibility of adapting this technology to many different types of ecosystems in both developed and developing countries. The term phytoremediation stems from the words "phyto," meaning plant, and the Latin suffix "remedium," meaning to clean or restore. *Phytoremediation* refers to a distinct set of plant-based technologies utilizing naturally occurring and/or genetically modified plants to remove contaminants, such as metals and hydrocarbons from soil,

sediments, or water systems (Padmavathiamma and Li, 2007; Amaya-Chavez et al., 2006). Plants accomplish this task by removal, transfer, stabilization, or decomposition of these contaminants in the environments listed above (Hughes et al., 1997). Heavy metals contaminate the major environmental systems of our planet: air, water, and soil; therefore, biogeochemical cycles can be severely disrupted (Tian et al., 2007). Heavy-metal pollution in soil differs from that of air- and water-based systems because heavy metals have a tendency to remain in the soil for very long periods of time. There are two major categories of contaminants that should be considered, elemental pollutants and organic pollutants, each of which has its own set of remediation strategies (Meagher, 2000). These will be discussed later in this chapter. Based on the type of contaminant, site conditions, quantity of contaminant to be removed, and the species of plants to be used for the process, four types of remediation technologies have been employed. They are classified based on the containment of metals (phytostabilization and phytodegradation) or the extraction of metals (phytofiltration and phytoextraction) (Padmavathiamma and Li, 2007). A brief description of these processes is as follows: (1) *phytostabilization* is the use of a plant's root system to stabilize the metal-contaminated soil thus preventing the spread of the contaminant; (2) *phytodegradation* is the process of using plants to convert toxic contaminants into less toxic forms; (3) *rhizofiltration* is the process of using plants to clean aquatic environments; and finally, (4) *phytoextraction* is the practice of using plants to take up metals from the soil and translocate them to the above-ground tissues which can then be harvested. In order for a plant to be listed as a good candidate for phytoremediation, several factors should be met. A plant must be tolerant to the environmental conditions of the contaminated site as well as be fast-growing and produce high quantities of biomass in harvestable tissue (Yang et al., 2005). With these conditions met, the phytoremediation process can begin.

7.2.1 Why Is Phytoremediation Important?

Taking into account the above-listed remediation techniques, the first thoughts that come to mind are cost and how environmentally sound the phytoremediation practices are at removing the contaminant from the environment. Traditionally, contaminated sites are remediated by physical, chemical, or biological processes (McEldowney et al., 1993). In the aftermath of the destructive treatments, irreversible effects may occur to soil properties. The destruction of biodiversity can render soils useless for the growth of plants that could potentially remove remaining contamination (Padmavathiamma and Li, 2007). By utilizing phytoremediation techniques, the environmental disruption is minimized, soil fertility is maintained, secondary air- and water-borne wastes are reduced, and these techniques are well received by the public as in situ methods (Tian et al., 2007; Amaya-Chavez et al., 2006; Padmavathiamma and Li, 2007). In some cases, phytoremediation may be the only solution for reducing contaminated soil and water systems that cover hundreds of thousands of square kilometers as a result of human activity (Meagher, 2000). The harvesting of plants that have accumulated large quantities

of usable metals in their tissues, such as nickel, zinc, and copper, could be recycled and used for other purposes, thus producing an economic incentive for using phytoremediation (Ow, 1996). In addition to being environmentally friendly, the phytoremediation process may also be cost-effective (Padmavathiamma and Li, 2007; Zhuang et al., 2007a,b; Yang et al., 2005). In the recent past, the cost to handle contaminated waste was approximately \$100 m^{-3} for incineration, \$60–\$300 m^{-3} for landfill, \$200–\$700 m^{-3} for special landfill requirements, and \$1,000–\$3,000 m^{-3} to dispose of radionuclides per year (Cunningham et al., 1995). Using the techniques of phytoremediation, these costs are reduced remarkably to levels of only \$5–\$40 t^{-1} and \$0.02–\$1.00 m^{-3} per year (Padmavathiamma and Li, 2007; Cunningham et al., 1995).

7.2.2 Remediation of Organic Contaminants

Over many decades, humans have added large volumes of contaminants to the soil, water, and atmosphere as a result of industrial manufacturing such as petroleum and chemical operations and private independent operations such as dry-cleaning. In addition to these processes, burning wood, coal, and fossil fuels, add a wide range of organic chemicals to the environment, potentially causing negative health effects for humans as well as wildlife. Further anthropogenic causes include car emissions, waste incineration, service stations, solvent use, cigarette smoking, and the use of pesticides.

Organic contaminants are carbon-containing compounds that are resistant to environmental degradation through chemical, biological, and photolytic processes. These compounds can be transported great distances, bioaccumulate in both human and other animal tissues, as well as biomagnify in food chains. Health agencies have identified several compounds as being extremely toxic to humans, wildlife, and the environment. This list is by no means complete, but it includes aldrin, chlordane, DDT, dieldrin, endrin, heptachlor, PCBs, and toxaphene (Fig. 7.1).

Some chemical properties of these organic toxins include decreased water solubility, increased lipid solubility, semi-volatility, and higher molecular weights. With their higher molecular weights and the addition of chlorine (Cl) substituents, the compounds are increasingly difficult to break down and remain persistent in the environment. Finally, their lipid solubility results in the ability of these molecules to pass through biological phospholipid membranes and bioaccumulate in fatty tissues. Humans may experience contamination through exposure from diet, environment, and accidental discharges into the atmosphere or spills into the soil. Deleterious health effects include damage to the endocrine system, the reproductive system, the immune system, neurological disorders, cancer, and ultimately, death. In contrast, compounds with lower molecular weights, less than 236 g mol^{-1}, are usually less toxic, thus reducing the health issues and environmental problems as a result of being less persistent (http://en.wikipedia.org/wiki/persistent_organic_pollutant, 2007).

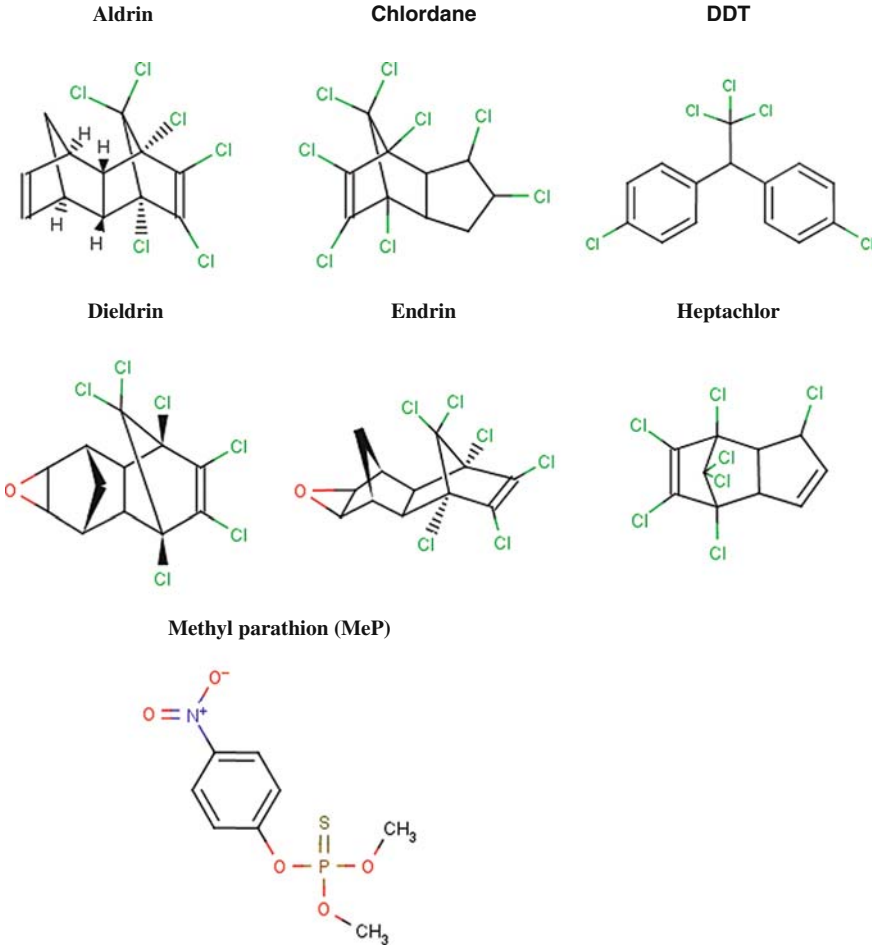

Fig. 7.1 Chemical structures of organic contaminates that are environmental risks to humans and wildlife

Current research suggests that there are two different ways by which organic contaminants can be removed from soil- and water-based systems, namely, phytodegradation and rhizofiltration. First we look at *phytodegradation* (Fig. 7.2), the process that utilizes plants and their associated microflora to convert hydrocarbons to non-toxic forms (Cunningham et al., 1995).

The conversion of contaminants by plants takes place in the following manner: First the plant releases root exudates that include organic and inorganic substances into the *rhizosphere* (soil–root–microorganism interface zone) during metabolism. These root exudates act as substrates for soil microorganisms, thus enhancing the uptake and degradation of toxic organic compounds by the plants. This principle has been used to remediate crude oil, motor oil, and diesel fuel from soils (Chaineau

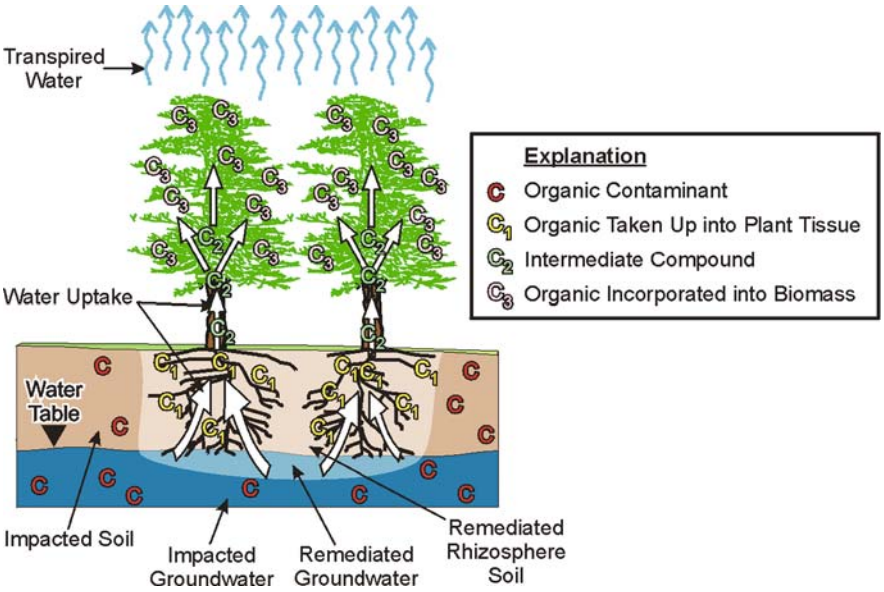

Fig. 7.2 Phytodegradation of organic contaminants. (Used with permission from Mueller et al., 2001)

et al., 2000). In a field study conducted by Palmroth et al. (2006) over a 39-month growth period, the initial soil concentration of contaminants was 11,400 mg kg^{-1} hydrocarbons in soil (dry weight). This soil contaminant component consisted of two-thirds lubricating oil, and the remaining one-third, diesel fuel. The field area was divided into four plots with two being fertilized with municipal biowaste, one plot with NPK fertilizer (16.6:4:25.3), and in the remaining plot, no fertilizer was used. The target concentration of phytoremediation was set at a level of 1,500 mg kg^{-1} hydrocarbons in dry soil, which translates to a reduction of hydrocarbons by 87% (Palmroth et al., 2006). Initially the hydrocarbon concentration did not decrease significantly in non-amended soil; however, there was a 30% decrease in the original concentration during the last 4 months of the experiment. In soil amended with either NPK fertilizer or biowaste compost, 65 and 60% of the hydrocarbons were removed, respectively, using a mixture of grasses (red fescue, *Festuca rubra*; meadowgrass, *Poa pratensis*; and ryegrass, *Lolium perenne*), Dutch white clover (*Trifolium repens*), Scots pine (*Pinus sylvestris*), and poplar seedlings (*Populus deltoides* x *Wettsteinii*). Ultimately, 57% of the hydrocarbons were removed in the plots amended with biowaste, clover, and grasses. Approximately 60% of the hydrocarbons were removed in the plot with grasses, clover, and trees. For the plot using NPK fertilizer, increased hydrocarbon removal was recorded compared to the biowaste plots, but during the last 4 months of the study there was no significant difference between the two amendments (Palmroth et al., 2006). This study shows positive potential for phytodegradation with approximately 50% or more of the hydrocarbons being removed by plants and associated microflora from contaminated

soil. The length of time for remediation can take several years to achieve treatment goals. It should be noted that in this study, the goal of achieving the 1,500 mg kg^{-1} hydrocarbons in dry soil weight was not achieved. This, however, does not mean that the process of phytodegradation is a non-viable technique; it does confirm the need for further research into optimizing the phytoremediation process and possibly using genetically modified (GM) plants.

7.3 Rhizofiltration

Rhizofiltration (Fig. 7.3) is the adsorption or precipitation of contaminants onto plant roots or the absorption into the roots of contaminants that are in solution surrounding the root zone due to biotic or abiotic processes.

The uptake, concentration, and translocation of contaminants by the plants may occur and will depend on the contaminant and the type of plant. Exudates from the plant roots may cause precipitation of some organics. Rhizofiltration first results in decontamination, a process by which the contaminants are immobilized or accumulated on or within the plant. Contaminants are then removed via plant harvesting (www.gsd.harvard.edu/users/yauanian/phyto_processes_main.html). Work done by Amaya-Chavez et al. (2006) has shown that cattails (*Typha latifolia* L.) have been successful in removing methyl parathion (MeP) (Fig. 7.1), an organophosphorus (OP) pesticide, from water systems and sediments. The cattails were subjected to various levels of MeP in the following concentrations: 0, 25, 50, 100, 150, and 200 mg L^{-1}. Photosynthetic potential, based on chlorophyll a and chlorophyll b concentration ratios, were used to determine the overall health of the plants. The basal mean total chlorophyll content was determined to be 32.9 mg ml^{-1} and the

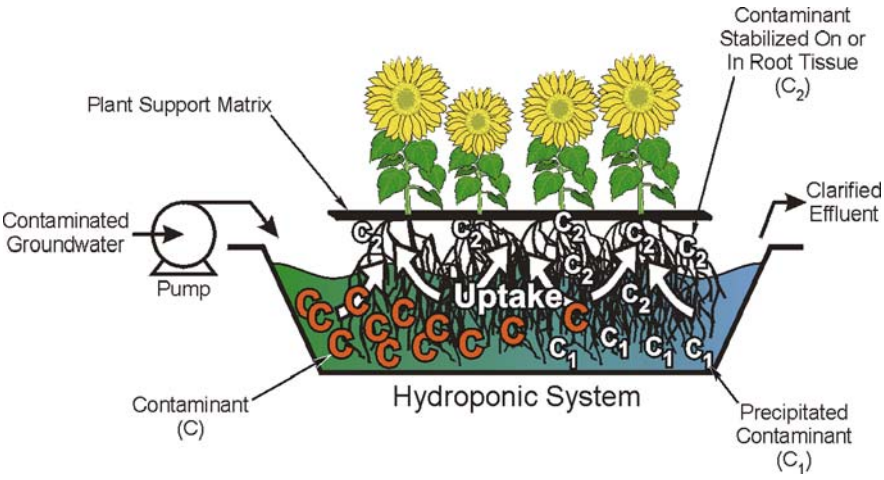

Fig. 7.3 Rhizofiltration of organic or inorganic contaminants. (Used with permission from Mueller et al., 2001)

chlorophyll a/b ratio was 2.8. After 10 days of exposure to MeP, no significant differences were shown in either total chlorophyll content or chlorophyll a/b ratio at the different MeP exposure levels (Amaya-Chavez et al., 2006). As a result, *T. latifolia* shows a low level of toxicity as a result of MeP uptake and a higher level of tolerance than other macrophytes tested to date. This higher tolerance could be due to *T. latifolia*'s ability to produce higher biomass with its rhizomatous/fibrous root system. As a result of these studies, *T. latifolia* has been determined to be quite efficient at removing MeP from water and sediment systems. Glick (2003) surmises that this efficiency could be due, in part, to a rhizosphere root/microorganism association that aids in the organic contaminant degradation with *T. latifolia*'s extensive rhizomatous/fibrous root system. Finally, given *T. latifolia*'s ability to tolerate a range of MeP concentrations without any loss of removal efficiency and minimal toxic effects to the plant, it should be seriously considered for remediation practices.

7.3.1 Remediation of Inorganic Contaminates

Inorganic contaminants (heavy metals or trace metals) compose much of the contamination at sites throughout the world. These higher atomic weight elements and some lower-weight elements can be called heavy metals as a group. Certain heavy metals or trace metals are required for the metabolic processes in organisms. Some of these trace metals, including iron, cobalt, copper, manganese, and zinc, however, become toxic at elevated levels (Alloway, 1995). As discussed in the Introduction section, some heavy metals have no biological use: these include mercury, lead, and cadmium. The question arises, then, as to how we can safely and effectively remove metals from our environment with as little destruction as possible, thus reducing or removing the threat to environmental health? This is where the utilization of phytoremediation techniques becomes important, particularly since they can be more cost-effective, less destructive, and at the same time, be more appealing to the public. There are a number of different ways in which phytoremediation can work. As discussed in the above section on rhizofiltration of organic types of contamination, *phytofiltration* is used in this section as a means to remove heavy metals from an aquatic environment. The processes are essentially the same.

Plants can also be used for *phytoextraction* (Fig. 7.4). This occurs when metal contaminants in the soil are taken up by roots and translocated to the above-ground tissues. The plants can then be removed from the site, or if removal of the entire plant is not practical, then the above-ground tissues can be removed for continual remediation of the soil. Removal of these tissues can occur multiple times during the growing season, thus increasing the rate of contamination removal. The harvested tissues can then be incinerated and the ash can be stored in a hazardous waste landfill. The volume of ash stored would be significantly less than excavating the soil and storing it in the same hazardous waste landfill.

Since there is no single plant species that can remove all contaminants at one site, several different species must be used for multiple contaminants. As these plants grow, they can accumulate high quantities of heavy metals such as lead. The normal

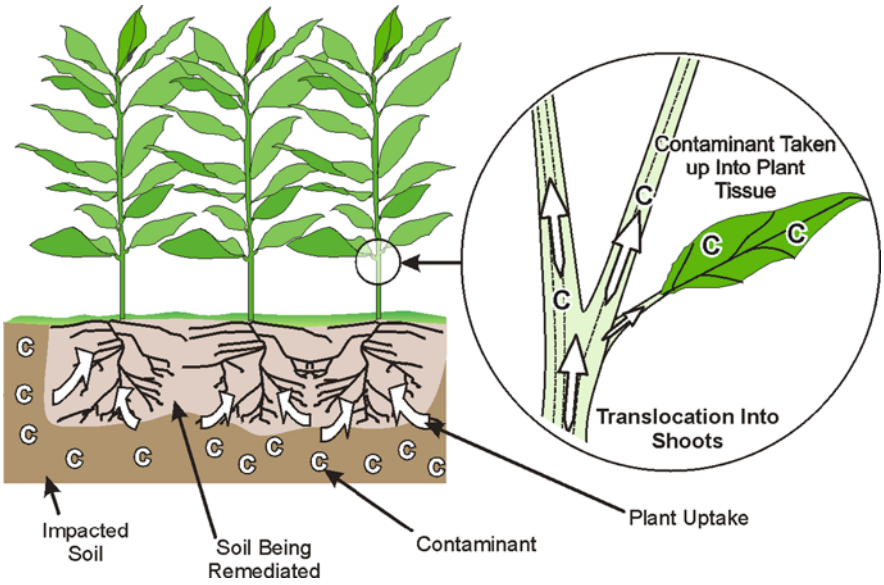

Fig. 7.4 Phytoextraction of inorganic contaminants. (Used with permission from Mueller et al., 2001)

range of lead a plant can accumulate is between 6.3 and 9.9 mg kg^{-1} (Outridge and Noller, 1991). Lead becomes toxic to the plants at levels above 27 mg kg^{-1} (Beckett and Davis, 1978). In addition to lead uptake, some plants are tolerant to increased levels of zinc, an essential mineral element. The mean level is 66 mg kg^{-1} and becomes toxic at levels of 230 mg kg^{-1} and higher (Borkert et al., 1998; Long et al., 2003). In some cases, plants can be classified as *hyperaccumulators* because of their ability to accumulate extremely high levels of the metal contaminant into their tissues. Hyperaccumulator status is achieved when a plant accumulates more than 1,000 mg kg^{-1} or 0.1% of the metal by dry weight for lead and 10,000 mg kg^{-1} or 1.0% of the metal by dry weight for zinc (Brooks et al., 1977). One example of a hyperaccumulator plant is *Brassica juncea* (L.) Czern. or Indian Mustard. *B. juncea* has been known to accumulate 1.5% Pb (lead) by dry weight with the addition of ethylenediaminetetraacetic acid (EDTA), a synthetic chelating agent used to increase the solubility of lead when grown in media that had a large quantity of lead available (Blaylock et al., 1997; Bouchier, 2003). Another plant that receives much attention as a hyperaccumulator of zinc (Zn) and cadmium (Cd) is *Thlaspi caerulescens* J. & C. Presl. or alpine penny cress. Research conducted by Baker et al. (1994, as cited in Brown et al., 1994) has shown that field samples collected at sites contaminated with cadmium (Cd) and zinc (Zn) had shoot concentrations as high as 164 and 21,000 mg kg^{-1} dry weight, respectively. Additionally, Brown et al. (1995) showed that when grown hydroponically in solutions containing 650 mg L^{-1} Zn and 22 mg L^{-1} Cd, *T. caerulescens* could accumulate Zn and Cd in shoots up to 33,600 and 1,140 mg kg^{-1} dry weight, respectively. These

heavy-metal concentrations far exceed the concentrations typically found in plant tissues.

In addition to *B. juncea* and *T. caerulescens* achieving hyperaccumulator status, *Typha latifolia* (the broadleaf cattail) is receiving attention due to its ability to grow in many different types of semi-aquatic and aquatic environments and tolerate high levels of contamination (Amaya-Chavez et al., 2006; Doucette et al., 2005). A greenhouse study of 12 weeks duration conducted by McDonald (2006), consisting of growing cattails in mason jars with lead levels of 0, 1,000, 2,000, and 4,000 mg kg^{-1}, has shown that there was no apparent reduction in growth caused by the different lead levels. It should be noted that any reduction in growth was attributed to the cattails being confined to a small growth area (McDonald, 2006). In addition, the only visible signs of exposure to consistently increasing levels of lead was the yellowing and burning of shoot tips subsequent to the addition of EDTA. To determine if *T. latifolia* could achieve hyperaccumulator status, ethylenediaminetetraacetic acid (EDTA) was added to the soil at the 10th week of the 12-week growth period.

Over the 12-week period, the cattails that were exposed to the varying levels of lead without the addition of EDTA revealed that the roots and rhizomes of the 4,000 mg kg^{-1} exposed cattails accumulated large quantities of lead rather than translocating lead to the above-ground tissues (McDonald, 2006). In this particular case, the cattails accumulated 1,515.2 mg kg^{-1} in root/rhizome tissue, showing that in the absence of a chelating agent, lead movement is significantly confined to a greater extent to the rhizomes and roots. On the other hand, with the addition of EDTA, the most promising results were obtained, as large quantities of lead accumulated in the cattail shoots that were exposed to the 4,000 mg kg^{-1} lead level. The roots were able to absorb 2,483.5 mg kg^{-1}, in which 2,418.5 mg kg^{-1} was transported into the shoots (McDonald, 2006), indicating that the chelating agent facilitated the translocation of lead to aerial portions of the plant. Based on these results, it was determined that *T. latifolia* can reach hyperaccumulator status with the assistance of a chelating agent that allows for the translocation of contaminates (lead in this case) from rhizomes and roots to the shoots.

Plants can also be used for *phytostabilization* which refers to use of plants to immobilize contaminants in the soil and/or ground water through absorption and accumulation by roots, adsorption onto roots, or the precipitation of contaminants within the rhizosphere (Fig. 7.5) (Phytoremediation Resource Guide, EPA, 1999; Brookhaven National Laboratory, 2000).

Additionally, this technique reduces the mobility of the contaminant(s) and prevents migration to groundwater, as well as reducing the bioavailability for entering the food chain. Using plants that are metal-tolerant can restore vegetation on contaminated sites where the threat of contaminant migration from soil erosion is high due to wind and water. Once a plant cover is established, the threat of direct contact with humans and animals is significantly reduced. The vegetative cover can provide a barrier between the contaminant(s) in the soil and the surrounding environment. This technique works in the following way: a study of the nature of the

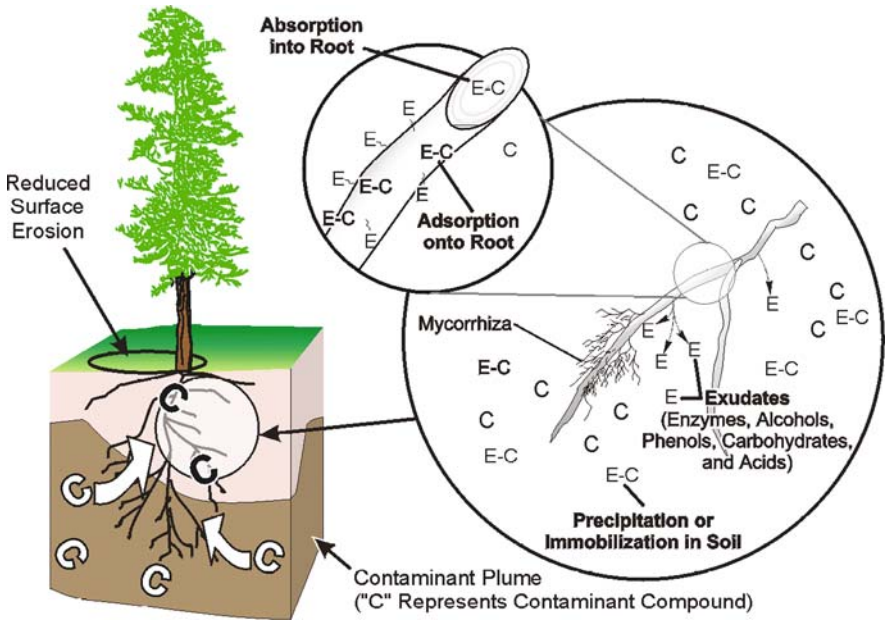

Fig. 7.5 Phytostabilization of inorganic or organic contaminants. (Used with permission from Mueller et al., 2001)

soil contaminant(s) is determined; next, traditional fertilizers or specific amendments are used to improve the soil conditions and render the contaminants immobile. Once this is achieved, a plant species is selected based on the conditions of the soil, the surrounding environment, the ability of the plant not to accumulate the contaminants into above-ground tissues, and the plant's tolerance to the specific site contaminants.

This technique can be thought of as a two-step process. First, a suitable soil amendment should be selected that is long-lasting if not permanent (Berti and Cunningham, 2000). This selection should be based on the following considerations: it must be inexpensive, easy to handle, safe for workers, be compatible and non-toxic with the plants selected, be available for use, and finally, not cause the environment further damage (Berti and Cunningham, 2000). In addition to the listed considerations, some amendments may have secondary benefits to the growth of plants by providing nutrients and by increasing soil water-holding capacity (i.e., phosphate fertilizers and organic materials). The most common amendments include phosphate fertilizers (e.g., superphosphate), organic matter or biosolids such as composts and manures, iron or manganese oxyhydroxides, clay minerals, or mixtures of these amendments (Cunningham and Berti, 2000; Berti and Cunningham, 2000). The second step is to determine a species of plant that is able to grow in the harsh environment of the contaminated site. These plants will be responsible for physically stabilizing the soil that they are growing on by having dense root

systems that prevent soil erosion by protecting the soil from human contact and rain. In addition, the root systems should reduce the incidence of water percolation, thus immobilizing the contaminant(s). Further considerations include these plants being poor translocators of heavy metals to the above-ground tissues (to reduce risk of entering the food chain via herbivory), their rapid growth rates, and their high transpiration rates that allow for more effective removal of soil water (Berti and Cunningham, 2000).

A study by Tang and Fang (2001) was conducted to determine the usefulness of two perennial herbs from the Polygonaceae family, *Polygonum microcephalum* D. Don (Red Dragon) and *Rumex hastatus* D. Don (Curly Dock), as plants that could be used for phytostabilization. Plant and soil samples (from the root zone) were taken from three different heavily contaminated copper mines of the Yunnan Province in China for analysis. Results showed that *P. microcephalum* accumulated high concentrations of copper in roots, stems, and shoots, averaging 491, 110, and 133 mg kg^{-1}, respectively. On the other hand, *R. hastatus* accumulated lower concentrations of copper in roots, stems, and shoots, averaging 33, 42, and 45 mg kg^{-1}, respectively. This, however, does show that both species can accumulate copper into tissues when grown in a copper-contaminated soil (Tang and Fang, 2001). Since *P. microcephalum* accumulated more copper in its tissues than *R. hastatus*, this could indicate that *P. microcephalum* has a higher level of tolerance than *R. hastatus*. When considering the higher levels of copper accumulated by *P. microcephalum* and lower accumulation amounts in *R. hastatus*, one can infer from these data that *R. hastatus* is excluding copper from the shoots. The result of the soil analysis revealed that copper concentrations for *P. microcephalum* and *R. hastatus*, averaged 1,494 and 2,105 mg kg^{-1} dry weight, respectively, confirming that these species can grow in highly contaminated soil. It should also be noted that there was a lack of soil fertility as confirmed by low soil organic carbon (humus). In conclusion, *P. microcephalum* appears to be more of an accumulator with respect to copper-type contaminants, whereas *R. hastatus* is more of an excluder based on the comparison of shoot/root metal ratios (Tang and Fang, 2001). This evidence shows that two species can grow side by side even though they have different uptake and transport characteristics, thus providing the potential for phytostabilization (Tang and Fang, 2001).

7.4 Case Study: Phytoremediation of Contaminated Air

There is also the concern today about how plants respond to air pollution and how they might be used to help alleviate this problem. This occurs mainly in urban areas as a result of industrialization and the burning of fossil fuels (Park et al., 2006). A large contributor to this pollution is the automobile, causing roadside damage to both plants and the underlying soil (Park et al., 2006). As fossil fuels are burned in the engines of vehicles, many greenhouse gases are emitted, including sulfur dioxide (SO_2), carbon monoxide (CO), and particulate matter (Kulshreshtha et al., 2003).

Since plants provide a large leaf surface area that is in contact with the surrounding environment, absorption, adsorption, and accumulation of these air pollutants can occur, thus reducing pollution's negative effect on the environment (Kulshreshtha et al., 2003; Sharma et al., 2005). Research conducted by Kulshreshtha et al. (2003) evaluated 30 species of plants to determine the tolerance to air pollution in a roadside (heavily polluted) environment and a park/garden (non-polluted) environment. In order to determine the tolerance of the plants, several factors were investigated. These included chlorophyll and ascorbic acid contents, relative water content, and leaf extract pH. These tolerance measures were then converted to an Air Pollution Tolerance Index (APTI) (Kulshreshtha et al., 2003). Plants that had an APTI that was high were considered to have a tolerance to pollutants, whereas plants with an APTI that was low indicated plants were not as tolerant to the pollutants. Of the 30 plants that were investigated, species that had an APTI of 30 or greater included *Catharanthus roseus* (L.) G. Don or Madagascar periwinkle (37), *Ficus religiosa* L. or Sacred fig (35), *Bougainvillea spectabilis* Willd. or Bougainvillea (32), and *Ficus glomerata* Roxb. or Bonsai tree (32), while plants that showed less tolerance (an APTI of 5 or less) to air pollution included *Delbergia sissoo* Roxb., or Shisham tree (3) and *Cansa carendes* (3). Further research with the plants that had high APTI value should be considered for the reduction of roadside pollution in urban areas.

Sharma et al. (2005) compared the use of bougainvillea plants as a means of reducing air pollution along roadsides in both high- and low-traffic areas. Plants of 11 cultivars grown in pots were subjected to a low-traffic-density area (LTDA) represented by the NBRI Botanical Garden and a high-traffic-density area (HTDA) represented by a median dividing roads in a high-traffic area (Sharma et al., 2005). To determine the effectiveness of the bougainvilleas at removing pollutants from the air, several different foliar aspects of health were studied. These parameters included cuticle, stomata, subsidiary cells, and trichomes. In the LDTA the cuticle was smooth, granular, or striate having inconspicuous wax at certain locations while the stomata were globose and present at the same level as other cells. The subsidiary cell walls were clear but slightly raised and the trichomes were non-glandular, uniseriate, and multicellular with a bolbus base and globular tip (Sharma et al., 2005). The HTDA foliage showed that the cuticle was wrinkled and striations were not present, while the stomata were raised from the surface of other cells and present in larger numbers. Subsidiary cell walls had fused and were irregularly shaped, while the number of trichomes increased, their overall length had decreased, and the cuticle over the trichomes had become cracked (Sharma et al., 2005). It was determined that because of morphological, anatomical, and physiological changes within the foliage, absorption of toxic pollutants was confined to the cell sap which neutralized these toxins, thus forming a stable complex within certain cellular compartments, thus allowing greater tolerance to pollutants. The conclusion reached was that bougainvilleas with their relatively thick leaves of large surface area can trap more dust and pollutants in the surrounding area of high traffic, reducing pollution, while at the same time, presenting a pleasing environment since these plants provide a wide array of flower colors (Sharma et al., 2005).

7.5 Conclusions

Since the beginning of time, humans have left their mark (in some cases, irreversibly) on the environment. During the industrial revolution, no one stopped to think about the long-term ramifications of constantly polluting air, water, and soil. The thinking during this period was that Earth's environment is going to be around forever and that it can clean itself. In addition to the industrial age releasing huge amounts of contaminants into the environment, the consumption of fossil fuels also increased as motor vehicles continued to grow in size and number. When humans discovered that the oil used for the production of gasoline was in short supply, they began to realize that our resources are limited. The same chain of events can be said about the logging industry, fishing industry, and the mining industry, just to name a few. As the realization became transparently clear, new ways to save our natural resources and to help repair the environment began to be developed. One of the outcomes of this greening revolution was the field or technology of phytoremediation, whereby plants that are already here can be used to assist in the rejuvenation of Earth's ecosystems. Through phytoremediation techniques, the environment can be "cleaned" in less destructive and most cost-effective ways by the virtue of the ability of plants' adaptation to many different types of contamination across many different ecosystems. Although phytoremediation seems like it is the answer to many problems of pollution, it is subject to some limitations such as requiring supplemental nutrients, having extended time requirements for remediation to occur, and only removing contaminants within the root zone (rhizosphere). However, the picture is not bleak because current research (from many different fields of study) is now coming together as a multidisciplinary effort for the discovery of new ways to better the phytoremediation processes. As long as there is a need for remediation (and there may likely be from now on), scientists will continue to come together and to work diligently to solve the problems of contamination in an attempt to make the world a safer and more healthy place to live.

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Chapter 8
Biotechnology of the Rhizosphere

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Abstract This chapter deals with the management of the rhizosphere as a living system, paying special attention to one of the three partners that define the rhizosphere: beneficial microorganisms (termed *PGPR* or the plant growth-promoting rhizosphere bacteria) that inhabit it. After that, several biotechnological approaches for management of the rhizosphere will be presented. These approaches relate to environment friendly agricultural practices, the production of high-quality foods with bioactive compounds (*phytonutrients*), and applications in the pharmaceutical industry.

The *rhizosphere* refers to the soil region that is subject to the influence of plant roots and their associated microorganisms. Among these microorganisms are plant growth-promoting rhizobacteria which are beneficial for plant health in many ways: by improving plant nutrition, protecting against other microorganisms, producing plant growth regulators, or enhancing plant secondary metabolic pathways that are directly related to a plant's defense. In some plant species, these secondary metabolites are useful to human health.

The biotechnology of the rhizosphere covers a wide array of applications that deal with sustainable agriculture (intensive or extensive): lowering of chemical inputs due to fertilizers and pesticides; improving crop productivity in saline and non-fertile soils; improvement of plant fitness for reforestation of degraded soils; and improvement in the bioactive levels of metabolites in medicinal plant species, among others. In this connection, the identification of *elicitors* (molecules that stimulate any of a number of defense responses in plants) appears to be an alternative to PGPR for unraveling limiting steps of secondary metabolism pathways.

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8.1 Introduction

The German agronomist Hiltner first defined the rhizosphere, at the end of the nineteenth century, as the "effect" of the roots of legumes on the surrounding soil, in terms of higher microbial activity, due to the organic matter released by the roots (Lynch, 1990). Until the end of the twentieth century, this "effect" was not considered to be an ecosystem in which the three components (plant, soil, and microorganisms) define a unique environment (Barriuso et al., 2008a). This environment changes depending on the conditions set up by the three components. Therefore, a deep knowledge of the interactions between the plant, the soil, and the microorganisms is vital to our understanding of how this complex rhizosphere system operates.

In this context, a second concept that needs to be addressed here is what we shall term biotechnology of the rhizosphere (Fig. 8.1).

Among the three components of the interaction shown in Fig. 8.1, microorganisms appear as the easiest element to manipulate, since we will usually select the plant of interest and the soil to work with. Microorganisms that inhabit the rhizosphere play a key role in plant physiology by affecting either directly or indirectly the plant's metabolism. These bacteria may increase nutrient availability in the soil, which will be reflected in better growth of the plant (indirect mechanisms), or may affect the plant's hormonal balance or its secondary metabolism (direct mechanisms) (Ramos Solano et al., 2008a). When secondary metabolism is affected, the plant's defense against pathogen or insect attack may be improved for better fitness. At the same time, in medicinal plant species, levels of phytopharmaceuticals are altered. In this case, either known metabolites increase or even new molecules may appear (Poulev et al., 2003). This role involves not only the direct effect of a single bacterial strain but also that of the molecular dialogue established among soil microorganisms and between microorganisms and the plant (Barriuso et al., 2008c).

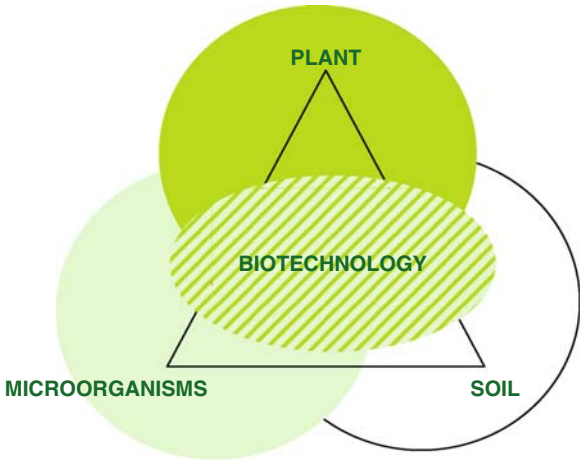

Fig. 8.1 Biotechnology of the *rhizosphere*. The *rhizosphere* is defined by the interaction of three components: plant, soil, and microorganisms. Biotechnology of the rhizosphere refers to management of any of the three factors and/or their interactions in order to obtain a certain effect

A thorough understanding of the PGPR action mechanisms is fundamental to manipulating the rhizosphere in order to maximize the processes within the system that strongly influence plant productivity. Therefore, the first goal of this chapter will be to examine rhizosphere microorganisms and then to explore their different biotechnological applications.

8.2 Plant Growth-Promoting Rhizobacteria (PGPR)

A large number of macroscopic organisms and microorganisms such as bacteria, fungi, protozoa, and algae coexist in the rhizosphere. The most abundant are bacteria. Plants release organic compounds via root exudates, which selectively attract beneficial bacteria (Lynch, 1990), creating a very selective low-diversity environment (Marilley and Aragno, 1999; Lucas García et al., 2001; Barriuso et al., 2005). Bacteria inhabiting the rhizosphere beneficial to plants are called *PGPR* (*Plant Growth-Promoting Rhizobacteria*) (Kloepper et al., 1980a). The rhizosphere of wild plant species appears to be the best source from which to isolate PGPR due to the co-evolution processes that have taken place over time (Lucas García et al., 2001; Gutiérrez Mañero et al., 2003; Barriuso et al., 2005; Ramos Solano et al., 2007).

PGPR have been reported as members of several genera including *Azotobacter*, *Acetobacter*, *Azospirillum*, *Burkholderia*, *Pseudomonas*, and *Bacillus* (Arshad and Frankenberger, 1998). The positive effect of PGPR occurs through various mechanisms.

Mechanisms used by PGPR have traditionally been grouped into direct and indirect mechanisms (for a recent review, see Ramos Solano et al., 2008a). Although the difference between them is not always obvious, *indirect mechanisms*, as a general rule, are those that happen outside the plant, while *direct mechanisms*are those that occur within the plant and directly affect the plant's metabolism. This means that the latter require the participation of the plant's defensive metabolic processes, which transduce the signal sent from the bacteria influencing the plant. Accordingly, indirect mechanisms are usually related to nutrient-related traits or defense against other microorganisms outside the plant, while direct mechanisms include those that affect the balance of plant growth regulators, either creating an outbound gradient from the roots to the soil (Glick et al., 1998) or because the microorganisms themselves release growth regulators that are integrated into the plant, leading to an improvement in its adaptative capacity (Gutiérrez Mañero et al., 1996, 2001). Two important phenomena are included in this group: systemic induction of secondary metabolism related to defense against plant pathogens and protection against high-salinity conditions (Barriuso et al., 2008b).

However, the existence of microorganisms able to prevent diseases from occurring in plants without the plant's direct participation is also known. This occurs by systems such as niche exclusion or pathogen-inhibiting substance production. When the physical contact of the pathogen and the protecting microorganism is required, it is known as *biocontrol* (Bloomberg and Lugtenberg, 2001; Compant et al., 2005).

A short review of the most relevant mechanisms follows and will be integrated into the subsequent case studies section later in this chapter.

8.2.1 PGPR That Utilize Indirect Mechanisms

The list of *indirect mechanisms* used by PGPR is substantial. A number of reports in the literature illustrate these types of mechanisms, and some are quoted herewith. Two groups can be devised, depending whether they are related to improvement of plant nutrition or pertain to pathogen performance. The first group includes (1) free nitrogen fixation, (2) siderophore production, and (3) phosphate solubilization. The second group includes (1) hydrolysis of molecules released by pathogens (e.g., Toyoda and Utsumi, 1991 reported the ability of two strains, *Pseudomonas cepacia* and *Pseudomonas solanacearum*, that are able to break down fusaric acid, a compound responsible for root rot caused by the fungus *Fusarium*); (2) synthesis of enzymes that are able to hydrolyze fungal cell walls (Lim et al., 1991); and (3) synthesis of cyanhydric acid (Voisard et al., 1989).

In addition, improvement of symbiotic relationships with rhizobia and mycorrhizae has been reported (Duponnois and Plenchette, 2003; Founoune et al., 2002; Garbaye, 1994; Marek-Kozackuk and Skorupska, 2001; Lucas García et al., 2004; Barriuso et al., 2008d), although further research will demonstrate whether these are direct or indirect.

Among indirect mechanisms, the most relevant for agricultural purposes are those involving nutrient mobilization (e.g., free nitrogen fixation, siderophore production, and phosphate solubilization). Such nutrient mobilization results in a lowering of chemical inputs to the environment, since the amount of chemical fertilizers necessary to achieve good crop yields would be lower. Interestingly, and for a proper and successful handling of this type of PGPR, it should be taken into account that there is increasing evidence that nutrient-related traits are inducible when the environmental conditions require such a need (Rainey, 1999). Otherwise, it may be the reason for the lack of success of some field inoculations (Ramos Solano et al., 2007). Moreover, if used appropriately, especially in low-fertility soils, these could be turned into better soils by increasing the culturable soil surface, which is one of the limiting factors currently needed to palliate world famine.

A short description of nutrient-related traits follows.

8.2.1.1 Free Nitrogen Fixation

These types of nitrogen-fixing bacteria were the first PGPR assayed to improve plant growth, especially crop productivity. The first report of these bacteria appeared before World War II, when they were widely used on cereal fields in the Soviet Union (Bashan and Levanony, 1990). They are free-living organisms able to fix nitrogen that inhabit the rhizosphere but *do not establish a symbiosis* with the plant. Although they do not penetrate the plant's tissues, a very close relationship is established; these bacteria live so close to the roots that the atmospheric nitrogen fixed

and not used by the bacteria is taken up by the plant, forming an extra supply of nitrogen. This relationship is described as an unspecific and "loose" symbiosis. Biological nitrogen fixation is a high-cost process in terms of energy. Bacterial strains able to perform this process do so to fulfill their needs, and thus, little nitrogen is left for the plant's use. However, difficulties may be overcome by biotechnological approaches based on genetic manipulations and other strategies to improve colonization capacities.

However, growth promotion caused by nitrogen-fixing PGPR was erroneously attributed to nitrogen fixation for many years, until the use of nitrogen isotopes occurred. This technique showed that the benefits of free nitrogen-fixing bacteria are due more to the production of plant growth regulators than to nitrogen fixation (Baldini, 1997). This kind of production of plant growth regulators is discussed later.

8.2.1.2 Production of Siderophores

Iron is an essential nutrient for plants. Iron deficiency is manifested in severe metabolic alterations due to its role as a cofactor for a number of enzymes essential to important physiological processes such as respiration, photosynthesis, and nitrogen fixation. Iron is quite abundant in soils, but it is frequently unavailable for the plant or soil microorganisms, since the predominant chemical species is Fe^{3+}, the oxidized form that reacts to form insoluble oxides and hydroxides, inaccessible to plants or microorganisms.

Plants have developed two strategies for efficient iron absorption. The first one consists of releasing organic compounds able to chelate iron, making it soluble; iron diffuses toward the plant where it is reduced and absorbed by means of an enzymatic system present in the cell membrane. The second strategy consists of absorbing the complex formed by the organic compound and Fe^{2+}, where the iron is reduced inside the plant and readily absorbed. Some rhizosphere bacteria are able to release iron-chelating molecules to the rhizosphere and, hence, serve the same function as in plants (Kloepper et al., 1980b).

Siderophores are low molecular weight compounds, usually below 1 kDa, which contain functional groups capable of binding iron in a reversible way. The most frequent groups are hydroximates and catechols, in which the distances among the groups involved are optimal to bind iron. Siderophore concentration in soil is around 10^{-30} M.

Siderophore-producing bacteria usually belong to the genus *Pseudomonas*, the most frequent being *Pseudomonas fluorescens*, which release the siderophores, pyochelin and pyoverdine. Rhizosphere bacteria release these compounds to increase their competitive potential, since these substances have antibiotic activity and improve iron nutrition for the plant (Glick, 1995).

Siderophore-producing rhizobacteria improve plant health at various levels: they improve iron nutrition, inhibit the growth of other microorganisms with their antibiotic molecules, and hinder the growth of pathogens by limiting the iron available for the pathogen, generally fungi, which are unable to absorb the iron–siderophore complex. Hence, siderophore-producing bacteria could be released to improve iron

nutrition at the same time that certain pathogens are controlled, resulting in lower chemical inputs due to pesticides and fertilizers.

8.2.1.3 Phosphate Solubilization

After nitrogen, phosphorous is the most limiting nutrient for plants. However, phosphorous reserves, although abundant, are not available in forms suitable for plants. Plants are only able to absorb the soluble forms, namely, monobasic and dibasic phosphates. Besides inorganic forms of phosphorous in soil, the phosphorous present in organic matter is of considerable importance. The organic forms of phosphorous are estimated to be between 30 and 50% of the total phosphorous in the soil. This reservoir can be mineralized by microorganisms, making it available to the plant as soluble phosphates. There are many bacteria from different genera that are able to solubilize phosphate. These include *Pseudomonas*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Achromobacter*, *Agrobacterium*, *Micrococcus*, *Aerobacter*, *Flavobacterium*, *Chryseobacterium*, and *Erwinia*. Bacteria use two mechanisms to solubilize phosphate: (1) releasing organic acids that mobilize phosphorous due to ionic interactions with the cations of the phosphate salt and (2) releasing phosphatases responsible for releasing phosphate groups bound to organic matter. Most of these bacteria are able to solubilize the Ca–P complex, and there are others which operate in the Fe–P, Mn–P, and Al–P complexes. Generally, these mechanisms are more efficient in basic soils.

Results with PGPR able to solubilize phosphate are sometimes erratic, probably due to soil composition, given the inducibility of nutrient-related traits. In fact, in order to have a good performance, they would have to be inoculated in soils with a phosphorous deficit and stored in insoluble forms. Hence, inoculations of these types of PGPR sometimes improve plant growth and sometimes they are completely inefficient. Without doubt, knowledge of their mechanisms and ecology in the rhizosphere will improve their use in sustainable agriculture (Gyaneshwar et al., 2002).

8.2.2 PGPR Using Direct Mechanisms

*Direct mechanisms*are those that occur inside the plant and directly affect the plant's metabolism (Ramos Solano et al., 2008a) by involving the plant's defensive metabolic processes, which transduce the signal sent from the bacteria that influence the plant. Plant growth regulators can be considered as participants in the principal PGPR mechanism, together with the induction of systemic resistance (ISR), which has in recent years become an important issue. Both involve the existence of bacterial eliciting molecules, receptor binding, and further signal transduction. When bacteria release a plant growth regulator, all three stages are known, because this process is the same in plants and bacteria. However, this is not the case for induction of systemic resistance, in which the eliciting molecules, the receptor, and the signal transduction mechanism, as a general rule, are still unknown.

8.2.2.1 PGPR That Modify Plant Growth Regulator Levels

Plant growth regulator production by bacteria was first described more than 40 years ago. This was determined in the 1960s using the biological assays then available. Nowadays, using modern techniques, it has been demonstrated that the production of plant growth regulators such as auxins and ethylene by bacteria is a common trait (Bent et al., 2001). Others, such as cytokinins, are less common, while gibberellins in high concentrations have only been described for two strains of the genus *Bacillus*, isolated in the rhizosphere of *Alnus glutinosa* (Gutiérrez Mañero et al., 2001), the amounts being 1,000 times higher than those reported for *Rhizobium* that is involved in forming the nodule.

Modification of a plant's physiology by plant growth regulator production is a very important mechanism, not only because it alters the principal mechanism of growth regulation and cell differentiation in the plant but also because it is based on the evolutionary development of common metabolic pathways in plants and bacteria. This implies interesting co-evolution aspects. Biosynthetic pathways of plant growth regulators share many steps with the classical secondary metabolism pathways. This suggests a common ancestor, which in the course of evolution has produced either a large diversion in the function, conserving the genetic homology, or the function has remained the same, but there has been a large genetic divergence. This is evident in the phenolic compound biosynthesis pathway (shikimic acid pathway), which is shared by both plants and microorganisms. It is essential for synthesis of amino acids such as tryptophan, the precursor in auxin biosynthesis. The same occurs in the biosynthetic pathway of terpenes, gibberellin precursors. Therefore, the existence of common biosynthetic pathways and metabolic products implies the possibility of creating a parallel evolutionary connection between plants and microorganisms. Furthermore, it is striking that secondary metabolites synthesized by plants for defense also target some human receptors that affect human physiology, making the interest in these compounds even more interesting.

The production and release of plant growth regulators by bacteria cause an alteration in the endogenous levels of plant growth regulators. This is dependent on several factors, including (1) plant growth regulator concentration; (2) the proximity of the bacteria to the root surface; (3) the ability of the growth regulator to diffuse in soil and be transported across plant cell walls to the interior compartments of the cells; and (4) the competitiveness of the bacteria to colonize and survive in areas where there is high root exudation.

Based on the above discussion, we see that the effect of bacteria on the plant growth regulators' balance depends on many factors, and because of this, results with these different types of PGPR may vary. Moreover, a PGPR producing more than one type of plant growth regulator can cause a synergistic effect when their action is coupled. The next logical points to consider here are the main physiological functions of each growth regulator. A short description for each follows.

The production of hormones such as *gibberellins* or *cytokinins* has been reported for a small number of bacteria able to produce these plant growth regulators (Timmusk et al., 1999; de Salomone et al., 2001). Cytokinins are known to induce

cell division (Salisbury, 1994) and have recently been reported in free-living bacteria (Arkhipova et al., 2007). Concerning gibberellins, there is little information regarding microorganisms that produce this type of plant growth regulator. However, it is known that symbiotic bacteria that form nodules in the plant to fix nitrogen (Rhizobia) are able to produce gibberellins, auxins, and cytokinins in very low concentrations when the nodule is forming at the time of high cell duplication rate (Atzorn et al., 1988). However, the production of gibberellins by PGPR is rare, with only two described strains able to produce gibberellins in relevant concentrations: *Bacillus pumilus* and *Bacillus licheniformis* (Gutiérrez Mañero et al., 2001).

Auxins are derived from tryptophan metabolism, and their effects depend on the concentration, the organ affected, and the physiological status of the plant. Auxins synthesized by the plant and the microorganisms only differ in the biosynthetic pathway, depending on the plant and/or the microorganisms. More than 80% of soil bacteria in the rhizosphere are capable of producing auxins. Thus, the potential of these microorganisms to affect the endogenous levels of this regulator, and therefore their effects on plant growth, is remarkable.

The reason there are so many bacteria in the rhizosphere that are able to produce auxins is still unknown. Some authors suggest that these bacteria have a tryptophan-related metabolism and that auxin biosynthesis represents a detoxification mechanism (Bar and Okon, 1992). Other authors propose that auxins have some cellular function because a clear relationship has been observed between auxin and cyclic AMP (adenosine monophosphate) levels, which regulate many metabolic processes (Katsy, 1997). However, the anthropomorphic view of this fact could be correct, namely, that auxin synthesis improves plant growth that results in more exudation and more nutrients for rhizobacteria. This hypothesis explains a mutualistic beneficial association between rhizospheric microorganisms and the plant. The plant controls the energy flux in the system because it has more genetic information and contributes most of the organic matter to the rhizosphere.

Auxins released by rhizobacteria mainly affect the root system, increasing its size and weight, branching number, and the surface area in contact with soil. All of these changes lead to an increase in the ability of roots to extract nutrients from the soil, therefore improving plant nutrition and growth capacity (Gutiérrez Mañero et al., 1996). Another important result of inoculation with auxin-producing bacteria is the formation of adventitious roots, which are derived from the stem. The auxins induce dedifferentiation of the stem tissues to dedifferentiate as root tissue. All the above effects can vary considerably depending on the auxin levels that reach the root system, including an excess, which could be inhibitory. In order to explain these inhibitory auxin effects, the relationship of auxin with ethylene has to be considered.

Ethylene is another growth regulator whose levels alter PGPR, in turn affecting physiological processes in the plant. It primarily functions in regulating plant development processes, including seed germination, root growth, leaf abscission, fruit development and ripening, as well as defense systems and stress responses. Factors such as light, temperature, salinity, pathogen attack, and nutrition can cause marked variations in ethylene levels. The influence of abiotic factors in ethylene

levels was deduced some time before biotic factors were discovered (Abeles et al., 1992; Morgan and Drew, 1997).

As ethylene levels decrease, root systems increase their growth, with the benefits already mentioned. Using PGPR to reduce ethylene levels in the plant could be an interesting method to improve certain physiological processes in the plant. Ethylene biosynthesis starts in the methionine cycle; one aminocyclopropanecarboxylic acid molecule (ACC) results from each turn of the cycle. The enzyme responsible for ACC production is ACC synthase, whose expression level and activity are regulated by a large number of signals such as auxin, ethylene, and environmental factors. The ACC is the substrate for ACC oxidase, also called ethylene-forming enzyme (EFE). This enzyme has been cloned from numerous species and belongs to a multigenic family which produces different types of ACC oxidases depending on the plant organ and development state.

The model proposed for ethylene regulation in the plant by PGPR is based on the ability of some bacteria to degrade ACC, the direct precursor of ethylene (Glick et al., 1994a). The degradation of this compound creates an ACC concentration gradient outbound, favoring its exudation and, hence, a reduction of the ethylene level inside. This, in combination with auxins that may be produced by the same microorganism, has a considerable impact on important physiological processes, such as root system development, since the bacterial ACC deaminase competes with the plant's ACC oxidase. This ACC deaminase enzyme has been isolated and identified in several bacterial and fungal genera, all having the ability to use ACC as the sole nitrogen source. Curiously, no microorganism has yet been found that is able to form ethylene from ACC (Glick et al., 1994b). Since ethylene and auxins are two related types of growth regulators and since the balance between them is essential for the formation of new roots, some effects attributed to auxin-producing bacteria are actually due to ACC degradation.

PGPR that reduce ethylene levels in plants are also able to improve nodule formation in legumes and mycorrhizae formation in many other types of plants. A temporary reduction of ethylene in the earlier stages of either of these processes is beneficial.

Case study: The aim of this case study involving two separate studies (Gutiérrez Mañero et al., 1996, 2001) is to highlight the synergistic effects of bacterial strains producing two types of plant growth regulators.

These bacteria were isolated from the rhizosphere of *A. glutinosa* and production of IAA-like compounds in the culture media was demonstrated by bioassay. This bioassay was set up by adding bacteria cultures media free of bacteria to alder seedlings in two different concentrations. When a bacterial strain tested positive for enhancement of shoot and root growth, the results were plotted against data for plants that were grown on media containing increasing concentrations of IAA (Gutiérrez Mañero et al., 1996). However, addition of synthetic IAA to plants did not reproduce exactly the same effects as obtained for compounds released by bacteria, when their growth parameters were studied. Higher shoot surface suggested the presence of gibberellin-type compounds. Hence, a second study was carried out to detect these compounds. First, a bioassay was performed and second, identification

of putative compounds by HRGC-MS was employed. Bacterial culture media free of bacteria were concentrated and added to the shoot tips of young, dwarf alder seedlings; a control with GA_3 was also used. The same bacterial medium that was free of bacteria was used for HRGC-MS identification. These strains have shown a capacity to produce large quantities of gibberellins (GA_1, GA_3, GA_4, and GA_{20}) in vitro. The gibberellins were identified by HRGC-MS, and the amounts detected reached 200 $ng \cdot mL^{-1}$, GA_1 being the most abundant (130–150 $ng \cdot mL^{-1}$). These amounts were 1,000 times higher than for any other example of fungal or bacterial gibberellin production reported. Furthermore, the combination of gibberellins produced caused a balanced physiological effect in the plant opposite to the effects of GA_3 alone. This resulted in excessively long stems with pale yellow leaves. The suggested reason for the pronounced effect of gibberellins released by the PGPR present in the rhizosphere is that these hormones can be translocated from the roots to the aerial parts of the plant. The effects in the aerial part are notable, and even more so, when the rhizobacteria also produce auxins that stimulate growth of the root system. This enhances the nutrient supply to the sink generated in the aerial parts.

Based on these results, rhizobacteria able to release plant growth regulators can be formulated in a *biofertilizer*, with its intended use being to strengthen plant growth without any chemical input to the system.

8.2.2.2 PGPR That Induce Systemic Resistance (ISR)

At the beginning of the 1990s, Van Peer et al. (1991) and Wei et al. (1991) made an important discovery about plant defense mechanisms and productivity. These investigators found that certain non-pathogenic bacteria were able to prevent a pathogen attack before the pathogen reached the plant. The difference with biocontrol is that the beneficial bacteria do not interact physically with the pathogen but instead trigger a response in the plant which is effective against subsequent attacks by a pathogen. This response is systemic; that is, the bacteria interact with the plant in a restricted area, but the response extends to the whole plant. This response is mediated by metabolic changes that are not evident at first glance. As a matter of fact, *priming* or *biopriming* is the physiological state of a plant that is systemically induced by non-pathogenic bacteria against subsequent pathogen attack; but, the effect is not detected until pathogen challenge occurs (Conrath et al., 2002). Since energetic metabolism is diverted to secondary metabolism, this physiological state is usually coupled to lower growth rates as compared to non-primed controls (van Hulten et al., 2006). For the protection to be effective, an interval is necessary between the PGPR–plant contact and the pathogen attack in order for the expression of the plant genes that are involved in the defense. This mechanism was first known as "rhizobacteria-mediated induced systemic resistance" (Liu et al., 1995), but it is now termed "induced systemic resistance" (*ISR*) (van Loon et al., 1998). ISR was reported in the plant–pathogen-beneficial bacteria model, *Arabidopsis thaliana–Pseudomonas syringae DC3000–P. fluorescens WSC417r*. Here, the defensive response induced by *P. fluorescens* WSC417r in *A. thaliana* against *P. syringae* DC3000 is mediated by JA (jasmonic acid) and ethylene. Since then, it has been described in many plant species, including bean,

tobacco, tomato, and radish, with different PGPR and pathogens, and an increasing number of signal transduction pathways. This finding is fundamental because it proposes an "immune" *response* in the plant, raising the possibility of "vaccination" for the plant.

The plant can also acquire immunity after a pathogen attack. This response has been described before the ISR. The acquisition of resistance by the plant after a pathogen attack, causing little damage or localized necrosis in response to a further pathogen attack, has been known for many years. The phenomenon is called *systemic acquired resistance* (*SAR*) (Ryals et al., 1996). During a pathogen attack, *reactive oxygen species* (*ROS*) are produced in necrotic areas, causing tissue death. If the plant survives the challenge, it remains protected for life.

In *A. thaliana*, the SAR and ISR responses are regulated by distinctly different pathways. SAR is associated with an increase in salicylic acid levels and the translation of an ankyrin-type protein called NPR1, located in the nucleus, which induces the transcription of the pathogenetic-related (PR) genes. These genes codify the PR proteins that are responsible for systemic resistance in the plant (Lawton et al., 1991; Uknes et al., 1993). In the ISR response, salicylic acid levels are not altered, but the response is mediated by other two growth regulators, namely, ethylene and jasmonic acid, which act as signal transductors and not as stress hormones. In ISR, the NPR1 protein is also involved. But here, it induces the expression of other proteins different from PRs (Conrath et al., 2002). In addition to these pathways, research with new beneficial agents and different pathways are being described, especially since the ability of a PGPR to induce systemic resistance depends on the plant-beneficial bacteria–pathogen system. There is evidence of certain PGPR that are able to elicit systemic protection against *P. syringae* DC3000 in *A. thaliana* involving the SA-mediated pathway (Ramos Solano et al., 2008b).

SAR and ISR responses lead to plant protection against different pathogen species, but there are species which overlap. However, both responses can coexist in the same plant at the same time (van Wees et al., 2000). Thus, the use of PGPR or PGPR mixes able to trigger both responses at same time would result in an important advance in the improvement of pest defense systems.

The induction of defense metabolism, in fact, involves an induction of secondary metabolism developed by sessile organisms that are adapted to survive any changes of a biotic and abiotic nature. Therefore, some PGPR may trigger secondary metabolism against pathogens that at the same time may also be effective against biotic stress, such as saline conditions in soils, a frequent situation in agriculture. Furthermore, when a medicinal plant is used, phytopharmaceutical levels may also be increased or even new molecules may appear. This topic will be discussed in Section 8.4.

8.3 Application of PGPR for Agricultural Purposes

The use of PGPR in agriculture is one of the most interesting alternatives for improving sustainable agricultural practices, as well as for recovering degraded ecosystems (Vessey, 2003). PGPR can be used for a wide range of purposes that include

biofertilizers, biocontrol agents, induction of systemic resistance, and elicitors of secondary metabolic pathways that lead to products of nutritional and pharmacological interest and, therefore, of economic interest.

In view of the mechanisms described in Section 8.2, three case studies will be discussed: (1) suitability of the PGPR for nutrient-related traits; (2) alteration of plant metabolism mediated through plant hormonal balance; and (3) alteration of soil communities and their relation to biological effects. They highlight important aspects for successful application in agriculture.

Case study: A screening for PGPR to improve the growth of *Cistus ladanifer* (Gum Rockrose) seedlings for reforestation of degraded Mediterranean ecosystems was carried out by Ramos Solano et al. (2007). This screening for PGPR was carried out in the rhizosphere of wild populations of *C. ladanifer*, with the aim being to identify putative strains that are able to enhance the mycorrhization ability of *Cistus* with its spontaneous mycorrhizal fungus, *Amanita ponderosa*. The aim of identifying these strains was two-fold: one due to the great economic interest in the edible fruiting body of *Amanita* and, the second objective, helping the mycorrhization for soil recovery. Two hundred and seventy bacteria were isolated, purified, and grouped by morphological criteria. Fifty percent of the isolates were selected and tested for aminocyclopropanecarboxylic acid (ACC) degradation, auxin and siderophore production, and phosphate solubilization. Fifty eight percent of the isolates showed at least one of the evaluated activities, with phosphate solubilization and siderophore production being the most abundant traits. This is consistent with the chemical composition of the soil sampled, since usually the areas where *C. ladanifer* grows consist of a degraded soil or one that is in a regeneration stage following strong perturbation. A genetic analysis was performed with all strains that showed at least one positive trait. After PCR-RAPDs (randomly amplified polymorphic DNA) analysis, 11 groups appeared with 85% similarity, revealing the low diversity in the system. One strain of each group was tested in a biological assay, and those that enhanced *Cistus* growth were identified by 16S rDNA sequencing.

Although 7 of the 11 assayed strains were phosphate solubilizers and able to produce siderophores, only one was really effective in increasing all biometric parameters in *C. ladanifer* seedlings. This suggests that other mechanisms apart from nutrient mobilization might be involved in growth promotion by this strain. The lack of effect of the other six strains was probably due to the rich substrate used (peat) that diminishes the putative beneficial effect of the bacterium. Since this trait is not useful for this condition, genes are not expressed. However, the low diversity, together with the high redundancy detected by PCR-RAPDs and the predominance of strains able to mobilize nutrients in the rhizosphere of *Cistus*, reveals that the plant selects for bacteria that can help to supply scarce nutrients. These types of plant growth-promoting rhizobacteria (PGPR) strains should be successful in reforestation practices or in agricultural soils where phosphate or iron is present but not available for plants. This occurs under extreme conditions, where the PGPR represent a selective advantage for the plant.

The above case study shows that nutrient-related traits are not always useful for growth promotion (indirect mechanisms). Inoculation of nutrient-mobilizing bacteria will only result in positive results where nutrients are scarce. Therefore, bacterial strains showing nutrient-related traits should be used to increase productivity in areas with low yields due to lack of nutrients, not in rich cropping soils where chemical fertilizers are used and would result in "silencing" of the bacterial effect. In these latter soils, they should be used to lower the input of chemical fertilizers into the system.

However, the same PGPR strain may act by several mechanisms or may use different ones. The following case study illustrates another aspect of agriculture where plant nutrition cannot be further improved and the bacterial strain is able to improve yield under intensive greenhouse conditions. This strain is able to produce plant growth regulators using direct mechanisms.

Case study: The aim of this case study is to show how the inoculation of one PGPR strain is able to produce plant growth regulators (gibberellin and auxin types), using intensive culture, that improve productivity and protect the plant. It is within the aim of this study to show that inoculation of such biologic agents can be useful and feasible in current agricultural practices.

The effects of inoculation with a strain of *B. licheniformis* on the growth of pepper and tomato were investigated in three experiments. Before field trials, the survival of the strain against pesticides used in greenhouse production was tested to discard any possibility of failure due to pesticides; the bacterium was tolerant to most of them, leaving a possibility to be a part of integrated pest management (IPM). Of the three experiments, one was carried out under seedbed conditions and two under greenhouse production conditions. In the first experiment, the bacterium significantly increased the height of the plants and the leaf area in both species and in both cultivars. This is interesting for the production of plantlets with better adaptative capacities that will have better performance when transplanted to production greenhouses. Effects were more marked on pepper than on tomato, revealing certain specificity between the strain and the plant species. In the second experiment, seedlings growing in sand and in hydroponic culture were studied. The number and diameter of tomato fruits produced in sand and in hydroponic medium were increased significantly by PGPR inoculation, revealing the effects of gibberellins released by the PGPR. Given the physiological effects of this PGPR on flowering, it is interesting that flowering took place 15 days earlier in inoculated plants. This would result in the fruit reaching the market 2 weeks before the expected time. Hence, one can see the putative benefits for producers using these types of inoculants. In addition, PGPR-treated plants showed a lower incidence of disease than non-treated plants (no pesticides were used in either block), revealing a systemic induction of defensive metabolism by the PGPR. The effect could also be attributed to the simple colonization that could be impeding soil pathogen colonization (termed *niche exclusion*). In the third experiment, the total weight of pepper fruits harvested from PGPR-inoculated plants increased significantly as compared with non-inoculated controls. In light of the considerable colonization

and competitive ability of this PGPR strain and its effects on growth and plant physiology, it could be used as a biofertilizer or biocontrol agent without altering normal management in greenhouses. This would allow for lower chemical inputs for pathogen control.

However, the effects reported for greenhouse production have been validated only for those conditions per se (that is, extreme nutrient control and hydroponic or sand support). When this same bacterial strain is inoculated into soil containing its natural communities, the effect can be different. As a matter of fact, among the number of studies conducted on this topic, two will specifically be addressed in the third case study. But the general rule is that a strong alteration of native soil communities results in a lack of effect on plant growth, while a slight alteration of soil communities after PGPR inoculation is coupled with good results for plant growth.

Case study: This study is concerned with the influence of an indigenous European alder (*A. glutinosa* L. Gaertn) rhizobacterium (*B. pumilus*) on the growth of alder and its rhizosphere microbial community structure in two soils (Ramos et al., 2003). The aim of this study is to show that alteration of native communities of soils is negatively related to biological effects. European alder seedlings were inoculated with a suspension of the putative plant growth-promoting rhizobacteria (PGPR) *B. pumilus* (CECT 5105), or left non-inoculated (controls) in two different soils, and grown under controlled conditions. Soil A showed a coarse texture, was slightly acidic, and possessed a high nitrogen content, while soil B showed a fine texture, was basic in pH, and possessed a lower nitrogen content. The bacterium was isolated from soil A. At each sampling time, over an 8-week period, shoot and root systems of the plants were measured, determining shoot and root length and surface area; the number of nodules produced were counted. In addition, changes in the microbial rhizosphere structure were evaluated by the phospholipid fatty acid (PLFA) profile after extracting directly from the rhizosphere soil.

The increases detected in shoot surface were significant only in soil A, while the root system was affected in both soils, revealing the ability to produce auxin-like compounds of this strain that elicited better growth of the root system. However, while in soil A inoculation with *B. pumilus* caused a perturbation that subsequently disappeared, the rhizosphere community structure was seriously altered in soil B. This effect is based on the different profiles of phospholipids/fatty acids that indirectly reveal changes in the composition of microbiota. All biometric parameters were enhanced to a greater extent in soil A, in which the PGPR inoculum did not alter the existing rhizosphere communities, and nutrient availability was better. This is consistent with the previous hypothesis that release of auxin-type plant growth regulators by inoculated PGPR will result in better growth of the root system, which improves plant nutrient absorption potential. If this is coupled to enhanced nutrient availability, plants will show an increase in their growth parameters. Also, results were worst in soil B, in which case the PGPR strain had been isolated from soil A. In addition to the problems that inoculation caused in rhizosphere communities, there might be a nutrient dependence from the original soil that could condition the synthesis of growth regulators or any other factors that could affect growth.

As a concluding remark from this case study, it is clear that the influence of the soil cannot be discarded as a factor that has a negative influence on the beneficial effects of PGPR. Thus, it should be considered so as to increase success when releasing inoculants in soils. Although this case study was developed for a tree, not an agronomic or vegetable crop, this concluding remark applies to any crop species where the soil nature may condition the success of an added biofertilizer.

8.4 PGPR Affects Secondary Metabolism

Because plants have evolved secondary metabolism strategies to survive changing biotic and abiotic conditions encountered during their existence, this has also allowed them to colonize most habitats. Given the number of possibilities of changing conditions, both for biotic and abiotic factors, the array of secondary metabolites designed for each situation is enormous. Secondary metabolites can be studied from different points of view, such as the above examples for agriculture, the evaluation of their role in a plant's defense against pathogens, and their effects on human health. The following examples have been selected to illustrate each of them.

8.4.1 Lowering Chemical Inputs by Enhancing a Plant's Defensive Responses

When secondary metabolism is studied from the plant's defense point of view, the main goal is to meet requirements for sustainable development so as to achieve the highest yields. The mechanisms involved can be direct or indirect. If the bacterium used triggers the plant's defensive metabolism, it is a direct mechanism; if the bacterium is not able to trigger the plant's metabolism, but is able to interact with other microorganisms (biocontrol), it is an indirect mechanism. Moreover, they can happen simultaneously, achieving even better results than if they rely on a single microorganism, or rely on different ones that may show complementary beneficial traits, as for example, those related to nutrient mobilization.

The *case study* presented here pertains to rice: (*Oryza sativa* L.) production in Southern Spain (Seville) (unpublished data) and brings together two aspects of induction of secondary metabolism: (1) protection against pathogens and (2) protection against salinity inherent to rice cropping in this area.

The study was set up in Seville by the Guadalquivir River. Two PGPR strains were tested in experimental plots in the area, namely, *Chryseobacterium balustinum* and *P. fluorescens*. Both of them have demonstrated their ability to induce systemic resistance in *A. thaliana* (L.) Heynh. against *P. fluorescens* DC3000, *C. balustinum* being more effective (Ramos Solano et al., 2008), and has also shown biocontrol activity against *Rhizoctonia* (Doménech et al., 2006). *C. balustinum* has, in addition, demonstrated its ability to induce protection against saline conditions in *A. thaliana* (L.) Heynh. (Barriuso et al., 2008b). With these two candidates, five treatments were set up: each of the strains alone, the two combined, and two controls, one under

regular phytochemical treatments and the other without any addition of chemicals. Interestingly, the addition of bacterial treatments, once in the seed and then followed by several aerial inoculations during the growing season, resulted in a small increase in yield, but in addition, caused a large decrease in disease incidence under natural conditions. It is striking that the combination of the two strains achieved the best results. In light of these results, a combined strategy is going to be proposed for their use in integrated pest management. In this case, chemical pesticides can be applied if disease incidence runs out of control when the biological agent is used.

8.4.2 Increase in Secondary Metabolites of Pharmaceutical Interest

Special interest has been paid to plant species of medicinal interest due to their role in human health. Ever since plants have been used for healing, plant collectors have selected those with a better effect on health. These differences, in effect, are attributable to either higher levels of phytopharmaceuticals or differences in their relative contents, which most of the time are affected by environmental conditions when harvested from the field. A third possibility to explain this is that, for a known medicinal plant species, new molecules with pharmacological interest may appear when it is grown under a new set of environmental conditions or with a stress factor. Therefore, this would account for the improvement in its beneficial effects for human health.

The variability of secondary metabolism is a problem for the pharmaceutical industry since field production is uncertain and may condition availability of final products. Sometimes, the problem may be solved by chemical synthesis, but it is not always possible or at least it usually lacks economic feasibility. Another alternative is cell culture, but for some plant species, it is not feasible, or yields achieved are too low because secondary metabolism is not necessary under such controlled and undifferentiated conditions. Therefore, one of the main goals in industry now is to obtain reproducible extracts of plants grown in field production or, even more challenging, grown in plant cell cultures. For this purpose, the use of *elicitors* appears to be an encouraging alternative (Radman et al., 2003). In support of this last statement, a recent study by Poulev et al. (2003) has reported the potential of elicitation to discover new molecules with pharmacological interest. But this study not only reports the presence of new molecules but also the use of elicitors has been able to duplicate the presence of these molecules and to increase the concentration of known compounds. Hence, unraveling the nature of elicitors and the elicited pathways remains an exciting challenge for the pharmaceutical industry.

Among these putative elicitors, PGPR appear as good candidates for upregulating secondary metabolism. The following case study (Gutiérrez Mañero et al., 2003) illustrates how PGPR strains isolated from the rhizosphere of wild populations of *Digitalis* are able to enhance levels of cardenolides in high-yielding varieties of *Digitalis lanata* Ehrh., grown either in field production or as in vitro cell cultures.

Case study: The 480 isolates from a bacterial screening assay carried out in the rhizosphere of two *Digitalis* species in two physiological stages were characterized at the generic level. *Bacillus* was the dominant genus in all cases. Fifty percent of the *Bacillus* strains isolated from each species were analyzed by PCR-RAPDs. At 85% similarity, 12 groups separated out for *D. thapsi* L. and 18 for *D. parviflora* Jacq. One strain of each group was selected for biological assay on high-yield selected varieties of *D. lanata* Ehrh., kindly provided by Boehringer Mannheim (Spain). The evaluated parameters were growth promotion and cardenolide content in leaves. Inoculation was performed in the root system so as to obtain a systemic induction of secondary metabolism. Only 17 strains caused significant increases in at least one of the parameters evaluated. The most striking result was that some strains promoted growth and increased cardenolide content at the same time. This effect was detected in leaves, while inoculation was carried out in roots. Interestingly, these two parameters are not enhanced simultaneously under regular conditions of pot culture or in tissue cultures. This result shows that the biotic agent employed was able to upregulate the plant's metabolism. Moreover, it was striking that bacterial strains selected from wild species were able to upregulate secondary metabolism in selected varieties, especially for terpenes and cardenolides. The implication of this study is that identification of eliciting agents may now make it possible to enhance secondary metabolism in undifferentiated tissue cultures. This could be of major economic importance for the pharmaceutical industry because it would make possible either field production with higher yields or biotechnological production in vitro with good yields.

8.4.3 Modification of Secondary Metabolite Profiles of Pharmaceutical Interest

The effects of secondary metabolites on human health may vary depending on several factors. One of them is how they are delivered and incorporated into the human body, either in a pharmaceutical formulation, as food supplements, or in the diet. A pharmaceutical formulation will provide known concentrations of well-characterized and identified compounds, while a food supplement will provide an extract of the plant that possesses variable concentrations of known and unknown compounds. The most variable input is seen through the diet. Some edible plants, such as soybeans or berries, contain bioactive compounds that provide more benefits than is attributed to their simple nutritional value. Levels of bioactive compounds do change depending on environmental conditions; hence, the lack of an effect on health may be due to a lack of reproducibility of the bioactive content. This lack of reproducibility may be overcome by elicitation with biotic agents. However, the effect of elicitation needs to be evaluated because variation in relative concentrations of bioactive compounds may change their effects on human health when delivered in the diet. Besides changing the relative profiles, some interesting metabolites can be increased. This may be a very interesting tool for pharmaceutical companies as a means to prepare normalized extracts of food supplements.

*Case study: Biotechnological elicitation of isoflavones in Glycine max with PGPR. Diploma de estudios avanzados (DEA) Elena Algar Parejo. Universidad San Pablo CEU (unpublished results).*The rationale for this study was to evaluate the ability of PGPR to elicit soybean growth and secondary metabolism in terms of isoflavone production .

In a study on biotechnological elicitation of isoflavone production in soybeans (*Glycine max* (L.) Merr.) with PGPR by Elena Algar Parejo at the Universidad San Pablo CEU (unpublished results), a battery of nine PGPR with diverse beneficial capacities on different plant species were evaluated. None of them increased significantly the concentration of isoflavones according to the isoflavone standards that were evaluated (daidzein, daidzin, malonyl daidzin, genistein, genistin, malonyl genistin). However, when considering daidzein and genistein, five PGPR caused a decrease in their concentration, especially for genistein; one strain increased levels of both isoflavones; and two increased daidzein and decreased genistein levels. Decreases in levels of isoflavones may be coupled to increases in levels of protective compounds such as pterocarpanes. Although isoflavones have been traditionally related to defense against pathogens, it seems that pterocarpanes, a family of compounds derived from daidzein, are the compounds responsible for effective defense. Another putative reason for this decrease may be that isoflavones are released from the roots to the surrounding soil, creating an outbound gradient. The rationale for this is that they are involved in establishment of the symbiosis with nitrogen-fixing *Rhizobia* bacteria. Irrespective of the reasons underlying the different behaviors of each PGPR, the fact is that there is a difference depending on the strain and that it is a reproducible effect. It remains to be seen if these changes are relevant for human health through the diet. If this were the case, this opens a new window for the food supply industry.

8.5 Bacterial Cell Wall Lipopolysaccharides Are Able to Mimick PGPR-Mediated Induction of Defense Metabolism

Communication between plants and microorganisms is a well-known fact consistent with the many reports in the literature. Relations between plants and microorganisms may be beneficial or harmful. Irrespective of their nature, there is a common "language" used by both partners which is now starting to be elucidated. Some plant secondary metabolites such as isoflavones are understandable signals for specific microorganisms, namely, rhizobia. Some plant growth regulators are released by soil microorganisms that affect the plant's metabolites by targeting the plant's receptor (see above). But still, there are other molecules, mainly bacterial or fungal surface molecules, that are able to induce some receptors in plant tissues to start a particular signaling pathway. The term "elicitor" includes all of those molecules of different chemical nature, either constitutive of the microorganism or released, that are able to upregulate a plant's defense metabolic pathways (SAR and ISR pathways). This is especially relevant in plants with medicinal applications.

Biotic elicitors are classified into several groups that include proteins, polysaccharides, lipopolysaccharides, and volatile compounds. Polysaccharides are the most frequent elicitors described. Identification of these elicitors is essential for the practical application of defense responses both for agricultural and industrial purposes, since some of the defensive compounds are molecules with pharmacological activity. Furthermore, they can be used to elicit plant tissues in vitro, opening a promising window for biotechnological production of phytopharmaceuticals.

Case study: Ramos Solano et al. (2008b) have recently reported on systemic disease protection elicited by plant growth-promoting rhizobacteria (PGPR) strains in order to examine the relationship between metabolic responses, systemic disease protection, and biotic elicitors. They were able to demonstrate that a fraction of the bacterial cell wall is able to reproduce the defensive effect of the whole bacteria inoculated on plant roots.

The rationale of this study was first to evaluate the ability of three PGPR to elicit defensive metabolism in the model plant *A. thaliana* Col 0 against *P. syringae* pv. *tomato* DC3000, focusing on the putative induction pathway by biochemical and molecular markers. Second, it was carried out in order to demonstrate that bacterial cell wall surface molecules were able to reproduce the effect of the bacterial strain. The PGPR employed included *C. balustinum* (AUR9), *Azospirillum brasilensis*, and *P. fluorescens* (AUR6). All three strains decreased disease severity when applied to *A. thaliana* prior to pathogen challenge. At a biochemical level, each of the three strains induced ethylene (ET) biosynthesis when incubated with 1-amino-cyclopropane-1-carboxylic acid (ACC) as well as salicylic acid production in the plant. Plants treated with each of the three strains showed lower levels of salicylic acid after pathogen challenge compared to untreated controls, revealing a previous contact with an eliciting agent, as described for plants that have survived a pathogen attack (SAR). This effect was more marked in plants treated with *C. balustinum* AUR9, the strain most effective in decreasing disease severity. When this response was evaluated at the molecular level, the expression level of *PR1*, a transcriptional marker of the SA-dependent pathway, in *C. balustinum* AUR9-treated plants is four-fold that of controls, while the expression of *PDF1.2*, a transcriptional marker for the SA-independent pathway, is not induced. This suggests that this PGPR strain is inducing the plant defensive pathway that is dependent on SA and one that has traditionally been associated with pathogenic microorganisms. Once this systemic protective effect was demonstrated *in vivo*, inoculating the bacterial strain *C. balustinum* on roots and evaluating disease incidence on leaves, cell wall lipopolysaccharides were tested as putative bacterial elicitor molecules on axenic cultures of *A. thaliana* (L.) Heynh. Putative elicitors were dissolved in the culture media and were able to reproduce this systemic induction effect at low doses (5 $\mu g \cdot mL^{-1}$), but failed to reproduce the effect at higher doses (50 $\mu g \cdot mL^{-1}$). From these observations, we can hypothesize that certain PGPR strains are capable of stimulating different systemic responses in host plants. With *C. balustinum* AUR9, the SA-dependent pathway is stimulated first, as indicated by increases in SA levels and *PR1* expression, followed by induction of the SA-independent pathway, as indicated by the increases in ET concentrations. The effects on both pathways

combined, with respect to disease suppression, appear to be additive. Irrespective of the defensive pathway involved, the ability of LPS to elicit the defensive pathway has been demonstrated. This provides a challenging alternative for elicitation of secondary metabolism pathways in medicinal plants.

8.6 Quorum Sensing Is Involved in PGPR-Mediated Systemic Resistance Against Abiotic Stress

Quorum sensing (QS) is a widespread, rapid means for bacterial communities to coordinately change genome expression patterns in response to environmental cues and population density. The term quorum sensing was first used in a review by Fuqua and Winans (1994), which essentially concerned the minimum threshold level of individual cell masses required to initiate a concerted population response. Bacteria that use QS produce and secrete certain signaling compounds called *autoinducers*, or normally, *N-acyl-homoserine lactone* (*AHL*). The bacteria also have a receptor that can specifically detect the inducer. When the inducer binds to the receptor, it activates transcription of certain genes, including those for autoinducer synthesis. When only a few other bacteria of the same kind are in the vicinity, diffusion reduces the concentration of the inducer in the surrounding medium to almost zero. So, as a result, the bacteria produce only small amounts of the inducer. When a large number of bacteria of the same kind are in the vicinity, the inducer concentration crosses a threshold, whereupon greater amounts of the inducer are synthesized. This forms a positive feedback loop in which the receptor becomes fully activated (Whitehead et al., 2001).

Many gram-negative bacteria utilize AHL in order to coordinate expressions of virulence in response to the density of the surrounding bacterial population. Several bacterial phenotypes essential for the successful establishment of symbiotic, pathogenic, or commensal relationships with eukaryotic hosts utilize motility, exopolysaccharide production, biofilm formation, and toxin production to show regulation by QS (Gonzalez and Keshavan, 2006). It has been demonstrated that bacterial AHLs are released into the rhizosphere, where they reach biologically active concentrations (Barriuso et al., 2008d). This study shows how QS is involved in disease incidence, where several pathogenic microorganisms such as *Xanthomonas campestris*, *Erwinia carotovora*, *Pseudomonas corrugata*, or *Burkholderia* sp. strains are the causal agents (Ahmad et al., 2008).

Interestingly, the production of quorum-sensing interfering (QSI) compounds by eukaryotic microorganisms has aroused immense interest among researchers, especially since such compounds can influence the bacterial signaling network positively or negatively. Hence, strategies addressing disruption of QS among bacterial strains appear to be an encouraging and novel strategy for plant health protection, especially when supported by the fact that some plant species do release AHL-like compounds to modulate bacterial communication in the rhizosphere (Teplistsky et al., 2000). On the contrary, synthesis of structural homologues to various QS signal molecules

has resulted in the development of additional QSI compounds that could be used to control pathogenic bacteria. Furthermore, the creation of transgenic plants that express bacterial QS genes is yet another strategy that could be utilized to interfere with bacterial behavior (Fray, 2002).

Case study: In a study on transgenic tomato plants that alter *quorum sensing* in plant growth-promoting rhizobacteria (PGPR), Barriuso et al. (2008d) showed that growth promotion and salt resistance are mediated by QS. This, then, extends the roles of QS and reinforces the concept of disruption of QS to control plant pathogen attack or to induce protection effects against different kinds of stresses. Details on this study are provided in the following account.

Two gram-negative plant growth-promoting rhizobacteria (PGPR), designated as M12 and M14, affiliated with *Burkholderia graminis*, could produce a variety of *N*-acyl-homoserine lactone (AHL) signaling molecules. The involvement of these molecules in plant growth promotion and induction of protection against salt stress was examined. AHL production was evaluated in vitro by thin-layer chromatography and bioindicator detection as well as by LC-MS/MS. In situ production of AHLs in the rhizosphere of *A. thaliana* (L.) Heynh. plants was detected by GFP (green fluorescent protein) biosensor constructs and confocal laser scanning microscopy. To determine if plant growth promotion and protection against salt stress were mediated by quorum sensing (QS), these PGPR were assayed on wild-type (wt) tomato plants as well as their corresponding transgenics expressing YenI (short-chain AHL producers) and LasI (long-chain AHL producers). In wt tomato plants, only M12 promoted plant growth, and this effect disappeared in both transgenic lines. In contrast, the strain M14 did not promote growth in wt tomatoes, but did in LasI. Resistance to salt stress was induced by strain M14 in wt tomato, but this effect disappeared in both transgenic lines. Strain M12, however, did not induce salt resistance in wt tomato, whereas it did in LasI tomato plants. These results reveal that QS AHL signal molecules mediate the ability of both PGPR strains, M12 and M14, to promote plant growth and to induce protection against salt stress.

8.7 Metagenomics: The Rhizosphere as a Source of Genes with Biotechnological Applications

A relative new perspective in the biotechnology of the rhizosphere is the *metagenomic approach* (Rolf, 2005). This approach is defined as the field of molecular biology whose purpose is to study the genome of entire communities of microorganisms instead of individual species. Also known as "communities genomics", this discipline examines the genetic material recovered from environmental samples derived from entire communities of microorganisms. Classical microbiology and genetics, in trying to isolate genes with new and valuable functions, basically are based on the isolation of single microorganisms and the sequencing of their genomes. Metagenomics allows the study of non-culturable, or difficult-to-culture, microorganisms. This approach gives a new estimation of microbial communities independent of their culture in the laboratory (Rolf, 2005).

Rhizosphere metagenomics examines the total DNA extraction from the rhizosphere, the preparation of a clonal library with this DNA, and the screening of the clones to select the genes of interest among the vast genetic reservoir derived from soil microbial communities.

The metagenomic approach has already been used for the identification of new biomolecules based on the finding of genes codifying proteins with new activities. This strategy has been validated by the isolation of novel genes that encode degradative enzymes (Henne et al., 1999), antibiotic resistance (Riesenfeld et al., 2004), and antibiotic production (Wang et al., 2000). However, association of a certain gene with its activity is quite difficult, and sometimes, the limiting factor is that the genes in question may not be expressed at detectable levels. Despite the tedious and long process of gene isolation and identification, rhizosphere metagenomics appears to be a very promising field to find new genes with novel activities and high biotechnological value, but also implicit are its own technical limitations that have to be overcome to assure new technical advances in the future.

8.8 Metabolic Engineering

The relevance of this perspective is fully described in Chapters 2 and 12. Nevertheless, a short discussion is needed here to examine the role of bacterial elicitors in metabolic engineering. Despite the efforts devoted to increase and diversify bioactive compounds in plants, it is still a challenge as to how to increase their content in vivo and, as mentioned before, how to obtain reproducibility of bioactives under field production conditions. These efforts rely on transgenic and non-transgenic approaches which involve complex regulation mechanisms that are required for increasing the levels of functional metabolites in plants. Bacterial elicitors may be used to determine the key genes limiting a metabolic pathway once the limiting step is identified. Transgenic approaches may allow us to overcome the low levels of target compounds produced. Finally, and this is a very attractive and encouraging challenge, upon elicitation, new molecules may appear after the activation of a given metabolic pathway.

8.9 Future Perspectives

The future of PGPR research should involve all aspects that are highlighted in this chapter. One of them is the selection of PGPR or PGPR mixes to help solve current agricultural problems, including limiting the use of highly contaminating pesticides and fertilizers and allowing for crop cultivation in low-fertility soils.

Although sustainable agriculture is directly related to human health, the use of PGPR to enhance levels of secondary metabolites that directly affect health through the diet may lead to the creation of *functional foods*, that is, foods having a beneficial effect on human health, that are superior to the benefits ascribed to their simple nutritional value.

Finally, identification of specific elicitors of bacterial origin may be used, either in agriculture, in the production of phytopharmaceuticals in vitro under controlled conditions, or at a physiological/molecular level, to study plant metabolism that allows us to unravel the limiting steps of metabolic pathways.

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Chapter 9
Plants as Sources of Energy

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Abstract This chapter is concerned with biotechnological applications involving the use of plants as sources of energy. Plants contain stored carbon captured from light-catalyzed carbon dioxide fixation via photosynthesis. This stored carbon from plants is available in oil and coal deposits that can be used as energy sources known as petrofuels. Living plants or plant residues can be used to generate biofuels such as methane from methane generators, wood fuel from wood chips, and alcohol from plant-based starch or cellulose in fermentation reactions. Topics that illustrate these applications include plant-based biofuels for engines – biodiesel and bioethanol; energy from woodchips (woodchip combustion, gazogen, or wood gasification); and methane (CH_4) or natural gas – methane gas production from landfills, methane gas produced in biodigesters using plant materials as substrate. We discuss the pros and cons of these applications with plant-derived fuels as well as the different types of value-added crops, including algae, that are currently being used to produce biofuels.

9.1 Introduction

Through the process of photosynthesis, plants have the capacity to capture and utilize energy, derived from the Sun, along with carbon from the Earth's atmosphere and nutrients from our soils to generate biomass. This biomass, in the form of roots, stems, leaves, fruits and seeds, is also consumed by animals and microorganisms, which in turn, generate their own forms of biomass. Manure, leaf litter, wood, garden waste, and crop residues are all common examples of biomass. Consequently, one definition of *biomass*is any organic/biological material which contains stored sunlight in the form of chemical energy. Typically, humans release this energy by burning the material, and humans have used biomass as an energy source in the form of solid biofuels for heating and cooking since the discovery of fire.

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Bioenergy is energy made available from organic materials and is often used as a synonym to biofuel. However, an important distinction between bioenergy and biofuel is that biomass is the fuel/biofuel and bioenergy is the energy contained in that fuel (Anderson, 2003; Agarwal, 2007; Drapcho et al., 2008). *Biofuel* can be broadly defined as any solid, liquid, or gas fuel derived from recently dead organic/biological material. This distinguishes it from fossil fuels such as coal, oil, and natural gas, which are derived from long dead, subterranean deposits of biological material. Unlike fossil fuel resources, which have an inevitable finite supply, biofuels are largely renewable energy sources based on a balance within the Earth's carbon cycle. As the human population continues to expand, and the demand for fossil fuels exceeds its supplies, pressure is mounting to find efficient and effective methods to produce renewable biofuels. Various plants and plant-derived materials are currently used for biofuel manufacturing, and biofuel industries are expanding in Europe, Asia, and the Americas. Agriculturally produced biomass fuels, such as biodiesel, bioethanol, and bagasse (often a by-product of sugarcane cultivation) can be burned in internal combustion engines and cooking stoves (Agarwal, 2007). However, there are many criticisms and concerns surrounding current practices for the production of biofuels. Consequently, research into more sustainable methods of generating biofuels will depend largely on the creation of environmentally responsible policies in farming, processing, and transporting of biofuels.

This chapter examines some of the pros and cons in the current methods used for generating various types of bioenergy, namely, energy derived from solid biomass, bioalcohol, biodiesel, biogas, and presents a critical look at how biotechnology can help to solve the world's current and future energy needs.

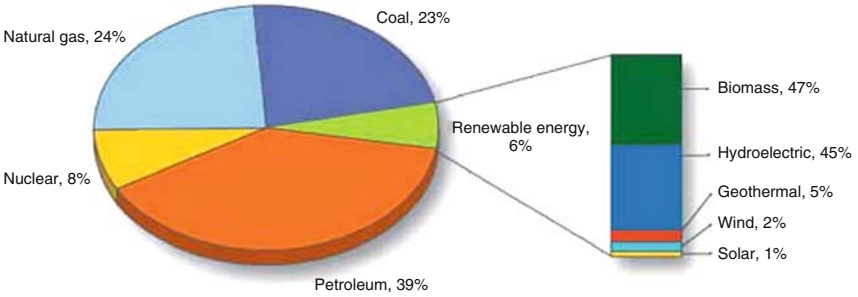
9.2 Energy Crisis and the Balance of Carbon

Biofuels were the first form of fuel used by human cultures around the world. Even up to the discovery of electricity and the start of the industrial revolution, fuels such as wood, whale oil, manure, and even alcohol were the primary sources of energy for heating, cooking, and lighting. However, the discovery and use of fossil fuels, including coal, oil, and natural gas dramatically reduced the emphasis on biomass fuel in the developed world (Peters and Thielmann, 2008). In the United States, for example, large supplies of crude oil were discovered in Pennsylvania and Texas in the mid- and late 1800s. This allowed petroleum-based fuels to become inexpensive. Because of these low costs, fossil fuels were widely used to promote the growing industrial age, especially for the production of power used to run factories and automobiles.

Despite the huge increase in the use of fossil fuels, most of the world continued to depend upon and make use of biofuels. Even in the United States, during the high-energy demand seen during wartime periods of World War II, biofuels were valued as a strategic alternative to imported oil. However, during the peacetime postwar period, inexpensive oil from the Middle East helped to trigger a worldwide shift away from biofuels. Since then, there have been a number of "energy crises" around

the world, caused by a variety of social and political factors. An *energy crisis*is any large-scale bottleneck (including price rises) in the supply of energy resources to an economy. Two of the best known ones occurred in 1973 and 1979, when geopolitical conflicts in the Middle East caused *OPEC* (*Organization of Petroleum Exporting Countries*) to cut exports. Consequently, non-OPEC nations experienced a very large decrease in their oil supply. This crisis resulted in severe shortages and a sharp increase in the prices of high-demand oil-based products, most notably gasoline. Throughout history, the fluctuations of supply and demand, energy policy, military conflict, and environmental impacts have all contributed to a highly complex and volatile market for energy and fuel. On the other hand, such problems always resurrect the principles of *green energy* and sustainable living. This has led to an increasing interest in alternate power/fuel research such as bioethanol, biodiesel, biogas, fuel cell technology, hydrogen fuel, solar/photovoltaic energy, geothermal energy, tidal energy, wave power, wind energy, and fusion power. Heretofore, only hydroelectricity and nuclear power have been significant alternatives to fossil fuels, which still dominate as energy sources (Fig. 9.1).

Although technology has made oil extraction more efficient, the world is having to struggle to provide oil by using increasingly costly and less productive methods, such as deep sea drilling and developing environmentally sensitive areas such as the Arctic National Wildlife Refuge. In addition, the world's population continues to grow at a rate of ~250,000 people/day, and while a small part of the world's population consumes most of the resources, the people of developing nations continue to



Biomass Consumption	Million dry tons/year
Forest products industry	
Wood residues	44
Pulping liquors	52
Urban wood and food & other process residues	35
Fuelwood (residential/commercial & electric utilities)	35
Biofuels	18
Bioproducts	6
Total	**190**

Fig. 9.1 Estimated world energy use from different sources. From the state energy conservation office web site (http://www.seco.cpa.state.tx.us/re_biomass-crops.htm). Source: The US Department of Energy's (DOE) Energy Information Agency (EIA), used with their permission

adopt more energy-intensive lifestyles. Currently, the United States, with its population of 300 million people, consumes far more oil than China, with its population of 1.3 billion people. But, this is also beginning to change, leading to an ever increasing demand for energy around the world. Many energy experts have concluded that the world is heading toward an unprecedented large and potentially devastating global energy crisis due to a decline in the availability of cheap oil and other fossil fuels and a progressive decline in extractable energy reserves.

To add to this problem, carbon emissions, including greenhouse gasses like carbon dioxide (CO_2), have been increasing ever since the industrial revolution. It is well documented that atmospheric CO_2 concentrations have risen by ~30% in the last 250 years. Data from monitoring stations, together with historical records extracted from ice cores, show that atmospheric CO_2 is now at a level higher than at any time in the last 650,000 years (Meehl et al., 2007). Such increases in CO_2 appear to be driven, in part, by the addition of 6–8 Pg (one Pg [petagram] = 1 billion metric tonnes = 1,000 × 1 billion kg) of carbon/year from human-derived sources, especially the burning of various fossil fuels which power our electricity and automobiles. Atmospheric CO_2 is predicted to continue to rise an additional 50% by 2050 (Meehl et al., 2007), and such rising levels of CO_2 are at the heart of the concerns over global warming and many of the associated environmental problems.

Biofuels and other forms of renewable energy aim to be carbon neutral or even carbon negative. *Carbon neutral* means that the carbon released during the use of the fuel is reabsorbed and balanced by the carbon absorbed by new plant growth during photosynthesis (Fig. 9.2). The plant biomass is then harvested to make the next batch of fuel, thus perpetuating the cycle of carbon in the Earth's atmosphere without adding to the problem. The Intergovernmental Panel on Climate Change (IPCC) estimates that between 46 and 56% of terrestrial carbon is found in forest biomes and that actions to preserve and enhance this carbon sink would likely increase the global terrestrial carbon by 60–87 Pg C by 2050, thereby offsetting ca. 15% of the anthropogenic emissions predicted for the same period (Saundry and Vranes, 2008). Using biomass to produce energy can reduce the use of fossil fuels, reduce greenhouse gas emissions, and reduce pollution and waste management problems (Agarwal, 2007). Therefore, carbon-neutral fuels, in theory, can lead to no net increases in human contributions to atmospheric CO_2 levels, thereby reducing the potential human contributions to global warming.

In addition to these arguments for biofuels, one of the strongest political drivers for the adoption of biofuel is "energy security." This means that a nation's dependence on oil is reduced and substituted with use of locally available sources, such as coal, gas, or renewable bioenergy sources. While the extent to which bioenergy can contribute to energy security and carbon balance will remain in active debate, it is clear that the dependence on oil is reduced. The US NREL (National Renewable Energy Laboratory) says that energy security is the number one driving force behind the US biofuels program (Bain, 2007) and the White House "Energy Security for the 21st Century" makes clear that energy security is a major reason for promoting bioenergy. Whether the driving forces behind a need for bioenergy is energy security, rising oil prices, concerns over the potential oil peak, greenhouse gas emissions

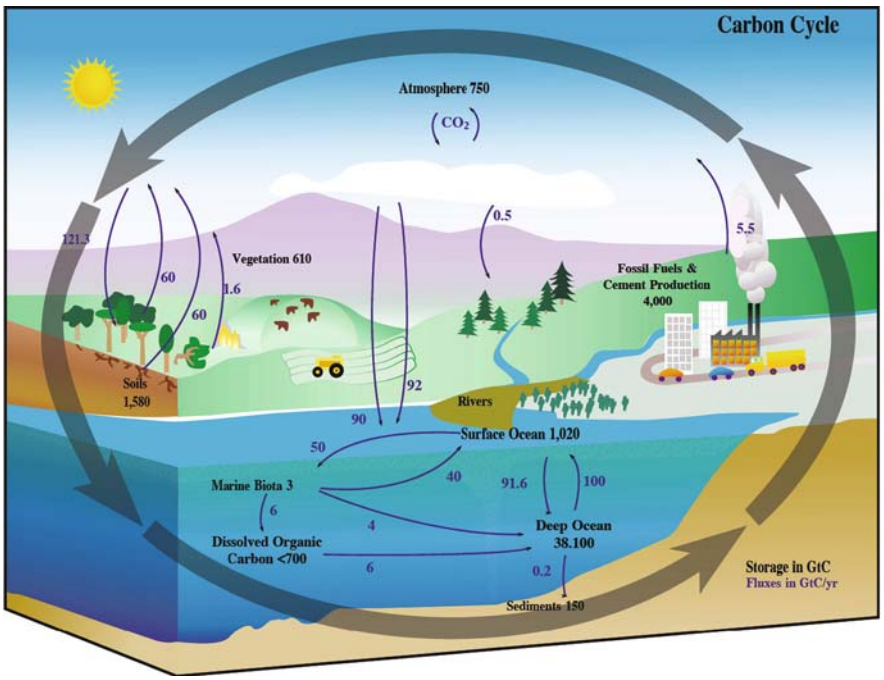


Fig. 9.2 The carbon cycle. Gigatons of carbon (GtC)/year, stored at various sites along the cycle. Illustration courtesy of NASA Earth Science Enterprise, available at Wikipedia public domain

(causing global warming and climate change), rural development interests, or instability in places such as the Middle East, it is clear that at some point, our global society is going to have to embrace the use of biofuels as a more stable, sustainable means of meeting our energy needs.

9.3 Disadvantages of Biofuels

While there are many potentially positive aspects to bioenergy and biofuels, there is growing international criticism because many biofuel energy applications take up large amounts of land, actually create environmental problems, or are incapable of generating adequate amounts of energy. While the plants that produce the biofuels do not produce pollution directly, the materials, farming practices, and industrial processes used to create this fuel may generate waste and pollution. Large-scale farming is necessary to produce agricultural biofuels, and this requires substantial amounts of cultivated land, which could be used for other purposes such as growing food, or left as undeveloped land for wildlife habitat stability. The farming of these lands often involves a decline in soil fertility. This is due to a reduction of organic matter, a decrease in water availability and quality due to intensive use of crops, and an increase in the use of pesticides and fertilizers (typically derived from

petroleum). The need for more energy crop land has been cited to cause deforestation, soil erosion, huge impacts on water resources and is implicated in the dislocation of local communities. Proponents of biofuels, however, point out that while the production of biofuels does require space, it may also reduce the need for harvesting non-renewable energy sources, such as vast strip-mined areas and slag mountains for coal, safety zones around nuclear plants, and hundreds of square miles being strip-mined for oil/tar sands.

As an example of such issues, the current alcohol-from-corn (maize) production model in the United States has come under intense scrutiny. When one considers the total energy consumed by farm equipment, soil cultivation, planting, fertilizers, pesticides, herbicides, and fungicides made from petroleum, irrigation systems, harvesting, transport of feedstock to processing plants, fermentation, distillation, drying, transport to fuel terminals and retail pumps, and lower ethanol fuel energy content, the net benefit does little to reduce unsustainable imported oil and fossil fuels required to produce the ethanol in the first place. The June 17, 2006, editorial in the Wall Street Journal stated, "The most widely cited research on this subject comes from Cornell University's David Pimental and University of Californa, Berkeley's Ted Patzek. They've found that it takes more than a gallon of fossil fuel to make one gallon of ethanol from corn – 29% more. That's because it takes enormous amounts of fossil-fuel energy to grow corn (using fertilizer and irrigation), to transport the crops and then to turn that corn into ethanol." Ethanol is also corrosive and cannot be transported in current petroleum pipelines; so, more expensive over-the-road stainless-steel tank trucks need to be used. This not only uses fuel but increases the cost to the customer at the pump. In addition, the subsidies paid to fuel blenders and ethanol refineries have often been cited as the reason for driving up the price of corn, in farmers planting more corn, and the conversion of considerable land to corn production, which generally consumes more fertilizers and pesticides than many other land uses and also leads to serious environmental consequences such as dead zones in the Gulf of Mexico (Ahring and Westermann, 2007).

There are many concerns that, as demand for biofuels increases, food crops are replaced by fuel crops, driving food supplies downward and food prices upward. This is especially true for biofuels derived from food crops such as corn and soybean, which impacts food security and food prices, especially in poorer countries where the inhabitants have barely enough money to purchase their food let alone any fuel for cars or even stoves they cannot afford. There are those, such as the National Corn Growers Association, who say biofuel is not the main cause of food price increases and, instead, point to government actions to support biofuels as the cause. Others say increases are just due to oil price increases.

Some have called for a freeze on biofuels. Others have called for more funding for second generation biofuels which should not compete with food production. Alternatives such as cellulosic ethanol or biogas production may alleviate land use conflicts between food needs and fuel needs. Instead of utilizing only the starch by-products from grinding corn, wheat, and other crops, cellulosic ethanol and/or biogas production maximizes the use of all plant materials. Critics and proponents both agree that there is a need for sustainable biofuels, using feedstocks that min-

imize competition for prime croplands. These include farm, forest, and municipal waste streams; energy crops engineered to require less water, fertilizers, and pesticides; plants bred to grow on marginal lands; and aquatic systems such as algae used to produce alcohol, oil, and hydrogen gas (Ahring and Westermann, 2007). In short, biofuels, produced and utilized irresponsibly, could make our environmental/climate problems worse, while biofuels, done sustainably, could play a leading role in solving the energy supply/demand challenges ahead.

9.4 What Are the Major Types of Biofuels (Solid, Liquid, and Gas)?

There are several common strategies of producing biofuels. Each strategy is derived from growing an "energy crop." This is a type of plant grown at low cost and low maintenance that is converted into solid, liquid, or gas biofuels. Where the energy crop will be burned directly to exploit its energy content, woody crops such as *Miscanthus*, *Salix,* or *Populus* are widely used. Liquid biofuels can be generated from energy crops that are high in sugars (sugarcane, sugar beet, and sweet sorghum) or starch (corn/maize) by using yeast (*Saccharomyces*) alcoholic fermentation to produce ethyl alcohol (ethanol). It is also possible to make cellulosic ethanol from non-edible plants (switchgrass, hemp, and timber) and plant parts (rice husks, corn stalks, or grass clippings). Other liquid biofuels are derived from plants that contain high amounts of vegetable oil, such as oil palm, soybean, *Jatropha* or even algae. When these oils are heated, their viscosity is reduced, and they can be burned directly in diesel engines or they can be chemically processed to produce fuels such as biodiesel (Agarwal, 2007). In fact, the diesel engine was originally designed to run on vegetable oil rather than fossil fuel. Finally, biogas (methane, CH_4) has been produced for hundreds of years from waste materials including manure and crop residues. If high carbohydrate content is desired for the production of biogas, whole-crops such as maize, sudan grass, millet, white sweet-clover, wood, and many others can be made into silage and also be converted into biogas.

Depending on geographic location in the world, the type of energy crop grown often varies. These include corn, switchgrass, and soybeans, primarily grown in the United States; rapeseed, wheat, and sugar beet primarily grown in Europe; sugarcane in Brazil; palm oil and *Miscanthus* grown in Southeast Asia; sorghum and cassava in China; and *Jatropha* in India. In many locations, biodegradable outputs from industry, agriculture, forestry, and households can also be used for biofuel production, either by the use of anaerobic digestion to produce biogas or by the use of second generation biofuels to make use of straw, timber, manure, rice husks, sewage, and food waste. It is unfortunate that most governments appear fixated on the liquid fuel paradigm. Refocusing and balancing policies and communications to support the development of other technologies, including biogas and methods to extract the most energy out of plant and waste material would be very prudent. How to use biotechnology to better access this stored energy is a hot topic in science these days.

9.4.1 Solid Biomass

As mentioned above, humans have used solid biomass as a fuel for cooking and heating since the discovery of fire. The most obvious examples are wood and grasses, which have been used in campfires for centuries. Many native cultures around the world have also used the burning of solid biofuels, not only to release stored energy in the form of heat but also to release stored nutrients used to fertilize fields for better plant growth. The Aborigines in Australia, for example, have routinely burned the native Spinifex grass (*Spinifex sericeus* R. Br.) to elicit better plant growth in the desert and to aid in hunting animals by driving them in a known direction. Other, more agricultural societies use burning to fertilize crop lands to this day. Cattle farmers in the United States still use fire to trigger the growth of new grasses for their cattle, not to mention their traditional uses of cow manure for fertilizer, heating, and cooking. In fact, cow manure is estimated to still contain two-thirds of the original energy consumed by the cow. Wood was the main source of energy in the United States and the rest of the world until the mid-1800s, and biomass continues to be a major source of energy in much of the developing world.

In modern societies, solid biomass continues to be used directly as a combustible fuel, producing 10–20 $MJ \cdot kg^{-1}$of heat. Its forms and sources include wood, the biogenic portion of municipal solid waste, or the unused portions of field crops. In the United States wood and wood waste (bark, sawdust, wood chips, and wood scrap) provide only about 2% of the energy we use today. About 84% of the wood and wood waste fuel used in the United States is consumed by the forest industry, electric power producers, and commercial businesses. The rest is used in homes for heating and cooking.

In addition to wood as a fuel, field crops may be used as fuel sources. For example, not only the field crops be grown intentionally as an energy crop but also the remaining plant by-products be used as a solid fuel. Sugarcane residue (also called *bagasse*), wheat chaff, corncobs, rice hulls, and other plant matter can be, and are burned quite successfully. Processes to harvest biomass from short-rotation poplars (*Populus* spp.) and willows (*Salix* spp.), and perennial grasses such as switchgrass (*Panicum virgatum* L.), *Phalaris*, and *Miscanthus*, require less frequent cultivation and less nitrogen than from typical annual crops. Pelletizing *Miscanthus* and burning it to generate electricity is being studied and may be economically viable.

Heating by wood is a more attractive option these days because technological improvements have made wood burning safer, more efficient, and cleaner. Options range from traditional wood stoves to pellet- and wood chipburning systems. While pellet fuel is manufactured by compressing ground wood and biomass waste into small, cylindrical pellets; woodchip fuel requires very little processing. In a typical woodchip heating system, a motor-driven conveyor system moves the chip fuel slowly and steadily from a chip hopper into a very efficient combustion chamber where the chips are burned (Fig. 9.3). As the chips burn, a fan blows hot air into a heat exchange boiler where water-filled tubes are heated. The hot water then circulates in pipes to provide heat to homes. In some commercial operations, steam can also be produced to power turbines that generate electricity. Many manufacturing

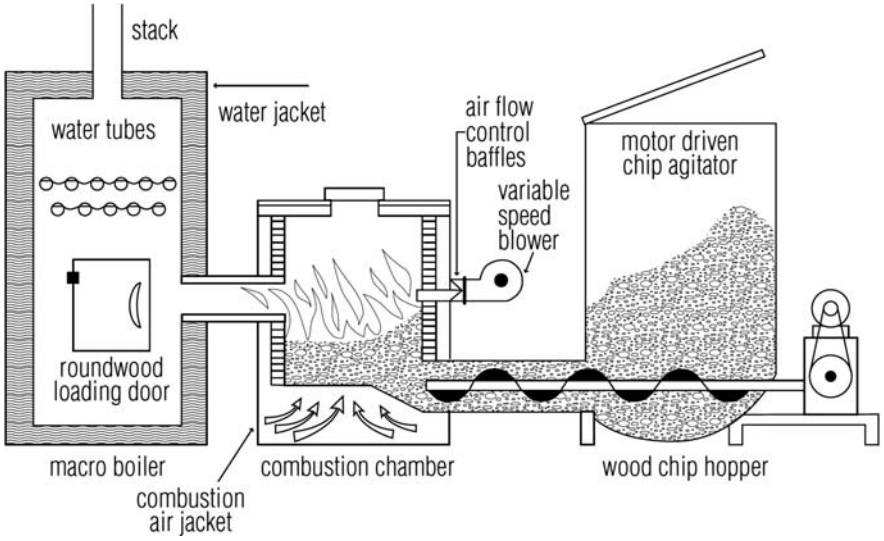


Fig. 9.3 An example of a modern woodchip heating system

plants in the wood and paper products industry use wood waste to produce their own steam and electricity. This saves these companies money because they do not have to dispose of their waste products and they do not have to purchase as much electricity.

Another advantage of solid biofuels is that the net carbon dioxide emissions that are added to the atmosphere by the burning process are only derived from the fossil fuels that were used to plant, fertilize, harvest, and transport the solid biomass. Likewise, chip combustion contributes less pollution and is a renewable resource. Modern woodchip combustion also gives the opportunity to use mill waste and lower grade wood from thinning operations. Wood chip fuel produced from such residues is cheaper than cordwood and pellet fuels. While the capital costs of wood chip heating systems are higher than oil-based systems, the operating costs are lower.

9.4.1.1 Combustion of Coal as a Biomass Energy Source: Pros and Cons

Coal is a solid fossil fuel formed in ecosystems where plant remains were preserved by water and mud during oxidization and biodegradation, thus sequestering atmospheric carbon present thousands or even millions of years ago. It is composed primarily of carbon and hydrogen along with small quantities of other elements, notably sulfur. Such elements are the primary source of pollution when the coal is finally burned. Since coal is the largest source of fuel for the generation of electricity worldwide, as well as the largest worldwide source of carbon dioxide emissions, its contribution to climate change and global warming is immense. In terms of carbon dioxide emissions, coal is slightly ahead of petroleum and about double that of natural gas. In addition, coal is extracted from the ground by coal mining, either

by underground mining or by open pit mining (surface/strip mining). The practices of mining coal are deleterious to the local environment as seen in mountain top removal with strip mining, pollution of streams and rivers, and destruction of ecosystems.

In recent years, there has been talk about "clean coal". This is an umbrella term used in the promotion of the use of coal as an energy source by emphasizing methods being developed to reduce its environmental impact. These efforts include chemically washing minerals and impurities from the coal, gasification (see also IGCC), treating the flue gases with steam to remove sulfur dioxide, and carbon capture and storage technologies to capture the carbon dioxide from the flue gas. These methods and the technology used are described as *clean coal technology*, and such technology is a popular conversational topic for politicians. Clean coal can certainly be beneficial to the energy security of a country, but it is unlikely that coal will ever be truly clean. The same is true for most solid biofuels. Over 2 billion people currently cook every day and heat their homes by burning biomass, and this process is not "clean." In the nineteenth century, for example, wood-fired steam engines were common and contributed significantly to unhealthy air pollution seen during the industrial revolution. Today, the black soot that is being carried from Asia to polar ice caps appears to be causing them to melt faster in the summer.

9.4.1.2 Does Wood as a Solid Biofuel Offer Any Benefits as a Transportation Fuel?

With current technology, solid biofuels are not ideally suited for use as a transportation fuel. Most transportation vehicles require power sources with high-energy density, such as that provided by internal combustion engines. These engines generally require clean burning fuels, which are in liquid form, and to a lesser extent, compressed gases. Liquid biofuels are more portable, and they can be pumped, which makes handling much easier. This is why most transportation fuels are liquids. Non-transportation applications such as boilers, heaters, and stoves can usually tolerate the low-energy density contained in solid fuels, but technologies are being developed to make better use of solid fuels. Wood and its by-products can now be converted through process such as gasification into biofuels such as wood gas (synthesis gas), biogas, methanol, or ethanol fuel; however, further development may be required to make these methods affordable and practical.

Because solid fuels have inherent problems of relatively high costs, air pollution on combustion, and production inefficiency, one has to look at other, less polluting, more efficient, lower cost fuel sources. These include bioalcohol and biogas, which are covered in the next two sections. In contrast to the above, energy harvesting via bioreactors (methane generators) is a cost-effective solution, as for example, when applied to the animal solid waste product (manure) disposal issues faced by the dairy farmer. They can produce enough biogas/natural gas (methane, CH_4) to run a farm and work quite well in internal combustion engines (see Section 9.4.4)

9.4.2 Bioalcohol

The most abundant source of ethanol is the hydration of ethylene ($CH_2{=}CH_2$) derived from petroleum and other fossil fuels. While bioalcohols (especially bioethanol) have been in use for hundreds of years, it is only relatively recently that ethanol from biological sources has become more substantial. Ethanol fuel is now the most common biofuel worldwide, particularly in Brazil and the United States. Alcohol fuels are produced by fermentation of sugars derived from energy crops, such as corn, sugarcane, sugar beets, sorghum, wheat, or any sugar or starch that alcoholic beverages can be made from, including potatoes and fruit waste. Creation of ethanol starts with the energy of the Sun, carbon dioxide from the atmosphere and nutrients from soil, which allow the feedstocks to grow. Plants produce sugars such as glucose through the process of photosynthesis ($6CO_2 + 6H_2O$ + light $\rightarrow C_6H_{12}O_6 + 6O_2$). During ethanol fermentation, performed primarily by yeast (*Saccharomyces* spp.), glucose is decomposed into ethanol and carbon dioxide ($C_6H^{12}O_6 \rightarrow 2C_2H_6O + 2CO_2$ + heat). During combustion, ethanol reacts with oxygen to produce carbon dioxide, water, and heat ($C_2H_6O + 3O_2 \rightarrow 2CO_2 + 3H_2O$ + heat). Since two molecules of ethanol are produced for each glucose molecule, there are equal numbers of each type of molecule on each side of the equation, and the net reaction for the overall production and consumption of ethanol is simply (light $\rightarrow$ heat). The heat of the combustion of ethanol can be used to drive the piston of an internal combustion engine (Agarwal, 2007). Ethanol is considered "renewable" because it is primarily the result of conversion of the Sun's energy into usable energy.

The most common steps in the production of bioalcohols are as follows: (1) enzymatic digestion (to release sugars from stored starches); (2) fermentation of the sugars through the action of microorganisms (yeasts that generate alcohol in the process); (3) distillation (to concentrate the alcohol); and (4) drying (to remove residual water that can prevent the liquid from being used as a fuel). The distillation process, in particular, requires significant energy input as heat (often using natural gas from fossil fuels). Likewise, we have already discussed some of the concerns over the amount of land needed to produce ethanol fuel crops and how land used for this purpose seems to be adversely impacting usable land for food resources (see Sections 9.2 and 9.3).

More recently, attention has focused on making use of non-food crops or the waste biomass leftover from other crops. Plant biomass high in cellulose (including wood and paper waste) can also be tapped for its stored sugar content. Once the cellulose is broken down through the action of enzymes and microorganisms (e.g., cellulose-decomposing fungi), it can be used as a starting material for fermentation and alcohol production. However, since cellulose is extremely stable, it is very difficult to break apart. In addition, it is commonly linked to lignin (another support molecule found in the cell walls of plants), and the resulting "lignocellulose" is one of the toughest plant materials to decompose. One good example of a plant high in both sugars and cellulosic biomass is sugarcane. The cane can be pressed to extract its juice which has high levels of sugar. The leftover bagasse, the waste left after

sugarcane is pressed, can also be dried and used as a solid biomass to provide heat for the distillation process after fermentation.

Ethanol can be used in automobile engines as a replacement for gasoline (Agarwal, 2007). It can be mixed with gasoline to any percentage; however, most existing automobile gasoline engines can only run on blends up to 15% bioethanol with petroleum/gasoline. Gasoline with ethanol added has a higher octane, which means that the engine can typically burn hotter, more efficiently, and more cleanly. In high-altitude (thin air) locations, some states mandate a mix of gasoline and ethanol as a winter oxidizer to reduce atmospheric pollution emissions (Agarwal, 2007). The top five producers of ethanol for fuel are the United States, Brazil, China, India, and France. Brazil and the United States accounted for ~70% of all ethanol production, with total world production of 13.5 billion US gallons (40 million tonnes).

9.4.2.1 History of Bioalcohol Use

Throughout the history of its use as a fuel, bioethanol has been at the crux of supply, demand, and often subtle price variations between ethanol and other liquid fuels. Since ancient times, ethanol has been used for lamp oil and cooking, along with plant and animal oils. Before the US Civil War, many US farmers had alcohol stills that could turn crop waste into virtually free lamp and stove fuel. In 1826, Samuel Morey, experimented with a prototype internal combustion engine that used ethanol (combined with turpentine and ambient air then vaporized) as fuel. At that time, his discovery was overlooked, mostly due to the success of steam power. And while ethanol was known of for decades, it received little attention as a fuel until 1860, when Nicholas Otto began experimenting with internal combustion engines. Such a use would have meshed well with the farmers' alcohol stills. However, the Industrial Age caused many farmers to move to city jobs, leaving their farms and ethanol fuel stills behind. Despite this, alcohol remained popular for lighting, cooking, and industrial purposes. In 1862, and again in 1864, a tax on alcohol was passed in the United States to help pay for the Civil War. This increased the price of ethanol dramatically, causing farmers not to be able to sell their ethanol due to reduced demand. Consequently, farmers used the ethanol themselves. Later in the 1890s, alcohol-fueled engines were used in farm machinery, train locomotives, and eventually cars in the United States and Europe. Henry Ford's first car, the Quadracycle, was released in 1896 and ran on 100% ethanol. Thus ethanol was the first fuel used by American cars before gasoline.

The early 1900s were an important time in the history of how gasoline eventually overtook alcohol fuels as the fuel of choice for automobiles. In 1902, the Paris alcohol fuel exposition exhibited alcohol-powered cars, farm machinery, lamps, stoves, heaters, laundry irons, hair curlers, coffee roasters, and many household appliances that were powered by alcohol. A few years later, the United States repealed the alcohol tax while under Theodore Roosevelt, who was strongly against fossil fuels like oil. This allowed the price of ethanol (~14 cents/US gallon) to fall below the price of gasoline (~22 cents/US gallon). Unfortunately, in 1907, the discovery of new oil fields in Texas caused the price of gasoline to drop to

between 18 and 22 cents/US gallon, and at the same time, alcohol fuel prices rose to around 25–30 cents/US gallon. Because of the struggle between the markets for alcohol and gasoline, Henry Ford introduced his Ford Model T in 1908. It had an engine that could run on either ethanol or gasoline or a mix of both. Ford continued to be an advocate for ethanol as a fuel, even during the prohibition. But in 1919, the prohibition police destroyed virtually all corn-alcohol stills, putting what appeared to be an end to the use of alcohol as a fuel in the United States.

It is interesting to note that in many other parts of the world, people believed that ethanol would be the fuel that would eventually replace petroleum. Experiments on the use of alcohol as fuel continued in these other parts of the world because there continued to be a battle between the prices of ethanol and gasoline. For example, in 1923, the price of alcohol from molasses was less than 20 cents/US gallon, while retail gasoline prices had reached an all-time high of 28 cents/gal. At about the same time, Standard Oil Co. experimented with a 10% alcohol/90% gasoline blend to increase octane and stop engine knocking. By the mid-1920s, ethanol blended with gasoline was standard in every industrialized nation except the United States. By 1925, France, Germany, Brazil, and other countries had already passed "mandatory blending" laws. During this time, Ford Motor Co. was building cars that could be changed slightly to run on gasoline, alcohol, or kerosene. It is noteworthy that the situation changed in the United States. In 2007, Portland, Oregon, became the first city in the United States to require all gasoline sold within city limits to contain at least 10% ethanol. As of January 2008, three states – Missouri, Minnesota, and Hawaii – require ethanol to be blended with gasoline motor fuel. Many cities are also required to use an ethanol blend due to non-attainment of federal air quality goals.

In 1933, faced with the 25% unemployment rate of the Great Depression, the US government considered tax advantages that would help ethanol production to increase employment among farmers. The "farm chemurgy" *movement*, supported by farmers, Republicans, and Henry Ford, searched for new crop-based products from farms (such as soybean-derived plastics) and supported alcohol fuel. From 1933 to 1939, The American Petroleum Institute argued that such government help would hurt the oil industry, reduce state treasuries, and cause an unhealthy criminal "bootlegger" atmosphere around fueling stations. They claimed alcohol fuel was in every way inferior to gasoline, and eventually, the government did not pass any alcohol fuel incentives. Pressure from the oil companies has also been blamed for the demise of various ethanol fuel companies. For example, in 1937, *Agrol*, an ethanol-gasoline blend, was sold at 2,000 service stations in the United States. Agrol plant managers complained of sabotage and bitter infighting elicited by the oil industry that resulted in cheaper gasoline prices. At this time, alcohol was 25 cents/gal, while gasoline was 17–19 cents/gal. In 1939, Agrol production shut down because of a lack of a viable market, and by 1940, the US Midwestern alcohol fuel movement had disintegrated.

Fuel pressures that arose during World War II resulted in yet another revival of alcohol as fuel, and new technologies were developed to make use of such a fuel. For example, on October 14, 1947, legendary test pilot Chuck Yeager became

the first man to fly faster than Mach 1, the speed of sound. He was piloting the Bell X-1, a bullet-shaped rocket plane (powered by liquid oxygen and alcohol fuel) that was the first in a series of secret high-speed research aircraft that were flown out of California's Edwards Air Force Base in the late 1940s and 1950s. Another boost for ethanol came in 1973, when a worldwide energy crisis began. This caused ethanol to once again become cheaper than gasoline. Gasoline containing up to 10% ethanol has been increasing in use in the United States since the late 1970s. By the mid-1980s, over 100 new corn-alcohol production plants had been built, and over a billion US gallons of ethanol were sold for fuel each year. However, the tide would turn against ethanol again when, in the late 1980s and 1990s, new oil wells were discovered and the price of gasoline once again became much cheaper than alcohol fuel. This time, however, ethanol plants were able to get subsidies from the US government to support farmers who were growing energy crops.

Between 1997 and 2002, three million US cars and light trucks were produced which could run on E85, a blend of 85% ethanol with 15% gasoline (Agarwal, 2007). Ford, DaimlerChrysler, and GM are among the automobile companies that sell "flexible fuel" cars, trucks, and minivans that can use gasoline and ethanol blends that range from pure gasoline up to 85% ethanol (E85). Such *flex-fuel vehicles* are now having a significant impact on an attempted alcohol fuel transition because they allow drivers to choose different fuels based on price and availability. The primary problem, however, is that there are almost no gas stations that sell E85 fuel, and the ones that do are mostly located in the Midwest part of the United States. During this time, the invasion of Iraq, and the subsequent turmoil it caused, allowed Americans to become aware of their dependence on foreign oil. In addition, the demand for ethanol fuel produced from field corn was spurred by the discovery that *methyl tertiary butyl ether* (*MBTE*) was contaminating groundwater. MBTE was the most common fuel oxygenate additive used to reduce carbon monoxide emissions. The groundwater contamination issue eventually led to MTBE being banned in almost 20 states by 2006. In 2003, California was the first state to start replacing MTBE with ethanol, and other states start switching soon afterward. This switch thus opened a new market for ethanol fuel, the primary substitute for MBTE. This event, coupled with worry over climate change, caused the leading alternative energy sources, including bioalcohol, solar and wind power, to expand ~20–30% each year (Agarwal, 2007). At a time when corn prices were around US $2 a bushel, corn growers recognized the potential of this new market and delivered accordingly.

Since 2003, crude oil prices have risen by as much as 80%, and gasoline and US diesel fuel prices have risen by as much as 50%, only to fall again in highly volatile markets. These rises are caused by hurricane damage to oil rigs in the Gulf of Mexico, attacks on Iraqi oil pipelines, disruptions elsewhere, and rising demand for gasoline in Asia, particularly as Asians buy more cars. Gasoline prices rise as ethanol prices stay the same, due to rapidly a growing ethanol supply and federal tax subsidies for ethanol production. In 2008, the United Nations urged that there be a cessation in the provision of subsidies for food-based biofuels, including ethanol,

due to rising controversies over fuel price fluctuations, production costs, and supply/demand variables.

9.4.2.2 Advantages and Disadvantages of Bioalcohol: Can Corn Do the Job?

As mentioned above, one advantage of bioalcohol is that it can be produced from a variety of feedstocks, including sugarcane, bagasse, miscanthus, sugar beet, sorghum, grain sorghum, switchgrass, barley, hemp, kenaf, potatoes, sweet potatoes, cassava, sunflower, fruit, molasses, corn, stover, grain, wheat, straw, cotton, biomass in general as well as many types of cellulose waste and harvestings. As discussed in Section 9.2, the primary advantage of biofuels such as bioalcohol is that they are relatively "renewable" or carbon neutral as compared to fossil fuels. Carbon dioxide, a greenhouse gas, is emitted during fermentation and combustion. However, this by-product is canceled out by the greater uptake of carbon dioxide by the plants as they grow to produce the input material for the alcohol. The replacement of MTBE (an environmental toxin) with ethanol as an oxygenate in gasoline has also reduced carbon monoxide emissions (Agarwal, 2007). However, ethanol is not a completely clean burning fuel. When burned in the atmosphere, harmful nitrous oxide gases are produced, including nitrogen dioxide which contributes to the formation of "brown smog." Acetaldehyde and other aldehydes are also produced when alcohols are oxidized. When only a 10% mixture of ethanol is added to gasoline (as is common in E10 gasohol), aldehyde emissions increase by as much as 40%, and these components are not regulated in emissions laws.

The use of alcohol in various mixes with gasoline is also cited as the reason for reducing prices. According to a 2008 analysis by Iowa State University, the growth in US ethanol production has caused retail gasoline prices to be 29–40 cents/gal lower than would otherwise have been the case. However, because alcohol mixes with both gasoline and with water, ethanol fuels are often diluted after the drying process by absorbing environmental moisture from the atmosphere. Water in alcohol-mix fuels reduces efficiency, makes engines harder to start, causes intermittent operation (sputtering), and oxidizes aluminum and steel components (Agarwal, 2007). Ethanol itself is also corrosive to standard fuel systems, rubber hoses and gaskets, aluminum, and combustion chambers. It also corrodes fiberglass fuel tanks such as those used in marine engines. For higher ethanol percentage blends, and 100% ethanol vehicles, engine modifications are required. In addition, corrosive ethanol cannot be transported in gasoline pipelines, so more expensive stainless-steel tank trucks are required to deliver ethanol to customers. Perhaps even more problematic, ethanol fuel has less BTU energy content, which means it takes more fuel to produce the same amount of work. Even dry ethanol has roughly one-third lower energy content per unit of volume compared to gasoline.

Current interest in ethanol fuel in the United States mainly lies in bioethanol, produced from corn, but there has been considerable debate about how useful bioethanol will be in replacing fossil fuels in vehicles. As described in Section 9.3, concerns relate to the large amount of arable land required for energy crops as well as energy and pollution balance of the whole cycle of ethanol production.

Large-scale farming is necessary to produce agricultural alcohol and this requires substantial amounts of cultivated land. Farming may also involve a decline in soil fertility due to reduction of organic matter, a decrease in water availability and quality, an increase in the use of pesticides and fertilizers, deforestation, and potential dislocation of local communities. Likewise, "food vs. fuel" is the dilemma regarding the risk of diverting farmland away from food crops and toward the production of biofuels. The "food vs. fuel" debate is internationally controversial, with good arguments on all sides. Recent developments with cellulosic ethanol production and commercialization may allay some of these concerns.

One rationale given for extensive ethanol production in the United States is its benefit to energy security by shifting the need for some foreign-produced oil to domestically produced energy sources. In the United States, the number of ethanol factories has almost tripled from 50 in 2000 to about 140 in 2008. A further 60 or so are under construction, and many more are planned. The debates surrounding bioalcohol production are needed to prevent too many resources being placed into a technology that could have too many problems to make energy issues any better. Such projects are being challenged by residents at courts in Missouri (where water is drawn from the Ozark Aquifer), Iowa, Nebraska, Kansas (all of which draw water from the non-renewable Ogallala Aquifer), central Illinois (where water is drawn from the Mahomet Aquifer) and Minnesota. With large current unsustainable, non-scalable subsidies, ethanol fuel still costs much more per distance traveled than current high gasoline prices in the United States.

The United States produces and consumes more ethanol fuel than any other country in the world. This is partly due to energy crisis issues and price battles between ethanol and gasoline as explained in Section 9.4.2.1. However, one of the main incentives has been legislation that has been passed. A senior member of the House Energy and Commerce Committee, Congressman Fred Upton, introduced the legislation to use at least E10 fuel by 2012 in all cars in the United States. Likewise, the US Energy Independence and Security Act of 2007 requires American "fuel producers" to use at least 36 billion gallons of biofuel in 2022. This is nearly a five-fold increase over current levels. Such legislation is at the heart of the push to use corn as fuel and causing a significant shift of resources away from food production. Essentially all ethanol fuels in the United States are now produced from corn. As described above, the amount of land used to generate such large amounts of corn ethanol is a central concern behind the food vs. fuel debate and other environmental issues. Unfortunately, corn is a very energy-intensive crop. In the current alcohol-from-corn production model in the United States, considering the total energy consumed by farm equipment, cultivation, planting, fertilizers, pesticides, herbicides, and fungicides made from petroleum, irrigation systems, harvesting, transport of feedstock to processing plants, fermentation, distillation, drying, transport to fuel terminals and retail pumps, and lower ethanol fuel energy content, the net energy content value added and delivered to consumers is very small. And, the net benefit (all things considered) does little to reduce unsustainable imported oil and fossil fuels required to produce the ethanol.

The problem here is that current processes for the production of ethanol from corn use only a small part of the corn plant. The corn kernels are taken from the corn plant and only the starch is transformed into ethanol. Corn is typically 66% starch and the remaining 33% is not fermented. This unfermented component is called distillers grain, which is high in fats and proteins, and makes good animal feed. US corn-derived ethanol costs 30% more because the corn starch must first be converted to sugar before being fermented into alcohol. Here enzymes are required to first liquefy the starch. A second enzyme converts the liquefied starch to sugars, which are fermented by yeast into ethanol and carbon dioxide. The released CO_2 can also be captured and sold for use in carbonating beverages and in the manufacture of dry ice; however, this is not always done. Despite the cost differentials in production, in contrast to Japan and Sweden, the United States does not import much Brazilian ethanol because of US trade barriers corresponding to a tariff of 54-cent/gal – a levy designed to offset the 51-cent/gal blender's federal tax credit that is applied to ethanol no matter its country of origin.

9.4.2.3 Ethanol Derived from Sugarcane

Sugarcane or sugar cane (*Saccharum*) is a genus of 6–37 species (depending on taxonomic interpretation) of 2–6 m tall perennial grasses (family Poaceae, tribe Andropogoneae). They are native to warm temperate to tropical regions of the world, having stout, jointed, fibrous stalks that are very rich in sugar. Sugarcane is one of the most efficient photosynthesizers in the plant kingdom. It is able to convert up to 2% of incident solar energy into biomass. All of the sugarcane species interbreed, and all of the major commercial cultivars are complex hybrids. Sugarcane originated from tropical South and Southeast Asia. Different species likely originated in different locations with *S. barberi* originating in India and *S. edule* and *S. officinarum* from New Guinea. The thick stalk stores energy as sucrose in the sap. This sap can be extracted by pressing, and sugar is extracted by evaporating the water from the resulting juice. The use of crystallized sugar has been reported for over 5,000 years in India. The methods of growing sugarcane and processing sugar were transferred to China from India in the seventh century, and around the eighth century C.E., Arabs introduced sugar to the Mediterranean, Mesopotamia, Egypt, North Africa, and Spain. By the tenth century, there was virtually no village in Mesopotamia that did not grow sugarcane, and sugarcane was among the early crops brought to the Americas by the Spaniards.

Currently, about 200 countries grow sugarcane to produce ~1,325 million tons of sugary biomass. As of 2005, the world's largest producer of sugarcane by far is Brazil, followed by India. Uses of sugarcane include the production of sugar, Falernum, molasses, rum, soda, cachaça (the national spirit of Brazil), and ethanol for fuel. Ethanol is produced most typically by yeast (*Saccharomyces* species) fermentation of the sugar extracted from the cane. The bagasse that remains after crushing the sugarcane may also be burned to provide heat both for distillation processes and for the production of electricity. Because of its high cellulose content, it may also be used as raw material for paper and cardboard, as a starting material for cellulosic

ethanol, and is branded as "environmentally friendly" because it is a renewable by-product of sugar production.

Brazil has the largest and most successful sugarcane biofuel programs in the world, and it is considered to have the world's first sustainable biofuels economy. In 2006, Brazilian ethanol provided ~18% of the country's transportation fuel, and by April 2008, more than 50% of the fuel used as a replacement to gasoline was derived from sugarcane. As a result of the increasing use of ethanol, together with the exploitation of domestic deep water oil sources, Brazil reached complete self-sufficiency in oil supply in 2006, whereas years ago, the country had to import a large share of the petroleum needed for domestic consumption. Since 1977, the government made it mandatory to blend 20% of ethanol (E20) with gasoline, requiring just minor adjustments on standard gasoline engines (Agarwal, 2007). Today, the mandatory blend is allowed to vary nationwide between 20 and 25% ethanol (E25), and it is used by all normal gasoline vehicles. In addition, three million Brazilian cars run on 100% anhydrous ethanol and six million flexible fuel vehicles are now active in Brazil. Introduced to the market in 2003, these flex-fuel vehicles became a commercial success, representing around 23% of Brazil's standard motor vehicles. The ethanol-powered and flex vehicles have also been manufactured to tolerate even *hydrated ethanol*, an azeotrope comprised of 95.6% ethanol and 4.4% water.

Together, Brazil and the United States lead the industrial world in global ethanol production, accounting for ~70% of the world's total production and nearly 90% of the ethanol used for fuel. However, Brazil's sugarcane-based industry is far more efficient than the US corn-based industry. Brazilian distillers are able to produce ethanol for less than 22 cents/l, compared with the 30 cents/l for corn-based ethanol. Sugarcane plantations cover 3.6 million ha of land for ethanol production, representing only 1% of Brazil's arable land, with a productivity of 7,500 l of ethanol/ha, as compared with the US maize ethanol productivity of 3,000 l/ha. However, as with corn in the United States, significant areas of land are likely to be dedicated to sugarcane in future years, as demand for ethanol increases worldwide. The expansion of sugarcane plantations is already placing pressure on environmentally sensitive native ecosystems, including rainforests in South America, where deforestation is contributing to the elevation of greenhouse gases, loss of habitat, and a reduction in biodiversity.

In some respects, it is good that sugarcane cultivation requires a tropical or subtropical climate, with a minimum of 24 in. of annual rainfall. This has limited its use in North America and has forced the development of technologies that are better suited to North America. However, sugarcane production in the United States is occurring in Florida, Louisiana, Hawaii, and Texas, and the first three ethanol plants to produce sugarcane-based ethanol are expected to go online in Louisiana by mid-2009.

9.4.2.4 Ethanol Derived from Biomass

Plant biomass is the most abundant renewable resource on Earth and is also a potential source of fermentable sugars for the production of bioalcohol. As in the production of other bioalcohols, fermentation of sugars derived from biomass can be accomplished through the action of microorganisms that generate alcohol, which then needs to be distilled and dried to remove residual water. However, conversion of plant biomass to fermentable sugars typically requires manual and/or chemical pretreatment and the hydrolysis of lignocellulose, a structural material that comprises most of the plant biomass. *Lignocellulose* is composed primarily of cellulose (a β-1,4-linked glucose polymer), hemicellulose (with various types of 5- and 6-carbon sugar polymers), and lignin (a polymer of phenolic compounds) (Table 9.1). Unfortunately, the use of lignocellulose as a fuel has been curtailed by its highly rigid structure. Consequently, an effective pretreatment is needed to liberate the cellulose from the crystalline structure of lignin so as to render it accessible for subsequent hydrolysis (also called *cellulolysis*).

In contrast to ethanol produced from corn and sugarcane starches and sugars, cellulose is contained in nearly every natural, free-growing plant, tree, and shrub, in every meadow, forest, and field all over the world. Since the components of lignocellulose cannot be digested by humans, the production of cellulosic ethanol does not have to compete with the production of food, and if marginal lands are used to grow cellulose-rich crops, it does not have to compete with the land used to grow food crops. According to US Department of Energy studies conducted by the Argonne National Laboratories and the University of Chicago, the major benefit of cellulosic ethanol is that it can reduce greenhouse gas emissions by as much as 85% over reformulated gasoline. By contrast, starch ethanol from corn most frequently uses natural gas to provide energy for processing and may not reduce greenhouse gas emissions at all, depending on how the starch-based feedstock is produced. In addition, cellulosic crops require fewer inputs, such as fertilizer, herbicides, and other

Table 9.1 Composition of various types of cellulosic biomass material (% dry weight)

Material	Cellulose	Hemicellulose	Lignin	Ash	Extractives
Softwood barks	18–38	15–33	30–60	0.8–1.0	4–6
Hardwood barks	22–40	20–38	30–55	0.8–1.0	6–8
Soft woods	42–44	27–29	28–31	0.5–0.6	3–5
Newspapers	40–55	25–40	18–30	–	–
Hard woods	45–47	30–35	20–24	0.6–0.8	5–8
Grasses	25–40	25–50	10–30	–	–
Wheat straw	37–41	27–32	13–15	11–14	7–9
Chemical pulps	60–80	20–30	2–10	–	–
Cornstalks	39–47	26–31	3–5	12–16	1–3
Cotton and flax	80–95	5–20	–	–	–
Algae	20–40	20–50	–	–	–

Modified from Demirbas et al. (2005).

chemicals that can pose risks to wildlife. Their extensive roots improve soil quality, reduce erosion, and increase nutrient capture. Herbaceous energy crops reduce soil erosion by greater than 90%, when compared to conventional food crop production. This can translate into improved water quality for rural communities. Additionally, cellulosic energy crops add organic material to depleted soils and can increase soil carbon as long as the land being used is not totally stripped of plant material. In addition, the price per ton of the raw cellulose material is much cheaper than for grains or fruits, and since cellulose is the main component of plants, the whole plant can be harvested. This results in much better yields per acre, up to 10 t, instead of 4 or 5 t for the best crops of grain. Thus, production of ethanol from lignocellulose has the advantage of having abundant and diverse resources that do not require agricultural effort or costs for growth; however, it does require a greater amount of processing to make the sugar monomers available to the microorganisms that produce ethanol during fermentation.

The first attempt at commercializing a process for ethanol from wood was undertaken in Germany in 1898. It involved the use of dilute acid to hydrolyze the cellulose to glucose and was able to produce 7.6 l of ethanol/100 kg of wood waste (18 gal/t). The Germans soon developed an industrial process optimized for yields of around 50 gal/t of biomass. This process soon found its way to the United States, where two commercial plants were put into operation in the southeast during World War I. These plants used what was called "*the American Process,*" a one-stage dilute sulfuric acid hydrolysis of wood products and waste. Although the yields were half that of the original German process (25 vs. 50 gal of ethanol/ton), the output of the American process was much higher. However, a drop in lumber production forced these ethanol plants to close shortly after the end of World War I. In the meantime, a small, but steady amount of research on dilute acid hydrolysis has continued at the USDA's Forest Products Laboratory in Madison, WI.

Currently, *corn stover* (leaves and stalks of maize left in the field after harvest), switchgrass, miscanthus, and woodchips are some of the more popular cellulosic materials for ethanol production. For example, switchgrass (*Panicum virgatum* L.) is a native prairie grass, known for its hardiness, rapid growth (from 2 to 6 ft tall), and high cellulose content. It can be grown in most parts of the United States, including swamplands, plains, streams, and along the shores and interstate highways. Since switchgrass yields twice as much ethanol per acre than corn, less land is needed for production, helping to prevent habitat fragmentation. It is unfortunate, however, that typical municipal practices discard the majority of cellulosic biomass. It is estimated that over 320 million tons of cellulose-containing raw materials, which could be used to generate ethanol, are thrown away each year. According to the International Energy Agency, this includes 36.8 million dry tons of urban wood wastes, 90.5 million dry tons of primary mill residues, 45 million dry tons of forest residues, and 150.7 million dry tons of corn stover and wheat straw. Likewise, organic waste makes up 71.5% of all landfill wastes deposited each day, consisting of large amounts of wood, envelopes, newsprint, grass, leaves, food scraps, office paper, corrugated cardboard, and agricultural composites as well as small amounts of manures, glossy paper, and paper ledger. All of these materials can be converted

into fuels, and transforming such leftovers into ethanol can actually reduce solid waste disposal costs and provide as much as 30% of the current fuel consumption in the United States. Thus, the raw material to produce cellulosic ethanol is basically free, and it may actually have a negative cost, where ethanol producers can get paid to take it away.

To date, the available pretreatment techniques include acid hydrolysis, steam explosion, alkaline wet oxidation, ozone pretreatment, and ammonia fiber expansion. Besides effective cellulose liberation, an ideal pretreatment has to minimize the formation of degradation products because of their inhibitory effects on subsequent hydrolysis and fermentation processes. The presence of inhibitors will not only complicate ethanol production, but also, increase the cost of production by adding detoxification steps. Even though pretreatment by acid hydrolysis is probably the oldest and most studied pretreatment technique, it produces several potent inhibitors including furfural and hydroxymethyl furfural (HMF) which are toxic compounds present in lignocellulosic hydrolysate. Ammonia fiber expansion (AFEX) is currently the only pretreatment which features promising efficiency with no inhibitory effect in resulting hydrolysate, although experiments using fungal organisms that naturally breakdown the biomass are showing some promise for the release of cellulose polymer from lignocellulose. In the hydrolysis process, these polymers are broken down to free the sugar before it is fermented for alcohol production.

There are two primary approaches to cellulose hydrolysis (*cellulolysis*): a chemical approach using acids, or an enzymatic approach. In the traditional method, hydrolysis is performed by attacking the cellulose with an acid. Dilute acid may be used under high heat and high pressure or more concentrated acid can be used at lower temperatures and atmospheric pressure. The product from this hydrolysis is then neutralized and yeast fermentation is used to produce ethanol. As mentioned, a significant obstacle to the dilute acid process is that the hydrolysis is so harsh that toxic degradation products can be produced that can interfere with fermentation. In enzymatic hydrolysis, cellulose can be broken into glucose molecules by cellulase enzymes. Such enzymes are commonly found in the digestive systems of ruminants, such as cows, sheep, and termites, where a collection of enzymes are produced by bacteria. They are also found in naturally occurring fungi and soil bacteria that are part of the global carbon cycle. Using a similar enzymatic system, lignocellulosic materials can be enzymatically hydrolyzed under relatively mild conditions (50°C and pH = 5), thus enabling effective cellulose breakdown without the formation of by-products that would otherwise inhibit enzyme activity. To be viable for large-scale fuel production, all major pretreatment methods, including dilute acid pretreatment, require some type of enzymatic hydrolysis step to achieve the high sugar yields required for ethanol fermentation. Various enzyme companies have already contributed significant technological breakthroughs in cellulosic ethanol production through the mass production of various cellulase enzymes at competitive prices. Iogen Corporation, for example, is a Canadian producer of enzymes for an enzymatic hydrolysis process that uses “specially engineered enzymes.”

Traditionally, baker's yeast (*Saccharomyces cerevisiae*) has long been used in the brewery industry to produce ethanol from hexoses (6-carbon sugars). Yeast cells are especially attractive for cellulosic ethanol processes because they have been used in biotechnology for hundreds of years. They are tolerant to high ethanol and inhibitor concentrations, and they can grow at low pH values, which avoids bacterial contamination. Due to the complex nature of the carbohydrates present in lignocellulosic biomass, a significant amount of xylose and arabinose (5-carbon sugars derived from the hemicellulose portion of the lignocellulose) is also present in the hydrolysate. For example, in the hydrolysate of corn stover, approximately 30% of the total fermentable sugars are xylose. Thus, the ability of the fermenting microorganisms to utilize the whole range of sugars available from the hydrolysate is vital to increase the economic competitiveness of cellulosic ethanol.

In recent years, metabolic engineering for microorganisms used in bioethanol production has shown significant progress. Besides *Saccharomyces*, bacteria such as *Zymomonas mobilis* and *Escherichia coli* have been targeted for metabolic engineering to improve their fermentation abilities, and thus, improve cellulosic ethanol production. Likewise, genetically engineered yeasts have been described that efficiently ferment xylose and arabinose sugars. Some species of bacteria have also been determined to be capable of the direct conversion of cellulose into ethanol. One example is *Clostridium thermocellum*, which utilizes a complex cellulosome to breakdown cellulose and synthesize ethanol. However, *C. thermocellum* also produces contaminating by-products during cellulose metabolism, including acetate and lactate, in addition to ethanol. While this lowers the efficiency of the process, further research into the ethanol-producing pathways of such organisms holds great potential for future improvements in the generation of bioalcohol. Enzymes from thermophilic organisms are also particularly well suited for industrial applications because they are typically thermostable and relatively tolerant of other stresses such as pH extremes. Genes for a variety of thermostable cellulase enzymes from both bacteria and fungi are currently being assessed for their ability to improve cellulosic ethanol efficiency.

Similarly, much effort has been devoted to developing transgenic plants as bioreactors to produce heterologous proteins, including industrial cellulase enzymes (Park et al., 2003). Such plants, expressing genes from other species, are typically fertile and grow normally, and they supply easy access to the enzymes needed when cellulose is to be broken into sugars. Manufacturing heterologous cellulases in crop plant bioreactors could significantly reduce costs associated with enzyme production and could offer a potentially high-volume alternative to traditional enzyme production methods. Other plant biotechnology approaches aim to improve the lignocellulose characteristics of the biomass crops themselves. This has been done in switchgrass, where alteration of gene expression in the lignin biosynthesis pathway has both increased and reduced the amount of lignin within the plant (Fig. 9.4A). The reduction of lignin in plant tissues allows easier access to the cellulose; however, the amount of reduction has to be carefully tailored so as not to cause the growing plant to collapse due to lack of support structure. Other approaches to improved cellulosic crops include more traditional breeding programs that identify

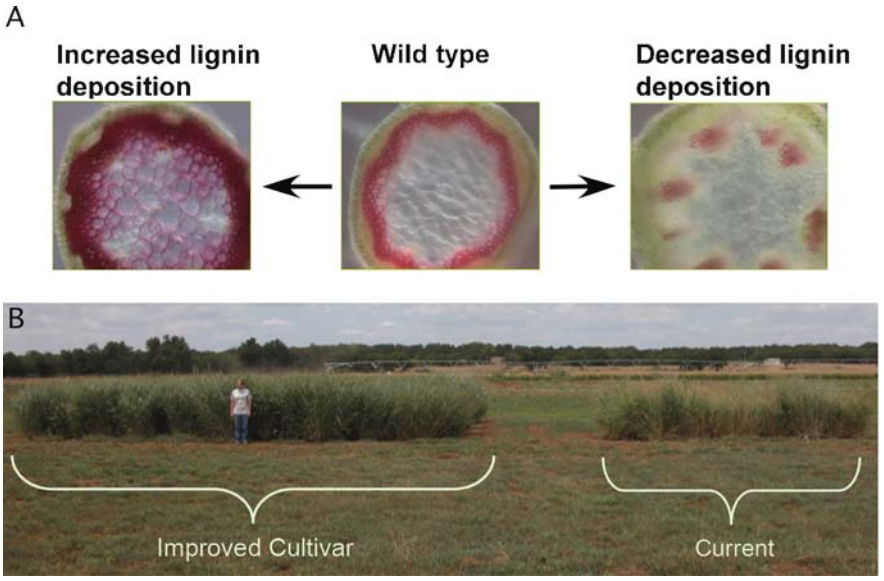


Fig. 9.4 Examples of improvements in cellulosic biomass. (**A**) Modifications in gene expression can result in both increased and decreased deposition of lignin in switch grass. (**B**) Genetic-based breeding programs can improve biomass in switch grass. Modified from Vermerris (2008) Genetic Improvement of Bioenergy Crops, Springer

useful traits to help develop superior varieties, including those that have enhanced biomass production (Fig. 9.4B).

It should be noted here that, while most efforts have focused on acid pretreatment and enzymatic hydrolysis of lignocellulose, gasification of the lignocellulosic raw material into gaseous carbon monoxide and hydrogen is also useful for ethanol production (Ahring and Westermann, 2007). The gasification process does not rely on chemical decomposition of the cellulose chain (cellulolysis). Instead of breaking the cellulose into sugar molecules, the carbon in the raw material is converted into *wood gas* (also called synthesis gas), using what amounts to partial combustion. The resulting carbon monoxide, carbon dioxide, and hydrogen may then be fed into a special kind of fermenter. Instead of sugar fermentation with yeast or bacteria, this process uses a bacterium named *Clostridium ljungdahlii*. *C. ljungdahlii* will ingest (eat) carbon monoxide, carbon dioxide, and hydrogen and produce ethanol and water. The ethanol can then be distilled and dried as usual. More recently, *C. thermocellum* (a thermophilic bacterium) has been found to be twice as efficient in making ethanol from carbon monoxide as *C. ljungdahlii*. Alternatively, the synthesis gas from gasification may be fed to a catalytic reactor where the synthesis gas is used to produce ethanol and other higher alcohols through a thermochemical process. Such technology development and the use of biotechnology will likely be key to the development of truly sustainable fuel sources in the future.

9.4.2.5 Biobutane as an Alternative Fuel

Biobutanol (also called *biogasoline*) has a longer hydrocarbon chain than ethanol. This causes it to be fairly non-polar, making it more similar to gasoline than ethanol. It is often claimed to provide a direct replacement for gasoline, because it can be used directly in internal combustion engines without modification. Butanol better tolerates water contamination and is less corrosive than ethanol, making it more suitable for distribution through existing pipelines for gasoline. In blends with diesel or gasoline, butanol is less likely to separate from the fuel than ethanol if the fuel is contaminated with water. There is also a vapor pressure co-blend synergy with butanol and gasoline containing ethanol. This better facilitates ethanol blending, thus allowing better storage and distribution of blended fuels. Butanol also has a high octane rating (over 100) and high energy content, is only about 10% lower than gasoline, and subsequently is about 50% more energy dense than ethanol (100% more so than methanol). Butanol's only major disadvantages are its high flashpoint (95°F or 35°C), potential toxicity (but not necessarily more than gasoline), and the fact that the distillation process requires a large energy input.

The feedstocks for biobutanol are the same as for bioethanol, including energy crops such as sugar beets, sugarcane, corn grain, wheat, and cassava as well as agricultural by-products such as straw, corn stalks, and various other biomass. Biobutanol is formed by acetone/butanol/ethanol fermentation (*ABE fermentation*) through the activity of the bacterium, *Clostridium acetobutylicum*, also known as the Weizmann organism. This process was first delineated by Chaim Weizmann in 1916 for the production of acetone from starch for making *cordite*, a smokeless gunpowder. At the time, the butanol was a by-product of this fermentation, forming twice as much butanol as acetone. The process also creates a recoverable amount of hydrogen gas and a number of other by-products, including acetic, lactic and propionic acids, acetone, and isopropanol.

Experimental modifications of the process have shown potentially high net energy gains with biobutanol as the only liquid product. However, the key research challenge that must be resolved is that butanol production inhibits microbial growth even at low concentrations. The Weizmann organism can only tolerate butanol levels up to 2%, compared to 14% for ethanol from yeast. Thus, the overwhelming constituent of the fermentation broth is water; so, an energy-intensive distillation step is required for purification. This may be acceptable if the goal is to produce butanol for use as a solvent, but if butanol is to gain traction as a fuel, energy inputs need to be minimal. Currently, biobutanol is far to expensive (~$4/US gallon) to be viable as a fuel. However, a number of companies are working on the problem. For example, DuPont and British Petroleum (BP) are working together to help develop biobutanol as a fuel source. According to DuPont, existing bioethanol plants can cost-effectively be retrofitted to produce biobutanol. Similarly, a Swiss company, Butalco GmbH, uses a special technology to modify yeasts in order to produce butanol instead of ethanol. Yeasts as production organisms for butanol production have decisive advantages compared to bacteria because they are much more tolerant to alcohol and contaminants that may inhibit fermentation.

9.4.2.6 Future Perspectives for Bioalcohol

In the United States, crops grown for biofuels are the most land- and water intensive of the renewable energy sources. In 2005, about 12% of the nation's corn crop (covering 11 million acres (45,000 km^2) of farmland) was used to produce 4 billion gallons of ethanol, which equates to about 2% of annual US gasoline consumption. For biofuels to make a much larger contribution to the energy economy, the industry will have to accelerate the development of new feedstocks, agricultural practices, and technologies that are more land- and water-efficient. The 200-page scientific roadmap cites recent advances in biotechnology that have made cost-effective production of ethanol from cellulose, or inedible plant fiber, an attainable goal, with federal loan guarantees for new cellulosic biorefineries. The report outlines a detailed research plan for developing new technologies to transform *cellulosic ethanol*– a renewable, cleaner burning, and carbon-neutral alternative to gasoline – into an economically viable transportation fuel. The US Department of Energy (DOE) has invested in research on enzymatic, thermochemical, acid hydrolysis, hybrid hydrolysis/enzymatic, and a variety of other technologies that are aimed toward achieving success in discovering an efficient and low-cost method of converting cellulose to ethanol. Already, the efficiency of biofuels production has increased significantly, and there are new methods being developed to boost biofuel production through the use of genetic engineering of both microorganisms and the plant feedstocks themselves (see Section 9.5). Many analysts suggest that, whichever ethanol fuel-production strategy is used, conservation efforts are also needed to make a large impact on reducing fossil fuel use, and biotechnology will likely play a central role in such conservation efforts by improving our ability to generate alternative fuels while also reducing energy inputs.

9.4.3 Biodiesel

*Biodiesel*is another type of liquid biofuel, commonly produced by the *trans*-esterification of the vegetable oil or animal fat feedstocks. This biofuel can be used directly in modern diesel engines. However, it is common to use various percentages of biodiesel blended with petroleum diesel (also called *petrodiesel*) so that modifications to the diesel engines can be avoided. Much of the world uses a system known as the "B" *factor* to state the amount of biodiesel in any fuel mix. Fuel containing 20% biodiesel is labeled B20, while pure biodiesel is referred to as B100. Blends of 20% biodiesel with 80% petrodiesel (B20) are generally used in unmodified diesel engines. Biodiesel can also be used in its pure form (B100), but may require certain engine modifications to avoid maintenance and performance problems. In many European countries, a 5% biodiesel blend is widely used and is available at thousands of gas stations (Agarwal, 2007).

A variety of plant oils can be used to produce biodiesel. Currently, rapeseed or canola (*Brassica napus* L.) and soybean (*Glycine max* L.) oils are most commonly used, where soybean oil alone accounts for about 90% of all biodiesel in

the United States. Other plant crops can also be used, including oil palm (*Elaeis guineensis* Jacq. and *Elaeis oleifera* Jacq.), sunflower (*Helianthus annus* L.), flax (*Linum usitatissimum* L.), mustard (*Brassica*spp.), mahua (*Madhuca longifolia*) (J. Konig, J.F. Macbr.), *Jatropha*, cotton (*Gossypium spp.)*, hemp (*Cannabis sativa* L.), field pennycress (*Thlaspi arvense* L.). Waste vegetable oil is also a useful starting material for biodiesel, as are animal fats including tallow, lard, yellow grease, chicken fat, and the by-products derived from the production of omega-3 fatty acids from fish oil. Each of these oils can, in theory, be used as fuel; however, to ensure that the fuel injectors atomize the fuel in the correct pattern for efficient combustion, vegetable oils must be heated to reduce its viscosity to that of diesel (Agarwal, 2007). This typically is done with electric coils or heat exchangers.

The *trans-esterification* step used to produce biodiesel generates a lower viscosity fuel that has combustion properties very similar to those of petroleum diesel. Chemically, *trans*-esterified biodiesel is a mix of mono-alkyl esters of long chain fatty acids. Thus, its chemical name is fatty acid methyl (or ethyl) ester (*FAME*). There are several methods for carrying out the *trans*-esterification reaction (Lachenmaier-Koelch and Meyer-Pittroff, 2005). These include the common batch process, supercritical processes, ultrasonic methods, and even microwave methods. In the most commonly used method, oils are mixed with sodium hydroxide and methanol (or ethanol), and the resulting *trans*-esterification reaction produces biodiesel and glycerol. Methanol (converted to sodium methoxide in the reaction) is normally used to produce methyl esters, as it is the cheapest alcohol available. However, ethanol can be used to produce ethyl ester biodiesel, and higher alcohols such as isopropanol and butanol have also been used. In addition, one part glycerol is produced for every 10 parts biodiesel, and this by-product can be used as a starting material for other processes. The glycerol by-product can be used as a humectant (hygroscopic moistening agent), solvent, sweetener, and food preservative and as a starting material in the production of nitroglycerin.

Biodiesel can also be used as a heating fuel in domestic and commercial boilers, where it is sometimes known as *bioheat*. In countries such as the United States, where more than 80% of commercial trucks and city buses run on diesel, biodiesel offers a promising alternative to petroleum-derived diesel. Since the feedstocks contain very little sulfur, biodiesel is a cleaner burning fuel than petrodiesel. Likewise, the solvent characteristics of biodiesel tend to keep engine deposits from forming, thus maintaining cleaner operation.

9.4.3.1 History of Biodiesel vs. Petrodiesel Production

Trans-esterification of a vegetable oil was conducted as early as 1853 by scientists E. Duffy and J. Patrick, many years before the first diesel engine became functional. It was not until 40 years later when Rudolf Diesel's invention, a single 10 ft (3 m) iron cylinder with a flywheel at its base, ran on its own power for the first time in Augsburg, Germany, on August 10, 1893. A few years later, Mr. Diesel also demonstrated his diesel engine. This engine was engineered to run on peanut oil (at the request of the French government) and built by the French Otto Company. It

was shown at the World Fair in Paris, France, in 1900, where it received the Grand Prix (highest prize). This engine stood as an example of Diesel's vision because it was powered by peanut oil – a biofuel. While peanut oil is not *trans*-esterified to a true diesel fuel, Rudolf Diesel believed that the utilization of biomass fuel was the real future of his engine. In a 1912 speech, Diesel said, "the use of vegetable oils for engine fuels may seem insignificant today but such oils may become, in the course of time, as important as petroleum and the coal-tar products of the present time."

During the 1920s, diesel engine manufacturers altered their engines to utilize the lower viscosity of petrodiesel, a fossil fuel, rather than vegetable oil, a biomass fuel. The petroleum industries were able to penetrate the fuel markets because their fuel was much cheaper to produce than the biomass alternatives at the time. The result, for many years, was a near elimination of the biomass fuel production. Only recently, environmental impact concerns and decreasing price differences have made vegetable oils an appealing alternative. Despite the widespread use of fossil petroleum-derived diesel fuels, interest in vegetable oils as fuels in internal combustion engines is reported in several countries during the 1920s and 1930s.

Later, during World War II, Belgium, France, Italy, the United Kingdom, Portugal, Germany, Brazil, Argentina, Japan, and China have been reported to have tested and used vegetable oils as fuels. As mentioned above, operational problems were reported due to the high viscosity of vegetable oils as compared to petroleum diesel fuel, which resulted in poor atomization of the fuel in the fuel spray and often leads to deposits and coking of the injectors, combustion chamber, and valves. Attempts to overcome these problems included heating of the vegetable oil, blending it with petroleum-derived diesel fuel or ethanol, pyrolysis, and catalytic cracking of the oils (Agarwal, 2007).

On August 31, 1937, G. Chavanne of the University of Brussels in Belgium was granted a patent for a "Procedure for the transformation of vegetable oils for their uses as fuels" (fr. 'Procédé de Transformation d'Huiles Végétales en Vue de Leur Utilisation comme Carburants' Belgian Patent 422,877). This patent described the *trans*-esterification of vegetable oils using methanol and ethanol in order to separate the fatty acids from the glycerol by replacing the glycerol by short linear chain alcohols. This appears to be the first account of the production of what is known as "biodiesel" today. More recently, in 1977, Brazilian scientist Expedito Parente produced biodiesel using *trans*-esterification with ethanol and filed a patent for the same process. Research into the use of *trans*-esterified sunflower oil, and refining it to low viscosity diesel fuel standards, was initiated in South Africa in 1979. By 1983, the process for producing fuel-quality, engine-tested biodiesel was completed and published internationally.

Since then, the benefits of the technology have been spreading. An Austrian company, Gaskoks, obtained the technology from the South African Agricultural Engineers. This company erected the first biodiesel pilot plant in November 1987 and the first industrial-scale plant in April 1989 (with a capacity of 30,000 t of rapeseed/year). Throughout the 1990s, biodiesel plants were opened in many European countries, including the Czech Republic, Germany, and Sweden. France launched local production of biodiesel fuel (referred to as *diester*) derived from rapeseed oil,

which is mixed into regular diesel fuel at a level of 5%, and into the diesel fuel used by some public transportation at a level of 30% (Agarwal, 2007). During the same period, nations in other parts of the world also saw local production of biodiesel starting up, and by 2000, over 21 countries had commercial biodiesel projects.

In September 2005 Minnesota became the first US state to mandate that all diesel fuel sold in the state contain part biodiesel, requiring a content of at least 2% biodiesel. The world's first biofuel-powered commercial aircraft took off from London's Heathrow Airport on February 24, 2008, and touched down in Amsterdam on a demonstration flight, hailed as a first step toward "cleaner" flying. The "BioJet" fuel for this flight was produced by Seattle-based Imperium Renewables, Inc.

In summary, Biodiesel is a clean burning fuel for diesel engines made from domestically produced, renewable fats and oils such as soybean oil. Biodiesel has no sulfur or aromatic compounds and already meets the new Environmental Protection Agency (EPA) ultra-low sulfur diesel fuel mandated for introduction in 2006. Biodiesel can be used in existing diesel engines without modification. Biodiesel burns substantially cleaner than petroleum-based diesel fuel. It is a powerful option for improving our environment while reducing dependence on foreign oil, stretching our fossil fuel reserves, and providing value-added markets for agricultural products.

9.4.3.2 Sources of Plant Oils

European production of biodiesel from energy crops has grown steadily in the last decade, principally focused on rapeseed used for oil and energy. In North America rapeseed was renamed *Canada Oil* or *Canola*. Production of oil/biodiesel from rapeseed covers more than 1.2 million ha in Germany alone and has doubled in the past 15 years. Typical yield of oil as pure biodiesel may be as much as 1,000 l/ha or more. This makes biodiesel crops economically attractive. They also provide sustainable crop rotations that are nutrient balanced and preventative of the spread of disease.

Soybeans are by far the main source of vegetable oil production in the United States and likewise biodiesel production. While US biodiesel is being produced from a diverse array of feedstocks, soybean oil is still used for up to 80% of US biodiesel production. Based on US Bioenergy program requirements, the Renewable fuel Standards (RFS) for biomass-based diesel is 500 million gallons in 2009 and ramps up to 1 billion gallons by 2012. Some experts estimate that if the biodiesel industry keeps its current momentum, over 10% of US soybean oil could be used for biodiesel production in the next few years. Biodiesel yield of soybeans is significantly lower than that of rape, as can be seen in Table 9.2.

Since none of the current crop plants produce enough oil to completely replace fossil fuel usage, there is ongoing research into finding more suitable crops and improving oil yields. For example, it is estimated that it would require twice the land area of the United States to be devoted to soybean production, or two-thirds to be devoted to rapeseed production, to meet current US heating and transportation needs. The use of alternative crops that do not make use of prime cropland may be one way to curb problems associated with crops that overlap with food demands.

Table 9.2 Amount (%) of oil in various crop plants

Crop plant	Scientific name	% Extractable oil
Copra	*Cocos nucifera*	62
Castor bean	*Ricinus communis*	50
Sesame	*Sesamum indicum*	50
Groundnut kernel	*Arachis hypogaea*	42
Jatropha	*Jatropha*spp.	40
Rapeseed	*Brassica napus*	37
Palm kernel	*Elaeis*spp.	36
Mustard seed	*Brassica*spp.	35
Sunflower	*Helianthus communis*	32
Palm fruit	*Elaeis*spp.	20
Soybean	*Glycine max*	14
Cotton seed	*Gossypium hirsutum*	13

Non-food crops, such as mustard, *Camelina*, and *Jatropha*, are used for biodiesel and can thrive on marginal agricultural land where many trees and crops will not grow, or would produce only slow growth yields. Specially bred mustard varieties can produce reasonably high oil yields and are very useful in crop rotations with cereals. Mustards have the added benefit that the meal leftover after oil has been extracted can act as an effective and biodegradable pesticide. Camelina (*Camelina sativa* L. Crantz) is virtually 100% efficient. It can be harvested and crushed for oil and the remaining parts can be used to produce high-quality omega-3-rich animal feed, fiberboard, and glycerin. Most camelina is grown in areas that were previously not utilized for farming. For example, camelina can be grown in areas that receive limited rainfall that cannot sustain corn or soybeans without the addition of irrigation.

Jatropha is a genus of approximately 175 succulent plants, shrubs, and trees (some are deciduous, like *Jatropha curcas L.*) from the spurge family, Euphorbiaceae. The name is derived from (Greek iatros = physician and trophe = nutrition), hence the common name physic nut. These plants are drought resistant and can share space with other cash crops such as coffee, sugar, fruits, and vegetables. It is well suited to semi-arid lands and can contribute to slowdown desertification, according to its advocates. The hardy *Jatropha* produces seeds containing up to 40% oil. When the seeds are crushed and processed, the resulting oil can be used in standard diesel engines, while the residue can also be processed into biomass to power electricity plants (Agarwal, 2007). However, estimates of *Jatropha* biodiesel yield vary, primarily due to a lack of research data, genetic diversity of different species, the range of environments in which the plants are grown, and *Jatropha's* perennial life cycle. Seed yields under cultivation can range from 1,500 to 2,000 kg/ha, corresponding to extractable oil yields of 540–680 l/ha (58–73 US gallons/acre).

Jatropha is native to Central America and has become naturalized in many tropical and subtropical areas, including India, Africa, and North America. Originating in the Caribbean, *Jatropha* was disseminated as a valuable hedge plant to Africa

and Asia by Portuguese traders. Cultivation and fruit picking by hand is labor intensive and needs ca. 1 person/ha. So, in parts of rural India and Africa, this provides much-needed jobs. About 200,000 people worldwide now find employment through the production of *Jatropha*. Moreover, villagers often find that they can grow other crops in the shade of Jatropha trees. Currently, the oil from *Jatropha curcas* seeds is used to make biodiesel in the Philippines, as promoted by a law authored by Philippine senators Miriam Defensor-Santiago and Miguel Zubiri. Likewise, *Jatropha* oil is being promoted as a biofuel crop in hundreds of projects throughout India and other developing countries. One good example of its ability to grow on marginal lands is its use along the railway lines between Mumbai and Delhi in India, where the train itself runs on 15–20% biodiesel. In Africa, cultivation of *Jatropha* is also being promoted and it is grown successfully in countries such as Mali.

Since food crops are not efficient sources for oil-based fuels, often having less oil content and requiring more input energy for growth, energy crops that can be grown on marginal lands may have higher oil content and thus be a much better choice.

9.4.3.3 Advantages and Disadvantages of Biodiesel

One of the primary advantages of biodiesel comes from feedstocks used to create it. Fossil fuels, including petrodiesel, contain minor contaminants, such as salts and sulfur compounds that end up in the refined diesel. When the fuel is burned, these compounds build up in the atmosphere, where they have been causing environmental problems for years. Since the feedstocks used to make biodiesel contain virtually no sulfur, biodiesel is a cleaner-burning fuel than petrodiesel. Pure biodiesel (B100) is by far the lowest emission diesel fuel, and it is often used as an additive to ultra-low sulfur diesel (ULSD) fuel (Agarwal, 2007). However, while B100 biodiesel has a viscosity similar to petrodiesel, it may become more viscous at lower temperatures, depending on the feedstock used, thus requiring vehicles to have fuel line heaters.

Biodiesel is also an oxygenated fuel, meaning that it contains a reduced amount of carbon and higher hydrogen and oxygen contents than fossil diesel. This improves the combustion of fossil diesel and reduces the particulate emissions from unburnt carbon (Agarwal, 2007; Armas et al., 2008). Similarly, biodiesel has better lubricating properties than other diesel fuels. Thus, the addition of biodiesel to various blends of petrodiesel fuels reduces engine wear, increases the life of the fuel injection systems, and effectively cleans the engine combustion chamber of carbon deposits, helping to maintain efficiency.

On the other hand, while biofuels are generally considered to improve emissions and engine efficiency, biodiesel still produces local air pollution, including nitrogen oxides, the principal cause of smog. Since biodiesel is an effective solvent and cleans residues deposited by fossil diesel, engine filters may need to be replaced more often, as the biofuel dissolves old deposits in the fuel tank and pipes. Likewise, while older furnaces can burn biodiesel without any required conversion, the biodiesel may cause problems because rubber parts are adversely affected by the solvent properties of this fuel.

Perhaps the most profound problem with biodiesel is that worldwide production of vegetable oil and animal fat is not yet of sufficient magnitude to replace liquid fossil fuel use. As described in Section 9.3, some people object to the vast amount of farming required for such crop-based biofuels and the resulting fertilization, pesticide use, and land use conversion that would be needed to produce the additional vegetable oil. Transitioning fully to biodiesel would require immense tracts of land if traditional food crops (such as rapeseed (canola) or soybean) are used. The problem would be especially severe for nations with large economies, where energy consumption is proportional to economic output. If using only traditional food plants, most of such nations do not have sufficient arable land to produce biofuel for the nation's vehicles. Nations with smaller economies (hence, less energy consumption) and more arable land may be in better situations. However, many regions cannot afford to divert agricultural land away from food production.

In some regions of the world, a combination of increasing demand for food, and increasing demand for biofuel, is causing deforestation and threats to biodiversity. The best reported example of this is the expansion of oil palm plantations in Malaysia and Indonesia, where rainforest is being destroyed at alarming rates to establish new oil palm plantations to keep up with growing biodiesel demand in Europe and other markets. It is an important fact that 90% of the palm oil produced in Malaysia is also used by the food industry in a wide variety of food products; therefore, biofuels cannot be held solely responsible for this deforestation. Palm oil is also used in the manufacture of detergents and in electricity and heat generation both in Asia and around the world. So, there is a pressing need for sustainable palm oil production for both the food and fuel industries. Fortunately, many organizations, such as the Roundtable on Sustainable Biofuels, are working to define criteria, standards, and processes to promote sustainably produced biofuels.

Many biodiesel advocates suggest that waste vegetable oil and animal fats are the best sources of oil to produce biodiesel, but since the available supply of these oils is drastically less than the amount of petroleum-based fuel that is burned for transportation and home heating in the world, this local solution can only account for a very small percentage of petrodiesel usage. It is likely that biodiesel sources that make use of marginal lands (where food crops cannot be grown) would make much more sense as a solution to land use issues (e.g., palm oil nuts grown along roads or *Jatropha*grown along rail lines, see Section 9.4.3.2).

9.4.4 Biogas

Unlike natural gas derived from fossil fuels, *biogas* is a renewable "natural gas" that is produced from organic/biological materials as they decay. There are two primary types of biogas. The most common biogas is produced by anaerobic digestion or fermentation of organic/biological materials such as manure or sewage, municipal waste, and energy crops through the action of anaerobic bacteria. The resulting biogas is comprised primarily of methane (also called *biomethane*) and carbon dioxide.

Methane bacteria are responsible for such biological sources of methane, including symbiotic relationships within other life forms such as termites, ruminants, and cultivated crops. Another type of biogas is *wood gas* (also called synthesis gas), created by the gasification of wood, wood chips, or other carbon-rich biomass. This type of biogas requires a gasifier or wood gas generator. This biogas is comprised primarily of nitrogen, hydrogen, and carbon monoxide, with trace amounts of methane. These gasses can be combusted with oxygen present in the atmosphere to release energy, thus allowing wood gas to be used as a fuel.

Both types of biogas can be utilized for cooking, space heating, and water heating. They can also be utilized in modern waste management facilities to run heat engines that generate either mechanical or electrical power (Fig. 9.5). If compressed, they can replace compressed natural gas for use in vehicles, where they can fuel internal combustion engines or fuel cells. Biogas can be produced easily and cost-effectively from current waste streams, such as paper production, sugar production, sewage, and animal waste. It is most commonly produced using agricultural waste, such as plants and manure. The gas can also be produced by separating organic materials from waste that otherwise goes to landfills. Using materials that would otherwise generate no income, or even cost money to dispose of, improves the profitability and energy balance of biogas production. Similarly, the solid by-product or digestate derived from the biogas process can typically be used as a biofuel or

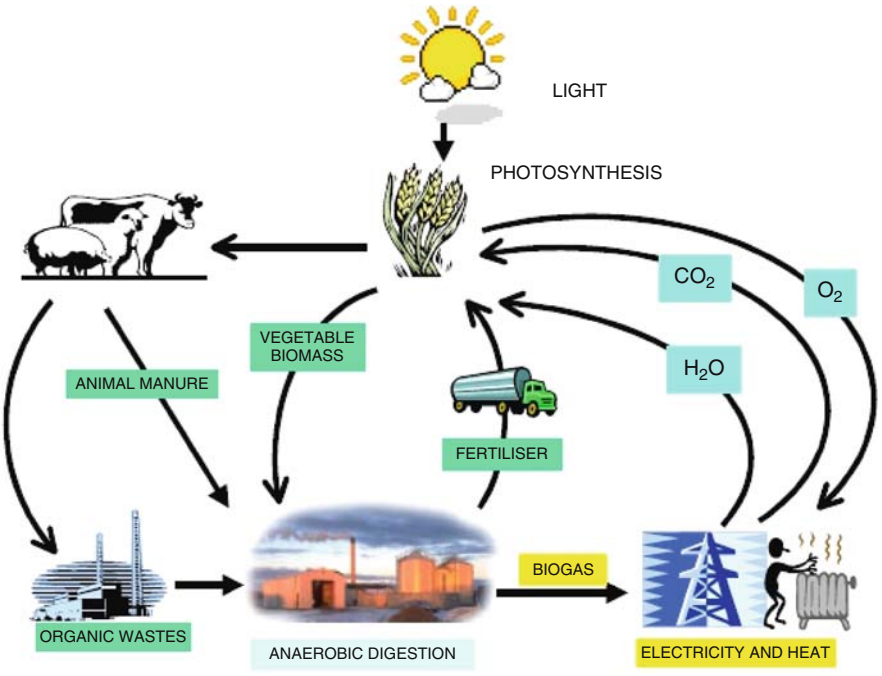


Fig. 9.5 A schematic of biogas formation. From www.makinemekanik.com

natural fertilizer. It is important to point out; however, that both carbon monoxide and methane are potent greenhouse gasses. Methane in particular is 21 times more reactive as a greenhouse gas than carbon dioxide (CO_2), and it is normally released into the atmosphere at most waste treatment facilities and landfills. Modern methods of biogas production have the advantage of keeping such gasses contained, avoiding potential environmental issues. Thus, biogas is considered to be one of the most climate-friendly sources of fuel.

9.4.4.1 History of the Use of Biogas

Some types of biogas, such as that derived from manure, have been used as a low-cost fuel for heating and cooking for hundreds of years. Anecdotal evidence indicates that biogas was used for heating bath water in Assyria during the tenth century BCE and in Persia during the sixteenth century CE. Some ancient Chinese literature also suggests that biogas was generated from sewage 2,000 to 3,000 years ago. However, Jan Baptita Van Helmont, in the seventeenth century, was credited as the first to determine that flammable gases could evolve from decaying organic matter. Likewise, Count Alessandro Volta concluded in 1776 that there was a direct correlation between the amount of decaying organic matter and the amount of flammable gas produced. In 1808, Sir Humphrey Davy determined that methane was present in the gases produced during the anaerobic decomposition of cattle manure.

It was not until the mid-1800s that the first biogas digesters were built. India has a quite long history of biogas development. The first unit usually referred to in literature is a biogas unit at the Mantunga Homeless Lepers Asylum near Mumbai in 1859. However, methane was first recognized as having practical and commercial value in England, where a specially designed septic tank was first used to generate gas for the purpose of lighting in the 1890s (Cheremisinoff et al., 1980). One other important development came from the development of microbiology as a science. This led to research by Buswell and others in the 1930s to identify anaerobic methane bacteria and the conditions that promote methane production by these organisms. Once the process became fairly well understood, the stage was set for the expansion of the use of biogas around the world. Such trends continue to this day, where India remains one of the biggest investors in biogas, making use of small biogas digester units installed in millions of homes.

9.4.4.2 Biogas Derived from Biodigesters

In India, biogas produced from the anaerobic digestion of manure in small-scale digestion facilities is called *gober gas*. This biogas is predominantly composed of methane and carbon dioxide. It is generated in more than 2 million household facilities. A typical gober gas digester consists of an airtight circular pit made of concrete with a pipe connection. The manure is directed to the pit, usually directly from a cattle shed, and the pit is then filled with a required quantity of wastewater. In this milieu, anaerobic bacteria create an oxygen-free environment in which they efficiently degrade the organic material and generate gas. The gas pipe can then be

connected to a kitchen fireplace through control valves, and the flammable methane gas generated out of this apparatus is largely odorless and smokeless. The residue left after the extraction of the gas is commonly used as fertilizer for crop plants. Owing to its simplicity in implementation and use of cheap raw materials in the villages, it is often quoted as one of the most environmentally sound energy sources for rural needs.

Similar biogas digesters are now used extensively in rural regions around the world, including China, Costa Rica, Nepal, and Vietnam. The Government of Pakistan provides 50% of funds needed for the construction of moveable gas chamber biogas plants. Farmers around the world are making use of such small-scale units to convert plant and animal wastes into a useful combustible gas like methane. In Colombia, experiments with diesel engine generators partially fuelled by biogas demonstrate that biogas can reduce electricity costs by 40% as compared with purchase from regional utilities.

Although based on the same principles as employed in the simple digesters above, larger municipal plants generate biogas in more advanced anaerobic digesters that recover the recyclable elements of household waste, sewage sludge, food wastes, farm wastes, or energy crops (such as maize silage) and process the biodegradable fraction in the anaerobic digesters. The methane contained in the resulting biogas can be concentrated to the same quality standards as fossil natural gas (typically called *biomethane*). If the local gas utility network grants permission, the producer of the biogas may then be able to utilize the local gas distribution networks to deliver the gas to consumers. Such biomethane, however, must be very clean and be of the correct composition to reach pipeline standards. Carbon dioxide, water, hydrogen sulfide, and particulates must therefore be removed if present. Biomethane can also be concentrated and compressed for use in vehicle transportation. Compressed biogas is becoming widely used in Sweden, Switzerland, and Germany as a renewable fuel source.

Sweden is cited as a particularly good example of a global leader in converting biowaste (largely agricultural material and residues) into usable biomethane. Facing oil shortages, waste management problems, and lacking any natural gas reserves of its own, Sweden was motivated to develop its biomethane industry under the Kyoto Accords. The resulting biogas is now used to generate electricity, residential heating, and transportation fuel. According to the Swedish Gas Association, more than 50% of the methane used to power Sweden's natural gas vehicles now comes from biological sources. More than 8,000 vehicles in Sweden are powered by a combination of natural gas and biomethane. The vehicles include transit buses, refuse trucks, and more than 10 different models of passenger cars. There are more than 25 biomethane production facilities in Sweden and 65 filling stations. A biogas-powered train has even been in service since 2005.

9.4.4.3 Biogas Derived from Landfills

Biogas is also produced in landfills from organic waste decomposing under anaerobic conditions. When a clay cap is placed atop the compacted waste materials within

the landfill, this prevents oxygen from penetrating the waste. As a consequence, anaerobic microorganisms like methane bacteria thrive under such conditions. One problem with such clay-capped landfills is that the resulting biogas builds up and is slowly released into the atmosphere. This gas contains a large portion of methane, which is 21 times more potent as a greenhouse gas as carbon dioxide. Therefore, uncontained landfill gas which escapes into the atmosphere may significantly contribute to the effects of global warming. In addition, *volatile organic compounds* (*VOCs*) contained within landfill gas contribute to the formation of unhealthy photochemical smog. However, if engineered properly, landfill sites can be made to capture such gases via pipes inserted into the clay cap that deliver the gases to gas clean-up and combustion facilities.

The European Union presently has some of the strictest legislation regarding waste management at landfill sites called the *Landfill Directive*. The United States legislates against landfill gas as it contains these VOCs. The US Clean Air Act and Title 40 of the Code of Federal Regulations (CFR) require landfill owners to estimate the quantity of *non-methane organic compounds* (*NMOCs*) emitted. If the estimated NMOC emissions exceeds 50 t/year, the landfill owner is required to collect the landfill gas and treat it to remove the NMOCs, which is usually done though combustion.

The composition of biogas varies depending upon the origin of the anaerobic digestion process. There are literally thousands of different types of anaerobic bacteria living in landfills. Their differing interactions, types of available waste, and resulting products generate different amounts of usable gas. Landfill gas typically has methane concentrations around 50%; however, advanced waste treatment technologies can produce biogas with 55–75% methane. This gas can be used to heat on-site buildings or to power engines for the generation of electricity, which can, in turn, be sold back to electricity utility companies for renewable energy subsidies. However, because of the remoteness of landfill sites, it is sometimes not economically feasible to produce electricity from this biogas. Still, it is estimated that a 3 MW landfill power plant can power 1,900 homes and at the same time prevent 6,000 t/year of methane from entering the environment. This is equivalent to eliminating 18,000 t/year of CO_2 derived from fossil fuel use or removing 25,000 cars from the road, planting 36,000 acres (146 km^2) of forest, or not using 305,000 barrels (48,500 m^3) of oil/year.

9.4.4.4 Wood Gas Derived from Carbon-Rich Biomass

Wood gas (or synthesis gas) is another form of biogas produced by thermal gasification of woody biomass or other carbon-rich materials in a gasifier or wood gas generator. Usable materials include wood chips, sawdust, charcoal, coal, rubber, or similar materials which are burned incompletely in a fire box that produces wood gas, tars, solid ash, and soot. The latter three by-products have to be removed periodically from the gasifier. In this case, the wood gas is filtered from tars and soot/ash particles, then cooled and directed to an engine or fuel cell to produce mechanical or electrical power. The gas is the result of two high-temperature reactions (above

700°C or 1,292°F): an *exothermic reaction,* where carbon burns to CO_2; and an *endothermic reaction,* where carbon reacts with steam to produce carbon monoxide (CO), hydrogen (H_2), and carbon dioxide (CO_2). Wood gas is flammable mainly because of the carbon monoxide and hydrogen content, but it also contains nitrogen and small amounts of methane, which is also flammable (Ahring and Westermann, 2007).

The first wood gasifier was apparently built by Bischof in 1839, and as a result, gasification became an important and familiar nineteenth and early twentieth century technology. "Town gas," produced by centralized gasifiers, was once quite popular and used primarily for lighting purposes. By the time World War II arrived in United States, large numbers of such generators were constructed and commercial generators were in production both before and after the war. The applicability of wood gas to the internal combustion engine was well understood from its earliest days of development, and the first vehicle powered by wood gas was built by Parker in 1901. Internal combustion engines were initially fueled by town gas during the nineteenth century; however, wood and wood chips can also be used to power cars with ordinary internal combustion engines if a wood gasifier is attached. This was actually quite popular during World War II in several European and Asian countries because the war prevented easy and cost-effective access to oil. Many of these early gas generators had the problem of generating soot and tar, which would in turn clog the engines if not first removed from the gas. This problem has only recently been solved through the use of modern heat-resistant filters that can separate practically all the particles, allowing easy disposal of clean, dry ash.

Modern gasifiers, especially those used to power gas turbines or fuel cells for the production of electricity, are quite efficient. The gasification process in modern designs is usually preceded by *pyrolysis*, where the biomass or coal is initially converted to char, releasing methane (CH_4) and tar rich in polycyclic aromatic hydrocarbons (PAH). This pyrolyzed char is then fed into another gasifier to generate the gasses described above. Such staged gasifiers, where pyrolysis and gasification occur separately, can also be engineered to produce essentially tar-free gas (less than 1 $mg \cdot m^{-3}$), while single reactor fluid-bed gasifiers may exceed 50,000 $mg \cdot m^{-3}$tar.

Contrary to general belief, exhaust gas emission levels of internal combustion engines are significantly lower for wood gas than for gasoline. The efficiency rate of the gasifier system is relatively high: it converts about 75% of fuel energy content into combustible gas that can be used directly as fuel for the engine. Based on long-term studies, comparing otherwise unmodified vehicles during real transportation and under similar driving conditions, the energy consumption for wood gas has been determined to be 1.54 times greater as compared to the energy demand of the same car powered by gasoline (not including the energy needed to extract, transport, and refine the oil from which gasoline is derived).

The primary *disadvantages* of wood gas generators are their typically large size and relatively slow starting speeds. In addition, while the carbon monoxide is an intentional fuel-product that is subsequently burned to safer carbon dioxide in the engine, it is poisonous to humans, even in small to moderate concentrations. However, if the system is well maintained, the chance of exposure to CO is extremely

low. Wood gas generators have several key *advantages* over other sources of fuel. They are relatively easy and inexpensive to build and can be used directly to run internal combustion engines using wood and other forms of carbon-rich biomass. This can reduce dependency on fossil natural gas, gasoline, and oil. Such generators have a closed carbon cycle. This means that the carbon released from the generator, in the form of CO_2, is absorbed by plants that via photosynthesis convert it to biomass. Gasifiers are also far cleaner burning than equivalent burning processes, such as a wood fire or even a gasoline-powered engine (without emissions controls).

In more recent times, wood gas has been suggested as a clean, cheap, and efficient method to heat and cook in developing countries or even to produce electricity when used in internal combustion engines, gas turbines, or fuel cells for maximal efficiency. Gasifiers have been built for remote Asian communities using rice hulls, which in many cases has no other use except for being utilized as a strengthener type additive in concrete. One installation in Burma uses an 80 kW modified diesel engine to supply power for about 500 people who are otherwise without power. The ash is also used as an efficient fertilizer.

So, in summary, it is clear that wood gas generators represent a promising technology based on its utilization of renewable biomass. Once improvements in this technology result in improved efficiency, their uses are expected to expand greatly.

9.4.4.5 Future Perspectives for Biogas

Whatever its source, biogas presents us with viable renewable energy opportunities for the following reasons: Anaerobic digesters allow us to sequester a potentially dangerous and environmentally unfriendly waste (biomethane) by using it as major biofuel. Of all the greenhouse gas emissions, biomethane is 21 times more harmful to the atmosphere than carbon dioxide. It is generated during natural processes of decay; so it is essentially free. Biogas is closer to carbon-neutral than any other source of fuel, making it a near perfect fuel. As biogas technologies such as anaerobic digesters and biomass gasification development increases and becomes more common, one of the fundamental questions is, what is the size of the potential biomass resource supply in the United States?

In April 2005, the US Department of Energy (DOE) and the US Department of Agriculture (USDA) co-published a report assessing the potential of the land resources in the United States for producing sustainable biomass entitled, "Biomass as Feedstock for a Bioenergy and Bioproducts Industry: The Technical Feasibility of a Billion-Ton Annual Supply." Looking at forest land and agricultural land, the two largest potential biomass sources, this study estimated that the United States can sustainably produce up to 1.3 billion tons of biomass feedstock by mid-century (2050). This would be enough feedstock to produce 60 billion gallons of B100 biodiesel and E100 ethanol with existing technologies. This is an impressive number. However, the study does not address the opportunities for biogas production from biomass feedstock or biomass gasification technologies. Some recent estimates indicate that biogas could replace up to 50% of present natural gas consumption in the United States. In some countries, such as Iceland, biogas already provides 100% of the

natural gas resources. Sweden currently obtains 51% of its methane from biogas, and Switzerland, 37%. Countries such as France, Norway, Germany, and Austria use smaller amounts for vehicles. China, India, Korea, the Ukraine, Spain, and Italy are other examples of countries now initiating projects where biogas will be used as a vehicle fuel.

When viewed in a broader perspective, biogas has an outstanding potential to help solve current environmental problems, including urban and agricultural waste management, water purification, and the need for cleaner air. By converting biomass waste, such as municipal solid waste, sewage sludge, crop residues, energy crops, and manure, into biogas, governments can address energy and environmental problems in a sustainable manner (Ahring and Westermann, 2007). However, most governments, including the United States, are perhaps too focused on liquid fuels to support the development of a biogas infrastructure. It is very unlikely that any one technology will come close to solving global energy needs. A combination of all technologies will therefore be required to address such problems, and the development of new technologies will clearly play an important role in this approach.

9.5 Future Technologies in Biofuels: Algae for Energy

The idea of using algae as a source of fuel is not new, but it is now becoming a promising technology because of the escalating price of petroleum and, more significantly, the emerging concern that burning fossil fuels is contributing to global warming (Chisti, 2007; Briggs, 2004). Like plant tissues, algae can provide several different types of renewable biofuels. These include methane produced by anaerobic digestion of the algal biomass; bioethanol fermented from algal cell walls; biodiesel derived from algal oil; and photobiologically produced biohydrogen. Unlike other plant energy crops, algae species can grow extremely rapidly and many are exceedingly rich in oil. Microalgae (single-celled algae) commonly double their biomass within 24 h, and biomass doubling times during exponential growth are commonly as short as 3.5 h. Thus, algae can develop huge amounts of biomass usable as starting material for both bioethanol and biogas production. An added benefit here is that algae do not produce lignin (Table 9.1); however, the amount of cellulose and hemicellulose is relatively low compared to other plants.

It is not yet feasible to collect algal biomass from natural sources. However, the US Department of Energy's Aquatic Species Program has experimented with "raceway ponds" for the cultivation of algae. These artificial, shallow ponds are divided into a rectangular grid, with each rectangle containing one channel in the shape of an oval, like an automotive raceway circuit. Each rectangle contains a paddle wheel to make the water flow continuously around the circuit. Under such conditions, nutrients can be delivered to algae crops, and biomass doubling rates can be optimized.

Another promising technology for algae growth involves photobioreactors. Unlike open raceways, photobioreactors permit essentially single-species culture of

algae for prolonged durations, and they have been successfully used for producing large quantities of microalgal biomass. A tubular photobioreactor consists of an array of straight transparent tubes that are usually made of plastic or glass. This tubular array, or the solar collector, is where algal broth is circulated from a reservoir and where sunlight is captured. Such reactors can be constructed in areas that are not suitable for plant growth (such as deserts), and they hold great promise for the large-scale production of algae oil and biohydrogen.

9.5.1 Biodiesel and Biopetroleum from Algae

Oil content in some algae species can exceed 80% by weight of dry biomass. Since many common algae (e.g., *Chlorella vulgaris*) have a natural oil content greater than 50%, they have a high potential to be low-input, high-yield feedstocks useable for biofuel production (Chisti, 2007). Oil-based algae fuel, also called *oilgae* or sometimes third generation biofuel, is a biofuel derived from oil content of algal biomass. A self-published article by Michael Briggs, at the UNH Biodiesel Group, offers estimates for the realistic replacement of all vehicular fuel with biodiesel by utilizing algae, which Briggs suggests can be grown on algae ponds at wastewater and sewage treatment plants. These oil-rich algae can then be extracted from the system and processed into biodiesel, with the dried remainder further reprocessed to create ethanol.

Algae fuel yields have not yet been accurately determined; however, the US Department of Energy is reported as saying that algae yield 30 times more energy per acre than land crops such as soybeans. The DOE estimates that if algae fuel replaced all of the petroleum fuel in the United States, it would require only 15,000 square miles (38,849 square km), which is roughly the size of Maryland. These estimates are very promising; however, algal oil is difficult to extract, and more research needs to focus on the development of efficient extraction protocols.

Many companies are pursuing algae bioreactors for various purposes, including high-yield oil production that can scale biodiesel production up to commercial levels. Alternative fuel companies such as Solazyme (South San Francisco, CA) and Solix Biofuels (Fort Collins, CO) are using algae to produce biodiesel (Fig. 9.6). While the cultivation of algae to harvest oil for biodiesel has not yet been undertaken on a commercial scale, one of the most appealing aspects of alga-culture is that – unlike crop-based biofuels – it does not entail a decrease in food production, since it requires neither farmland nor fresh water. Unfortunately, like ethanol, biodiesel (including that extracted from algae) attracts water and thus cannot be shipped in existing pipelines. Both ethanol and biodiesel also have lower energy density than traditional gasoline and diesel fuels.

Some companies, such as Sapphire Energy (formally launched in May of 2007) have developed molecular platforms that converts sunlight and CO_2 into renewable, carbon-neutral alternatives to conventional fossil fuels without the numerous downsides of current biofuel efforts. The end product is not ethanol – and not biodiesel. The end product is "green crude," which is chemically identical to molecules in

Fig. 9.6 Colorado's Solix Biofuels tackles the difficult task of harvesting algae for oil with a field of bioreactors that take a kind of painter's drop cloth (inset) to bubble CO through its system. From Popular Mechanics online article "Pond-Powered Biofuels: Turning Algae into America's New Energy" 2007 (http://www.popularmechanics.com/science/earth/4213775.html)

crude oil, making the products entirely compatible with the current energy infrastructure. Such green crude is said to have the same energy density as gasoline and can be shipped in existing pipelines and refined the same way gasoline and diesel are. Such technologies are at the forefront of an entirely new industry with the potential to profoundly change America's energy and petrochemical landscape. However, the use of such technology will be dependent on the enactment of government policies that pressure oil companies to turn their attention away from foreign oil.

9.5.2 Biohydrogen

Hydrogen is regarded as the fuel of the future, being easy to handle and extremely clean burning. The efficiency of conversion of hydrogen to useable energy is especially high in fuel cells for the production of electricity, with water being the sole end product. Presently, hydrogen is produced from fossil reserves with the concomitant release of anthropogenic carbon dioxide. Therefore, new hydrogen production technologies, making use of renewable resources such as the production of biohydrogen from biomass through the action of microorganisms, are being pursued.

In 1939, a German researcher named Hans Gaffron, while working at the University of Chicago, observed that the algae he was studying, *Chlamydomonas reinhardtii* (a green alga), would sometimes switch from the normal production of oxygen to the production of hydrogen. Gaffron never discovered the cause for this

change and for many years other scientists failed in their attempts at its discovery. However, in the late 1990s professor Anastasios Melis, a researcher at the University of California at Berkeley, discovered that if the algae culture medium is deprived of sulfur it will switch from the production of oxygen (normal photosynthesis) to the production of hydrogen. He found that the enzyme responsible for this reaction is hydrogenase, but that the hydrogenase is not functional in the presence of oxygen.

As a result of these findings, photobioreactors, harboring various algae species, are currently being used for biological hydrogen production (Rupprecht et al., 2006). Initially, the yields were not very high; however in 2006, researchers from the University of Bielefeld and the University of Queensland used genetic modifications to changed *C. reinhardtii* in such a way that it produces an especially large amount of hydrogen, producing five times the volume made by the wild form of the algae with up to 2.0% energy efficiency. Later in 2007 while studying mutants of *C. reinhardtii*, Anastasios Melis achieved 15% energy efficiency, demonstrating that truncated chlorophyll antenna size would minimize wasteful dissipation of sunlight by individual cells. Such results hold great promise for alternative fuels, but other areas of research need to be address, including the development of oxygen-tolerant hydrogenases and increasing hydrogen production rates through improved electron transfer.

Biohydrogen can and is also produced in bioreactors that utilize feedstocks other than algae, the most common feedstock being waste streams. The hydrogen is produced by bacterial species such as *Rhodobacter sphaeroides* and *Enterobacter cloacae* through dark fermentation either by mixed cultures of hydrogen-producing sludge or pure cultures of anaerobic bacteria, such as *Clostridium butyricum*. This process involves allowing the bacteria to feed on hydrocarbons under anoxic conditions so that they release hydrogen and CO_2. Under natural conditions, this hydrogen is usually consumed as soon as it is being produced by methanogenic bacteria, releasing methane as the end product. However, under bioreactor conditions, the CO_2can be sequestered successfully by several methods, and the methanogens can be omitted from the system, allowing hydrogen gas to be captured. A prototype hydrogen bioreactor using waste as a feedstock is in operation at Welch's grape juice factory in North East, Pennsylvania.

By uncoupling methane production from hydrogen production, more hydrogen can be harvested. In such systems, hydrogen and acetic acid are first produced from biomass by thermophilic bacteria. Then in a second stage, the acetic acid in the effluent is converted to hydrogen by purple non-sulfur bacteria (Zheng et al., 2008). Biohydrogen can also be produced from glucose in upflow biofilm reactors with plastic carriers under extreme thermophilic conditions (>70°C). Biological hydrogen production derived from these systems is also promising but needs to address the following issues: (1) conversion of biomass from an energy crop or an organic waste stream to fermentable feedstock; (2) selection of thermophilic and/or photoheterotrophic microorganisms and design of optimal growth and production conditions; (3) design of an integrated bioprocess; and (4) development of recovery and purification methods for upgrading the gas to fuel cell specifications.

9.6 How Can Plant Biotechnology Contribute to Bioenergy?

Technology tends to change in cycles that last approximately 50 years. The Russian economist Nikolai Kondratiev (1892–1938) was the first to bring this observation international attention in his book *The Major Economic Cycles* (1925), and others have expanded upon his observations (Fig. 9.7). Starting with the industrial age, when the expansion of steam power changed the world, this source of power (fueled by biomass) led to the era of the railroads. Railroads in turn promoted the development of petrochemicals (including coal and oil), which gave ample energy required to develop information technology. Currently, the information age is in full swing, and without this technology, modern biology and biotechnology would not be possible. Computers play an essential role in the generation and analysis of the gene sequences used in most modern biotechnological applications. With the invention of new processes for large-scale gene sequencing (e.g., *pyrosequencing* approaches) and even faster computer technology, it appears that we have only scratched the surface of the potential held within a new biological age. This new age will likely be driven by the human need for food and energy, and plant biotechnology will play an essential role in meeting these human needs while finding ways to address land and water usage issues and potential environmental problems including global carbon balance.

The use of biotechnology for improving plant-derived materials for bioenergy production can be achieved at different levels. At the front end of potential improvements are improvements to the plant feedstocks themselves. High-energy yield can

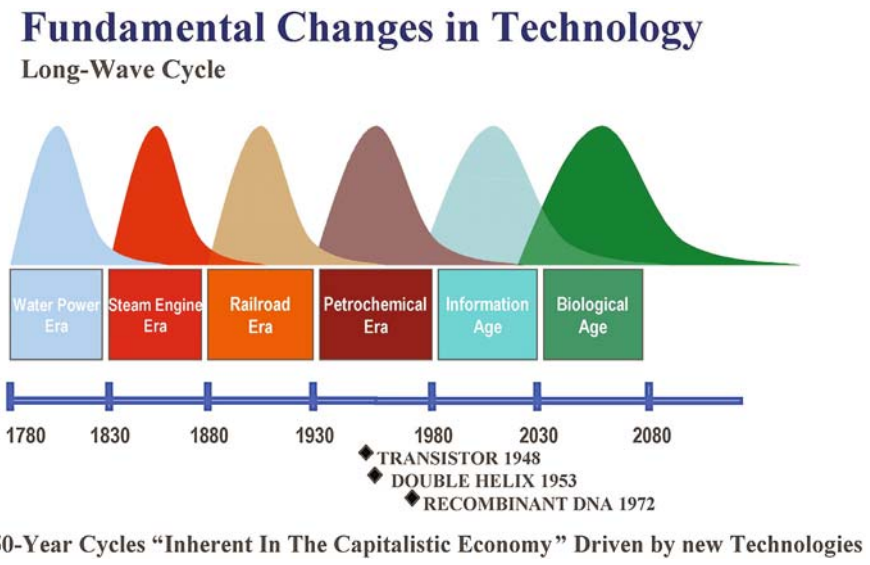


Fig. 9.7 Progressive cycles of technology: 1780–2080 CE based on the work of Nikolai Dimitriyevitch Kontratyev (CE refers to "common era" that replaces "AD")

be realized through plant breeding programs designed to identify genetic markers for beneficial traits and to enhance these traits in existing energy crops, especially sugarcane, sorghum, switchgrass, and non-traditional oil crops having especially high levels of oil (e.g., *Camelina*, mustard, and *Jatropha*). Genetic modification of biosynthetic pathways (metabolic engineering) and the application of biotechnology will likely play the biggest role because of the promise of significant and rapid improvements. Plants will very likely be manipulated to create greater yields of oils and biomass; show greater resistance to abiotic and biotic stresses; require less water and grow in harsher environments; and reduce associated costs of growth and processing. Some such modifications have already been accomplished, such as the reduction of lignin biosynthesis, allowing easier extraction of cellulose and hemicellulose polymers in preparation for processing (see Section 9.4.2.4) or altering plant oil profiles so that specific types of plant oils are produced.

Improvements in bioenergy can also be achieved at the level of processing plant materials. This can include (1) improvements to extraction and pretreatment stages of plant oil and ethanol production; (2) improvements to chemical and enzymatic processes used to extract stored energy (including mass production of tailor-made cellulase enzymes that are more efficient or by genetically engineering plants and fungi to produce desired cellulase, xylanase, and/or other hydrolase enzymes that can help degrade plant polymers); or (3) improvements to the efficiency of collecting and transporting the plant oil tissues or cellulosic biomass to the biofuel refinery. Efforts are also being directed toward improving the efficiency of the biosynthetic pathways of the microorganisms (yeast and bacteria) that perform ethanol fermentation or ABE fermentation (see Section 9.4.2.4), as well as methanogen processes that generate biogas.

Improvements can also be made at the tail end of bioenergy production. This may include improvements to distillation efficiency by engineering microorganisms to be more resistant to bioalcohol (allowing higher concentrations of ethanol or butanol) or by simply improving the ability of the biofuels to be used in existing networks of pipes for delivery to the consumer (see Section 9.5.1). The point here is that there is room for biological improvement at virtually every stage of bioenergy production. Almost all of these improvements, however, are dependent on an in-depth understanding of the biological processes and biosynthetic pathways that control each of these stages.

Sorghum (*Sorghum spp.*), which is related to corn and sugarcane, provides a good example because it has a number of characteristics that make it an attractive biomass crop for ethanol production. Sorghum requires little water and little fertilizer compared to traditional bioenergy crops. It is tolerant to heat and drought, has high biomass yield, and bioalcohol can be produced from sugars stored in the stems, starch from the grain as well as the lignocellulose from the leftover stalks. Two traits of particular interest are the "sweet sorghum trait," which results in the accumulation of fermentable sugars in the juice of the stems (similar to sugarcane), and the "brown midrib trait," which changes the chemical composition (and hence color) of the vascular tissue resulting in higher yields of fermentable sugars obtained after enzymatic processing of the lignocellulosic biomass. The genetic basis of these

traits, however, is poorly understood and impedes the full exploitation of sorghum for bioenergy production.

Consequently, research is being directed toward identification of regions of the genome (through quantitative trait loci [QTL] and genomics) that contain genes associated with the sweet sorghum trait and other traits of interest for bioenergy production. In addition, identification of the genes that are responsible for the accumulation of sugars in the stem (through genetic mapping, cloning, and elucidation of biochemical functions) will aid in the understanding of the brown midrib trait. Without an understanding of the biochemical and molecular mechanisms behind such traits, improvements to subsequent ethanol production are limited.

Perhaps the most major improvements can be made at the front end of bioenergy production, i.e., improvement of cellulosic biomass/cell wall production in herbaceous grasses such as sorghum, sugarcane, and switchgrass. Likewise, improvements to woody biomass may include the manipulation of wood productivity and carbon sequestration as well as improved cellulose biosynthesis in poplar trees (*Populus* species) for use as energy crops (Joshi and Mansfield, 2007). Attention to front end production is especially important because it can benefit multiple types of biofuels at the same time, including the production of solid biomass, bioalcohol and biogas.

There are a number of traits that are especially beneficial for the biological improvement of bioenergy. An ideal energy crop plant would have a combination of (1) a high yield of biomass resulting from a high rate of growth and high photosynthetic capacity; (2) a higher fuel to mass ratio resulting from efficient nutrient uptake from the soil and high levels of stored sugars or oils; (3) reduced need for soil nutrients and soil moisture resulting from the growth of deep roots; (4) resistance to pests, disease, and the extremes of cold and hot temperatures; (5) tolerance to salt, high and low pH, and heavy metal contaminants in the soil; (6) a perennial grow habit to allow collection of biomass each year without fresh planting; and (7) the silencing of flowering to help avoid the spread of foreign genes to native populations (Fig. 9.8). Many of these traits have already been studied in model plant species such as *Arabidopsis*, rice, soybean and poplar trees, and some of the genes that are orchestrating molecular events behind such traits have been identified (Fig. 9.9). Therefore, the stage is already set for the use of plant biotechnology to genetically engineer plants.

While genetic modification of algae has received little attention until just recently, metabolic engineering will also likely have an impact on improving the economics of biomass, oil production, and biohydrogen formation from algae species. Molecular level engineering can be used to potentially (1) increase the photosynthetic efficiency to enable increased biomass yield and rate of production; (2) improve temperature tolerance to reduce the expense of cooling within photobioreactors; (3) eliminate the light saturation phenomenon so that growth continues to increase in response to increasing light level; (4) reduce photoinhibition that actually reduces growth rate at midday light intensities; and (5) reduce the susceptibility to photooxidation that can damage the cells; and (6) increase oil content in biomass.

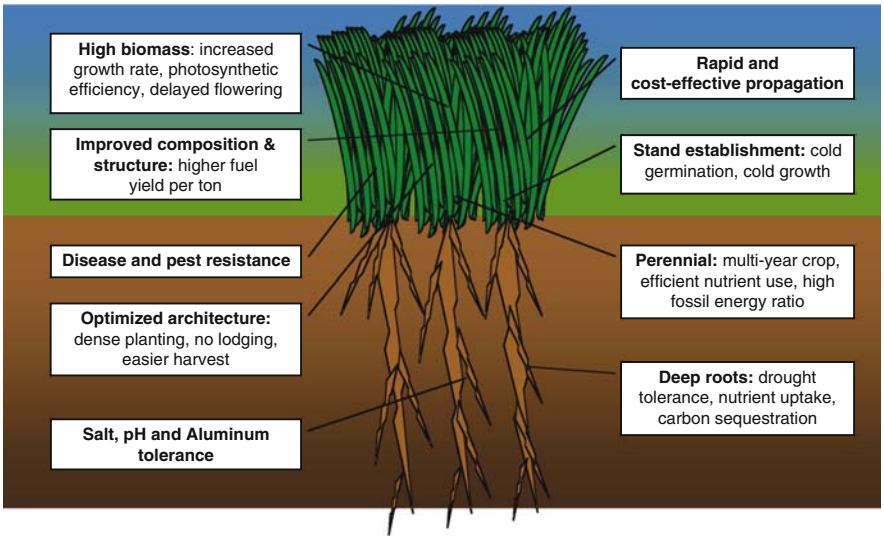


Fig. 9.8 Traits of the perfect energy crop. Modified from Ceres Technologies "The Promise of Dedicated Energy Crops" 2008

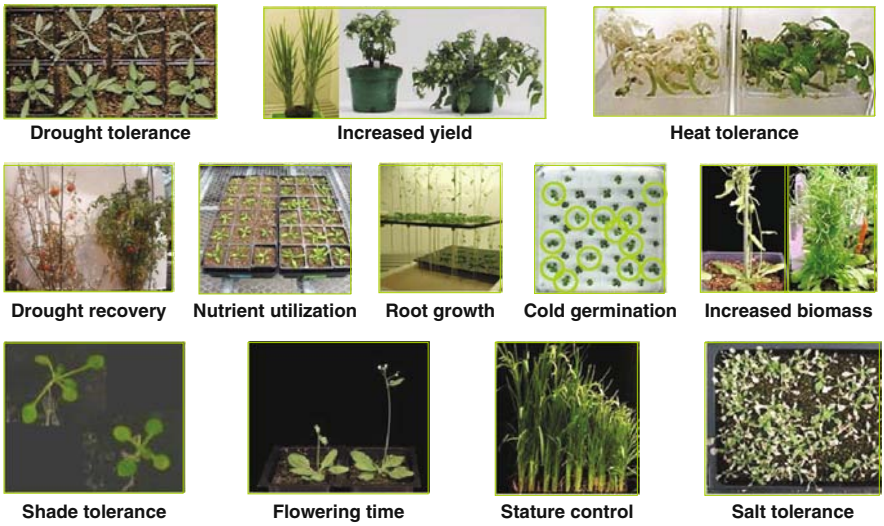


Fig. 9.9 Traits for which genes have been identified that offer improvements in biofuel production. Modified from Ceres Technologies "The Promise of Dedicated Energy Crops" 2008

As far as plant oil is concerned, development of knowledge-based approaches to generate high-yielding oil crop species is key to manipulating oil biosynthesis through plant biotechnological methods (Abbadi et al., 2004; Dyer et al., 2008). Based on our current understanding of seed oil production, improvements

can be made for increasing oil content and composition in oil crops. For example, triacylglycerol (TAG) is the plant oil product that is chemically most similar to fossil oil and therefore has the most potential to replace petroleum-based oil (Agarwal, 2007). Thus, manipulation of the biosynthetic pathways leading to TAG is a useful endeavor. In addition, the study of high-yield oil crops that can be grown on marginal lands, such as *Camelina*, mustard, and *Jatropha,* will also be important (Table 9.2). These crops offer benefits to small-scale farmers, especially those in under-developed countries, by providing new sources of income using land that they would otherwise not have developed. Since oil biosynthesis pathways and genes that can be manipulated to improve oil yield and composition have been tested in model crops such as soybean and castor bean, the application of altered oil biosynthesis can now be implement in new marginal land energy crops.

While there are still limitations on the widespread application of plant biotechnology for bioenergy production, expanding information on the genes involved in plant biosynthetic pathways, compartmentation of intermediates within plant cells, and the genomics of bioenergy-producing plant species are helping to design new approaches for improving yields and composition in a variety of plant species (Allen et al., 2007). It remains to be seen if such a push can be accomplished. However, it is clear that the world is moving in the direction of bio-based energy, focusing on renewability and efficiency in energy consumption. Genetic improvements of energy crops require knowledge of desired quality attributes; the relative economic value of the quality parameters in relation to yield, genetic variation for the desired traits, or for molecular breeding; knowledge of genes to suppress or add; and knowledge of any associated negative consequences of biomass quality manipulation. Finding the optimal direction forward in the new biological age will

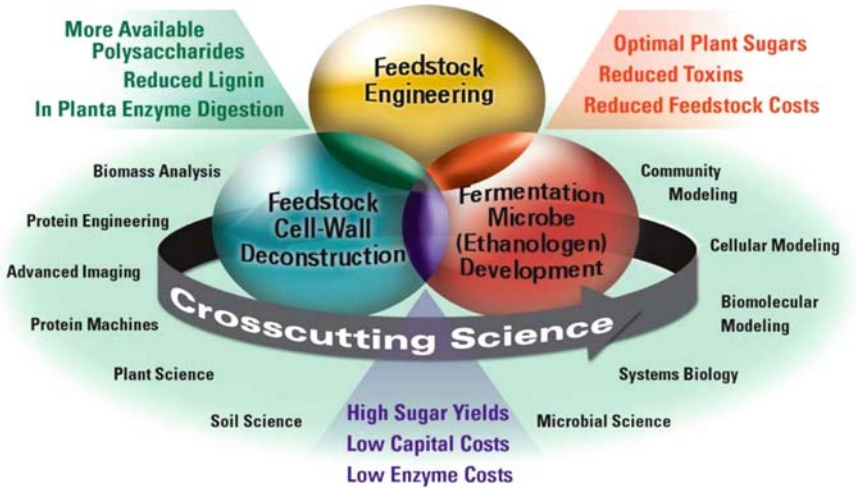


Fig. 9.10 A multidisciplinary approach to improvements in bioenergy. DOE's Bioenergy Roadmap for Biomass. From the DOE Genome Program (http://doegenomes.org)

certainly require a multidisciplinary approach that combines and spans many different fields of research (Fig. 9.10). Because plant bioenergy technology is still under development, desirable plant feedstock characteristics have not yet been completely delineated, but there is no question that plant biotechnology and modern biological techniques (such as genomics, proteomics, metabolomics, and fluxomics) will be at the heart of the improvements to bioenergy.

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Part III
Use of Plant Secondary Metabolites in Medicine and Nutrition

Chapter 10
Interactions of Bioactive Plant Metabolites: Synergism, Antagonism, and Additivity

John Boik, Ara Kirakosyan, Peter B. Kaufman, E. Mitchell Seymour, and Kevin Spelman

Abstract Drugs are commonly used in mixtures, also called cocktails, to treat disease, particularly cancer and viral infections. Any two or more drugs, or for that matter, two or more bioactive plant compounds, will either interact in some way or fail to interact. If an interaction produces an effect greater than that expected for each individual drug, the interaction is termed synergistic. If the effect is less than expected, it is termed antagonistic. If the effect is equal to the expected effect (i.e., there is no interaction), the interaction is termed additive (see Greco et al., 1995; Spelman, 2007, in Cseke et al., 2006). In most therapeutic situations, the hope is that mixtures will produce a synergistic effect, but additivity can also be useful and should not be neglected.

Our focus in this chapter is on interactions between bioactive plant compounds used in food and medicine. In particular, we are interested in plant compounds that have potential therapeutic effects, but also exhibit low systemic toxicity, and thus do not pose a high risk of producing adverse effects. Thousands of such compounds are known to exist, and more are being discovered each year. Even a single plant can contain dozens of bioactive compounds. With such a large pool to draw from, there is nearly an unlimited number of ways in which compounds can be combined, either with each other or with market-approved drugs. Clearly many opportunities exist to find mixtures that exhibit synergism or additivity.

In the following sections we explore physical models of drug interaction, discuss a mathematical model that can be used to assess interactions, and provide a number of examples of plant compounds that have been shown to interact in a synergistic fashion. In particular, we look at ways by which mixtures of plant compounds may bind to proteins and affect signaling pathways, as well as ways by which plant compounds could alter receptors indirectly by affecting the plasma membrane. The mathematical model discussed provides an accurate method to estimate interaction indices as well as to construct confidence intervals of the indices. An interaction index is of little use if it is not accompanied by confidence intervals. Technical

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aspects of the model are presented in order to provide a full description, but publically available software for the model can be used without a complete understanding of the mathematics involved.

10.1 Introduction

We start this chapter by noting that most drugs approved for the market were not developed with synergism in mind. The conventional regulatory process requires that the safety and efficacy of a drug be based on the merits of the drug used alone. It is only *after* market approval that assessment of synergistic interactions with other approved drugs begins in earnest. The current guiding philosophy in drug development is one of *targeted therapy*, whereby a single drug is designed to affect a single protein target. This target could be a cell surface receptor or an intracellular protein. The goal is to develop drugs that bind with high affinity to a target, but have little affinity for off-target proteins. In this way, some adverse effects can be avoided.

When developing bioactive plant compounds as drug mixtures, nutraceuticals, or medicinal foods, an alternative philosophy is needed. The development process for a mixture of plant bioactive compounds must necessarily be different from that for a single-target drug. At least four primary differences between the two types of products stand out. First, rather than a single active constituent, there could be several or even many dozens of active constituents in a plant extract. Indeed, a given meal rich in plant compounds could contain hundreds of bioactive compounds, albeit in small doses. Second, within the class of (reasonably) nontoxic plant compounds that are the focus of this chapter, the binding affinity for known drug targets is often modest to low. Rather than using a low dose of a single high-affinity compound, the optimal clinical effect for many of these plant compounds might be seen when either a relatively high dose of a single compound is administered or, as preferred, modest doses of numerous compounds are given in a complex mixture that takes advantage of additive and synergistic effects. Third, many plant compounds are promiscuous, in that they bind with multiple targets, which may exist in different signaling pathways within the cell (Frantz, 2005; Aggarwal and Harikumar, 2009). Fourth, many bioactive plant compounds, such as flavonoids and curcuminoids, are not soluble in water, are metabolized to less-active conjugates or other products after oral administration, or in some other way exhibit nonideal *absorption, distribution, metabolism, or excretion* (*ADME*) characteristics. The fact that many bioactive plant compounds bind with relatively low or modest affinity to known targets, bind with multiple targets, and/or exhibit poor pharmacokinetics rules them out as useful drugs according to the conventional mode of thinking.

However, this does not mean that bioactive plant compounds do not and could not play a highly useful role in health and medicine. Indeed, in spite of the fact that most do not resemble a “silver bullet” drug, it is likely that such compounds are responsible for much of the disease-prevention effects seen in human populations that consume a diet rich in plant compounds (Liu, 2003). To understand how their

beneficial properties can best be exploited, we must redefine, or at least expand, our definition of a model drug.

Within the class of nontoxic bioactive plant compounds, the characteristics that appear to limit their use in medicine also provide us with opportunities. The fact that a plant extract or mixture of extracts may have a large number of bioactive constituents means that there are abundant opportunities for additivity and synergism. It should be emphasized that although synergistic interactions tend to receive the most research attention, additive interactions are more common, and in large mixtures they may play an even more important role than synergism. For example, in a cytotoxicity study on doxorubicin and nine natural compounds against human lung cancer cells, Boik and Newman (2008) found that synergism in smaller mixtures could allow a tenfold reduction in the concentration of doxorubicin needed to produce a given effect level. Importantly, larger mixtures that exhibited less synergism (and more additivity) allowed a similar degree of reduction in doxorubicin concentrations. In the larger mixtures, each drug was used at a lower concentration.

Additivity and synergism arise in mixtures through their ability to affect multiple targets. The importance of affecting multiple targets cannot be overemphasized when dealing with complex diseases such as cancer, chronic inflammatory diseases, chronic viral infection, and many others. In these diseases, numerous macro homeostatic systems may be affected, as well as numerous intracellular and intercellular signaling pathways. A large number of proteins can be involved, and each may be considered as a potential target. For complex diseases, acceptance is growing in the pharmaceutical industry for the design of mixtures or drugs capable of affecting multiple targets (Tortora et al., 2004; Roth et al., 2004; Frantz, 2005; Zimmermann et al., 2007).

Taking common solid tumors as an example, multiple signaling pathways can be critically under- or overregulated (Chandran et al., 2007; Chan et al., 2008). As tumors progress, the genetic machinery of tumor cells tends to become increasingly unstable. When this occurs, more proteins can become involved and the tumor population becomes better able to adapt to drug therapy, immune attack, or other obstacles to growth. It would seem unlikely that any single drug, particularly a drug that affects a single target, would have a lasting effect on such a flexible cell population. If the drug does not kill 100% of cells, there is a reasonable chance that the surviving cells will reestablish a population resistant not only to the applied drug but also to other drugs. This characteristic is termed *multidrug resistance*. Cells may become resistant through gene amplification and overproduction of the target protein, production of proteins that pump the drug out of the cell, underproduction of proteins that allow the drug to enter the cell, improved repair of drug-induced DNA damage, production of proteins that inactivate the drug, and/or production of proteins that serve the same function as the target protein but that are not affected by the drug. Literally, hundreds of proteins could be involved in the progression of cancer and developed resistance to drugs.

While plants may offer a rich source of bioactive compounds for use in mixtures, do these compounds need to bind to their targets with high affinity in order to be effective? Csermely et al. (2005) have proposed that in some cases, partial inhibition

of multiple signaling pathways may be more efficient than complete inhibition of a single pathway (see also Ágoston et al., 2005). This suggests that compounds that exhibit modest binding affinity could still be useful.

Could administration of multiple compounds lead to adverse effects? Of course, adverse effects are possible with any pharmacologic therapy. Thus, mixtures will need to be designed carefully. But the risk of adverse effects can be minimized through the use of relatively nontoxic compounds. In addition, keeping doses as low as possible can reduce risks. Because of additivity and synergism in a well-designed mixture, relatively low doses of individual compounds may still be capable of producing a desired effect without excessive risk of toxicity.

Pharmacokinetic issues are also important when considering the medical and health effects of plant compounds. While many otherwise interesting compounds exhibit poor ADME characteristics, a good number of these could still be useful. For example, the vehicle used to deliver the compounds could be altered to improve the pharmacokinetics. In some cases, enteric-coated tablets, emulsifiers, complexing agents, or lipid-based formulations might be useful. In addition to manipulations based on physical pharmacy, the act of using mixtures of compounds can in some cases affect ADME characteristics. For example, the ability of grapefruit juice to affect drug metabolism is now well established. This is discussed more in Chapter 14 in relation to adverse drug interactions. But beneficial effects on ADME characteristics are also possible. For example, hypericin, thought to be one of the active constituents of *Hypericum perforatum* (St. John's wort), is nearly insoluble in water. Jürgenliemk and Nahrstedt (2003) showed that some phenolic constituents typical for *Hypericum* extracts increased the concentration of hypericin in the water phase by up to 400-fold. Butterweck et al. (2003) showed that the oral bioavailability of hypericin was increased if administered with hyperoside, also found in *Hypericum* extracts. In another example, Gawande et al. (2008) found that oral administration of a black grape extract along with (–)-epigallocatechin gallate (EGCG), a green tea component, increased the systemic availability of EGCG in humans.

We see then that interactions in a mixture may be due to *pharmacodynamic* or *pharmacokinetic* events (Spinella, 2002). In the former, the effects are due to two or more drugs acting on single or multiple regulatory proteins. Such interactions are often directly related to the binding affinity or membrane-altering ability of the drugs. In contrast, pharmacokinetic interactions are due to influences of a compound on another's ADME characteristics.

10.2 Physical Models of Drug Interaction – Protein Binding and the Plasma Membrane

As discussed above, drugs in a mixture may interact by binding to target proteins. For convenience, we use the term *drug* here and in the remaining portions of this chapter to refer to both approved drugs and bioactive plant compounds. The binding of a drug to a protein may affect the function of that protein through a number of

mechanisms. For example, if the protein were an enzyme that binds substrate S, the drug could bind near the active site of the enzyme, thereby sterically inhibiting the binding of S. If the enzyme contains an allosteric binding site, distant from the active site, the drug may bind to the allosteric site and thereby affect the conformation of the active site and its ability to bind S. When multiple drugs affect a single protein, the complexity of binding patterns and the mechanical alteration of protein function will influence the type of interaction produced: additive, synergistic, or antagonistic. If multiple proteins are involved, the complexity of the (signaling) network in which the target proteins exist will also influence the type of interaction produced.

As a simple example, targets could be two intracellular enzymes serially connected in a signaling pathway. In this case, inhibition of an enzyme by each drug in a binary mixture might produce additive effects. In more complex cases, drugs may affect multiple proteins in a signaling pathway that contains positive and/or negative feedback loops or drugs may affect proteins that are involved in distinct but connected signaling pathways. As the number of bound proteins and the complexity of the signaling pathways increase, there are increasing opportunities for antagonistic or synergistic interactions.

In addition to intracellular proteins, drugs may also bind to protein targets on the plasma membrane. In particular, they may bind to transmembrane receptors embedded in the lipid bilayer. Many signals that originate outside of the cell enter the cell via cell surface receptors. Growth factors, such as *epidermal growth factor* (*EGF*), are an example of an extracellular signal. Extracellular EGF binds to EGF receptors (EGFR) on the plasma membrane, and the resulting signal is propagated into the cell, eventually reaching the nucleus and causing proliferation.

Drugs can directly bind to receptor proteins on the plasma membrane, in some cases stimulating signal transduction and in other cases inhibiting it. For example, plant compounds that bind weakly with estrogenic receptors may physically block the binding of more potent ligands, thereby producing an antiestrogenic effect. Genistein, from soybean, is reported to act in part by this mechanism in some experimental models (Kogiso et al., 2006). If multiple therapeutic compounds are used and multiple receptor types are affected, downstream signaling pathways may interact in additive, antagonistic, or synergistic ways.

The interaction of drugs can be influenced by the properties of the plasma membrane that contains the receptors. Although the membrane has been described as a system driven by thermodynamic equilibrium (Aon et al., 1996), it is more accurately seen as an emergent structure consisting of highly asymmetrical structures and undergoing dynamic transitions (Perillo, 2002). Typically, mammalian cellular plasma membranes consist of about eight major classes of lipids (Simons and Vaz, 2004) and also include a variety of proteins embedded in the bilipid structure. The plasma membrane serves a number of purposes, including protection, endocytosis, signaling, and mechanical stability. It must be rigid enough to protect the cell and offer stability, but at the same time, it must be dynamic and pliable enough to allow cell deformation and promote adaptation to diverse environmental messages. Either directly or indirectly, the characteristics of the lipid membrane affect nearly every activity that occurs in a cell.

One way that the membrane affects signaling is by supporting the dynamic creation and movement of *lipid rafts* (also known as membrane rafts), which are clusters of proteins that horizontally "float" in the membrane. One report has identified as many as 250 proteins that exist in lipid rafts (Patra, 2008). A good number of these proteins, including *ras* (ras is a signal transduction protein that belongs to a large superfamily of low-molecular-weight G proteins) and EGFR, are cell surface receptors. Several authors have postulated or shown that receptors in a raft can cooperate to affect each other's conformation, thereby coordinating the overall response to a ligand (Duke and Bray, 1999; Graham and Duke, 2005; Fuxe et al., 2008; Sourjik, 2004). For example, consider a receptor that switches probabilistically between two conformations, active and inactive, and binding of a ligand stabilizes the active state. Certain cellular responses, such as chemotaxis, in response to a chemoattractant, are most useful if they are binary. For example, it might be beneficial if a cell moves toward a weak stimulus with the same force that it moves toward a stronger stimulus. This requires that as a group, the receptors act like an *on/off switch*. One way to accomplish this is by allowing adjacent receptors in a raft to influence the conformation, active or inactive, of one another. Above a critical but low ligand concentration, a small percentage of receptors are bound, but these cause unbound receptors to switch to the active conformation. In a more complicated scenario, receptors may simultaneously bind two ligands, and in this case, receptor–receptor interactions may produce the equivalent of *AND/OR logic gates*. For example, if a cell senses both poison and chemoattractant in the same location, it is beneficial if the cell does not move toward the chemoattractant.

The switching characteristics mentioned above are dependent on receptor–receptor interactions, which in turn are dependent upon the characteristics of lipid rafts. The notion that membrane characteristics may influence the type of drug interaction (additive, antagonistic, or synergistic) begs the question of whether some drugs may act directly on the plasma membrane itself, in addition to or in contrast to protein binding. Indeed, this seems to occur for a good number of drugs and bioactive plant compounds. It is well known that hydrophobic drugs tend to interact with biological membranes (Schreier et al., 2000). At high concentrations, they can act like detergents and disrupt membranes, while at low concentrations, they tend to stabilize membranes, such as protecting red blood cells from hemolysis. Many bioactive plant products are also hydrophobic and can be expected to interact with membranes. For example, some flavonoids have been shown to affect lipid viscosity. In addition, flavonoids preferentially located in the hydrophobic portion of the bilayer have been shown to initiate the formation of raft-like domains, whereas those located in the polar interface region can fluidize membranes and have a raft-breaking effect (Tarahovsky et al., 2008). In a study on human colon cancer cells, the flavonoid quercetin was shown to induce the accumulation of cell death receptors in lipid rafts and thereby facilitate *apoptosis* (programmed cell death) in response to death-inducing signals (Psahoulia et al., 2007). Adachi et al. (2007) reported that EGCG from green tea inhibited the binding of EGF to EGFR and the subsequent activation of EGFR by altering membrane organization related to lipid rafts. As a last example, omega-3 fatty acids (EPA, eicosapentaenoic acid, and DHA,

docosahexaenoic acid) have been shown to inhibit the proliferation of human breast cancer cells in vitro, in part by reducing EGFR levels in lipid rafts (Schley et al., 2007).

In summary, the type of interaction produced by a drug mixture is influenced by the complexity of protein binding patterns, the complexity of the network in which the bound proteins interact, the degree of receptor–receptor interactions, and the effects of drugs on the plasma membrane, particularly on the formation and composition of lipid rafts. In the interest of brevity, other mechanisms by which a drug can affect cellular function have not been discussed. For example, some drugs can bind directly to DNA molecules. Many of these drugs, however, tend to exhibit high systemic toxicity. Drugs can also affect cellular function by acting as pro- or antioxidants.

10.3 Quantifying Synergism Using Nonlinear Mixed-Effects Modeling

In the following discussion on mathematical models of drug interaction, we shift to a more technical tone. In particular, the reader is given a complete mathematical description of the MixLow method for assessing interaction indices developed by Boik et al. (2008). An implementation of the MixLow method is currently available in the R language (package *mixlow*) and can be downloaded from the CRAN web site (http://cran.r-project.org/). Although details of the model are presented here, the R package can be used without a complete understanding of the mathematics involved. For those readers who are not biostatisticians, the details given here should provide a general insight into the many issues involved in estimating an interaction index, and for those who do become users of the *mixlow* package or other software, the details should provide useful reference material.

10.3.1 Background

Over the last few decades, several mathematical methods have been proposed to assess synergism between drugs in a mixture.[1] All of these are based on some index of additivity (or null interaction). There has been disagreement on a strict mathematical definition of additivity and reviews have been published discussing the various proposals (Berenbaum, 1989; Greco et al., 1995; Merlin, 1994; Tallarida, 2001). Two indices that have gained widespread acceptance are those for *Loewe*

[1]Where convenient and not confusing, the continuum of antagonism/additivity/synergism is referred to as *degrees of synergism*. The term *method* is used to refer to the combination of a model to estimate concentration–response curve parameters, an interaction index, and a procedure to calculate confidence intervals. The term *confidence interval* is used to refer to the nominal 95% confidence interval of the Loewe index or other interaction indices.

additivity and *Bliss independence* (Greco et al., 1992). The Loewe additivity index forms the basis for the present work, as well as the basis for isobolograms (Poch, 1990), which are graphical assessments of additivity. In contrast to other indices, particularly that of Bliss independence, the Loewe index produces the intuitively reasonable result that a *sham* mixture, a mixture of a drug with itself, is additive. In the MixLow method, developed by Boik et al. (2008), estimation of the Loewe index is a three-step process: (1) estimate parameter values that define the shape of concentration–response curves for the mixture and its component drugs; (2) use the estimated parameters in calculating the Loewe index; and (3) generate confidence intervals of the index.

Methods to assess synergism can be distinguished not only by the interaction index used but also by the experimental design used to obtain data, the model used to estimate parameters of the concentration–response curves, and the dimensions of the resulting interaction index plot (two- or three-dimensional). Experimental designs are usually either *factorial*, where concentrations of each drug are crossed (or partially crossed) with concentrations of other drugs, or *fixed-ratio*, where concentrations of all drugs are fixed at a constant ratio. If fixed-ratio concentrations in a two-drug mixture are graphed, where each axis represents the concentration of one drug, then the plotted points fall on a straight line, or ray, extending out from the origin. With the fixed-ratio design, synergism can be assessed either along one ray or along a three-dimensional response surface based on a series of rays. The current MixLow method assesses data from a single ray.

The factorial design is used primarily when a three-dimensional response surface (additivity surface) is desired. An example of a factorial design is given by Martinez-Irujo et al. (1996). Examples of response surface methods for multiray data include those by White et al. (2003, 2004), Minto et al. (2000), and Fidler and Kern (2006). While response surface methods do provide more information than can be obtained from the analysis of single-ray experiments, they require that more data be collected. For this reason, single-ray experiments remain common.

The de facto standard for assessing synergism in single-ray experiments is the *median-effect method* of Chou and Talalay (1984). This method estimates concentration–response curve parameters by using log linearization and ordinary least squares. The method presented here, dubbed the *MixLow* (Mixed-effects Loewe) *method* for convenient reference, is similar to the median-effect method in that it assesses data from single-ray experiments, presents results in graphical form (as a plot of fraction affected versus combination index; see below), and uses the Loewe index to define additivity. Unlike the median-effect method, however, it employs a mixed-effects model to estimate parameters of concentration–response curves. This approach allows more accurate estimation of concentration–response curve parameters and can also produce confidence intervals with improved coverage. *Coverage* is the probability that a confidence interval method captures the true parameter. If the coverage differs markedly from the nominal confidence coefficient (typically 0.95), then the confidence intervals are of questionable value. Confidence intervals for the interaction index, as well as accurate parameter estimators, are vital to fully assess whether drugs in a mixture interact synergistically, antagonistically,

or additively. By extension, knowledge of the coverage of these intervals is also vital. As reported in Boik et al. (2008), in a series of simulations, the MixLow method produced confidence intervals with excellent coverage properties.

The approach described here is applicable to the common situation where within-unit and between-unit measurements are available, responses follow a sigmoidal pattern,[2] and ratios between drugs in a mixture are fixed (that is, various dilutions of the mixture and its component drugs are tested). While such data could be generated in many types of experiments, the discussions here focus on data obtained from in vitro cytotoxicity experiments, where cancer cells are exposed to a drug for a specified length of time (typically 72 hours) and then cell viability is indirectly measured, usually via fluorescence readings after addition of a suitable dye. Such cytotoxicity assays use multiwell incubation trays, where each tray receives one drug or mixture, each column of the tray typically receives a different drug concentration, and replicate trays are tested for each drug and mixture. The experimental unit in this situation is the incubation tray.

Regardless of the drugs tested and assay employed, cytotoxicity responses occur in a nonlinear relationship with drug concentration. If a parametric model is employed, either the relationship can be linearized prior to estimation of concentration–response curve parameters, as is done in the median-effect method, or parameters can be estimated using a nonlinear model, as is done in the MixLow method. Furthermore, when using a nonlinear (or linear) model, effects can be either fixed or random. A mixed-effects model contains both fixed and random effects. Random effects are commonly associated with observations sharing the same level of a classification factor. In this paper, that factor is incubation tray. Nonlinear mixed-effects models are widely used in medicine, particularly to assess pharmacokinetic data. Their use is rare, however, with cytotoxicity data.

In the median-effect method, raw data must be preprocessed. This is accomplished by (1) scaling data by control-well means – responses are averaged across in-tray replicates and divided by average responses of in-tray control wells – and (2) taking the log of a function of the scaled data. Each of these steps can have detrimental effects on the precision of parameter estimators, and the MixLow method does not require any preprocessing.

10.3.2 MixLow Method

Let the random variable *Fa* signify the fraction of cells affected by a drug concentration. Define $\phi = \mathrm{E}[Fa]$, where $\mathrm{E}[\bullet]$ is the expected value. In some contexts, ϕ is estimated based on concentration–response data, and in other contexts, a concentration is estimated that results in a fixed value of ϕ. Denote by $\psi_{d,\phi}$ the ϕ-effective log concentration of drug d. This is the log concentration that produces a fraction

[2] A sigmoidal pattern is relatively flat at high and low concentrations, with a smooth transition linking the two. The overall shape is that of an elongated S.

affected equal to a fixed ϕ. For example, the log concentration of drug d that inhibits proliferation of a cell population by 10% relative to controls is denoted by $\psi_{d,0.1}$. By convention, $\exp(\psi_{d,0.1})$ is referred to as the IC10 (10% inhibitory concentration).

The MixLow method fits a sigmoid curve to concentration–response data and uses the resulting parameter estimates to estimate an interaction index. The sigmoidal curve is parameterized by two constants, $\psi_{d,0.5}$ and a shape parameter denoted by γ_d. The shape parameter indexes the steepness of the concentration–response curve. At the IC50, the slope of the curve is $0.25\gamma_d \big/ \exp(\psi_{d,0.5})$.

The *Loewe index*, which is used by both the median-effect and the MixLow methods, provides a measure of drug interaction. For two drugs, the Loewe index and its estimator[3] are

$$L_\phi = \sum_{d=1}^{2} \frac{\exp(m_{d,\phi})}{\exp(\psi_{d,\phi})} \text{ and } \hat{L}_\phi = \sum_{d=1}^{2} \frac{\exp(\hat{m}_{d,\phi})}{\exp(\hat{\psi}_{d,\phi})}, \tag{10.1}$$

respectively, where $m_{d,\phi}$ is an unknown constant signifying the log concentration of drug d in the mixture when the mixture is at its ϕ-effective log concentration and $\psi_{d,\phi}$ is the unknown ϕ-effective log concentration of drug d alone. The mixture is antagonistic, additive, or synergistic at ϕ depending on whether the value of the Loewe index is greater than 1, equal to 1, or less than 1, respectively. Chou and Talalay (1984) were apparently the first to use plots of $\hat{L}_\phi$ versus ϕ as a summary of drug interactions.

10.3.3 Basic MixLow Model

For clarity of presentation, the basic MixLow model is introduced first. Later, a modified model is discussed. The MixLow model uses a nonlinear mixed-effects framework to represent the concentration–response curve. As a skeleton description, responses are modeled as the expected mean of control wells times a sigmoidal function, plus an error term. Sigmoidal models of this type are sometimes referred to as Hill models. Formally, responses, $\{Y_{d,t,w}\}$, obtained from unprocessed data are modeled as a sigmoidal function of the concentration:

$$Y_{d,t,w} = \exp(\mu + b_t)\left(1 - \phi_{d,t,w}\right) + \varepsilon_{d,t,w}, \tag{10.2}$$

where

$$\phi_{d,t,w} = 1 - \frac{1}{1 + \left(\frac{\exp(c_{d,t,w})}{\exp(\psi_{d,0.5})}\right)^{\gamma_d}}, \tag{10.3}$$

[3]Throughout this discussion the *hat notation* is used to denote parameter estimators and estimates.

and the subscripts *d, t, w* refer to the *d*th drug, *t*th tray, and *w*th well, respectively.[4] Here, drug *d* could refer to a single drug or a mixture, and $c_{d,t,w}$ refers to the log of the drug concentration for the *d*th, *t*th, and *w*th observation, a known constant. The expected value of $\exp(\mu + b_t)$ refers to the expected mean of control wells from all trays, where b_t is a random deviate specific to tray *t*. Values $\{b_t\}$ are independently distributed as $b_t \sim \mathrm{N}(0, \sigma_b^2)$. The error terms in Model (10.2) are independently distributed as $\varepsilon_{d,t,w} \sim \mathrm{N}\left(0,\ f(\sigma^2, \mathrm{E}\left[Y_{d,t,w}|b_t\right])\right)$, where f(•) is a function discussed later.

Differences in control-well means across trays occur for a variety of reasons. For example, if one tray in a group of replicates is seeded with a higher density of cells, the responses in the tray will tend to be higher than those of the other trays. Differences can also occur because of differential handling of trays, differential growth conditions (e.g., incubating trays on different days), differential assay procedures (e.g., allowing one tray to incubate for a slightly longer time before reading results), and/or random biologic variations in cell proliferation.

Having explained the model, the procedures for calculating the Loewe index and its confidence intervals are described. An *n*-drug generalization of the Loewe index in (10.1) is given by

$$L_\phi = \sum_{d=1}^{n} \exp\left(m_{d,\phi} - \psi_{d,\phi}\right) = \sum_{d=1}^{n} \tau_d \exp\left(\psi_{m,\phi} - \psi_{d,\phi}\right), \tag{10.4}$$

where $\psi_{d,\phi}$ is the ϕ-effective log concentration of drug *d* alone, $\psi_{m,\phi}$ is the ϕ-effective log concentration of the mixture, and τ_d is the fraction of the mixture that is composed of drug *d*. Note that $m_{d,\phi} = \log(\tau_d) + \psi_{m,\phi}$.

If $c_{d,t,w}$ in (10.3) is equated to the ϕ-effective log concentration, $\psi_{d,\phi}$, then $\phi_{d,t,w}$ becomes ϕ. That is,

$$\phi = 1 - \frac{1}{1 + \left(\frac{\exp(\psi_{d,\phi})}{\exp(\psi_{d,0.5})}\right)^{\gamma_d}}. \tag{10.5}$$

To write the Loewe index as an explicit function of $\psi_{d,\phi}$, first solve (10.5) for $\psi_{d,\phi}$ to obtain

$$\psi_{d,\phi} = \log\left(\left(\frac{\phi}{1-\phi}\right)^{\frac{1}{\gamma_d}}\right) + \psi_{d,0.5}. \tag{10.6}$$

Second, substitute the expression for $\psi_{d,\phi}$ in (10.6) into (10.4) to obtain

[4]The notation $(1 - \phi)$ is used to denote the expected fraction unaffected, rather than introducing a new symbol for the latter. The exponent term in Model (10.2) is used to ensure that the expected response in control wells is always positive.

$$\begin{aligned}\hat{L}_\phi &= \sum_{d=1}^{n} \tau_d \exp\left(\hat{\psi}_{m,\phi} - \hat{\psi}_{d,\phi}\right) \\ &= \sum_{d=1}^{n} \tau_d \exp\left(\log\left(\left(\frac{\phi}{1-\phi}\right)^{\frac{1}{\hat{\gamma}_m}}\right) + \hat{\psi}_{m,0.5} - \log\left(\left(\frac{\phi}{1-\phi}\right)^{\frac{1}{\hat{\gamma}_d}}\right) - \hat{\psi}_{d,0.5}\right),\end{aligned} \tag{10.7}$$

which is written here as the estimator of the index, where

$$\hat{\psi}_{d,\phi} = \log\left(\left(\frac{\phi}{1-\phi}\right)^{\frac{1}{\hat{\gamma}_d}}\right) + \hat{\psi}_{d,0.5}. \tag{10.8}$$

Confidence intervals of L_ϕ can be based on the standard error of $\hat{L}_\phi$ or its log transformation $\mathrm{SE}\left(\log\left(\hat{L}_\phi\right)\right)$. The latter approach ensures that confidence interval limits are positive and this is the approach used here. The standard error of the transformed index is obtained using the Delta method as follows:

$$\mathrm{SE}\left(\log(\hat{\mathrm{L}}_\phi)\right) \approx \left(\left(\begin{array}{c}\frac{\partial \log\left(\hat{L}_\phi\right)}{\partial \hat{\Psi}} \\ \frac{\partial \log\left(\hat{L}_\phi\right)}{\partial \hat{\gamma}}\end{array}\right)^{\mathrm{T}} \hat{\mathrm{var}}\begin{pmatrix}\hat{\psi} \\ \hat{\gamma}\end{pmatrix} \left(\begin{array}{c}\frac{\partial \log\left(\hat{L}_\phi\right)}{\partial \hat{\Psi}} \\ \frac{\partial \log\left(\hat{L}_\phi\right)}{\partial \hat{\gamma}}\end{array}\right)\right)^{1/2}, \tag{10.9}$$

where the superscript T refers to transpose. The term $\hat{\mathrm{var}}\begin{pmatrix}\hat{\psi} \\ \hat{\gamma}\end{pmatrix}$ is obtained from the observed information matrix, which is produced when Model (10.2) is fit by maximizing the likelihood function. The confidence interval for the Loewe index at ϕ is calculated as

$$\exp\left(\log\left(\hat{L}_\phi\right) \pm t_{df,1-\alpha/2} \quad \mathrm{SE}\left(\log\left(\hat{L}_\phi\right)\right)\right), \tag{10.10}$$

where $t_{df,1-\alpha/2}$. is a multiplier obtained from the t-distribution with df degrees of freedom and α significance level.

Within-group errors for cytotoxicity data tend to be heteroscedastic, with smaller errors at low $\mathrm{E}\left[Y_{d,t,w}|b_t\right]$ values and larger errors at high ones. To allow for this, the error term in Model (10.2), $\varepsilon_{d,t,w}$, can be modeled as a function of σ and $\mathrm{E}\left[Y_{d,t,w}|b_t\right]$. Table 10.1 lists four error functions, e1–e4, which are useful for cytotoxicity data. Note that all functions listed belong to a single family of functions. For example, e4 and e3 are identical when β_d is equal to 1.0 for all drugs. The error functions listed

Table 10.1 Error functions

Error function	Formula $\varepsilon_{d,t,w} \sim \mathrm{N}\left(0,\sigma^2_{d,t,w}\right)$, where $\sigma_{d,t,w}$ equals
e1	σ
e2	$\sigma\,\alpha_d$, where the scaling parameter α_d is drug dependent
e3	$\sigma\,\mathrm{E}\left[Y_{d,t,w}|b_t\right]$
e4	$\sigma\,\mathrm{E}\left[Y_{d,t,w}|b_t\right]^{\beta_d}$, where the power parameter β_d is drug dependent

in Table 10.1 stem from straightforward application of variance options for the nlme function in R (http://cran.r-project.org/).

10.3.4 Modified MixLow Model

The MixLow model can be modified to allow for a "double" sigmoid response pattern. The extension is similar in spirit to modification of a single Hill model to a "double" one. A double-sigmoid response pattern has been observed for a number of drugs in various cell lines (Levasseur et al., 1998; Yeung et al., 1999). The modification to the basic MixLow model is straightforward. Model (10.2) is modified to represent a weighted sum of two sigmoidal curves, which is as follows:

$$Y_{d,t,w} = (1-\lambda_d)\exp(\mu+b_t)(1-\phi'_{d,t,w}) + \lambda_d \exp(\mu+b_t)(1-\phi''_{d,t,w}) + \varepsilon_{d,t,w}, \tag{10.11}$$

where

$$\phi'_{d,t,w} = 1 - \frac{1}{1+\left(\frac{\exp(c_{d,t,w})}{\exp(\psi'_{d,0.5})}\right)^{\gamma'_d}}, \quad \phi''_{d,t,w} = 1 - \frac{1}{1+\left(\frac{\exp(c_{d,t,w})}{\exp(\psi''_{d,0.5})}\right)^{\gamma''_d}}, \tag{10.12}$$

and $0 \le \lambda_d \le 0.5$ is a drug-dependent weight.

As illustrated in Fig. 10.1, each of the component curves and their weighted sum converge to zero as the drug concentration increases. The term $\phi'_{d,t,w}$ refers to the expected fraction affected for the first weighted curve; the corresponding IC50 is denoted by $\exp\left(\psi'_{d,0.5}\right)$ and the slope by γ'_d. Parameters for the second curve are defined analogously. The maximal expected response of the weighted sum is $\mathrm{E}\left[\exp(\mu+b_t)\right]$. The maximal expected responses for the first and second weighted curves are $(1-\lambda_d)\,\mathrm{E}\left[\exp(\mu+b_t)\right]$ and $\lambda_d\,\mathrm{E}\left[\exp(\mu+b_t)\right]$, respectively. If λ_d is equal to zero, then Model (10.11) collapses to Model (10.2).

Denote the fraction affected according to Model (10.11) by ϕ. That is,

$$1-\phi_{d,t,w} = \frac{\mathrm{E}\left[Y_{d,t,w}\right]}{\mathrm{E}\left[Y_{0,t,w}\right]} = (1-\lambda_d)(1-\phi'_{d,t,w}) + \lambda_d(1-\phi''_{d,t,w}). \tag{10.13}$$

If $c_{d,t,w}$ in (10.12) is equated to the ϕ-effective log concentration $\psi_{d,\phi}$, then $\phi_{d,t,w}$ becomes ϕ and

$$(1-\phi) = \frac{(1-\lambda_d)}{1+\left(\frac{\exp(\psi_{d,\phi})}{\exp(\psi'_{d,0.5})}\right)^{\gamma'_d}} + \frac{\lambda_d}{1+\left(\frac{\exp(\psi_{d,\phi})}{\exp(\psi''_{d,0.5})}\right)^{\gamma''_d}}. \tag{10.14}$$

The solution to (10.14) for $\psi_{d,\phi}$ is the root of the polynomial

$$(1-\phi)\exp(\psi_{d,\phi})^{\gamma'_d+\gamma''_d} + a\exp(\psi_{d,\phi})^{\gamma'_d} + b\exp(\psi_{d,\phi})^{\gamma'_d} + c = 0, \tag{10.15}$$

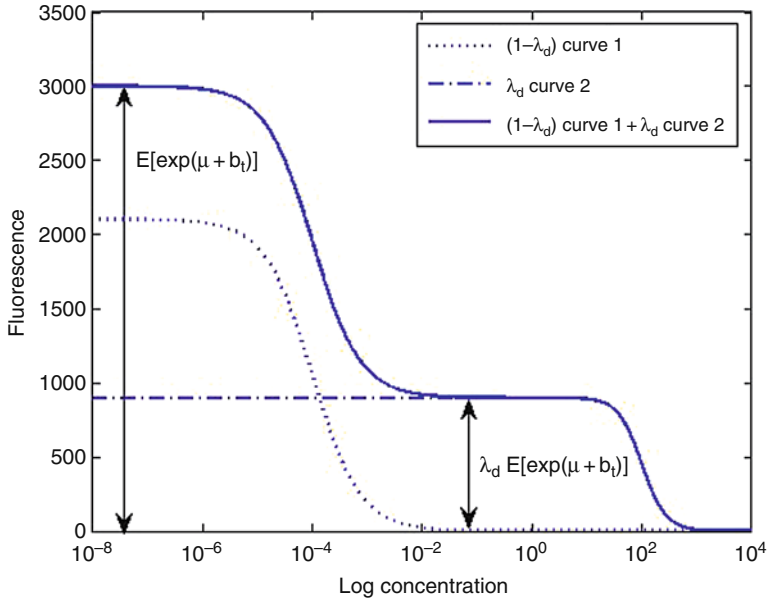

Fig. 10.1 Example of double-sigmoid curve

where $a = (1 - \phi - \lambda_d) \exp(\psi''_{d,0.5})^{\gamma''_d}$, $b = (\lambda_d - \phi) \exp(\psi'_{d,0.5})^{\gamma'_d}$, and $c = -\exp(\psi''_{d,0.5})^{\gamma''_d} \exp(\psi_{\prime d,0.5})^{\gamma'_d}\phi$. Equation (10.15) has at most one real root because the weighted sum of curves is monotonic decreasing by construction. To obtain an estimator of $\psi_{d,\phi}$, namely $\hat{\psi}_{d,\phi}$, substitute estimators $\left(\hat{\lambda}_d, \hat{\gamma}'_d, \hat{\gamma}''_d, \hat{\psi}'_{d,0.5}, \hat{\psi}''_{d,0.5}, \hat{\psi}_{d,\phi}\right)$ for parameters $\left(\lambda_d, \gamma'_d, \gamma''_d, \psi_{\prime}d, 0.5, \psi''_{d,0.5}, \psi_{d,\phi}\right)$ in (10.15) and label the root of (10.15) as $\hat{\psi}_{d,\phi}$.

The estimator $\hat{\psi}_{d,\phi}$ is substituted into (10.4) to obtain the Loewe index as a function of ϕ. Although the estimator is not in closed form, the implicit function theorem can be used to obtain derivatives for computing confidence intervals using equations similar to (10.9) and (10.10).

In practice, drug solubility or other issues can limit the concentration at which a drug can be tested. If the drug produces a double-sigmoid response, but high enough concentrations were not tested to see the full curve, then $\psi''_{d,0.5}$ is higher than the highest concentration tested and it can appear as if the drug produces a nonzero asymptotic response. This situation is not uncommon in practice. It presents a problem in that $\psi_{d,\phi}$ cannot be estimated for $\phi > 1 - \max_d(\hat{\lambda}_d)$. When ϕ is greater than $1 - \max_d(\hat{\lambda}_d)$, the data only tell us that $\hat{\psi}_{d,\phi}$ is not lower than the largest concentration tested. To account for this, $\psi''_{d,0.5}$ can be fixed at infinity and Model (10.11) changed to

$$Y_{d,t,w} = (1 - \lambda_d)\ \exp(\mu + b_t)(1 - \phi'_{d,t,w}) + \lambda_d\ \exp(\mu + b_t) + \varepsilon_{d,t,w}, \quad (10.16)$$

where the Loewe index and its confidence intervals are computed only for $\phi < 1 - \max_d(\hat{\lambda}_d)$. An equation analogous to (10.15) is used to obtain an expression for $\hat{\psi}_{d,\phi}$.

10.3.5 Use of the MixLow Method

While the equations given above may appear complex to some readers, the use of the MixLow method is relatively straightforward. The R package *mixlow* can be downloaded from the CRAN website, and a paper describing the package and its use has been submitted (Boik and Narasimhan, 2008). In brief, the following six steps are performed in sequence:

1. Design the experiment, collect the data, and prepare the data file. An example data file is included with the R package.
2. Read the data file using the function *readDataFile*.
3. Prepare and subset the data using the function *prepareData*.
4. Obtain starting values for the fixed-effects parameters of the nonlinear mixed-effects (NLME) model by using nonlinear least squares (NLS) analysis. This is done using the function *doNls*.

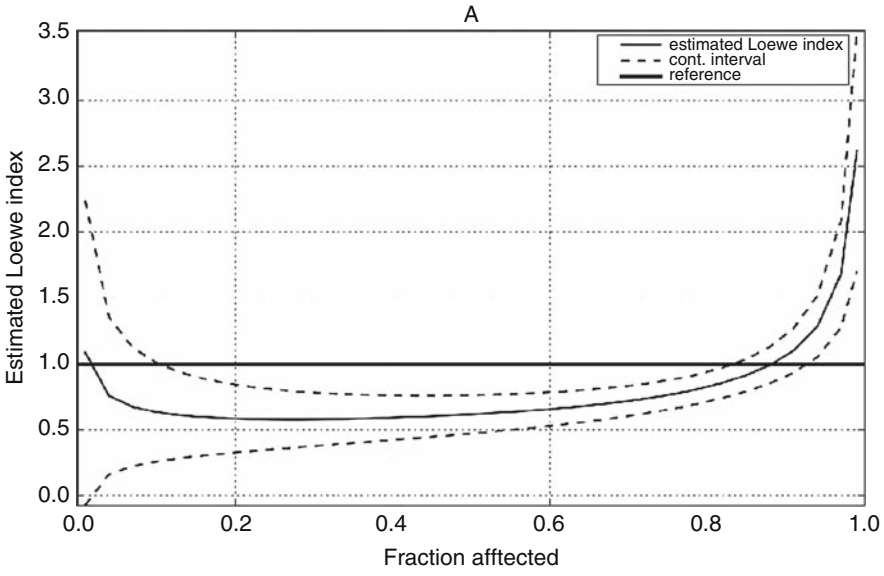

Fig. 10.2 Example of Loewe index versus fraction affected

5. Use the estimated NLS parameters as starting values for the NLME model. The mixed-effects model is estimated using the function *doNlme*.
6. Use the estimated NLME parameters to estimate the Loewe index. This is done using the function *doLoewe*.

In summary, the process is: read data → prepare data → NLS → NLME → Loewe. Figure 10.2 shows the Loewe index versus fraction-affected values for an example data set. Confidence intervals of the index are shown by dotted lines. In the figure, statistically significant synergism is occurring between fraction-affected values of about 0.1–0.85 (the upper confidence interval is less than 1.0).

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Chapter 11
The Use of Selected Medicinal Herbs for Chemoprevention and Treatment of Cancer, Parkinson's Disease, Heart Disease, and Depression

Maureen McKenzie, Carl Li, Peter B. Kaufman, E. Mitchell Seymour, and Ara Kirakosyan

Abstract In this chapter, we present recent advances on the use of several different kinds of medicinal herbs to treat cancer, Parkinson's disease (PD), heart disease, and depression. These include recent studies on the use of *Vaccinium* spp. (blueberries and relatives) for cancer treatment and prevention; blueberries in the diet to improve motor skills and cognitive ability in patients with PD; digitalis (foxglove) to treat patients with heart disease; and St. John's wort that is used to treat patients with mild-to-moderate depression. The basic conclusion from these studies is that rigorous, well-designed clinical trials are needed to validate the safe use of these and other medicinal herbs for treatment of these and other diseases.

11.1 Introduction

In the last few years, medicinal plants with promise to impact human health have undergone extensive laboratory and clinical testing. Many scientific methods of analysis have been developed for the investigation of the constituents and biological activities of these constituents of plants. Various chromatographic, spectroscopic, and biological (e.g., anticancer, anti-inflammatory, immunostimulant, antioxidant, antiprotozoal, and antimicrobial) techniques are being used for medicinal plant research (Cseke et al., 2006). Advances in scientific methodology have been made that contribute to our understanding of the mechanisms of action of herbal constituents (see Chapter 10). Examples of active constituents of different medicinal plants and their known activities are listed in Table 11.1 and can also be found in Duke, J.A. Phytochemical and Ethnobotanical Database; http://www.ars-grin.gov/duke/.

Although medicinal plants have been known for thousands of years and have been used for a variety of medicinal purposes, understanding of the activity and

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Table 11.1 Examples of constituents and their activities from different medicinal plants

Constituents	Activity
Dianthrone derivatives: hypericin, pseudohypericin, frangula-emodin anthranol	Photodynamic, anti-depressive (MAO inhibitor), antiviral
Phloroglucinols derivatives: hyperforin, secohyperforin	Anti-depressant and antibacterial
Flavanols: (+)-catechin (+polymers: condensed tannins), (–)-epicatechin, proanthocyanidins	Astringent, anti-inflammatory, styptic, antiviral, heart disease
Flavonoids: hyperoside (hyperin), quercetin, isoquercetin, rutin, methyhespericin, iso-quercitrin, quercitrin, I-3/II-8-biapigenin, kaempferol, myricetin	Capillary-strengthening, diuretic, antidiarrheal, cholagogic, dilated coronary, anti-inflammatory, arteries, sedative, tumor inhibition, antitumor, blood glucose lowering
Anthocyanins: cyanidin, delphinidin, malvidin, pelargonidin, petunidin, and peonidin	Antioxidants and anti-inflammatory
Isoflavones: genistein, genistin, daidzein, daidzin and puerarin	Antiosteoporosis, phytoestrogen, anti-alcoholism, anti-colon cancer
Lignans: podophyllotoxin, α- and β-peltatin	Anti-cancer, antioxidants, phytoestrogen
Xanthones: xanthonolignoid compound	Generally, xanthones exhibit anti-depressant, antitubercular, choleretic, diuretic, antimicrobial, antiviral, and cardiotonic activity
Coumarins: umbelliferone, scopoletin	
Phenolic carboxylic acids: caffeic acid, chlorogenic acid, genistic acid, ferulic acid	Antioxidants
Phloroglucinol derivatives: hyperforin	Anti-bacterial (*Staphylococcus aureus*)
Essential oil components: *monoterpenes* α-pinene, β-pinene, myreene, limonene camphor, borneol, menthol, geraniol, and terpineol	Antifungal, disinfectant, deodorant, pain reliever, counterirritant, anesthetic, expectorant, and antipruritic
Sesquiterpenes: caryophyllene, humulene	
Terpens: *Sesquiterpenes*; farnesol, artemisinin	Anti-cancer, anti-malaria
Diterpenes: examples of diterpenes are cafestol, kahweol, cembrene, and taxadiene (precursor of taxol). Diterpenes also form the basis for biologically important compounds such as retinol, retinal, and phytol	They are known to be antimicrobial and anti-inflammatory. The herb Sideritis contains diterpenes Anti-inflammatory, anti-hypertensive
Triterpenes: lanosterol, cycloartenol, and soyasaponins	Antioxidants
Tetraterpenes: Biologically important tetraterpenes include the acyclic lycopene, the monocyclic gamma-carotene, and the bicyclic α- and β-carotenes	
n-Alkanols: 0.42% of total dried herb: 1-tetracosanol (9.7%), 1-hexacosanol (27.4%), 1-octacosanol (39.4%), 1-triacontanol (23.4%)	Health products including octacosanol are sold in Japan and the United States as “metabolic stimulants” (Japanese studies show it stimulates feeding of silkworm larvae; studies with neurological disorders (Parkinson’s, ALS, MS) show mixed results)

Table 11.1 (continued)

Constituents	Activity
Carotenoids: epoxyxanthophylls, lutein, zeaxanthin, lycopene, β-carotene	Available oxygen in xanthophylls may explain burn-healing activity, eye pigment protection from blue light, prostate health, pro-vitamin A activity
Phytosterols: β-sitosterol	Anticancer, hearing loss, benign prostatic hypertrophy, hypercholesterolemia

mechanisms of action of their bioactive constituents is relatively new and not well understood, particularly in connection with applications for human health benefits.

11.2 Cancer

A body of now firmly established research and epidemiological evidence has shown overwhelmingly that dietary intake of berry fruits has a positive and profound impact on human health, performance, and disease (Seeram, 2008a). Evidence from tissue culture, animal models, and human studies suggests that flavonoid-rich fruits, in particular, deeply colored berries, have promise to limit the development and severity of diseases based on inflammatory processes including atherosclerosis and ischemic stroke, neurodegenerative diseases of aging, and certain cancers. The first report of the anticancer properties of "anthocyan" flavonoids from fruits and vegetables was published over 40 years ago and cited their significance as cell respiratory activators for cancer prophylaxis and therapy (Seeger, 1967). Early studies also proposed enzymatic modulatory and anti-inflammatory activities and related processes, including inhibition of prostaglandin biosynthesis, platelet-activating factor (PAF)-induced exocytosis, and inflammatory cyclooxygenase activities, as well as numerous therapeutic benefits of berry "anthocyanosides" and other flavonoids in traditional medicine and the clinic (Cluzel et al., 1970; Amouretti, 1972; Lietti et al., 1976; Jonadet et al., 1983; Tunon et al., 1995; Middleton et al., 2000).

11.2.1 Case Study on and Cancer

The anticancer effects of berries are hypothesized to be mediated through many mechanisms mostly associated with their flavonoid content (Seeram, 2008b). Although berries from numerous families and included genera provide an array of flavonoid compounds that could contribute to cancer chemoprevention and therapy, species from the family Ericaceae, and especially the genus *Vaccinium,* are widely favored for their anticancer attributes. A number of informative reviews published in the literature cover this subject, as well as the cancer chemopreventive properties of specific *Vaccinium* components and metabolites (Prior and Wu, 2006; Neto, 2007a,b; Neto et al., 2008; Seeram, 2008b).

The principal *Vaccinium* species discussed in this chapter include *Vaccinium corymbosum* L. (cultivated blueberry), *Vaccinium ashei* Reade (southern rabbiteye blueberry), *Vaccinium angustifolium* Ait. (lowbush blueberry), *Vaccinium myrtillus* L. (European bilberry), *Vaccinium uliginosum* L. (bog bilberry or whortleberry), *Vaccinium macrocarpon* Ait. (North American cranberry), *Vaccinium oxycoccus* L. (European cranberry), and *Vaccinium vitis-idaea* L. (lingonberry).

All species in the genus *Vaccinium* are replete with flavonoids such as anthocyanins (flavylium ion moieties that contribute the blue, purple, and red colors to fruits and flowers which are primarily glycosylated derivatives of the anthocyanidins, cyanidin, delphinidin, peonidin, malvidin, and petunidin), proanthocyanidins, tannins, catechin (and epicatechin, gallocatechin and epigallocatechin units), flavonols (myricetin, quercetin, and kaempferol), phenolic acids (gallic acid, p-hydroxybenzoic acid, caffeic acid, ferulic acid, and ellagic acid), substituted cinnamic acids, and stilbenes such as resveratrol, pterostilbene, and piceatannol, and triterpenoids such as ursolic acid and its esters, oleanic acid, alpha-amyrin and beta-amyrin, steroidal, and iridoid glycoside compounds. Extensive work has focused on phytochemical and chemotaxonomic investigations with the goal of isolating and identifying constituents of not only fruits but also flowers, leaves, stems, and roots that have been used for food and traditional medicinal purposes (Ramstad, 1954; Thieme et al., 1969; Schonert and Friedrich, 1970; Friedrich and Schonert, 1973; Nees et al., 1973; Sticher et al., 1979; Dombrowicz et al., 1991; Fraisse et al., 1996; Sun et al., 1997; Prior et al., 2001; Dugo et al., 2001; Nyman and Kumpulainen, 2001; Gu et al., 2002; Jensen et al., 2002; Kandil et al., 2002; Du et al.,2004; Ichiyanagi et al., 2004c, 2004d; Rimando et al., 2004; Vvedenskaya et al., 2004; Migas et al., 2005; Zadernowski et al., 2005; Ek et al., 2006; Seeram et al., 2006; Burdulis et al., 2007; Harris et al., 2007; Pyka et al., 2007; Szakiel and Mroczek, 2007). The data that emerged from these investigations demonstrated strong similarities in the chemical composition of species within the genus *Vaccinium*.

Nonetheless, clear differences could be observed in the relative and absolute amounts of flavonoids, in particular anthocyanins, and in their species-dependent, unique "fingerprints". By comparison, the main phenolics found in widely consumed fruits from the family Rosaceae were ellagitannins, phenolic acids, and anthocyanins. Many *Vaccinium* fruits contain 15–25 distinct anthocyanins (based on the anthocyanidins, delphinidin, cyanidin, petunidin, peonidin, and malvidin) in conjunction with abundant proanthocyanidins and a diverse array of polyphenolic compounds. Both *V. myrtillus* and *V. ashei* contained 15 identical anthocyanins with different distribution patterns, as elucidated by high-performance liquid chromatography (HPLC) coupled with photodiode array detection and electrospray ionization – mass spectrometry (LC/PDA/ESI-MS) (Nakajima et al., 2004). Distinctive similarities in the distribution of conjugated forms of phenolic compounds among berry species of the same family were confirmed, but differences in chromatographic profiles of conjugates and compositions of aglycones were also observed, especially in the case of anthocyanins (Määttä-Riihinen et al., 2004). One report delineated anthocyanins as the main phenolic constituents in *V. myrtillus*, *V. uliginosum*,

and *V. macrocarpon*, but in *V. vitis-idaea*, belonging also to the family Ericaceae genus *Vaccinium*, flavanols and proanthocyanidins predominate in the composition (Kähkönen et al., 2001). Proanthocyanidins of various degrees of polymerization (DP) have been identified in many types of foods, but *Vaccinium*species contain oligomeric (DP $\leq$ 10) and polymeric proanthocyanidins (DP > 10), in both A- and B-type linkages (Gu et al., 2003). Later experiments employing advanced analytical techniques, including liquid chromatography-time-of-flight mass spectrometry (LC-TOFMS), liquid chromatography-tandem mass spectrometry (LC-MS/MS), and nuclear magnetic responance spectrometry (NMR) to identify *V. vitis-idaea* polyphenolics revealed a total of 28 flavonols, anthocyanidins, catechins and their glycosides, and different caffeoyl and ferulic acid conjugates (Ek et al., 2006). This appears to be the first report of coumaroyl-hexose-hydroxyphenol, caffeoyl-hexose-hydroxyphenol, quercetin-3-*O*-alpha-arabinofuranoside, kaempferol-pentoside, and kaempferol-deoxyhexoside, and the flavonol acylglycosides quercetin-3-*O*-[4′′-(3-hydroxy-3-methylglutaroyl)]-alpha-rhamnose and kaempferol-3-*O*-[4′′-(3-hydroxy-3-methylglutaroyl)]-alpha-rhamnose. Compounds from parts of *Vaccinium* plants, other than fruit flesh, including essential fatty acids from seeds and seed oils, and fibers, such as microcrystalline cellulose, pectins, lignins, cutin-like polymers, and condensed tannins, have been suggested to have potential health benefits and cancer chemopreventive attributes (Parry et al., 2006; Wawer et al., 2006).

Although little direct data uniquely link berry consumption with lower cancer risk, evidence is mounting that berry extracts and berry phytochemicals modulate biomarkers of DNA damage and indicators of malignant transformation in vitro and in vivo (Hou, 2003; Duthie, 2007; Seeram, 2008b). The anticancer effects on macromolecules, in particular DNA, and cells, tissues, and organ systems involve (1) protection from genotoxicity; (2) regulation of carcinogen and xenobiotic metabolizing enzymes; (3) ability to prevent and mitigate damage resulting from oxidative stress; (4) inhibition of cancer cell proliferation and induction of apoptosis; (5) regulation of subcellular signaling pathways and modulation of transcription factors; and (6) inhibition of growth factors and inflammatory cytokines linked to tumor angiogenesis and invasiveness. In addition, berry phytochemicals may induce sensitivity of tumor cells to chemotherapeutic agents by inhibiting pathways that lead to drug resistance and ameliorate therapy-associated toxicities.

11.2.1.1 Protection from Genotoxicity

The initial step in the transformation of a normal, somatic cell to a malignant one is damage to the genome resulting in a mutation. Mutagenic agents may be chemical, radioactive, or biological (e.g., viruses) in nature. Chemical mutagens cause DNA modifications through base pair substitutions, frameshifts, and strand breaks. Carcinogens are mutagens that have been documented to cause progression to a cancerous state. Carcinogens are typically classified as (1) direct acting and possess a chemical structure that is sufficient to cause DNA damage or (2) require metabolic activation to convert a prescursor to an active form. Mutation of

a particular oncogene or a tumor-suppressor gene may enhance susceptibility to development of specific types of cancer.

There is evidence that *Vaccinium* preparations may preserve DNA integrity or promote repair of DNA damage. Juice of *V. corymbosum* suppressed mutagenicity of the polycyclic aromatic hydrocarbons 2-amino-3-methyl[4,5-f]-quinoline and, in part, of 2-amino-3,4-dimethylimidazo-[4,5-f]quinoline or 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline in Ames tester strains *Salmonella typhimurium* TA98 and TA100 (Edenharder et al., 1994). Ethanol extracts of *V. ashei* (cv. Premier) significantly inhibited mutagenesis by both direct-acting and metabolically activated carcinogens (Wedge et al., 2001). Similar results were obtained with juices from *V. ashei* (cv. Tifblue and cv. Premier), shown to inhibit the production of mutations by the direct-acting mutagen, methyl methanesulfonate, and the metabolically activated carcinogen, benzo[a]pyrene (Hope Smith et al., 2004). Moreover, a *V. asheis* extract reduced oxidative DNA damage in mouse brain tissue in vitro (Barros et al., 2006).

11.2.1.2 Regulation of Carcinogen and Xenobiotic Metabolizing Enzymes

The metabolism of carcinogens (and other xenobiotics defined as "foreign" chemical substances) by the body is often divided into three phases: (1) modification; (2) conjugation; and (3) excretion. These reactions act in concert to detoxify and remove them from cells. In Phase I, a variety of enzymes and isozymes in the cytochrome P-450-dependent mixed-function oxidase system (CYP450) act to introduce reactive and polar groups into their carcinogen or xenobiotic substrates. These enzyme complexes incorporate an atom of oxygen into non-activated hydrocarbons, which can result in either the introduction of hydroxyl groups or oxygen (O-), nitrogen (N-), and sulfur (S-)mediated dealkylation of substrates. A typical reaction mechanism of the CYP450 oxidases proceeds through the reduction of cytochrome-bound oxygen and the generation of an oxyferryl species, according to the general scheme:

$$\mathrm{NADPH} + \mathrm{H}^{+} + \mathrm{RH} \rightarrow \mathrm{NADP}^{+} + \mathrm{H_2O} + \mathrm{ROH}$$

In ensuing Phase II reactions, these activated metabolites are conjugated with charged species such as glutathione (GSH), sulfate, glycine, or glucuronic acid. A large group of broad-specificity transferases catalyze these reactions which, in combination, can metabolize almost any hydrophobic compound that contains nucleophilic or electrophilic groups. The principal of these are glutathione S-transferases (GSTs) and are responsible for the addition of large anionic groups (such as GSH) to detoxify reactive electrophiles and produce more polar metabolites that cannot diffuse across membranes and may, therefore, be actively transported by specialized systems for their removal. During Phase III, conjugates may be further metabolized prior to excretion. A common example is the processing of glutathione conjugates to acetylcysteine (mercapturic acid) conjugates in which the gamma-glutamate

and glycine residues in the glutathione molecule are removed by gamma-glutamyl transpeptidase and dipeptidases. In the final step, the cystine residue in the conjugate is acetylated. Through another Phase II mechanism, conjugates and their metabolites can be excreted from cells as a result of the anionic groups acting as "affinity tags" for membrane-associated transporters of the multidrug resistance protein (MRP) family. These proteins are members of the larger family of ATP-binding cassette transporters that catalyze the ATP-dependent transport of a huge variety of hydrophobic anions across cell membranes. Thus, further metabolism may result in removal or excretion of Phase II products across the plasmalemma to the extracellular medium.

Many polyphenols, including phenolic acids, anthocyanins, stilbenes, catechins, and other flavonoids, which constitute a large fraction of phytochemicals in all *Vaccinium* species, modulate components of the detoxification systems and cellular levels of endogenous antioxidants, such as glutathione (Rodeiro et al., 2008). Experiments with Chinese hamster lung fibroblasts, genetically engineered for the expression of rat CYP450 (also known as *cytochrome P450-dependent monooxygenase*) and rat sulfotransferase 1C1 (V79-rCYP1A2-rSULT1C1 cells), were designed to seek possible protective effects of berries and other fruits, vegetables, spices, and plant-derived beverages against genotoxicity induced by 2-acetylaminofluorene (AAF) or 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) (Edenharder et al., 2002). Applying alkaline single-cell gel electrophoresis (comet assay), which detects DNA strand breaks and abasic sites, the genotoxicity of PhIP could be demonstrated only in the presence of hydroxyurea and 1-[beta-D-arabinofuranosyl]cytosine, known inhibitors of DNA repair synthesis. AAF and PhIP predictably were unable to induce any genotoxic effects in the parent V79 cells. Genotoxic activity of PhIP was strongly reduced in a dose-related manner by *V. myrtillus* and many other plant preparations to a lesser extent, but *Vaccinium* did not inhibit the genotoxicity of N-OH-PhIP metabolite or of another benzo[a]pyrene, benzo[a]pyrene-7,8-dihydrodiol (BaP-7,8-OH), whereas the genotoxicity of AAF was strongly reduced by other fruits. Through presentation of N-OH-PhIP and benzo[a]pyrene-7,8-dihydrodiol (BaP-7,8-OH) as substrates for enzymes of the rSULT 1C1 and CYP450-1A family, respectively, these results demonstrate enzyme inhibition as the mechanism against genotoxicity of heterocyclic aromatic amines. This inhibition may take place within metabolically competent mammalian cells and under the conditions of the Salmonella/reversion assay, as demonstrated previously by some of these workers.

A number of genes important for expression of detoxification and antioxidant defense enzymes, proteins, and endogenous cofactors induced by environmental stress may provide health benefits by deployment of such defense responses. One Phase II detoxification enzyme, *NAD(P)H:(quinone-acceptor) oxidoreductase* (*QR*), belongs to the flavoprotein clan in the human genome and is encoded by two genes, *NQO1*and *NQO2* (Vasiliou et al., 2006). QR functions to inactivate electrophilic forms of carcinogens, particularly quinones, providing a mechanism for the inhibition of carcinogenesis. QR catalyzes the beneficial two-electron reduction of quinones to hydroquinones, thereby preventing the unwanted one-electron

reduction of quinones by other quinone reductases. One-electron reduction results in the formation of reactive oxygen species (ROS), generated by redox cycling of semiquinones in the presence of molecular oxygen. Both mammalian *NQO1* and *NQO2* genes are upregulated as a part of the oxidative stress response and are inexplicably overexpressed in particular types of tumors. In early investigations, extracts of fruit from four *Vaccinium* species, *V. angustifolium, V. myrtillus, V. macrocarpon, and V. vitis-idaea*, and a hydrophobic subfraction of *V. myrtillus* were tested for their ability to induce QR in vitro in Hepa 1c1c7 human liver cells and to serve as possible dietary anticarcinogens (Bomser et al., 1995; 1996). The crude extracts, as well as anthocyanin and proanthocyanidin fractions, were not highly active or were inactive in QR induction, whereas the ethyl acetate extracts were potent QR inducers. The concentrations required to double QR activity (designated CDqr) for the ethyl acetate extracts of *V. angustifolium, V. macrocarpon, V. vitis-idaea*, and *V. myrtillus* were 4.2, 3.7, 1.3, and 1.0 μg tannic acid equivalents (TAE), respectively. The *V. myrtillus* ethyl acetate extract was processed into a hexane/chloroform subfraction, a step that revealed the majority of inducer potency (Cdqr = 0.3–70 ng TAE). Analysis of this subfraction of the *V. myrtillus* ethyl acetate extract was required to elucidate the compounds responsible for the induction of QR.

Anthocyanins from *Vaccinium* have been shown to inhibit oxidative stress and unregulated cell proliferation, although regulation of apoptosis and Phase II detoxifying enzymes QR and glutathione-S-transferase (GST) are other potential mechanisms through which anthocyanins and other flavonoids may prevent cancer. *V. myrtillus* anthocyanins and other phenolics have been shown to upregulate mRNA transcripts of the oxidative stress defense enzymes, heme oxygenase 1 (HO-1) and glutathione-S-transferase-pi (GST-pi), in cultured human retinal epithelial cells. This suggests that they stimulate signal transduction pathways influencing genes controlled by the antioxidant response element, at least in this tissue type in vitro (Milbury et al., 2007). Interestingly, anthocyanins from preparations of all four *V. ashei* cultivars (cv. Tifblue, cv. Powderblue, cv. Brightblue, and cv. Brightwell) significantly lowered QR activity in treated cells as compared to untreated control cells (Srivastava et al. 2007). The activity decreased gradually when treated with increasing concentrations of anthocyanin fractions (50–150 $\mu g \cdot mL^{-1}$) from cv. "Tifblue" and cv. "Powderblue". Similarly, GST activity was lower in cells treated with anthocyanin fractions from all of the cultivars and at all tested concentrations as compared to untreated controls; however, in HT-29 colon cancer cells, apoptosis was induced by treatment with anthocyanins from all *V. ashei* cultivars but, at the same concentrations, Phase II QR and GST activities decreased rather than demonstrating induction in this cell line. Polyphenolic flavonoids and other plant phytochemicals are thought to transactivate detoxification and genes containing electrophile response elements (EpREs) within their promoters. A product of one of these genes, gamma-glutamylcysteine synthetase, has previously been shown to be positively regulated by quercetin, a flavonoid found in high concentrations *V. myrtillus,* diverse *Vaccinium* species, and other foods, through EpRE transactivation (Myhrstad et al., 2006).

11.2.1.3 Prevention of Damage from Oxidative Stress

According to the "free-radical theory of aging", oxidative damage intiated by reactive oxygen species (ROS) is a major contributor to the functional decline that is characteristic of senescence and chronic disease. ROS form as by-products of the normal metabolism of oxygen (e.g., food metabolism and respiration) and have important roles in cell signaling and immune function; however, the presence of unpaired valence shell electrons causes high reactivity so these same free radicals can participate in unwanted side reactions resulting in cumulative cell damage. In addition to endogenously generated sources in the body, ROS are also generated by exposure to exogenous sources such as ionizing radiation (e.g., ultraviolet light exposure leading to sunburn among other environmental exposures, cigarette smoke, radon gas, to name a few). During times of environmental stress, ROS levels can increase dramatically and result in significant damage to cell structures. Harmful effects of reactive oxygen species on the cell are most often observed as (1) damage of DNA; (2) oxidations of unsaturated fatty acids in lipids; (3) oxidations of amino acids in proteins; and (4) inactivation of specific enzymes through oxidation of catalytic cofactors. Many forms of cancer are thought to be the result of reactions between oxygen-free radicals and DNA, resulting in mutations that can adversely affect the cell cycle and other growth regulatory mechanisms that potentially lead to malignancy.

ROS associated with cell damage include superoxide (O_2^{*-}) (a term used interchangeably with *superoxide anion*), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), peroxyl (ROO^*) and hydroxyl (OH^*) radicals, and peroxynitrite ($ONOO^-$), formed in vivo through reaction of the free-radical superoxide with the free radical, nitric oxide, that are derived from molecular oxygen under reducing conditions. Because free radicals are necessary for life, the body has a number of mechanisms to minimize free radical-induced damage and to repair damage which does occur, such as through the action of the enzymes, superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase. In addition, antioxidants, such as vitamin A, vitamin C, and vitamin E, play a key role in these defense mechanisms. For years, the antioxidant power of fruits was thought to be attributable to conventional vitamin content, but far more complexity is now attributed to total reactive oxygen scavenging capacity. Studies on antioxidant capacities of flavonoids revealed that they could scavenge free radicals, chelate metals, bind specific proteins, and act through other mechanisms that involve inhibition of oxidative enzymes.

11.2.1.4 *In Vitro* Antioxidant Protection

Fruits – especially berries – have been examined extensively in vitro for antioxidant capacity with *Vaccinium* species being no exception (Vinson et al., 2001; Neto, 2007a; Vinson et al., 2008; Seeram, 2008a). Some of these experiments revealed extracts of *Vaccinium* protect against oxidation of lipids (methyl linoleate) and protein tryptophan (Trp) residues (Kähkönen et al., 2001; Viljanen et al., 2004; Salminen and Heinonen, 2008). Mechanisms of antioxidative action of phenolic

compounds from *Vaccinium* and fruits from other genera toward the oxidation of biomolecules were distinct, as the pattern of oxidation products varied with different phenolic compounds. The extent of protein oxidation was measured by determining the loss of tryptophan fluorescence and formation of protein carbonyl compounds, and that of lipid oxidation, by conjugated diene hydroperoxides and hexanal analyses. *V. myrtillus* phenolics possessed some of the best overall antioxidant activity toward protein oxidation. Anthocyanins found in *V. myrtillus* contributed most to the antioxidant effect by inhibiting the formation of both hexanal and protein carbonyls. *V. macrocarpon* proanthocyanidins were also found to provide potent antioxidant protection toward oxidation of Trp residues. The antioxidant protection toward lipid oxidation was best provided by *V. vitis-idaea*and *V. myrtillus* phenolics, whereas proanthocyanidins, especially the dimeric and trimeric molecules, from *V. vitis-idaea*, were among the most active phenolic constituents toward both lipid and protein oxidation.

Crude extracts of *Vaccinium* were shown to be potent scavengers of chemically generated O_2^{*-} and possessed inhibitory activity toward the enzyme xanthine oxidase (Constantino et al., 1992). Tannins isolated from *V. vitis-idaea* exhibited O_2^{*-} scavenging and multiple antioxidant activities (Ho et al., 1999). Cinnamtannin B1 displayed the strongest anti-lipid peroxidation activity, proanthocyanidin A-1 displayed the strongest superoxide scavenging activity, and epicatechin-(4beta → 6)-epicatechin-(4beta → 8, 2beta → O → 7)-catechin had the strongest anti-superoxide formation effect. Subsequent work marked distinctions among various antioxidants in their abilities to scavenge different reactive oxygen species (Wang and Jiao, 2000). Juice from different cultivars of *V. corymbosum, V. angustifolium,* and *V. macrocarpon*, as well as from various species in the family Rosaceae, was assessed for antioxidant activities against O_2^{*-}, H_2O_2, $^1O_2$, and OH* radicals. *Vaccinium* cultivars had high antioxidant capacity against all four reactive oxygen moieties but, in general, were lower in antioxidant capacity inhibition of scavenging activity than Rosaceae juices. *V. macrocarpon* had the lowest inhibition of hydrogen peroxide moieties, while *V. corymbosum* had the lowest antioxidant capacity against OH* and $^1O_2$.

The reactivities of 12 major anthocyanins identified in *V. myrtillus* extracts toward nitric oxide (NO) and $ONOO^-$ were studied in vitro using capillary zone electrophoresis (Ichiyanagi et al., 2004b). With the exception of delphinidin glycosides, the reactivities of anthocyanins toward $NO^{\cdot}$ were weaker than that of (+)-catechin as a reference antioxidant under anaerobic conditions. Aglycon structure or type of sugar moiety did not significantly affect the reactivities of other anthocyanins. Conversely, all anthocyanins and catechin showed significant enhancement of reactivity under aerobic conditions, indicating that they reacted with other reactive species secondarily generated from NO. Delphinidin glycosides showed rather comparatively high reactivity toward $ONOO^-$ compared to other anthocyanins, which also showed approximately two times lower reactivity than catechin. These results were corroborated, in part, by others (Rahman et al., 2006). This group found that antioxidant activities of 15 purified *V. myrtillus* anthocyanins, together with pelargonidin 3-*O*-beta-D-glucopyranoside and 4′-*O*-methyl delphinidin 3-*O*-beta-D-

glucopyranoside, the major metabolite of delphinidin 3-*O*-beta-D-glucopyranoside, were evaluated in order to study the structure-antioxidant activity relationship and any synergism between them in the mixture. Both the aglycone structure and the attached sugar moiety affected the superoxide radical- and peroxynitirite-scavenging activities, although the effect of the attached sugar moiety was smaller than that of the aglycone structure. The potency of activity toward the superoxide radical was in the following order: delphinidin > petunidin > malvidin = approximately cyanidin > (+)-catechin > peonidin > pelargonidin. The activity toward $ONOO^-$ was in the following order: delphinidin > cyanidin = approximately petunidin > malvidin = approximately (+)-catechin > peonidin > pelargonidin. It was confirmed that methylation of 4′-OH markedly reduced the antioxidant activity of anthocyanin. Further, it was revealed that synergism occurred in both $O2^{*-}$ and $ONOO^-$ scavenging activities among the anthocyanins in the mixture.

Kinetic parameters of 12 major anthocyanins identified in *V. myrtillus* extracts toward 2,2′-azobis (2-amidinopropane) (AAPH) radicals, tert-butylhydroperoxides (t-BuOOH), and H_2O_2 were studied in vitro using capillary zone electrophoresis (Ichiyanagi et al., 2004a). The reactivity of anthocyanins toward H_2O_2 was not significantly affected by aglycon structure or by the type of sugar moiety, with no marked difference observed in reaction rates among various anthocyanins. Reactivity toward t-BuOOH was essentially the same as toward H_2O_2, although the reaction rate was several times smaller. Also, the reaction rate of anthocyanin toward H_2O_2, compared to that of (+)-catechin, was relatively high (approximately 30 times larger) when measured as a reference antioxidant. Conversely, reactivity toward AAPH radicals was determined principally by the aglycon structure instead of the type of sugar moiety. Delphinidins carrying three-hydroxyl groups on the B-ring were most reactive followed by cyanidins, with two-hydroxyl groups. Further, methylation of the hydroxyl groups reduced reactivity toward AAPH radicals. The reactivities of anthocyanins and (+)-catechin toward AAPH radicals were similar.

Over the past decades, more specific antioxidant assays were developed and numerous reports on the radical-scavenging capacity of *Vaccinium* were added to the literature. Some of the most widely used were (1) oxygen radical absorbing capacity (ORAC) (otherwise known as Trolox-equivalent antioxidant capacity (TEAC)) based on fluorescence decay of Trolox® (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water soluble analogue of vitamin E sensitive to peroxyl (ROO^*) radicals; (2) total oxyradical-scavenging capacity (TOSC), which measures the decrease in ethylene production caused by antioxidants; (3) scavenging capacity against the artificial free-radical 1,1-diphenyl-2-picrylhydrazyl ($DPPH^*$); and (4) ferric-reducing/antioxidant power (FRAP), also known as ferric-reducing antioxidant of plasma (Klouwen, 1962; Cao et al., 1993; Winston et al., 1998; Regoli and Winston, 1999; Lichtenthäler and Marx, 2005; Tomer et al., 2007).

In a TOSC assay, *V. vitis-idaea* extracts were shown to scavenge efficiently three ROS, peroxyl and hydroxyl radicals, and peroxynitrite (Lichtenthäler and Marx, 2005). Others confirmed that fruit of *V. vitis-idaea* contains high antioxidant activity and potent-free radical-scavenging activities for $DPPH^*$, ROO^*, OH^*,

and O_2^{*-}, despite the fact that soluble solids, titratable acids, antioxidant capacity, and anthocyanin and phenolic contents varied between cultivars (Wang et al., 2005). Among ethanol extracts of 10 edible berries, that from *V. myrtillus* fruit contained the largest amounts of phenolic compounds, including anthocyanins, and showed the greatest DPPH*-scavenging activity (Katsube et al., 2003). Cold-pressed *V. corymbosum* seed oil, along with seed oils from Rosaceae genera, was evaluated for its fatty acid composition, carotenoid content, tocopherol profile, total phenolic content (TPC) as gallic acid equivalents per gram, oxidative stability index (OSI), peroxide value, and antioxidant properties (Parry et al., 2005). All tested seed oils contained significant levels of alpha-linolenic acid, ranging from 19.6 to 32.4 g per 100 g of oil, along with a low ratio of n–6/n–3 fatty acids (1.64/3.99). The total carotenoid content ranged from 12.5 to 30.0 μmoles·kg^{-1} oil. Zeaxanthin was the major carotenoid compound in all tested berry seed oils, along with beta-carotene, lutein, and cryptoxanthin. Total tocopherol was 260.6–2276.9 μmoles·kg^{-1} oil, including alpha-, gamma-, and delta-tocopherols. The lowest OSI was attributed to *V. corymbosum* oil, and the highest TPC and ORAC values were achieved by various Rosaceae seed oils. All tested berry seed oils directly reacted with and quenched DPPH* in a dose- and time-dependent manner. These data suggest that the cold-pressed berry seed oils may serve as potential dietary sources of tocopherols, carotenoids, and other natural antioxidants. Seed flours from *V. macrocarpon* and other fruits were also examined for their total fat content, fatty acid composition, total phenolic content (TPC), and total anthocyanin content (TAC), against the peroxyl (ORAC) and stable DPPH radicals, and chelating capacity against Fe^{2+} (Parry et al., 2006). Significant levels of fat were detected in the fruit seed flours, and their fatty acid profiles may differ from those of the respective seed oils. *V. macrocarpon* seed flour, compared to that from other fruits, had the highest level of alpha-linolenic acid (30.9 g/100 g fat) and the lowest ratio of n–6/n–3 fatty acids (1.2/1). The fruit seed flours also differed in their TAC values and Fe^{2+}-chelating capacities; ORAC, which correlated significantly to TPC values in this report, was not the highest in the flour of *V. macrocarpon* seed flour.

A number of novel compounds, such as ortho-benzoyloxyphenyl acetic acid ester, also called vaccihein A, isolated from the fruit of *V. ashei*, rare A-type proanthocyanidin dimers and trimers from *V. vitis-idaea*, *V.oxycoccus*, *V. myrtillus*, *V. macrocarpon*, and *V. uliginosum*, and uncommon anthocyanin derivatives, such as anthocyanin-pyruvic acid adducts and vinylpyranoanthocyanin-catechins (portisins) from *V. myrtillus*, have been identified and contribute to antioxidant capacity, as measured in DPPH* scavenging and FRAP assays (Gu et al., 2003; Ono et al., 2002; Faria et al., 2005; Määttä-Riihinen et al., 2005). The A-type proanthocyanidins inhibited the oxidation of methyl linoleate emulsion and human LDL, whereas anthocyanin derivatives were able to inhibit lipid peroxidation induced by 2,2′-azobis (2-methyl-propanimidamide) dihydrochloride, in a liposomal membrane system.

The radical-scavenging activity of a *V. macrocarpon* extract, composed primarily of flavonol glycosides, was the greatest compared to those with other components derived from the whole fruit (Yan et al., 2002). Seven flavonol glycosides

were isolated and purified from whole fruit for further evaluation; the anthocyanin cyanidin 3-galactoside was also purified for comparison with the flavonoids. Three flavonol monoglycosides were newly identified by ^{13}C NMR as quercetin 3-xyloside, 3-methoxyquercetin 3-beta-galactoside (isorhamnetin), and myricetin 3-alpha-arabinofuranoside; the other four isolated were the previously identified quercetin 3-beta-galactoside, quercetin 3-alpha-arabinofuranoside, quercetin 3-alpha-rhamnopyranoside, and myricetin 3-beta-galactoside. These compounds were evaluated in vitro for DPPH*-scavenging activity. Most of the flavonol glycosides showed antioxidant activity comparable or superior to that of vitamin E; cyanidin 3-galactoside showed activity superior to that of the flavonoids as well as vitamin E or Trolox (the reference compound for the ORAC assay) in both antioxidant assays. The antioxidative activities of proanthocyanidins from *V. macrocarpon* were found to be much stronger than vitamin C or vitamin E in aqueous systems; the mechanisms for their antioxidative actions were shown to involve radical scavenging, quenching, and enzyme-inhibiting actions (Ariga, 2004). Other authors identified 20 compounds in *V. macrocarpon* fruit, but those with potent antioxidant activity in the μmolar range were quercetin, 3,5,7,3′,4′-pentahydroxyflavonol-3-*O*-beta-D-glucopyranoside, 3,5,7,3′,4′-pentahydroxyflavonol-3-*O*-beta-D-galactopyranosi-de, and 3,5,7,3′,4′-pentahydroxyflavonol-3-*O*-alpha-l-arabinofuranoside (He and Liu, 2006).

Although anthocyanins were the main components, specific compounds such as chlorogenic acid in *V. corymbosum* (cv. Sierra), and quercetin glycosides in *V. macrocarpon* (cv. Ben Lear) and *V. vitis-idaea* (cv. Amberland) were found to be present in relatively high concentrations (Zheng and Wang, 2003). Chlorogenic acid, peonidin 3-galactoside, and cyanidin 3-galactoside were the most important antioxidants in *V. corymbosum, V. macrocarpon,* and *V. vitis-idaea*, respectively. The point has been made that the major metabolite of cyanidin, protocatechuic acid, is largely responsible for its antioxidant and other effects in humans (Galvano et al., 2007; Vitaglione et al., 2007). The total antioxidant capacity was generally dependent on the structure of individual phenolics and content in the berries, and variability was considerable. Important phenolics from *Vaccinium*, such as quercetin and cyanidin, with 3′,4′-dihydroxy substituents in the B-ring and conjugation between the A- and B-rings, had highly effective radical-scavenging structures. Furthermore, strong iron-binding properties have been confirmed for polyphenolic compounds, but especially for those containing the "iron-binding motifs" identified in their structures (Guo et al., 2007). A build-up of iron in biological systems is believed to result in the production of free radicals, leading to oxidative stress, cellular damage and eventual cellular death via apoptotic signaling (*apoptosis* is a process also known as "programmed cell death"). Quercetin, both at μmolar levels, and in the presence of major cellular iron chelators like ATP or citrate, could suppress completely Fenton chemistry, described as (1) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\cdot + OH^-$ and (2) $Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + OOH\cdot + H^+$, where ferrous iron (II) is oxidized by hydrogen peroxide to ferric iron (III), a hydroxyl radical, and a hydroxyl anion. Iron (III) is then reduced back to iron (II), a peroxide radical, and a proton by the same hydrogen peroxide (disproportionation). However, the radical-scavenging activity of quercetin

provides only partial protection against Fenton chemistry-mediated damage, while iron chelation by quercetin can completely inhibit Fenton chemistry, indicating that the chelation may be the key to its antioxidant activity.

A cellular antioxidant activity (CAA) for quantifying antioxidant activity in cell culture was developed recently to meet the need for a more biologically representative method than the popular chemistry antioxidant capacity measurements (Wolfe and Liu, 2007). CAA accounts for some aspects of uptake, metabolism, and location of antioxidant compounds within cells. This method measures the ability of test compounds to prevent 2,2′-azobis (2-amidinopropane) dihydrochloride (ABAP)-generated peroxyl radicals from forming oxidized, fluorescent dichlorofluorescein (DCF) from its non-fluorescent precursor in human hepatocarcinoma cells (HepG2). The decrease in cellular fluorescence generated from the precursor dichlorofluorescein probe, when compared to the control cells, indicates the antioxidant capacity of the compounds. *V. angustifolium* and *V. corymbosum* had some of the highest CAA values, followed by *V. macrocarpon*, among 25 commonly consumed fruits (Wolfe et al., 2008). Of the pure tester compounds, quercetin had the highest CAA value, followed by kaempferol, epigallocatechin gallate (EGCG), myricetin, and luteolin (expressed in μmoles of quercetin equivalents). These authors also point out that flavonoid structures with the most antioxidant activity in the CAA assay possessed a 3′,4′-*O*-dihydroxyl group in the B-ring, a 2,3-double bond combined with a 4-keto group in the C-ring, and a 3-hydroxyl group (Wolfe and Liu, 2008). Flavanols with a galloyl moiety had higher antioxidant activity than those without, and a B-ring 3′,4′,5′-trihydroxyl group further improved their efficacy. On the other hand, isoflavones had no cellular antioxidant activity. Interestingly, chemically based ORAC values for flavonoids were not related to their CAA values.

The primary conclusion reached in these in vitro studies was that a high correlation exists between antioxidant potency and flavonoids, in particular polyphenolics, anthocyanins, and proanthocyanidins (Moyer et al., 2002; Sellappan et al., 2002; Sanchez-Moreno et al., 2003; Ehala et al., 2005; Seeram, 2008b). One group pointed out the importance of careful chemical analysis of these compounds, since they occur in many derivative forms (Sun et al., 2002). Their report describes, as an example, the underestimation of total phenolics because bound forms were not quantified with soluble forms in many analyses. Similarly, bioactive anthocyanins and derivatives must be differentiated from inactive anthocyanidins in assessments of composition and antioxidant potency. A further critical consideration for evaluating the potential health benefits of any *Vaccinium* antioxidants is their capacity to function in vivo as ROS scavengers. In vitro antioxidant potency does not prove in vivo biological activity, although there is clinical evidence of antioxidant potency for the most potent beverages (e.g., red wine) correlating with positive health benefits.

11.2.1.5 *In Vivo* Antioxidant Protection

A body of literature indeed documents effects of consumption of *Vaccinium* on postprandial antioxidant status in animal models, including orchidectomized rats and strains with hereditary defects in oxidative metabolism, as well as exercising dogs

(Shabalina et al., 2001; Ariga, 2004; Dunlap et al., 2006; Kolosova et al., 2006; Sinitsyna et al., 2006; Deyhim et al., 2007; Villarreal et al., 2007).

Mouse models have been employed to measure restraint-stress oxidation in liver tissue (Bao et al., 2008b). Restraint stress may induce serious liver damage, with an increase in plasma alanine aminotransferase (ALT) level. A concomitant increase in malondialdehyde (MDA) levels and lowered ORAC values in plasma and liver were observed in restraint mice compared with starved mice. Oral administration of a *V. myrtillus* extract containing ~42% anthocyanins remarkably decreased plasma ALT level and, thus, alleviated stress-induced liver damage. In addition, the extracts increased glutathione GSH and vitamin C levels and significantly decreased MDA and nitric oxide (NO) levels in the liver tissues. These results suggest that *V. myrtillus* extract plays an important role in protecting against restraint-stress-induced liver damage by both free radical-scavenging activity and a lipid peroxidation inhibitory effect. This group also examined chemically induced organ damage of the kidney by potassium bromate ($KBRO_3$), an oxidizing agent used as a food additive (Bao et al., 2008a). The mechanism of potent nephrotoxicity has been hypothesized to occur through the generation of oxygen free radicals. A single intraperitoneal administration to mice could induce serious kidney damage, with an increase in serum blood urea nitrogen (BUN) and creatinine levels. Intervention with *V. myrtillus* extract resulted in a reversal in serum BUN and creatinine to normal levels and decreased kidney MDA, NO, and the enzyme, xanthine oxidase, levels. Also, the extract improved ORAC levels in kidney tissue, which showed that it reduced the degree of oxidative stress and kidney damage induced by $KBrO_3$.

Sophisticated methods have been designed for analysis of ORAC and total antioxidant status (TAS) values in plasma. In humans, in a single-blinded crossover study performed with a group of eight middle-aged male subjects (38–54 years), ingestion of freeze-dried *V. angustifolium* resulted in a significant increase in ORAC and TAS (Kay and Holub, 2002). Post-prandial plasma antioxidant capacity changes differed depending on the food consumed, and *Vaccinium* was shown to influence hydrophilic and hydrophobic ORAC values in human plasma (Prior et al., 2003; 2007). Conversely, consumption of an energy source of macronutrients containing no antioxidants was associated with a decline in plasma antioxidant capacity.

In other investigations, *Vaccinium* species increased vitamin C and quercetin concentrations in human plasma, and some revealed correlation with FRAP, electron spin resonance (ESR) values, and suppression of serum levels of advanced oxidation protein and lipoprotein products (Erlund et al., 2003; 2006; Ruel et al., 2005; Duthie et al., 2006; Valentova et al., 2007). Nonetheless, a comprehensive study in healthy female human subjects compared the total phenol, anthocyanin, and catechin content of *V. macrocarpon* supplements prior to ingestion and in the plasma following ingestion, as well as the total antioxidant ability determined by ESR spectrometry and by the FRAP assay (Duthie et al., 2006). Vitamin C, homocysteine (tHcy), and reduced glutathione (GSH) were measured by HPLC. Glutathione peroxidase (GSH-Px), catalase (CAT), and superoxide dismutase (SOD) activities

were measured in erythrocytes. Urine was collected for analysis of malondialdehyde (MDA) by HPLC and 8-oxo-deoxyguanosine (8-oxo-dG) by ELISA. Endogenous and induced DNA damage were measured by single-cell gel electrophoresis in lymphocytes. Also measured were plasma total cholesterol, high-density lipoprotein (HDL), and low-density lipoprotein (LDL) cholesterol and triglycerides (TG). *V. macrocarpon* juice, as compared with the placebo, contained higher vitamin C, total phenol, catechin, and anthocyanin concentrations. Vitamin C increased significantly in volunteers consuming juice, but no anthocyanins (plasma) or catechins (plasma or urine) were detectable and plasma total phenols were unaffected. The antioxidant potential of the plasma, GSH-Px, CAT and SOD activities, and MDA were similar for both groups and changes were not noted in tHcy, TC, TG, HDL, or LDL. Supplementation with cranberry juice did not affect endogenous or H_2O_2-induced DNA damage in lymphocytes or appearance of 8-oxo-dG in urine. Thus, the authors concluded that juice consumption, compared to placebo, did not affect plasma or cellular antioxidant status and had no effect on basal or induced oxidative DNA damage, or several biomarkers of lipid status. Although these results seem to be inconsistent with those of others, they highlight the importance of distinguishing between in vitro and in vivo antioxidant and other bioactivities of dietary anthocyanins in relation to human health.

11.2.1.6 Inhibition of Cancer Cell Proliferation and Induction of Apoptosis

Unlike normal cells, cancer cells proliferate rapidly and fail to respond to growth inhibitory signals. In the latter, apoptosis does not occur in a regulated manner. The polyphenolic extracts and flavonols, proanthocyanidin oligomers, and triterpenoids isolated from *Vaccinium* inhibit the growth and proliferation of several types of tumor cells lines in vitro and may act in a complementary fashion to limit this aspect of the carcinogenic process (Neto, 2007a,b).

Studies in tumor cell lines. In early work, fruit extracts of four *Vaccinium* species (*V. angustifolium, V. myrtillus, V. macrocarpon, and V. vitis-idaea*) were screened in vitro for anticarcinogenic compounds by a combination of fractionation and ability to inhibit the induction of ornithine decarboxylase (ODC), the rate-limiting enzyme in polyamine synthesis, by the tumor promoter, phorbol 12-myristate 13-acetate (also known as 12-*O*-tetradecanoyl phorbol-13-acetate (TPA)) (Bomser et al., 1996). In contrast to their effects on the enzyme quinone reductase (QR), crude extracts of *V. angustifolium, V. macrocarpon, and V. vitis-idaea* were active inhibitors of ODC activity. The IC_{50} values were 8.0, 7.0, and 9.0 μg TAE, respectively. The greatest activity in these extracts appeared to be contained in the polymeric proanthocyanidin fractions of these fruits (IC_{50} = 3.0, 6.0, and 5.0 μg TAE, respectively). A proanthocyanidin fraction from these fruits inhibited ODC and suppressed the formation of polyamines typically enhanced in rapidly proliferating cells characteristic of cancer. The anthocyanidin and ethyl acetate extracts of the four *Vaccinium* species were either inactive or relatively weak inhibitors of ODC activity. Different authors also reported significant chemopreventive activity, as measured in a TPA tumor promoter-induced ODC assay (as well as antioxidant activity in

a wide range of fractions generated from a crude extract) that localized to one particular proanthocyanidin-rich fraction from *V. macrocarpon* (Kandil et al., 2002). The active anticarcinogenic fraction was found to contain the following components: a series of oligomeric proanthocyanidins, seven flavonoids, mainly quercetin, myricetin, the corresponding 3-*O*-glycosides, (–)-epicatechin, (+)-catechin, and dimers of both gallocatechin and epigallocatechin types.

In further investigations, a proanthocyanidin-rich extract of *V. angustifolium* was separated into fractions and was characterized by mass spectrometry and NMR spectroscopy. One fraction, with an average degree of polymerization (DP) of 5.65, had significant antiproliferation activity against human prostate and mouse liver cancer cell lines (Schmidt et al., 2004). A significant positive correlation was established between proanthocyanidin content of different fractions and biological activity. Proanthocyanidin-rich fractions from *Vaccinium* fruits demonstrated differential inhibitory effects on the proliferation of LNCaP, an androgen-sensitive prostate cancer cell line, and DU145, a more aggressive androgen-insensitive prostate cancer cell line. Two similar proanthocyanidin-rich fractions from *V. corymbosum* significantly inhibited LNCaP growth in the $mg \cdot mL^{-1}$ range (Schmidt et al., 2006). Only one fraction modestly inhibited the growth of DU145 cells. Differences in cell growth inhibition of LNCaP and DU145 cell lines by *V. corymbosum* fractions rich in proanthocyanidins indicate that its proanthocyanidins exert an effect through mechanisms characteristic of androgen-dependent growth in prostate cancer cells. An extract of *V. myrtillus* was effective at inhibiting the growth of HL60 human leukemia cells and HCT116 human colon carcinoma cells in vitro (Katsube et al., 2003). The extract induced apoptotic cell bodies in both, but to a far lesser extent in HCT116 than HL60 cells, and caused nucleosomal DNA fragmentation only in HL60 cells. Likewise, pure delphinidin and malvidin induced apoptosis in HL60 cells, as did related glycosides isolated from the extract. Only pure delphinidin and its glycoside isolated from the *V. myrtillus* extract, but not malvidin and its glycoside, inhibited the growth of HCT116 cells.

Polyphenol-rich *V. vitis-idaea* extracts were screened for their antiproliferative effectiveness in human cervical cancer (HeLa) cells (McDougall et al., 2008). In this system, *V. vitis-idaea* and other berry extracts were effective with EC_{50} values in the range of 25–40 $\mu g \cdot mL^{-1}$ relative to phenol content. These extracts were also effective against the human colon cancer cell line, Caco-2, which was generally more sensitive at low concentrations, but conversely, less sensitive at higher concentrations. Although some of the extracts share common polyphenol constituents, especially the ellagitannins, shown to be effective antiproliferative agents, the bioactive components of *V. vitis-idaea* extracts are not known. Although anthocyanin-enriched fractions were considerably less effective than the crude extract, antiproliferative activity was retained in the tannin-rich fraction composed almost entirely of proanthocyanidins of type A and B linkages. Others found, through statistical analyses, that anthocyanin chemical structure affected chemoprotection, with non-acylated monoglycosylated anthocyanins having greater inhibitory effect on proliferation of another colon cancer cell line, HT-29, whereas anthocyanins with pelargonidin, triglycoside, and/or acylation with cinnamic acid exerted

the least effect (Jing et al., 2008). They concluded that anthocyanins played a major role in chemoprotection and exerted an additive interaction with the other phenolics present.

Freeze-dried preparations of two *V. ashei* cultivars (cv. Tifblue and cv. Premier) were sequentially extracted with solvents of various polarities and shown to possess in vitro antiproliferative activity against CaSki and SiHa cervical cancer cell lines and MCF-7 and T47-D breast cancer cell lines (Wedge et al., 2001). Proliferation inhibitory and apoptosis-inducing effects of polyphenolic compounds from *V. ashei* (cv. Briteblue, cv. Tifblue, and cv. Powderblue) were also assessed in a systematic study of Caco-2 and HT-29 (Yi et al., 2005). Extracts were further separated into phenolic acid, tannin, flavonol, and anthocyanin-enriched fractions, and some individual phenolic acids and flavonoids were identified by HPLC with >90% purity in anthocyanin fractions. The dried extracts and fractions were tested for antiproliferation activities and induction of apoptosis by addition to the cell culture medium. Flavonol and tannin fractions resulted in 50% inhibition of cell proliferation, whereas the phenolic acid fraction showed relatively lower bioactivities with 50% inhibition at higher concentrations of test preparations in both cell lines. The greatest antiproliferation effect among all four fractions was from the anthocyanin fractions, which significantly inhibited cell growth by >50% at concentrations in the $\mu g \cdot mL^{-1}$ range. Anthocyanin fractions also induced apoptosis resulting in —two to seven times increase in DNA fragmentation. Anthocyanin fractions from *V. ashei* cultivars, principally containing delphinidin, cyanidin, peonidin, petunidin, and malvidin, increased apoptosis as determined by DNA fragmentation and cysteine–aspartic acid protease, caspase-3, activity assays (Srivastava et al. 2007). DNA fragmentation increased at anthocyanin concentrations from 50 to 150 $\mu g \cdot mL^{-1}$ with cv. Tifblue and cv. Powderblue, but a prominent ladder was apparent in cells treated with 50–100 $\mu g \cdot mL^{-1}$ of the anthocyanin fraction of cv. Brightblue and cv. Brightwell as compared to cells treated with 150 $\mu g \cdot mL^{-1}$. Apoptosis related caspase-3 activity in the control cells and the cells treated with anthocyanins from all four cultivars demonstrated a significant positive difference.

Extracts of six popularly consumed berries, including *V. corymbosum*, *V. macrocarpon*, as well as *Rubus* and *Fragaria* species, were analyzed for their phenolic constituents using high-performance liquid chromatography with ultraviolet detection (HPLC-UV) and electrospray ionization mass spectrometry (LC-ESI-MS) detection, and evaluated ability to inhibit the growth of human oral (KB, CAL-27), breast (MCF-7), colon (HT-29, HCT116), and prostate (LNCaP) tumor cell lines (Seeram et al., 2006). At concentrations in the $\mu g \cdot mL^{-1}$ range, increasing concentration of berry extract was shown to increase inhibition of cell proliferation in all of the cell lines tested, but with different degrees of potency between cell lines. All berry extracts were also evaluated for their ability to stimulate apoptosis of the inflammatory cyclooxygenase (specifically, COX-2) expressing HT-29 cells, but *Rubus* and *Fragaria* were most effective. *V. corymbosum* (cv. Bluecrop) leaf extract was highly inhibitory in vitro against a drug-sensitive promyelocytic HL60 human cell line, although it was much less effective against multi-drug resistant sublines exhibiting two different MDR phenotypes: HL60/VINC (overexpressing

P-glycoprotein) and HL60/DOX (overexpressing multi-drug resistance protein, MRP1) (Skupien et al., 2006).

Antiproliferation assays *in vitro* with HepG2 human liver cancer cells showed a high inhibitory effect of *V. macrocarpon*, followed by many other types of popular fruits (Sun et al., 2002). Extracts of whole *V. macrocarpon* fruit were assayed for radical-scavenging activity and tumor growth inhibition using seven tumor cell lines (Yan et al., 2002). Selective inhibition of K562 human leukemia cells and HT-29 colon cancer cells was observed from a methanolic extract. In a further of investigation of tumor cell inhibitory components of *Vaccinium*, a total *V. macrocarpon* extract (TCE) was analyzed, quantified, and separated into fractions enriched with respect to sugars (39.4%), organic acids (30.0%), total polyphenols (10.6%), proanthocyanidins (5.5%), and anthocyanins (1.2%) (Seeram et al., 2004). The antiproliferative effects of the TCE (200 $\mu g \cdot mL^{-1}$) versus all fractions were evaluated against human oral (KB, CAL27), colon (HT-29, HCT116, SW480, SW620), and prostate (RWPE-1, RWPE-2, 22Rv1) cancer cell lines using a luminescent ATP cell viability assay. The total polyphenols fraction was the most active fraction against all cell lines with 95 and 96.1% inhibition of CAL27 and KB oral cancer cells, respectively. For the colon cancer cell lines, the antiproliferative activity of TCE was greater against HCT116 (92.1%) than against HT-29 (61.1%), SW480 (60%), and SW620 (63%). TCE and all fractions showed $\geq$50% antiproliferative activity against prostate cancer cells, but total polyphenols was the most inhibitory fraction, with efficacy against RWPE-1 (95%), RWPE-2 (95%), and 22Rv1 (99.6%). Conversely, the sugars' fraction did not inhibit the proliferation of any cancer cell lines. The authors concluded that enhanced antiproliferative activity of total polyphenols compared to TCE, and its individual phytochemicals (and with a compositional majority of sugars and organic acids), suggests synergistic or additive antiproliferative interactions of the anthocyanins, proanthocyanidins, and flavonol glycosides within the extract.

A *V. macrocarpon* proanthocyanidin-rich extract (PAC) was evaluated for chemoprevention of esophageal adenocarcinoma (recognized through its precursor lesion, Barrett's esophagus) in model SEG-1 human esophageal adenocarcinoma cells (Kresty et al., 2008). PAC pretreatment significantly inhibited the viability and proliferation of SEG-1 cells in a time- and dose-dependent manner. Moreover, PAC significantly inhibited acid-induced cell proliferation of SEG-1 cells and induced cell cycle arrest at the G1 checkpoint with a significant reduction in the percentage of SEG-1 cells in S-phase following 24 and 48 h of exposure. PAC treatment also resulted in significant induction of apoptosis. The authors propose that PAC modulates cell cycle regulation, aberrant proliferation, and apoptosis, all key biological processes altered during progression to esophageal adenocarcinoma.

Extracts of *V. macrocarpon* significantly inhibited MCF-7 cell proliferation at doses of 5–30 $mg \cdot mL^{-1}$ (Sun and Hai Liu, 2006). Doses from 10 to 50 $mg \cdot mL^{-1}$ arrested MCF-7 cells at G_0/G_1 phase, and a constant increasing pattern of the G_1/S index was observed in the treatment group, whereas the G_1/S ratio of the control group decreased concomitantly between 10 and 24 h of treatment. Following 24 h exposure to extracts, the G_1/S index of MCF-7 cells was approximately six times

higher than that of the control group. Induction of apoptosis in MCF-7 cells was observed in a dose-dependent manner after exposure to extracts for 4 h. A dose of 50 $mg \cdot mL^{-1}$ resulted in a 25% higher ratio of apoptotic cells to total cells as compared to the control groups. These results suggest that extracts of *V. macrocarpon* possess the ability to suppress the proliferation of MCF-7 cells, which can be attributed, at least in part, to both the initiation of apoptosis and the G_1 phase arrest.

Novel purified triterpene cinnamates from *V. macrocarpon*, identified as *cis* (1) and *trans* (2) isomers of 3-*O*-*p*-hydroxycinnamoyl ursolic acid, were bioassayed in human tumor cell lines in vitro (Murphy et al., 2003). The *cis* isomer showed slightly greater activity than the trans moiety in most cell lines, with a $GI_{50}$of approximately 20 μM in MCF-7, ME180 human cervical epithelial, and PC3 androgen-independent human prostate tumor cell lines. The GI_{50}value is a redefinition of the IC_{50} value, the concentration that causes 50% growth inhibition, corrected for the cell count at time zero (http://dtp.nci.nih.gov/docs/compare/compare_methodology.html). Quercetin was slightly less active than *cis*-3-*O*-*p*-hydroxycinnamoyl ursolic acid, while cyanidin-3-galactoside exhibited much lower cytotoxicity, with greater than 250 μM in all cell lines. Antiproliferative activities of isolated *V. macrocarpon* compounds against MCF-7 and HepG2 human liver cancer cells were also evaluated through bioactivity-guided fractionation (He and Liu, 2006). Among the compounds isolated, ursolic acid, quercetin, and 3,5,7,3′,4′-pentahydroxyflavonol-3-*O*-beta-D-glucopyranoside showed potent inhibitory activity toward the proliferation of MCF-7 cells, with EC_{50} values of 11.7 ± 0.1, 137.5 ± 2.6, and 23.9 ± 3.9 μM, respectively. Ursolic acid, quercetin, and 3,5,7,3′,4′-pentahydroxyflavonol-3-*O*-beta-D-glucopyranoside showed potent antiproliferative activities against HepG2 cell growth, with EC_{50} values of 87.4 ± 2.7, 40.9 ± 1.1, and 49.2 ± 4.9 μM, respectively.

In hormone-dependent tumor cell lines, an extract of *V. macrocarpon presscake* (material remaining after squeezing juice from the berries) containing flavonoids inhibited proliferation of eight human tumor cell lines of multiple origins (Ferguson et al., 2004). The androgen-dependent prostate cell line LNCaP was the most sensitive of those tested, but other human tumor lines originating from breast (MCF-7), skin (SK-MEL-5), colon (HT-29), lung (DMS114), and brain (U87) were less sensitive. An estrogen-independent breast line (MDA-MB-435) and an androgen-independent prostate line (DU145) were the least sensitive and required comparatively high doses of extract to inhibit proliferation. Nonetheless, the extract was able to block cell cycle progression in MDA-MB-435 cells and induce cells to undergo apoptosis in a dose-dependent manner as demonstrated by using flow cytometric analyses of DNA distribution (cell cycle) and annexin V-positivity (apoptosis marker). These authors also reported that *V. macrocarpon* presscake was shown to decrease the growth and metastasis of tumors in mice bearing human breast tumor MDA-MB-435 cells. Subsequently, explants of human tumor cell lines glioblastoma multiforme (U87), colon carcinoma (HT-29), and androgen-independent prostate carcinoma (DU145) were shown to be sensitive to a flavonoid-rich fraction and a more purified proanthocyanidin-rich fraction of *V. macrocarpon*

(Ferguson et al., 2006). Both significantly slowed the growth of explant tumors of U87 in vivo, but the proanthocyanidin-rich fraction inhibited growth of HT-29 and DU145 explants, inducing complete regression of two DU145 tumor explants. Flow cytometric analyses of in vitro-treated U87 cells indicated that both fractions also could arrest cells in G_1 phase of the cell cycle and induce cell death within 24–48 h of exposure, consistent with in vivo results. *V. macrocarpon* seed flour extracts were found also to significantly inhibit HT-29 cell proliferation, although specific compounds were not cited for this effect (Parry et al., 2006).

Studies in animal models. The chemopreventive efficacy of *V. macrocarpon* juice concentrate in an experimental in vivo model of urinary bladder cancer was evaluated using female Fischer-344 rats (Prasain et al., 2008). The animals received *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine (OH-BBN) and, following treatment, a dose-dependent preventive effect of juice concentrate was observed, with a reduced number of urinary bladder cancers (38%) versus those observed in the control group. Serum and urine were collected after the administration of the juice concentrate, and quercetin, as well as its methylated derivative, was detected in the urine samples. As a consequence of poor bioavailability, no quercetin was detected in the serum samples. Although quercetin moieties were detected predominantly, the authors conclude that many components of *V. macrocarpon* juice concentrate may be responsible for some of the observations.

Experiments designed to study the inhibitory effect against the formation of colonic aberrant crypt foci (ACF) pre-neoplastic lesions of pterostilbene, an important compound in *Vaccinium* fruits, were conducted in Fisher 344 male rats (Suh et al., 2007). Animals were treated with the colon carcinogen, azoxymethane (AOM), and were fed experimental diets with or without pterostilbene. At sacrifice, colons were evaluated for ACF formation, for inhibition of inducible nitric oxide synthase (iNOS) and proliferating cell nuclear antigen, and for effects on mucin glycoprotein (MUC2). Administration of pterostilbene significantly suppressed AOM-induced formation of ACF and multiple clusters of aberrant crypts. Importantly, dietary pterostilbene also suppressed AOM-induced colonic cell proliferation and iNOS expression, with the latter effect being confirmed in cultured human colon cancer cells. To test directly the chemopreventive potential of fruit rich in pterostilbene, another study examined the possible effects of *V. corymbosum* and *V. macrocarpon* juice, as well as other fruit preparations, on AOM-induced ACF in Fisher 344 male rats (Boateng et al., 2007). The rats received subcutaneous injections of AOM and, upon sacrifice, total ACF numbers assessed in the rats fed control diet, *V. corymbosum,* and *V. macrocarpon* were, respectively, 171.67 ± 5.6, 11.33 ± 2.85, and 39.0 ± 15.31, with numbers from other types of flavonoid-rich fruits ranging from 15.67 ± 1.86 to 33.67 ± 0.89. Total glutathione-*S*-transferase (GST) activity in the liver of the rats fed fruit preparations was significantly higher as compared with the control. Although juice from *V. macrocarpon* was effective, among all fruits and fruit juices, *V. corymbosum* juice induced the most significant reductions in the formation of AOM-induced ACF.

The chemoprotective activity of anthocyanin-rich extracts (AREs) from *V. myrtillus* and other fruits was assessed with multiple biomarkers of colon cancer

in male rats treated with AOM (Lala et al., 2006). Fischer 344 male rats were fed the AIN-93 diet (control) or AIN-93 diet supplemented with AREs for 14 weeks. Biomarkers that were evaluated included the number and multiplicity of colonic aberrant crypt foci (ACF), colonic cell proliferation, urinary levels of oxidative DNA damage, and expression of *COX-2* genes. To assess the bioavailability, levels of anthocyanins in serum, urine, and feces were evaluated; total ACF were reduced in all treatment groups compared with the control group. The number of large ACF, colonic cellular proliferation, and COX-2 mRNA expression was decreased by *V. myrtillus* in ARE-fed rats. High levels of fecal anthocyanins and increased fecal mass and fecal moisture occurred in ARE-fed rats. There was also a significant reduction in fecal bile acids in ARE-fed rats. The levels of urinary 8-hydroxyguanosine were similar among rats fed different diets. These results are consistent with other studies and suggest a protective role of AREs in colon carcinogenesis through multiple mechanisms of action. Collectively, these observations provide insights into pivotal mechanisms of anthocyanin- and stilbene-mediated antitumor effects and support recommending consumption of fruits and preparations rich in these for colon cancer chemoprevention and, potentially, for treatment of human gastrointestinal tract cancer.

Prevention studies in the estrogen-sensitive female ACI rat model allowed identification of agents that are effective against estrogen-induced mammary tumorigenesis (Aiyer et al., 2008). Compared with the control group, *V. corymbosum* powder showed a 40% reduction in tumor volume, whereas pure ellagic acid reduced tumor volume by 75% and tumor multiplicity by 44%. This is the first report showing the significant efficacy of both ellagic acid and berries in the prevention of solely estrogen-induced mammary tumors.

11.2.1.7 Regulation of Subcellular Signaling Pathways and Modulation of Transcription Factors

Studies in tumor cell lines. Commercially prepared anthocyanin-rich extracts were shown to inhibit proliferation of colon cancer-derived HT-29 cells at low concentrations that did not affect non-tumorigenic colonic NCM460 cells (Zhao et al., 2004). The effects of berry extracts containing different phenolic profiles on cell viability and expression of markers of cell proliferation and apoptosis were studied in HT-29 cells by another group (Wu et al., 2007). Anthocyanins were the predominant phenolic compounds in *V. myrtillus* and other extracts (including those from *V. vitis-idaea*). Among these, *V. myrtillus* extract was the most potent. An increase in the expression of p21WAF1, an inhibitor of cell proliferation and a member of the cyclin kinase inhibitors, was seen in cells exposed to all extracts. The pro-apoptosis marker, Bax, was increased 1.3-fold in *V. myrtillus*-treated cells, whereas the pro-survival marker, Bcl-2, was detected only in control cells. The results demonstrate that berry extracts inhibit cancer cell proliferation mainly via the p21WAF1 pathway. As other berries with comparatively very low anthocyanin content were potent inhibitors of cell proliferation, it was concluded that, in addition to anthocyanins

found in *V. myrtillus* and other deeply pigmented fruits, an array of phenolic or non-phenolic phytochemicals is responsible for the antiproliferative activity of berries.

The juice of 14 different berries, including four *Vaccinium* species, was evaluated for antioxidant capacity, antiproliferative activity, induction of apoptosis and cell cycle arrest, and anti-inflammatory activity (Boivin et al., 2007). The growth of various cancer cell lines, including those of stomach, prostate, intestine, and breast, was strongly inhibited by *V. angustifolium, V. myrtilloides,* and *V. macrocarpon* juices, but not (or only slightly) by *V. corymbosum* juice. No correlation was found between the antioxidant capacity and antiproliferative activity of the juice. The inhibition of cancer cell proliferation appeared to involve cell cycle arrest, not caspase-dependent apoptosis, as evidenced by downregulation of the expression of calmolulin-dependent kinases, cdk4 and cdk6, cyclin D1 and cyclin D3. Approximately half of the berries evaluated, including those of *Vaccinium*, significantly inhibited the tumor necrosis factor (TNF)-induced activation of COX-2 expression and activation of NF-kappaB. Interestingly, berry juices have a pronounced distinction in their potential chemopreventive activity, and thus, consumption of a variety of berries may prove useful for preventing or delaying the onset of tumor development.

V. vitis-idaea extracts produced a dose-dependent inhibition of transcription activator protein-1 (AP-1) and NF-kappaB induced by either TPA tumor promoter or ultraviolet-B (UVB) radiation in JB6 P+ mouse epidermal cells (Wang et al., 2005). Both proteins play an important mechanistic role in ultraviolet (UV)-induced skin carcinogenesis in mice. Pretreatment of cells with extracts blocked UVB-induced phosphorylation of the mitogen-activated protein kinase (MAPK)-signaling members, extracellular kinase signal-regulated kinases (ERK1 and ERK2), stress-activated protein kinase (p38), and extracellular signal-regulated ERK kinase (MEK1/2), but not c-Jun N-terminal kinase (JNK). The *c-Jun protein* is synonymous with AP-1 and is an important regulator of cell cycle progression and apoptosis. The extract also prevented TPA-induced phosphorylation of ERK1, ERK2, and MEK1/2 and TPA-induced neoplastic transformation of JB6 P(+) cells was also suppressed in a dose-dependent manner in soft agar assays. In addition, extracts promoted apoptosis of human leukemia HL-60 cells in a dose-independent manner. These results suggest that ERK1, ERK2, and MEK1/2 may be the primary targets of *V. vitis-idaea* extracts that result in suppression of AP-1, NF-kappaB, and neoplastic transformation in JB6 P(+) cells and that cancer cell death is caused by an apoptotic mechanism in human leukemia HL-60 cells. In other mouse epidermal cells, methanol extracts of *Vaccinium* were unable to inhibit AP-1 and NF-kappaB activation by UVB and short UV radiation, UV-C (Huang et al., 2007). Different berry extracts were able to exert this inhibitory effect. These results suggest that berries differ in their composition, and hence, ability to influence signaling pathways leading to activation of NF-kappaB and AP-1 when using UV light as the inducer. Another group investigated whether *V. myrtillus* and quercetin, notably abundant in this fruit, have the ability to induce transcription of Fos-related antigen 1 (Fra-1), which contains two EpREs in its promoter (Myhrstad et al., 2006). Fra-1 is a member of the AP-1 family of transcription factors and, due to the lack of transactivation domain Fra-1, can

suppress activation of AP-1. Their work demonstrated that *V. myrtillus* preparations and pure quercetin were able to induce the Fra-1 promoter as well as the cellular content of Fra-1 mRNA, and suggested that induction is mediated through EpREs.

Naturally occurring stilbenes, resveratrol, pterostilbene, and piceatannol, occur in *Vaccinium* and are known to be strong antioxidants and anti-inflammatory agents with cancer chemopreventive activities (Rimando et al., 2004). Pterostilbene was able to inhibit cell proliferation and induce apoptosis of human gastric carcinoma AGS cell line (Pan et al., 2007). Pterostilbene-induced cell death was characterized with changes in nuclear morphology, DNA fragmentation, and cell morphology. The results showed that caspase-2, -3, -8, and -9 are all activated by pterostilbene, together with cleavage of the downstream caspase-3 target DNA fragmentation factor (DFF-45) and poly(ADP-ribose) polymerase. Moreover, activation of the caspase cascade, the Bcl-family of proteins, and the mitochondrial pathway is responsible for pterostilbene-induced apoptosis. Pterostilbene markedly enhanced the expression of growth arrest of DNA damage-inducible gene 45 and 153 (GADD45 and GADD153), blocked cell cycle progression at G_1 phase, increased the p53, p21, p27, and p16 proteins, and decreased levels of cyclin A, cyclin E, cyclin-dependent kinases Cdk2, Cdk4, and Cdk6, but the expression of cyclin D1 was not affected. Also, the degree of phosphorylation of retinoblastoma protein (Rb) was decreased. Collectively, pterostilbene induced apoptosis in AGS cells through activating the caspase cascade via the mitochondrial and Fas/FasL pathway, GADD expression, and by modifying cell cycle progress and changes in several cycle-regulating proteins.

Studies in animal models. Mirtoselect, a 36% anthocyanin mixture from *V. myrtillus* (available from Indena, S.p.A., http://www.mirtoselect.info) or isolated cyanidin-3-glucoside (C3G), the most abundant anthocyanin in the diet, was evaluated for intestinal adenoma formation in the Apc-mutated multiple intestinal neoplasia (Min/+) mouse, a genetic model of human familial adenomatous polyposis (Cooke et al., 2006). Min/+ mice ingested Mirtoselect or C3G at <0.3% of the diet, and intestinal adenomas were counted at sacrifice. Plasma, urine, and intestinal mucosa were analyzed for presence of anthocyanins by high-pressure liquid chromatography (HPLC) with detection by UV spectrophotometry (520 nm) or tandem mass spectrometry (multiple reaction monitoring). Total anthocyanin levels in mice on C3G or Mirtoselect were 43 ng and 8.1 $\mu g \cdot g^{-1}$ tissue, respectively, in the intestinal mucosa, and 7.2 and 12.3 $\mu g \cdot g^{-1}$ in the urine. Anthocyanins were found at the analytical detection limit in the plasma and at quantifiable levels in the intestinal mucosa; glucuronide and methyl metabolites were identified in intestine and urine. Ingestion of either C3G or Mirtoselect reduced adenoma load dose dependently. At the highest doses of C3G and Mirtoselect, adenoma numbers were decreased significantly by 45 or 30%, respectively, compared to controls.

Subsequently, *V. myrtillus* and *V. vitis-idaea*, along with other berry preparations, natural ellagitannins, and pure ellagic acid were evaluated for their effects on adenoma formation in the intestinal tract of Min/+ mice (Misikangas et al., 2007; Mutanen et al., 2008). The mice were fed high-fat AIN93-G diets containing test substances for 10 weeks. All of the berries significantly reduced tumor number

(15–30%), but *V. vitis-idaea*also reduced tumour size by over 60% and, as compared to the control, resulted in a larger proportion of small adenomas with a smaller proportion of large adenomas. On the molecular level, beta-catenin and cyclin D1 protein levels in the adenomas and in the normal-appearing mucosa were determined by Western blotting and immunohistochemistry. Early changes in gene expression in the normal-appearing mucosa were analyzed by Affymetrix microarrays. *V. vitis-idaea* increased the level of cyclin D1 in the large adenomas. Affymetrix microarrays revealed changes in genes implicated in colon carcinogenesis, including the decreased expression of the adenosine deaminase, ecto-5f-nucleotidase and prostaglandin receptor PGE2 subtype EP4. Ellagic acid had no effect on the number or size of adenomas in the distal or total small intestine, but it increased adenoma size in the duodenum when compared with the control diet. The ellagitannins did not have any effect on the adenoma formation. Taken together, the results of these three groups, the efficacy of berry preparations, Mirtoselect from *V. myrtillus*, and C3G in the Min/+ mouse, warrant the further development of anthocyanins as potential human colorectal cancer chemopreventive agents.

11.2.1.8 Inhibition of Growth Factor-Dependent Processes, Inflammation, and Tumor Angiogenesis and Metastasis

Inflammatory processes mediated by COX-2 and associated growth factors have been implicated in the invasiveness of various types of tumors. COX-2 inhibitors, such as nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit carcinogenesis, reduce blood flow through the tumor tissue and, thereby, inhibit angiogenic activity within the tumor. A crude hydroalcoholic extract from *V. corymbosum* was assessed in anti-inflammatory and antinociceptive models (Torri et al., 2007). Inflammation was reduced significantly in the carrageenan test (rat paw edema), histamine assay, and myeloperoxidase (MPO) assay after injection of carrageenan. For the abdominal constriction test, inhibition observed for the extract was almost as potent as that for indometacin. In the formalin test, the *V. corymbosum* extract and indometacin similarly inhibited only the second phase. With the granulomatous tissue assay, the steroidal anti-inflammatory, dexamethasone, displayed significant activity, whereas the test extract was inactive. Consumption of *V. corymbosum* displayed anti-inflammatory, as well as antinociceptive activity, and it may be helpful for the treatment of inflammatory disorders, some of which participate in the etiology of cancer.

However, *V. corymbosum* and *V. macrocarpon* preparations were found to be inactive against the COX enzyme system (Seeram et al., 2001). A possible explanation is the absence in *Vaccinium* species of compounds (i.e., cyanidin-3-glucosylrutinoside and cyanidin-3-rutinoside) to which cyclooxygenase inhibition was attributed in these experiments. Subsequently, commercial extracts of *V. angustifolium* (VitaBlue^{TM}) were shown to selectively inhibit COX-2 in vitro and to inhibit proliferation of an unspecified human prostate tumor cell line (VDF FutureCeuticals, www.futureceuticals.com). Potent in vitro inhibition of COX-2 was observed, with no effect of the extract on the related enzyme, COX-1. The

importance of this finding relates to the overexpression of COX-2 in neoplasias, and that increased activity of COX-2 promotes tumor vascularization and angiogenesis. In later investigations, the effect of anthocyanidins on expression of COX-2 was investigated in lipopolysaccharide (LPS)-activated murine macrophage RAW264 cells. Delphinidin and cyanidin aglycones inhibited LPS-induced COX-2 expression, but pelargonidin, peonidin and malvidin were ineffective (Hou et al., 2005). Delphinidin was the most potent inhibitor and possesses an ortho-dihydroxyphenyl structure on the B-ring. A dose-dependent inhibition by delphinidin of COX-2 expression at both mRNA and protein levels was observed. Suppression of degradation of an inhibitor of the nuclear transcription factor NF-kappaB (IkappaB-alpha), nuclear translocation of a subunit of NF-kappaB (p65) and CCAAT/enhancer-binding protein (C/EBP) delta, and phosphorylation of c-Jun, but not CRE-binding protein (CREB), was demonstrated by Western blotting. Moreover, delphinidin suppressed the activations of MAPK including JNK, ERK, and p38 kinase. MAPK inhibitors (U0126 for MEK1/2, SB203580 for p38 kinase and SP600125 for JNK) specifically blocked LPS-induced COX-2 expression. Thus, it appears LPS-induced COX-2 expression by activating MAPK pathways, and delphinidin suppressed COX-2 by blocking MAPK-mediated pathways with the attendant activation of NF-kappaB, AP-1, and C/EBPdelta. These authors provide the first molecular basis that anthocyanidins with ortho-dihydroxyphenyl structure may have anti-inflammatory properties through the inhibition of MAPK-mediated COX-2 expression.

Various *Vaccinium* extracts and a commercial mixed berry powder (Optiberry®) exhibited a high ORAC value, low cytotoxicity, and superior anti-angiogenic properties (Atalay et al., 2003; Bagchi et al., 2004; InterHealth Nutraceuticals, http://www.interhealthusa.com). Anti-angiogenic approaches to treat cancer represent a priority area in vascular tumor biology, as invasive tumors develop new blood vessels to fuel their rapid proliferation by a process known as *angiogenesis*. The mixed berry powder, as well as that derived from *V. angustifolium*, significantly inhibited expression of both H_2O_2 and TNF-α-induced vascular endothelial growth factor (VEGF), a key regulator of tumor angiogenesis, in human keratinocytes. Optiberry significantly inhibited inducible monocyte chemotactic protein 1 (MCP-1) and NFkappaB transcription associated with angiogenesis in endothelioma cells. When pre-treated with berry powders, endothelioma cells showed diminished hemangioma formation, a powerful model to study in vivo angiogenesis, and markedly decreased tumor growth by more than 50%. Matrigel assay, using human microvascular endothelial cells, showed that OptiBerry impaired angiogenesis. Histological analysis demonstrated substantially decreased infiltration of macrophages in hemangioma of treated mice compared to placebo-treated controls. These studies provided the first in vivo evidence to substantiate the anti-angiogenic property of edible berries.

Cancer cells invade normal tissue with matrix-metalloproteinase enzymes. Regulation of these matrix-metalloproteinases (MMPs), the major mediators of extracellular matrix (ECM) degradation, is crucial to regulate ECM proteolysis, which is important in metastasis. In a study of three flavonoid-enriched fractions prepared from *V. angustifolium*, a crude fraction, an anthocyanin-enriched fraction,

and a proanthocyanidin-enriched fraction were compared for inhibitory effects on MMP activity in DU145 human prostate cancer cells in vitro (Matchett et al., 2005). All fractions elicited a downregulation of MMP-2 and MMP-9, but the proanthocyanidin-enriched fraction was found to be the most effective. No induction of either apoptotic or necrotic cell death was noted in response to treatment of DU145 cells. The activity of the endogenous tissue inhibitors of metalloproteinases (TIMPs) from these cells was also evaluated (Matchett et al., 2006). Increases in TIMP-1 and TIMP-2 activity were observed in response to these fractions. The possible involvement of protein kinase C (PKC) and mitogen-activated protein (MAP) kinase pathways in the flavonoid-mediated decreases in MMP activity was observed. These findings indicate that *Vaccinium* flavonoids may employ multiple mechanisms for downregulation of MMP activity in these cells, which may decrease overall ECM degradation. These effects may be important in controlling tumor metastatic processes.

V. myrtillus extracts were tested for effects on angiogenesis *in vitro* and *in vivo* (Matsunaga et al., 2007). The extracts, at low $\mu g \cdot mL^{-1}$ concentration and GM6001, a MMP inhibitor (0.1–100 μM), inhibited in a dose-dependent manner both tube formation and migration of human umbilical vein endothelial cells (HUVECs) induced by vascular endothelial growth factor-A (VEGF-A). Furthermore, the extracts inhibited VEGF-A-induced proliferation of HUVECs and VEGF-A-induced phosphorylations of extracellular signal-regulated kinase 1/2 (ERK 1/2) and serine/threonine protein kinase family protein kinase B (Akt). Phosphorylation of phospholipase Cgamma was not affected by treatment. In an in vivo assay, intravitreal administration of extracts inhibited the formation of neovascular tufts during oxygen-induced retinopathy in mice. Thus, inhibition of phosphorylations of ERK 1/2 and Akt by extracts may be responsible, at least in part, for inhibition of angiogenesis both in vitro and in vivo. In order to evaluate further the molecular basis of anti-inflammatory function and underlying genes targeted by *V. myrtillus*, gene expression profiling through DNA microarray was performed on extract-treated macrophages (Chen et al., 2008). Utilizing "Panther" group analysis, 308 genes affected by the extract with $\geq$1.5-fold change were classified into 43 categories relating to signaling pathways (26), biological processes (97), and molecular functions (186). The genes categorized as "defense, inflammatory response, cytokines activities, and receptor activities" were further identified, and some of them were confirmed by real-time polymerase chain reaction. These findings indicate that *V. myrtillus* may be effective against diseases involving angiogenesis, such as cancer, although investigations will be needed to clarify the major angiogenesis-modulating constituent(s) in the extracts.

A point that must be considered in the vigorous pursuit of dietary compounds as chemopreventives, particularly based on the success of therapeutic anti-inflammatory compounds to inhibit, delay, and reverse colon carcinogenesis, is the metabolic transformation of these food-derived compounds in the gut that may affect their bioactivity (Russell et al., 2007). An example was made of esterified ferulic acid and its 5-5′-linked dimer, one of many commonly consumed dietary phenolics which have the potential to undergo predominant microbial transformations

(de-esterification, hydrogenation, demethylation, dehydroxylation, and dimer cleavage). Following incubation with human intestinal flora, potential anti-inflammatory properties were compared by measuring the ability of the parent compounds and their metabolites to modulate inflammatory prostanoid production in a responsive cell line following a cytokine-induced insult. Various metabolites demonstrated highly different effects, suggesting that microbial transformation of dietary compounds will have important anti-inflammatory implications in chemoprevention, especially with respect to colon cancer.

11.2.1.9 Protection from Toxicity of Chemotherapeutic Agents

The overall protective properties of *Vaccinium* preparations and compositions have led to proposals of their potential protective effects from cancer chemotherapeutic agents (Seeram, 2008b). In C57BL/6 mice, anthocyanin-rich preparations demonstrated protective effects from myelotoxicity caused by a single dose of the cancer chemotherapeutic agent, 5-fluorouracil (5-FU), expressed as induced severe peripheral erythrocytopenia, thrombocytopenia, and leucopenia as well as hypocellularity of the spleen and bone marrow (Choi et al., 2007). Furthermore, treatment with a monomeric anthocyanin did not interfere with, but rather, enhanced the chemotherapeutic efficacy of 5-FU in vitro. These results suggest that *V. myrtillus* components may have protective potential against 5-FU-induced myelotoxicity and/or the ability to enhance the chemotherapeutic effectiveness of 5-FU.

Anecdotal evidence from chemotherapy patients in a clinic providing transfusions for thrombocytopenia was suggestive of a potent effect on platelet recovery of a commercial *Vaccinium* preparation, AuroraBlue® (Denali BioTechnologies, www.denalibiotech.com). AuroraBlue is a proprietary blend of whole, wild Alaska *Vaccinium ovalifolium* Sm. (Alaska early blueberry), *V. alaskaense* How. (Alaska black huckleberry), *V. uliginosum* L. subsp. *alpinum* (Bigel.) Hult. (Alpine blueberry), and *V. uliginosum* L. subsp. *microphyllum* Lange (Bog Bilberry), dried by Refractance Window® technology (MCD Technologies, http://www.mcdtechnologiesinc.com). These berries are comparable or higher in levels of flavonoids reported for *V. myrtillus* on a fresh weight basis, and those compounds are preserved during the gentle drying process. Patients who voluntarily consumed recommended daily doses of AuroraBlue received fewer transfusions over a 6-week period than in a former comparable preceding period without intervention. In preliminary studies, AuroraBlue was also observed to have significant inhibitory effects on HUVECs, in particular on MCP-1 and epithelial cell-derived neutrophil-activating peptide-78 (ENA-78/CXCL5), consistent with some of the observations of others. Interestingly, such pro-inflammatory and angiogenic chemokines are potential factors that contribute to the aggressive biology of many malignancies. Furthermore, a natural product preparation containing, in part, *Vaccinium* phytochemicals, has been shown to protect against oxidative stress in hematopoietic and stem cell lines (Shytle et al., 2007). Collectively, these observations clearly merit further investigation in controlled clinical trials.

11.2.1.10 Studies on Novel and Wild *Vaccinium* Species

Only a small part of the wild plant flora has been tested for any kind of bioactivity. Yet, many of these plants have been essential to humans as food and medicine throughout the ages. More than 400 species in the genus *Vaccinium* have played an important role in human cultures worldwide. Wild and novel *Vaccinium* species have been shown to possess similar health-promoting compositions as the commercially important species (Sakakibara et al., 1973; Sticher et al., 1979; Shang and Chen, 1992; Tu et al.,1997; Chkhikvishvili and Kharebava, 2001; Meyer et al., 2002; Lee et al., 2004; Taruscio et al., 2004; Ayaz et al., 2005; Chukarina et al., 2007; Hosseinian and Beta, 2007; Nicoue et al., 2007; Wei et al., 2007; Latti et al., 2008; Rieger et al., 2008; Zhao et al., 2008). Despite variability between cultivars of commercially important species, comparisons of conventionally cultivated versus organically grown crops consistently show organic fruits to contain the higher levels of flavonoids. In conventionally cultured fruit, the average values for the ORAC, total anthocyanin, and total phenol content were 30.8 μmol of TE (Trolox equivalents)·g^{-1} of fwt, 82.4 mg·100 g^{-1} of fwt, and 190.3 mg·100 g^{-1} of fwt, respectively. In organically cultured fruit, the average values for ORAC, total anthocyanins, and total phenolic content were 46.14 μmol of TE (Trolox equivalents)·g^{-1} of fresh weight (fwt), 131.2 mg·100 g^{-1} of fwt, and 319.3 mg·100 g^{-1} of fwt, respectively. The organic culture also produced fruit with higher contents of myricetin 3-arabinoside, quercetin 3-glucoside, delphinidin 3-galactoside, delphinidin 3-glucoside, delphinidin 3-arabinoside, petunidin 3-galactoside, petunidin 3-glucoside, and malvidin 3-arabinoside than conventional culture. Notably, the levels of flavonoids in wild species are, on average, two- to ten-times higher than those of cultivated and organic relatives (Kalt et al., 2001; Wang and Stretch, 2002; Taruscio et al., 2004; Brambilla et al., 2008; Wang et al., 2008).

Investigations of wild *Vaccinium* species revealed exceptional compositions and antioxidant properties (Taruscio et al., 2004). Of particular note were *V. ovatum* Pursh (Evergreen Huckleberry) and *V. ovalifolium* Sm. In another study, two western North American species, *V. ovatum* and *V. membranaceum* Douglas ex Torr. (Thinleaf Huckleberry), were evaluated for their total, and individual, anthocyanin and polyphenolic compositions (Lee et al. 2004). Total anthocyanins (ACY), total phenolics (TP), ORAC and FRAP were greater in *V. ovatum* than in *V. membranaceum*. Anthocyanin fractions of each species had the highest amount of ACY, TP, and antioxidant activity. Similar to *V. myrtillus*, each species contained 15 anthocyanins (galactoside, glucoside, and arabinoside of delphinidin, cyanidin, petunidin, peonidin, and malvidin) but in different proportions. Polyphenolic profiles differed but, in both species, were composed mainly of cinnamic acid derivatives and flavonol glycosides. The major polyphenolic compound in *V. ovatum* was chlorogenic acid, but in *V. membranaceum*, was neochlorogenic acid. An ethanol extract of *Vaccinium parvifolium* Sm. (Red Huckleberry) was tested in the DPPH* assay and compared to nine other North American edible plant extracts, although the results from this work on *V. parvifolium* were unremarkable (Acuña et al., 2002).

Compared to juices derived from 43 small fruits, *Vaccinium smallii* A. Gray strongly inhibited the proliferation of multiple cancer cell lines examined; and yet, all of these juices were substantially less cytotoxic toward normal human cell lines (Yoshizawa et al., 2000a). Furthermore, *V. smallii* clearly induced, in a concentration-dependent manner, differentiation of HL-60 cells to monocyte/macrophage characteristics, as indicated by histochemical and biochemical examinations (Yoshizawa et al., 2000b). In a later study, fruit of *Vaccinium stamineum* L. (Deerberry) was evaluated for antioxidant capacity and anticancer properties in JB6 P (+) mouse epidermal cells, human lung, and leukemia cells (Wang et al., 2007). AP-1 and NF-kappaB induced by either 12-*O*-tetradecanoylphorbol 13-acetate (TPA) or UVB were inhibited by pretreatment of JB6 P (+) mouse epidermal cells with fruit extracts. The extracts also blocked TPA- or UVB-induced phosphorylation of ERKs and MEK 1/2, two upstream regulators of AP-1. Furthermore, the extracts inhibited proliferation of human leukemia HL-60 cancer cells and human lung epithelial cancer A549 cells and induced apoptosis in HL-60 cells. These results suggest ERKs and MEK 1/2 signal pathway mediate inhibition of TPA- or UVB-induced AP-1 and NFkappaB activity, inhibit proliferation by HL-60 cells and cancer A549 cells, but promote apoptosis in human leukemia HL-60 cancer cells.

11.2.2 Intervention Studies in Humans

Data from numerous cell culture and animal models indicate that *Vaccinium* flavonoids are potent anticarcinogenic agents and are protective at several sites in the carcinogenic development pathway. In the majority of in vitro and in vivo studies, the concentration of extract or phytochemical employed is non-nutritional (Duthie, 2007). Precisely which berry constituents are cancer chemopreventive remains uncertain, and evidence for an anticarcinogenic effect in human studies also remains weak, albeit promising for future work.

Intervention studies in healthy human subjects with *V. corymbosum*/blended fruit juice compared to one of their principal components, quercetin, have demonstrated protection in vitro and ex vivo against induction of oxidative DNA damage from hydrogen peroxide (H_2O_2)and formation of bulky DNA adducts of benzo(a)pyrene (B(a)P) formed through its diol-epoxide metabolite (BPDE) (Wilms et al., 2005). Human lymphocytes were pre-incubated with various concentrations of quercetin, followed by incubation with hydrogen peroxide, and protection against oxidative DNA damage was evaluated by the use of the single-cell gel electrophoresis (Comet) assay. Quercetin-treated human lymphocytes were challenged by treatment with B(a)P, and adduct formation was measured by ^{32}P-post-labeling. In these assays, a significant dose-dependent protection by quercetin was observed against both the formation of oxidative DNA damage and of BPDE–DNA adducts. Also, lymphocytes from female volunteers who consumed a quercetin-rich juice mixture for 4 weeks were treated ex vivo with an effective dose of H_2O_2 and B(a)P, respectively, before the intervention. Juice intervention led to a significant increase in the total

antioxidant capacity of plasma, as reflected by the increase of the Trolox equivalent TEAC value and an increase in plasma quercetin content. After intervention, the level of oxidative damage upon exposure to H_2O_2 and BPDE–DNA adduct level induced ex vivo was not significantly decreased; nonetheless, taken together with the quercetin results, the authors suggest a protective role of dietary fruit against destructive DNA modifications.

In a subsequent human intervention study, these authors assessed the antioxidative and possible anti-genotoxic properties of fruit-borne antioxidants in individuals bearing genetic polymorphisms for genes related to quercetin metabolism, B(a)P metabolism, oxidative stress, and DNA repair, and evaluated differences in their response to DNA protective effects of increased antioxidant intake (Wilms et al., 2007). Healthy volunteers consumed juice containing, in part, *V. corymbosum*, that provided known quantities of quercetin and ascorbic acid each day. After an intervention period, plasma concentrations of quercetin and ascorbic acid and TEAC were significantly increased. Further, a significant decrease was noted in ex vivo H_2O_2-provoked oxidative DNA damage, measured by comet assay. Notably, the level of ex vivo-induced BPDE–DNA adducts significantly increased upon intervention. Statistical analysis of 34 biologically relevant genetic polymorphisms revealed that six significantly influenced the outcome of the intervention. Lymphocytes from individuals bearing variant genotype for cytochrome P-450 isoform 1B1 5 (Cyp1B1 5) seemed to benefit more than wild types from DNA damage-protecting effects upon intervention. Variants for catechol-*O*-methyltransferase (COMT) tended to benefit less, or even experienced detrimental effects, from intervention. With respect to glutathione S-transferase theta 1 (GSTT1), the effect is ambiguous; variants respond better in terms of intervention-related increase in TEAC, but wild types benefit more from its protecting effects against oxidative DNA damage. These results support the possibility that genotyping for relevant polymorphisms may enable the selection of subgroups among the general population that benefit more from DNA damage-modulating effects of micronutrients and opens many interesting opportunities in nutrigenomic research.

Another intervention study was performed with an anthocyanin/polyphenolic rich, mixed berry juice with a high TEAC value, and a corresponding polyphenol-depleted juice (polyphenols largely removed, low TEAC value) serving as a control (Weisel et al., 2006). The study design includes a run-in period, an intervention period, followed by a washout in healthy male humans. Samples were collected to analyze DNA damage (Comet assay), lipid peroxidation (plasma malondialdehyde: HPLC/fluorescence; urinary isoprostanes: gas chromatography–mass spectrometry), blood glutathione (photometrically), DNA-binding activity of NF-kappaB (enzyme-linked immunosorbent assay) and plasma carotenoid/alpha-tocopherol levels (HPLC-diode array detection). During intervention with the fruit juice, but not control juice, a significant decrease in oxidative DNA damage and an increase of reduced glutathione and of glutathione status were observed, which returned to the run-in levels in the subsequent washout phase. The other biomarkers were not significantly modulated by the juice supplement. These authors concluded that the fruit juice clearly reduces oxidative cell damage in healthy probands.

11.2.2.1 Conclusions

Compiled results show promise for the cancer chemopreventive and possible therapeutic applications of *Vaccinium* preparations and derived phenolic acids, anthocyanins, catechins, stilbenes, and several other flavonoids. Although promising, the differences in occurrence and amounts of flavonoids between species make appropriate selection of preparations difficult for a given application. Likewise, effects on cancer, both positive and negative, and the various proposed mechanisms through which the chemicals exert their effects, may obfuscate future directions for research, health-care professional recommendations for use of *Vaccinium*-based products, and public confidence in their benefits. Although most of the work done to date indicates a chemopreventive activity of these compounds, there are some studies that show cancer-inducing or no effects. Perhaps the most fundamental of these concerns is the ability to distinguish between the in vitro and the in vivo antioxidant activities of dietary anthocyanins in relation to human health. But, despite outstanding questions regarding the relevance of in vitro antioxidant capacity to in vivo antioxidant protection from deleterious ROS and health consequences, *Vaccinium* flavonoids have demonstrated other mechanisms for anti-aging and anticancer potential (Wilson et al., 2006; Neto, 2007). These include effects on proliferation, apoptosis, cellular differentiation, and effects on proteins and enzymes that are involved in these processes at a molecular level, and other various effects through altered immune function and chemical metabolism (Nichenametla et al., 2006). Several common mechanisms by which these chemicals exert their effects appear to be conducive to additive, synergistic, or antagonistic interactions. Further long-term clinical trials will be required to translate these experimental observations and resulting hypotheses into a potential decreased risk of aging, chronic disease, or cancer chemoprevention and treatment in the general population.

11.3 Parkinson's Disease

Parkinson's disease (also known as *Parkinson disease* or *PD*) is a degenerative disorder of the central nervous system that often impairs the sufferer's motor skills and speech. Parkinson's disease belongs to a group of conditions called movement disorders. It is characterized by muscle rigidity, tremor, a slowing of physical movement (bradykinesia) and, in extreme cases, a loss of physical movement (akinesia). The primary symptoms are the results of decreased stimulation of the motor cortex by the basal ganglia, normally caused by the insufficient formation and action of dopamine, which is produced in the dopaminergic neurons of the brain. Secondary symptoms may include high-level cognitive dysfunction and subtle language problems. PD is both chronic and progressive. Parkinson's disease (PD) affects over 1.2 million Americans.

So far, there have been no known human clinical trials conducted that attempt to unambiguously answer the question: Do blueberries in the diet have any positive benefits for patients afflicted with early stages of Parkinson's disease? This is

remarkable in light of the fact that manifold studies conducted by Dr. James Joseph USDA, Agricultural Research Service Research Physiologist at the Neuroscience Laboratory at the Jean Mayer USDA Human Nutrition Research Center on Ageing at Tufts University, Boston, MA and colleagues, using a rat model system, have shown how a blueberry-rich diet, in comparison with placebo, elicits significant improvement in motor skills and cognitive ability in rats induced to manifest PD symptoms (Lau et al., 2006; Emborg, 2004). Similar results have been reported by Strömberg et al. 2005 following injury of the rat nigrostriatal dopamine system.

What is the basis for blueberries having beneficial effects for PD, aside from their nutritional value? Blueberries contain an array of anthocyanins and polyphenols which have high antioxidant activity and anti-inflammatory activity. It is possible that these kinds of constituents act synergistically at target sites of action (Cseke et al., 2006) as we have shown recently for sour cherry products. However, no one yet knows how blueberries act at target sites in the *human* brain to ameliorate the severe deleterious effects of PD on cognitive ability and motor skills. A well-designed human-based clinical trial using blueberries (as whole berries or blueberry juice) versus placebo is certainly needed to help answer this question.

V. myrtillus preparations are documented for their anthocyanin content and improvement of cerebral functions, neurotransmitter levels, emotional stress, and progression of neurodegenerative conditions such as Parkinson's and Alzheimer's diseases (Rahman et al., 2008).

11.4 Heart Disease

11.4.1 Case Study with Digitoxin from Digitalis purpurea *L. 'Used as a Heart Stimulant*

Digoxin is one of the most commonly prescribed cardiac glycosides by physicians, a closely related group of drugs that can affect the myocardium or heart muscle (Carey et al., 1998; Sanborn, 2007). The term "digitalis" is used to describe the entire group of cardiac glycosides (Sanborn, 2007). Digitalis is more commonly prescribed as digoxin, tradename Lanoxin^{TM}, an extract from the leaves of *Digitalis lanata* Ehrh. (Sanborn 2007), or sometimes as digitoxin, from foxglove or the leaves of *Digitalis purpurea* L. (Wilkins et al., 1985).

11.4.1.1 History

For centuries, cardiac glycosides have been used to treat congestive heart failure, since it has the ability to increase the power of contraction of the failing heart (Norn and Kruse, 2004). However, ancient Romans and Syrians might have used squill or sea onion, a seashore plant, to treat their patients with edema, or water in their tissues (Norn and Kruse, 2004).

For many centuries, foxglove had been used for various purposes, but had fallen out of use by 1745 due to inappropriate use (Wilkins et al., 1985). Dr. William Withering introduced foxglove back to the medical profession in *An Account of the Foxglove and Some of Its Medical Uses: With Practical Remarks on Dropsy, and Other Diseases* in 1785. In this book, he took the first steps in transforming digitalis into a modern drug with his observation that foxglove, *Digitalis purpurea* L., was the active ingredient chosen from more than 20 substances that could treat many diseases, including dropsy, or congestive heart failure (Drury, 1984; Hauptman and Kelly, 1999; Breckenridge, 2006). Unaware of its effect on heart failure, he believed that digitalis was a diuretic (Eichhorn and Gheorghiade, 2002a).

Early in the 20th century, digitalis was found to be useful for treating patients with heart failure and normal cardiac rhythm (Eichhorn and Gheorghiade, 2002a,b). The pharmaceutical activity of foxglove was found to be influenced by when the leaves were harvested and how the plant was grown (Goldman, 2001). In 1906, the potencies of 16 commercial digitalis preparations were found to vary over a fourfold range (Goldman, 2001). In the 1927, animal and human studies demonstrated the hemodynamic effects of digitalis on failing myocardium (Eichhorn and Gheorghiade 2002a,b). Ultimately, digoxin was found to be the preferred form of digitalis for treating heart problems in humans (Goldman, 2001).

During the 1970s, the effects of digoxin on heart failure were further elucidated (Eichhorn and Gheorghiade, 2002b). However, three challenges to digoxin therapy were discovered: an increase among patients with digoxin intoxication due to elevated serum levels, the development of newer drugs to treat heart failure, and a perceived increase in mortality due to the use of digoxin. During the 1980s, interest in using digoxin was renewed with a decrease in cases of digoxin intoxication, newer heart failure drugs demonstrating poorer survival rates, and the discovery that digoxin might benefit patients with heart failure and normal cardiac rhythm (Eichhorn and Gheorghiade 2002a). During the late 1980s, digoxin was discovered to have, in addition to its hemodynamic effects, neurohormonal modulating effects. However, the utility of digoxin was questioned because of the development of new neurohormonal modulators for heart failure and the lack of mortality data on digoxin (Eichhorn and Gheorghiade 2002b).

In the late 1980s and early 1990s, several randomized placebo-controlled trials demonstrated the utility of digoxin in patients with heart failure and normal cardiac sinus rhythm (Eichhorn and Gheorghiade 2002a). By 1990, one of the first systematic reviews of seven large-scale randomized controlled trials concluded that digoxin prevented clinical deterioration in patients with heart failure (Hood et al., 2004). In 1997, a large clinical trial showed digoxin had no effect on mortality but it decreased hospitalizations for heart failure (Eichhorn and Gheorghiade, 2002b). By the 1990s, digoxin became the most commonly prescribed cardiac glycoside by physicians for both heart failure and atrial fibrillation due its flexible routes of administration, the ability to measure its serum levels, and an intermediate duration of action (Hauptman and Kelly, 1999). Because digoxin is inexpensive and well tolerated, it may result in considerable medical cost savings (Eichhorn and Gheorghiade, 2002a).

11.4.1.2 Heart Failure

With heart failure more common in the elderly, heart failure represents a major public health problem in industrialized nations since the prevalence of heart failure is likely to increase dramatically as the population continues to age (Fauci, 1998). Heart failure is becoming an increasingly common condition associated with high morbidity and mortality (Carey et al., 1998). In the United States, heart failure is responsible for almost one million hospital admissions and 40,000 deaths per year (Fauci, 1998).

Heart failure is the pathophysiologic state in which an abnormality of cardiac muscle function results in the failure of the heart to pump blood out to the body at a rate commensurate with its metabolic requirements (Fauci, 1998). As a result, the heart is unable to maintain a cardiac output of blood adequate to meet the metabolic demand of the body (Carey et al., 1998). Cardiac output is defined as a function of the heart rate and stroke volume. Hypertension, i.e., high blood pressure, and coronary artery disease are the most frequent causes of heart failure (Carey et al., 1998). Often, heart failure is caused by a defect in myocardial contraction (Fauci, 1998). Heart failure manifests itself as organ hypoperfusion and tissue hypoxemia, i.e., inadequate delivery of oxygen to the tissues, as a result of low cardiac output leading to decreased cardiac reserve and pulmonary and systemic whole body venous congestion (Carey et al., 1998).

11.4.1.3 The Evidence for Heart Failure

Digoxin is widely used to treat mild-to-moderate heart failure in normal sinus rhythm, i.e., normal heart beat (Sanborn, 2007). In one of the more comprehensive systematic reviews of the literature, the results of 13 randomized placebo-controlled double-blinded trials of digitalis for treating individuals with heart failure who are in normal cardiac rhythm were analyzed (Hood et al., 2004). Patients treated with digitalis compared to placebo had an odds ratio (OR) for mortality of 0.98 with a 95% confidence interval (CI) from 0.89 to 1.09, for hospitalization of 0.68 (95% CI 0.61–0.75), and for clinical deterioration of 0.31 (95% CI 0.21–0.43). This means that digitalis had 31% probability of resulting in clinical deterioration, or it decreased the probability of clinical deterioration by 69%, preventing deterioration in the clinical status of the patient. Confidence intervals measure the robustness and stability of the estimate of the odds ratio, and if the confidence interval excludes 1.0, then there is a statistically significant association. This means that digitalis had no effect on long-term death rates, significantly decreased the need for hospitalization for worsening heart failure, and significantly improved clinical cardiac symptoms (Hood et al., 2004).

From one of the more definitive clinical studies of digoxin, it was reported that digoxin when used in patients with heart failure and normal sinus rhythm decreased the number of heart failure-related hospitalizations and emergency care visits, while having no effect on mortality, i.e., death due to heart failure (The Digitalis Investigation Group, 1997; Sanborn, 2007). This means that digoxin most likely affects

the frequency of hospitalizations rather than survival for patients with heart failure in normal rhythm (The Digitalis Investigation Group, 1997).

11.4.1.4 Atrial Fibrillation

Atrial fibrillation, a type of heart arrhythmia defined as an irregular heart beat or loss of rhythm is a common heart arrhythmia that can occur with or without heart failure. Atrial fibrillation, often describing the heart as a bag of worms, begins as a loss of atrial contraction, irregular rhythm, and increased heart rate leading to a decreased cardiac output.

Digoxin is effective for treating heart failure accompanied by chronic atrial fibrillation with a rapid ventricular rate, because digoxin slows the ventricular rate and results in a positive inotropic effect (Carey et al., 1998; Fauci, 1998). Digoxin decreases the rate of beating of the heart ventricles, the chambers of the heart that push blood out to the body, in most patients with atrial fibrillation (Eichhorn and Gheorghiade, 2002b). Because digoxin is taken once a day simplifying management, is well tolerated by the patient, is inexpensive, and can be measured in the blood if intoxication is suspected, it remains a useful drug for heart rate control in the management of atrial fibrillation (Eichhorn and Gheorghiade, 2002a). But because digoxin does not appear to restore normal cardiac rhythm in patients with atrial fibrillation without heart failure (Eichhorn and Gheorghiade, 2002b), it is likely that in patients that have atrial fibrillation with a rapid ventricular response, digoxin should be combined with beta-blockers (Eichhorn and Gheorghiade, 2002b). Digoxin may also be prescribed in combination with other heart drugs, such as calcium-channel blockers or beta-adrenergic antagonists, as adjunctive therapy for heart rate control of chronic atrial fibrillation without heart failure or left ventricular dysfunction (Sarter and Marchlinski, 1992; Carey et al., 1998).

For many years, digitalis was the only drug available for rate control in patients with atrial fibrillation resulting in much of the clinical experience with rate control being based on the sole use of this drug (Bjerregaard et al., 2004). As a result, current clinical treatment guidelines for ventricular rate control in patients with atrial fibrillation are based primarily on the clinical experience with digitalis rather than the results of clinical trials (Bjerregaard et al., 2004).

11.4.1.5 Mechanism

The underlying physiologic mechanism for digoxin is that it provides positive inotropic support, meaning it increases the contractility of heart muscle by preventing the movement of sodium and potassium across the cellular membrane by inhibiting sodium-potassium adenosine triphosphatase (ATPase), a cell surface transport enzyme that regulates the quantity of sodium and potassium inside cells (Carey et al., 1998; Fauci, 1998; Sanborn, 2007). Inhibition of this enzyme leads to an increase in the intracellular concentration of sodium and then an increase in the intracellular concentration of calcium (Sanborn, 2007). The increased myocardial

uptake of calcium during excitation of the myocardial cells invokes a positive inotropic response (Fauci, 1998).

At the cellular and organ level, digoxin has direct mechanical action on cardiac muscle and indirect electrophysiologic action on the cardiovascular system mediated through the autonomic nervous system, its neurohormonal effect (Sanborn, 2007). The pharmacologic consequences of these actions on the myocardium are an increase in the force and velocity of myocardial contraction (positive inotropic action), a decrease in the activation of the adrenergic sympathetic nervous system mediated through norepinephrine and the renin-angiotensin system originating in the kidneys (both neurohormonal deactivating effects), and the slowing of the heart rate and decreased nerve impulse conduction velocity through the atrio-ventricular (AV) node (vagomimetic effect) in the heart (Sanborn, 2007). The effects of digoxin on heart failure are related to the positive inotropic and neurohormonal deactivating effects. The effect of digoxin on atrial arrhythmias, such as atrial fibrillation, is related to its vagomimetic actions (Sanborn, 2007).

Ultimately, digoxin's inotropic action increases the amount of blood pushed out the heart, the left ventricular ejection fraction, improving ventricular emptying and increasing cardiac output. The increased cardiac output improves the clinical symptoms resulting from heart failure as evidenced by increased exercise capacity on a treadmill (DiBianco et al., 1989).

11.4.1.6 Current Status

Current controversies include the determination of the most efficacious and least toxic serum level for digoxin (Hauptman and Kelly, 1999; Hood et al., 2004) and the concern over interaction of digoxin with other prescribed drugs (Eichhorn and Gheorghiade, 2002a,b). Because current clinical treatment guidelines for rate control in patients with atrial fibrillation is based primarily on clinical experience with digitalis, more studies are needed to demonstrate the clinical significance of various treatment strategies, including newer drugs like beta-receptor blockers, for rate control in patients with chronic atrial fibrillation (Bjerregaard et al., 2004). Further, as new therapies for heart failure are developed, such as angiotensin-converting enzyme (ACE) inhibitors, beta receptor-blocking agents, aldosterone inhibitor spironolactone™ or angiotensin-receptor blockers like valsartan™ (Hood et al., 2004), it is not known whether digoxin will remain as the standard therapy for heart failure (Hauptman and Kelly, 1999).

In the future, there might be other uses for digitoxin. There is evidence that digitoxin might have utility in cancer therapy. Recent reports suggest that digitoxin might inhibit the growth or promote apoptosis, or cell death, of cancer cells. The inhibition of glycolysis has been proposed as the mechanism for digitoxin's anticancer effect (Lopez-Lazaro, 2007). Other mechanisms include regulating angiogenesis, or the creation of blood vessels, promoting factors, like fibroblast growth factor-2, or inhibiting the activation of a transcription factor, such as NF-kappaB (Winnicka et al., 2006). More studies are needed to further evaluate the antineoplastic effects of digitoxin.

After some 200 years of use, digoxin still appears to have utility in treating heart failure and atrial fibrillation (Eichhorn and Gheorghiade, 2002a,b). In the mid-1990s, the results of several studies prompted the Food and Drug Administration to approve the current regulations for physicians to prescribe digoxin for the treatment of heart failure (Eichhorn and Gheorghiade, 2002a), which is in agreement with a recent American College of Cardiology/American Health Association (ACC/AHA) Guidelines for Chronic Heart Failure (Young, 2005). Physicians prescribe digoxin for patients with symptomatic heart failure despite the availability of angiotensin-converting enzyme (ACE) inhibitors or diuretics, or for patients with atrial fibrillation with or without heart failure for rate control (Eichhorn and Gheorghiade, 2002b).

11.5 Depression

*11.5.1 Case Study with Polyketides (Hypericin, Pseudohypericin, Hyperforin) in St. John's Wort (*Hypericum perforatum *L.)*

Hypericum, an extract of the flower of St. John's wort, has been used for the treatment of depression for centuries (Mischoulon, 2007). St. John's wort is the common name for the flowering plant, *Hypericum perforatum* L. (Gaster and Holroyd, 2000), a member of the Hypericaceae family. Its yellow flower has been gathered for the feast of St. John the Baptist and the word "wort" is the Old English word for plant (Gaster and Holroyd, 2000).

11.5.1.1 History

The use of St. John's wort has risen dramatically in the United States (Gaster and Holroyd, 2000) as well as worldwide (Mischoulon, 2007). European physicians have used hypericum to treat mild-to-moderate depression for some time (Mischoulon, 2007). In the United States, St. John's wort accounts for about 10% of all herbal medicine sales (Williams et al., 2000) and currently is one of the largest selling "natural" remedies (Mischoulon, 2007). The three top-selling herbal products in the United States are *Ginkgo biloba* L., St. John's wort, and ginseng (Ernst, 2002).

11.5.1.2 Depression

Depression is an international public health issue that will continue to grow in importance. According to the World Health Organization (WHO), the prevalence of major depressive disorder is increasing internationally, with an estimated 120 million persons experiencing a depressive episode in a year (Sarris, 2007). The American Psychiatric Association defines major depressive disorder, or unipolar depression, as depressed mood or reduced interest or pleasure accompanied by at

least four other additional symptoms such as weight or appetite change, fatigue, psychomotor agitation, insomnia or hypersomnia, i.e., excess sleep, lack of concentration, suicidal ideation, feelings of worthlessness or guilt, and low libido (Sarris, 2007). However, major depression has also been defined as a clinical syndrome with at least 2 weeks of depressed mood and at least five additional symptoms, such as depressed mood most of the day, nearly everyday; loss of interest in nearly all activities most of the day; significant weight loss or gain or appetite disturbance; insomnia or hypersomnia; psychomotor agitation or retardation; inappropriate guilt; difficulty in concentrating or making decisions; or recurring thoughts of death, including suicidal ideation (Snow et al., 2000).

The use of complementary medicines by patients with psychiatric disorders has been estimated to range from 8 to 57%, with use most frequently reported for those with depression or anxiety (Werneke et al., 2006). In one study from the United States, seven percent of respondents reported severe depression with 54% of these individuals using complementary medicines (Kessler et al., 2001).

11.5.1.3 The Evidence

The use of extracts from the plant *H. perforatum* L., commonly called St. John's wort, to treat clinical depression has been reviewed by The Cochrane Collaboration, authoritative specialists in systematic reviews (Linde et al., 2005a).

The major objective of the review was to ascertain the effectiveness of St. John wort for treating depressive disorders. Cochrane reviewers searched for results from clinical trials in computerized databases and from projects conducted by manufacturers and researchers. Clinical trials were included if they met the following inclusion criteria: (1) if they were double-blinded randomized controlled clinical trials; (2) if they included adults with mild, moderate, or major depression; (3) if they compared extracts of St. John's wort with controls, including placebo or antidepressant drugs; and (4) if they measured outcomes with questionnaires assessing depressive symptoms, such as the Hamilton Depression Scale (HAMD) and the Clinical Global Impression Index (CGI).

Of 68 potential studies found, 37 clinical trials met inclusion criteria and demonstrated heterogeneous results with 26 trial results compared to placebo and 14 compared to prescribed antidepressants. In the larger more precise trials restricted to major depression, the response rate ratio was 1.15 with a 95% confidence interval (CI) of 1.02–1.29, signifying minimal effect. A response rate ratio was defined as the ratio of the number of patients classified as responders to treatment divided by the number of patients randomized to the respective group. Response rate ratios greater than 1.0 demonstrated a better response to St. John's wort (Linde et al., 2005a,b). Confidence intervals measure the robustness and stability of the estimate of the response rate ratio, and if the confidence interval excludes 1.0, then there is a statistically significant association. In the larger more precise clinical trials not restricted to major depression, the response rate ratio was 1.71 (95% CI 1.40–2.09), signifying a higher level of effectiveness (Linde et al. 2005a; Linde et al., 2005b). In clinical trials comparing St. John's wort to older antidepressants, such as amitriptyline™ and

imipramine™, the rate ratio was 1.03 (95% CI 0.93–1.14), signifying no difference in effect. In clinical trials comparing St. John's wort to selective serotonin reuptake inhibitors (SSRIs), such as sertraline™ and fluoxetine™, the rate ratio was 0.98 (95% CI 0.85–1.12), signifying no difference in effect (Linde et al., 2005a).

One major limitation of this systematic review is that publication bias could not be excluded (Linde et al., 2005a). Publication bias is the likelihood that published studies, likely to have positive results, might in aggregate have different results from unpublished studies, which are likely to have negative results. It is possible that the published studies included in the systematic review might overestimate the effect of St. John's wort. However, a search of unpublished trials revealed few additional relevant trials (Linde et al., 2005b).

The results of the systematic review suggest non-uniform findings because of the "inconsistent and confusing" data (Linde et al., 2005a). When clinical trials did not use a strict diagnosis of major depression, St. John's wort demonstrated greater effectiveness when compared to placebo (Mischoulon, 2007). Clinical trials that closely adhered to the *Diagnostic and Statistical Manual of Mental Disorders*, 4th edition (DSM-IV) criteria for depression demonstrated less robust findings (Mischoulon, 2007). Extracts of St. John's wort had minimal effects on treating major depression, a more severe form of depression, when compared to placebo and likely no benefit among patients with chronic depression (Linde et al., 2005a). However, extracts of St. John's wort had similar effectiveness as standard anti-depressants, such as selective serotonin reuptake inhibitors (SSRIs) and tri- or tetracyclic antidepressants, but were more effective than placebo for treating mild-to-moderate depression in adults (Linde et al., 2005a; Linde et al., 2005b).

The Cochrane reviews are an internationally known highly regarded source of evidence about the effects of health-care interventions. Since 1996, systematic reviews prepared and maintained by the Cochrane Collaboration have been published in The Cochrane Library. The Cochrane Collaboration, a loose-knit organization of experts in conducting systematic reviews who voluntarily contribute to the Collaboration, is an international attempt to develop evidence-based research guidelines about different treatment modalities. The Cochrane Library, also called The Cochrane Database of Systematic Reviews (Starr, 2003), contains more than 5,000 randomized controlled clinical trials (RCTs) and more than 60 systematic reviews of complementary and alternative medicine (CAM) therapies (Hughes, 2001). The Cochrane Database of Systematic Reviews, available at the web site http://www.update-software.com/cochrane, provides high-quality information to health-care providers and patients and those in research, teaching, funding, and administration.

The randomized controlled clinical trial (RCT) has become the objective scientific standard for evaluating the efficacy of therapeutic procedures in humans (Talalay, 2001). The ultimate source of evidence is the double-blinded randomized placebo-controlled clinical trial (RCT) since the research subjects are randomly allocated into the treatment group and a control group that receives placebo. Appropriate randomization should result in the treatment and control groups being uniform

with respect to the distribution of known and unknown characteristics, including known and unknown biases (systematic errors) and confounders (extraneous variables that can confuse any association between treatment exposure and disease outcome), except for the treatment exposure. If neither the investigators nor the study subjects know who was allocated into the treatment group or the control group, then the RCT was "double-blinded." Neither investigator nor study subject can influence, or bias, the assessment of the effect of the treatment on disease outcome. RCT study design features of particular importance include the following: an appropriate and large enough sample size to detect small effects of the treatment, uniform entry criteria for study subjects allocated to the treatment or control groups, objective measurable clinical disease endpoints, inclusion of placebo-controlled or standard-of-care controlled groups, reproducible administration of the treatment interventions, and compliance by the study subjects preventing "crossing over" by patients in the placebo study arm into the treatment arm by surreptitiously taking the study supplement (Berman and Straus, 2004). The RCT is considered the "gold standard" because it provides high-quality data that have a high degree of validity and include a minimum of bias and confounding.

Systematic reviews summarize the existing evidence from groups of RCTs. Assessing the methodologic quality of primary studies refers to aspects of study design, performance, and analysis, with a focus on randomization of the study subjects, blinding of the investigators and study subjects, and the handling of dropouts and withdrawal of study subjects (Linde et al., 2001). Herbal therapies have been submitted to systematic reviews more frequently than any other complementary or alternative medicine therapy (Ernst, 2003).

In another systematic review of 14 clinical treatment trials for adults with depression, 8 studies compared St. John's wort to placebo and six studies compared the results to first-generation tricyclic antidepressants, such as amitriptyline™, desipramine™, and imipramine™. Mild-to-moderate depression was defined as fewer symptoms standardized to the 17-time Hamilton Depression Rating scale (Williams et al., 2000). St. John's wort was found to be more effective than placebo for mild–to-moderate depression with a risk ratio (RR) of 1.9, meaning that the probability of an improvement in symptoms with treatment increased by 90%, with a 95% confidence interval (CI) from 1.2 to 2.8, using confidence intervals as a measure of robustness and stability of the estimate of the risk ratio. However, extracts of St. John's wort were as effective as the older tricyclic antidepressants (RR = 1.2, CI 1.0–1.4, with a risk ratio of 1.0 implying no statistical significance) (Williams et al., 2000). Tests for publication bias were statistically significant, meaning that publication bias might have spuriously overestimated the treatment benefits (Williams et al., 2000). The authors conclude that extracts of St. John's wort appear to be more effective than placebo for short-term treatment of mild-to-moderate depression, though they state that more research on clinical efficacy still needs to be conducted (Williams et al., 2000).

In contrast, in another systematic review of eight studies examining tricyclic antidepressants of moderately high methodologic quality, St. John's wort was found to be more effective than placebo in treating mild-to-moderate depression (Gaster

and Holroyd, 2000). The response rate to St. John's wort ranged from 23 to 55% higher than placebo, but compared to imipramine™ and amitriptyline™, the response rate ranged from 6 to 18% lower than the response rate to these tricyclic antidepressants (Gaster and Holroyd, 2000). This means that St. John's wort was more effective than placebo, but less effective than the tricyclic antidepressants for treating mild-to-moderate depression (Gaster and Holroyd, 2000).

In a review of systematic reviews and randomized controlled clinical trials, the evidence suggests that St. John's wort is superior to placebo for the short-term treatment of mild-to-moderate depression (De Smet, 2002). One of the studies demonstrated that St. John's wort was significantly more efficacious than placebo and as effective as imipramine™, an antidepressant, for treating moderate depression (De Smet, 2002). However, in studies of major depression, two methodologically rigorous randomized controlled trials of an 8-week treatment with St. John's wort did not demonstrate statistically significant differences when compared to placebo for outpatients with major depression of moderate severity (De Smet, 2002). Limitations of this review included the methodologic quality of the randomized controlled clinical trials under review and potential publication bias (De Smet, 2002). In addition, most trials excluded severely depressed patients (Goldman, 2001). This researcher concluded that based on the evidence cited, St. John's wort should not be substituted for a conventional antidepressant in patients with moderately severe or severe major depression (De Smet, 2002).

In the most recent review of systematic reviews and randomized controlled clinical trials in 2007, the evidence suggests that St. John's wort has greater efficacy than placebo and equal efficacy to low-dose tricyclic antidepressants, such as imipramine™, in most trials for treating mild-to-moderate depression (Mischoulon, 2007). In the last 5–6 years, about 10 randomized controlled double-blinded studies compared St. John's wort to the newer antidepressants, the selective serotonin reuptake inhibitors (SSRIs), as well as placebo. St. John's wort demonstrated equal efficacy compared to SSRIs and placebo in more recent studies, but this is likely because more severely and/or chronically depressed patients were recruited to the more recent studies (Mischoulon, 2007). Several other studies indicated that St. John's wort was as effective as selective serotonin reuptake inhibitors (SSRIs) for treating depression (Jorm et al., 2002).

In summary, most of the evidence suggests that St. John's wort was more effective than placebo, but as effective as standard antidepressants, for treating mild-to-moderate depression (Werneke et al., 2006; Sarris, 2007). The evidence for major depression is less well defined.

11.5.1.4 Mechanism

The mechanism of action for St. John's wort is still under investigation (De Smet, 2002; Mischoulon, 2007). Constituents of extracts of St. John's wort under investigation as the effective pharmacologic agents include hypericins, hyperforin (De Smet, 2002), polycyclic phenols, and pseudohypericin (Mischoulon, 2007). Some

studies indicate that the antidepressant activity of St. John's wort might be related to hyperforin (Goldman, 2001; Werneke et al., 2006; Mischoulon, 2007). It has been proposed that hyperforin inhibits the synaptic reuptake of several neurotransmitters, including serotonin, dopamine, and norepinephrine (Mischoulon, 2007). Hypericin has not been confirmed as the active ingredient for St. John's wort (Goldman, 2001). Hypericin might decrease the production of cortisol or inhibit reuptake of neurotransmitters, such as serotonin, norepinephrine, and dopamine (Mischoulon, 2007).

11.5.1.5 Safety and Tolerability

Uncommon and mild adverse events reported with use of St. John's wort as monotherapy include gastrointestinal symptoms such as constipation, dizziness or confusion, fatigue, dry mouth, restlessness, headache, allergic skin reactions, sexual dysfunction, frequent urination, swelling, and photosensitivity (De Smet, 2002; Mischoulon, 2007). Other possible adverse effects have also been reported, including serotonin syndrome or serotonin overload (De Smet, 2002), mania, or hypomania (Mischoulon, 2007).

In the past few years, adverse events between St. John's wort and other drugs have been increasingly reported in the literature (Mischoulon, 2007). St. John's wort has been found to interact with a number of conventional drugs, such as amitriptyline™ (an antidepressant), paroxetine™ (a selective serotonin reuptake inhibitor [SSRI] antidepressant) leading to serotonin syndrome, and simvastatin™ (an anticholesterol drug) (Goldman, 2001; De Smet, 2002; Eggertsen et al., 2007). St. John's wort has been reported to decrease the activity of several drugs including warfarin™ (an anticoagulant used to prevent blood clotting), oral contraceptives, theophylline™ (a drug to treat chronic obstructive pulmonary disease [COPD]), digoxin (a drug for the heart) (Mischoulon, 2007), and HIV protease inhibitors (drugs to treat human immunodeficiency virus [HIV] disease).

The Cochrane Library, Embase and phytobase databases, case reports, case series, clinical trials, or other types of human investigations relating to herbal supplement and prescription medication interactions were included in a literature review using Medline (an electronic scientific literature database) (Izzo and Ernst, 2001). The results indicated that St John's wort lowers blood concentrations of cyclosporine (an immunosuppressive drug used to prevent rejection of transplanted organs), amitriptyline™, digoxin™ (a cardiovascular drug), warfarin™, and theophylline™; and causes menstrual bleeding, delirium, or mild serotonin (a neurotransmitter) syndrome when used concomitantly with oral contraceptives, loperamide™ (an antidiarrheal drug), or selective serotonin-reuptake inhibitors (sertraline™, paroxetine™, nefazodone™, all are anti-depression drugs), respectively (Izzo and Ernst, 2001).

In a recent study, when healthy volunteers added St. John's wort to a regimen of the HIV protease inhibitor indinavir™, the serum level of indinavir™ decreased below the therapeutic concentration necessary for antiviral activity leading to

potential HIV treatment failure (Piscitelli et al., 2000). Following this report, the Food and Drug Administration (FDA) issued a public health advisory warning that St. John's wort appeared to induce cytochrome P-450 enzymes, liver enzymes responsible for the metabolism of many prescription medications including those used to treat heart disease, depression, seizures, and cancers, or to prevent transplant rejection or pregnancy (oral contraceptives). These prescription medications lose their therapeutic effects when given with St. John's wort (Talalay, 2001).

In an authoritative systematic review of 35 randomized controlled double-blinded studies, part of an update of a meta-analysis by the authoritative Cochrane Collaboration, experts on systematic reviews, on the use of hypericum extracts for depression, the rate of dropouts and adverse effects for patients receiving hypericum extracts was similar to placebo, less than that for older antidepressants such as amitriptyline™ and imipramine™, and similar to selective serotonin-reuptake inhibitors (Knuppel and Linde, 2004). Data from published case reports and national drug surveillance agencies suggest that the most relevant adverse effects of hypericum extracts were related to drug interactions and dermatologic reactions (Knuppel and Linde, 2004). Hypericum extracts had documented interactions with cyclosporine™ in transplant patients, warfarin™ (an anti-clotting drug), and selective serotonin reuptake inhibitors (SSRIs) resulting in serotonin syndrome (Knuppel and Linde, 2004). A potential mechanism for this drug interaction is the induction of a hepatic enzyme through the activation of the P450 cytochrome system which metabolizes drugs (Ernst, 2002) and the induction of P-glycoprotein which results in increased drug excretion (Knuppel and Linde, 2004). This mechanism could lead to decreased plasma levels of the drugs. The majority of other adverse effects were related to skin and allergic reactions, such as erythema (redness), dermatitis, urticaria (hives or itching edematous wheals), hyperesthesia (abnormally acute sensitivity to sensation such as touch or pain), and neuropathy (Knuppel and Linde, 2004). The authors conclude that hypericum extracts are well tolerated and safe if they are taken under the supervision of a physician who is aware of its potential risks. Further, the authors suggest that self-medication by patients might be acceptable for those with very mild depressive symptoms and who are not taking any other medication (Knuppel and Linde, 2004).

11.5.1.6 Current Status and Recommendations

In 2000, the American College of Physicians-American Society of Internal Medicine published a guideline stating that St. John's wort might be considered for short-term treatment of mild acute depression, though patients should be warned that this treatment is neither approved nor regulated by the Food and Drug Administration (FDA) and that the constituents in the extracts of St. John's wort may vary substantially from those tested in the randomized trials (Snow et al., 2000). Furthermore, the quality of the extract might be considerably different. Because the composition of St. John's wort extracts might vary among preparations that are produced and sold, the results of the systematic reviews apply only to those preparations analyzed in the studies that were reviewed. Because the FDA does not regulate

St. John's wort, unlike all medications prescribed by physicians, St. John's wort is not subject to randomized clinical trial testing for efficacy. Unlike prescription medications, no animal investigations, clinical trials, or post-marketing surveillance are required before herbal treatments are marketed to the public. Dietary supplements can be sold to the public without Food and Drug Administration approval.

With the high potential for adverse effects and drug interactions, clinicians should treat patients in a safe, evidence-based fashion. Because extracts of St. John wort might have adverse interactions with other drugs, individuals taking other drugs should consult their physician before using St. John wort (Linde et al., 2005a,b; Mischoulon, 2007). With nearly 70% of patients who use alternative therapies not informing their health-care providers about their use of herbal products (Eisenberg et al., 1993) it is imperative that health-care providers inquire into their patients' use of herbal treatments.

Health-care providers and their patients should proactively discuss the use or avoidance of complementary and alternative medicine (CAM) therapies. They should formally discuss patient's preferences and expectations, and in order to monitor for toxicity of CAM therapies; they should ask patients to maintain a symptom diary and see patients in follow-up visits (Eisenberg, 1997). Both patients and health-care providers must acknowledge that data on CAM therapy efficacy and toxicity remain incomplete and that recommendations remain a matter of best judgment and not fact.

Health-care providers should be vigilant about potential interactions between herbal products and prescription medications. Health-care provider can report potential interactions by calling FDA's MedWatch hotline at 1-800-FDA-1088 or using the web site http://www.fda.gov/medwatch/report/hcp.htm. The *MedWatch program* allows health-care providers to report problems possibly caused by FDA-regulated products such as drugs, medical devices, and medical foods in addition to dietary supplements. The identity of the patient is kept confidential. Patients may also report an adverse event or illness they believe to be related to the use of a dietary supplement by calling the FDA at 1-800-FDA-1088 or using the web site http://www.fda.gov/medwatch/report/consumer/consumer.htm. Health-care providers should recognize and report suspected interactions between herbal therapies and prescription medications since this leads to increasing knowledge and awareness of herbal treatment and medication interactions and ultimately, to improvement in the quality of patient care.

Future well-designed controlled studies of St. John's wort should address further clinical issues such as standardization of dosing and extracts of St. John's wort (Werneke et al., 2006), more specific reporting of adverse effects (Werneke et al., 2006), comparison with other antidepressant agents, including other selective serotonin reuptake inhibitors (Snow et al., 2000), effects of acute short-term and long-term therapy, effects on measures of functional status and quality of life (Gaster and Holroyd, 2000), efficacy in treating severe depression (Gaster and Holroyd, 2000), and efficacy in treating adolescents (Sarris, 2007) and minority populations. More studies are needed to test the efficacy of St. John's wort for treating major depression and its efficacy compared to other antidepressants (Gaster and Holroyd,

2000). Future well-designed controlled studies of St. John's wort should address epidemiologic methods issues such as using an improved definition of depression as an inclusion criteria (Werneke et al., 2006), strict use of the diagnostic criteria for defining major depression using DSM-IV criteria (Mischoulon, 2007), having longer observation periods, and further elucidating mechanisms of action.

In summary, studies of St. John's wort show promise as a potential treatment for mild-to-moderate depression (De Smet, 2002; Werneke et al., 2006; Mischoulon, 2007). However, St. John's wort might be less effective for treating severe depression or chronic depression though more research should be conducted (Mischoulon, 2007). The current lack of regulation by the Food and Drug Administration and lack of standardization of commercially available preparations remain a major barrier to implementing recommendations for physicians to prescribe St. John's wort to treat patients with depression (Gaster and Holroyd, 2000).

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Chapter 12
Regulating Phytonutrient Levels in Plants – Toward Modification of Plant Metabolism for Human Health

Ilan Levin

Abstract Plants constitute a major component of our diet, providing pigments and additional phytonutrients that are thought to be essential for maintenance of human health and are therefore also referred to as *functional metabolites*. Several fruit and vegetable species already contain high levels of several of these ingredients, while others do not. Nevertheless, efforts have been devoted to increasing and diversifying the content of phytonutrients, such as carotenoids, flavonoids, and vitamins, even in plants that normally produce high levels of such nutritional components. These efforts rely on transgenic and non-transgenic approaches which have exposed complex regulation mechanisms required for increasing the levels of functional metabolites in plants. The study of these regulatory mechanisms is essential to expedite improvement of levels of these metabolites in fruits, vegetables, cereals, legumes, and starchy roots or tubers. Such improvement is important for the following reasons: (1) to increase the efficiency of the industrial extraction of these compounds that are later being used as natural food supplements or fortifiers and as a source of natural colors to replace the chemical alternatives; (2) to improve and diversify the diet in populations of developing countries, where malnutrition may occur through lack of variety in the diet; (3) to provide fresh agricultural products such as fruits and vegetables highly enriched with certain phytonutrients to possibly substitute the chemically synthesized food supplements and vitamins; and (4) to provide an array of new and attractive colors to our diet.

Three basic approaches to modifying a biosynthetic pathway to increase amounts of desirable phytonutrients are available: (1) manipulation of pathway flux, including increasing, preventing, or redirecting flux into or within the pathway; (2) introduction of novel biosynthetic activities from other organisms via genetic engineering; and (3) manipulation of metabolic sink to efficiently sequester the end-products of particular metabolic pathways. These approaches have been effectively demonstrated in relation to the flavonoid and carotenoid biosynthetic pathways in

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tomato (*Solanum lycopersicum*). This chapter is therefore focused on carotenoids and flavonoids, their importance to human nutrition, and approaches used to induce, regulate, and diversify their content in tomato fruits. In addition, several examples of outstanding approaches employed to modulate carotenoid content in other plant species will also be given.

12.1 Introduction

Plants synthesize and accumulate an excess of 200,000 natural products (Fiehn, 2002). Plants also constitute a major component of our diet, providing fiber (i.e., cellulose, hemicellulose, and starch), carotenoids, flavonoids, vitamins, minerals, and additional pigmented and non-pigmented metabolites thought to promote or at least maintain good health (Willcox et al., 2003; Fraser and Bramley, 2004, Davies, 2007). These metabolites are referred to as *phytonutrients*, *functional metabolites*, *phytochemicals*, and lately also *nutraceuticals* (Davies, 2007), defined as certain organic components of plants that are thought to promote human health (The American National Cancer Institute drug dictionary at http://www.cancer.gov/drugdictionary/). Major examples of phytonutrient-rich plant foods and the principle phytonutrients which they accumulate are listed in Table 12.1.

Phytochemicals have been used, even as drugs, for centuries (Yonekura-Sakakibara and Saito, 2006). For example, Hippocrates (ca. 460–370 BC) used to prescribe willow tree leaves to abate fever. The active ingredient, salicin, with potent anti-inflammatory and pain-relieving properties was later extracted from the White Willow Tree (*Salix alba*) and eventually synthetically produced to become the staple over-the-counter drug called *Aspirin*. Noteworthy, the initial conceptual link between food and human health is also related to Hippocrates, who has been referred to as the "father of modern medicine". He stated, "Let thy food be thy medicine and thy medicine be thy food".

The recent completion of the human genome sequence and the advances made in high-throughput technologies brought about the area of *nutragenomics* that is predicted to uncover more precisely the possible relationship between human genetic makeup and nutrients, including phytonutrients. Meanwhile, efforts have been invested in increasing and diversifying the content of nutrients, such as carotenoids, flavonoids, tocopherols, minerals, fatty acids, phytosterols, and vitamins in both model and agricultural plant species (extensively reviewed with selected examples by Galili et al., 2002; Levin et al., 2006; Davies, 2007). While it is not at all clear whether these efforts would necessarily lead to agricultural products with better functional properties for human health benefits, they have exposed regulation mechanisms important for increasing and maintaining high levels of functional metabolites in plant products. The study of these regulatory mechanisms will have an important role in delivering functional attributes through foods, once better relationships between these ingredients and human health will be unraveled.

Table 12.1 Examples of phytonutrient-rich plant foods and the principle phytonutrients they accumulate

Plant food	Phytonutrients
Soybean	Protease inhibitors, β-sitosterol, saponins, phytic acid, isoflavones
Red apples, grapes, blackberries, blueberries, raspberries, red wine	Anthocyanins
Tomato	Lycopene, β-carotene, vitamin C
Broccoli	Vitamin C, 3,3′-diindolylmethane, sulforaphane, lignans, selenium
Garlic	Thiosulfonates, limonene, quercitin
Flax seeds	Lignans
Citrus fruits	Monoterpenes, coumarin, cryptoxanthin, vitamin C, ferulic acid, oxalic acid, flavanones
Corn, watercress, spinach, parsley, avocado, honeydew melon	Lutein, zeaxanthin
Broccoli, Brussels sprouts, kale	Glucosinolates, indoles
Garlic, onions, leeks, chives	Allyl sulfides
Blueberries	Tannic acid, lignans, anthocyanins
Sweet potatoes, carrots, mangos, apricots, pumpkin, winter squash	α-Carotene, β-carotene
Chilli peppers	Capsaicin
Cantaloupe, peaches, tangerines, papaya, oranges	b-Cryptoxanthin, flavonoids
Celery	Flavones
Tea, apple, cocoa	Flavanols
Beans, peas, lentils	Omega fatty acids, saponins, catechins, quercitin, lutein, lignans

Several plant foods already contain high levels of certain phytonutrients, while others do not (Davies, 2007). Nevertheless, efforts have been invested in increasing and diversifying the content of phytonutrients, such as carotenoids, flavonoids, and vitamins in several plant species, even in those that already contain high levels of one or several of these ingredients. The tomato fruit, for instance, is considered to be a good source of lycopene, vitamin C, β-carotene, folate, and potassium (Davies and Hobson 1981; Willcox et al., 2003). The tomato could also potentially be a good source for flavonoids as well (Jones et al., 2003; Willits et al., 2005; van Tuinen et al., 2006; Sapir et al., 2008). Nevertheless, efforts have been invested in increasing the content and diversifying phytonutrients, such as carotenoids and flavonoids, in the tomato fruit (Verhoeyen et al., 2002; Fraser and Bramley, 2004; Levin et al., 2004).

Increasing the levels of phytonutrients, such as lycopene in the tomato fruit, is highly justified from the perspective of the extraction industry due to cost-effectiveness reasons (Levin et al., 2006). Further enriching phytonutrients in plant species that already contain high levels of such ingredients is also directed to possibly substitute the chemically synthesized food supplements and vitamins in human populations that normally consume such supplements (Sloan, 2000; Levin et al.,

2006). Diversifying phytonutrients, including those that contribute to fruit color, can provide an array of new and attractive colors to our diet and also harness synergistic effects among phytonutrients which are important to human health. Increasing the levels of phytonutrients in plant species that normally do not contain high levels of these ingredients, including cereals, some legumes, and starchy roots or tubers/tuberous roots, is important in order to improve the diet in populations of people in developing countries, where nutrition is not diversified enough to provide all of the essential metabolites, primarily vitamins and minerals needed to maintain proper health (Davies, 2007). Due to these reasons, there is now a growing interest in the development of food crops with enhanced levels of phytonutrients. The tomato is an excellent candidate for the following reasons: (1) it is a major crop; (2) it is already a good source of several phytonutrients such as lycopene and vitamin C; (3) it contains many accessions with modulated levels of essential metabolites; (4) it can be easily modified by both classical genetic and transgenic means; and (5) it has been a subject of many studies aimed at increasing and diversifying the content of fruit phytonutrients, mainly carotenoids and flavonoids. Also, excellent analytical and genomics tools have been developed for tomatoes which can facilitate the molecular analysis of a certain gene modification. This chapter will therefore focus on factors that induce, regulate, and diversify carotenoids and flavonoids in tomato (*Solanum lycopersicum*) and their importance to human nutrition. A few outstanding examples of similar factors in other plant species will be also given.

Strategies to increase and diversify the content of either carotenoids or flavonoids in tomato fruits are reviewed here. These efforts rely on transgenic and non-transgenic approaches (i.e., use of spontaneous or induced mutations and/or quantitative trait loci affecting levels of these phytonutrients). The tomato light-responsive *high-pigment* (hp) mutations are an outstanding example of the latter alternative (Levin et al., 2003; 2004) and will therefore be presented in more detail. Due to their impact on fruit lycopene content, these hp mutations were already introgressed into elite tomato germplasm (Levin et al., 2003; 2006). Introgression of one of these hp mutations, hp-2^{dg}, into elite processing cultivars, characterized by an average fruit lycopene concentration of 80–90 $\mu g \cdot g^{-1}$ FW, resulted in cultivars with an average fruit lycopene concentration of up to 280 $\mu g \cdot g^{-1}$ FW, representing an up to 3.5-fold increase in fruit lycopene content. Most notably, recent studies also reinforce earlier ones suggesting that plants carrying these mutations are also characterized by higher levels of other health-promoting metabolites, such as flavonoids and vitamins (Bino et al., 2005). Further, and more recently, it was shown that cross-hybridizing light-responsive hp mutant plants with plants carrying either the Anthocyanin fruit (Aft) or the *atroviolacium* (atv) mutations, known to cause anthocyanin expression in tomato fruits, displayed a significant more-than-additive effect on the production of fruit anthocyanidins and flavonols (van Tuinen et al., 2006; Sapir et al., 2008). This effect was manifested and quantitatively documented as a remarkable ~5-, 19-, and 33-fold increase of petunidin, malvidin, and delphinidin, respectively, in the hp-1/hp-1 Aft/Aft double mutants compared to the cumulative levels of their parental lines (Sapir et al., 2008). These results underlie the importance of

light-responsive hp mutations in modulating phytonutrient content in plants, either on their own or in combination with other gene mutations.

Up to date, five light-responsive hp mutations have been discovered (Lieberman et al., 2004; Galpaz et al., 2008). These mutations, i.e., hp-1, hp-1^{w}, hp-2, hp-2^{j}, and hp-2^{dg}, were initially marked as lesions in structural genes of the carotenoid biosynthetic pathway (Stevens and Rick, 1986). However, more recent studies have demonstrated that they represent mutations in two evolutionary conserved regulatory genes active in light signal transduction, known also as *photomorphogenesis* (Mustilli et al., 1999; Levin et al., 2003; Lieberman et al., 2004). The identification of the genes that encode these hp mutant phenotypes has therefore created a conceptual link between photomorphogenesis and biosynthesis of fruit phytonutrients and suggests that manipulation of light signal transduction machinery may be very effective toward the practical manipulation of an array of fruit phytonutrients (Levin et al., 2003; 2006; Liu et al., 2004). Recent studies focusing on the manipulation of light signaling genes in tomato plants, cited in this chapter, support this approach.

12.2 Carotenoids

Carotenoids are orange, yellow, and red pigments that exert a variety of critical functions in plants. They comprise a class of lipid-soluble compounds within the isoprenoid family, which is one of the largest classes of natural products in the plant kingdom with over 22,000 known constituents (Connolly and Hill, 1992; Britton, 1998).

The isoprenoid family also includes gibberellins, phytosterols, saponins, tocopherols, and phylloquinones. Chlorophylls also contain an isoprenoic component, formed from the same precursor of the carotenoid metabolism, geranylgeranyl diphosphate (GGDP) (Fig. 12.1). In addition to their many functional roles in photosynthetic organisms, carotenoids have many industrial applications as food and feed additives and colorants, in cosmetics and pharmaceuticals, and as nutritional supplements (Galili et al., 2002). Carotenoids are C_{40} hydrocarbons with polyene chains that contain 3–15 conjugated double bonds. These double bonds are responsible for the absorption spectrum, and therefore the color of the carotenoid, and for the photochemical properties of the molecule (Britton, 1995).

The carotenoid backbone is either linear or contains one or more cyclic β-ionone or ε-ionone rings or, less frequently, the unusual cyclopentane ring of capsanthin and capsorubin that impart the distinct red color to peppers. Non-oxygenated carotenoids are referred to as *carotenes*, whereas their oxygenated derivatives are designated as *xanthophylls*. The most commonly occurring carotenes are β-carotene in chloroplasts and lycopene as well as β-carotene in chromoplasts of some flowers and fruits, e.g., tomatoes. The most abundant xanthophylls in photosynthetic plant tissues (lutein, violaxanthin, and neoxanthin) are key components of the light-harvesting complexes.

Carotenoids are synthesized in the membranes of nearly all types of the plant plastids and accumulate to high levels in chromoplasts of many flowers, fruits,

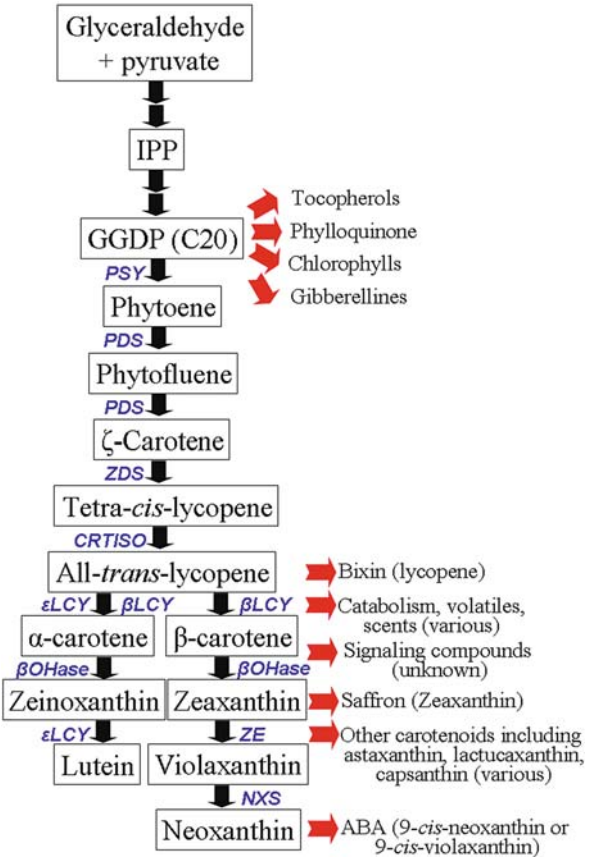

Fig. 12.1 A schematic presentation of the carotenoid biosynthetic pathway and its structural genes. Gene abbreviations: *CRTISO* = carotenoid isomerase, *βLCY* = *β*-lycopene cyclase, *εLCY* = *ε*-lycopene cyclase, *NXS* = neoxanthin synthase, *βOHase* = *β*-carotene hydroxylase, *PDS* = phytoene desaturase, PSY = phytoene synthase, *ZDS* = *ζ*-carotene desaturase, *ZE* = zeaxanthin epoxidase; Metabolite abbreviations: GGDP, geranylgeranyl diphosphate; IPP, isopentenyl diphosphate

and roots (Howitt and Pogson, 2006). They are involved in photosystem assembly, light harvesting and photoprotection, photomorphogenesis, non-photochemical quenching, lipid peroxidation, and affect the size and function of the light-harvesting antenna and seed set (Pogson et al., 1998; Havaux and Niyogi, 1999; Niyogi, 1999; Davison et al., 2002; Kulheim et al., 2002; Lokstein et al., 2002; Holt et al., 2004, 2005; Cuttriss and Pogson, 2006; Wang et al., 2008). In chromoplasts, carotenoids serve as pigments that furnish fruits and flowers with distinct colors in order to attract insects and animals for pollination and seed dispersal (Fraser and Bramley, 2004).

Animals as well as humans are unable to synthesize carotenoids de novo and rely upon the diet as a source of these compounds. Over recent years there has been considerable interest in dietary carotenoids with respect to their potential in alleviating age-related diseases in humans, propelling a market with an estimated yield of 100 million tons and a value of about US $935 million per annum (Fraser and Bramley, 2004). Although key carotenoids can be chemically synthesized, there is an increasing demand for the natural alternatives mainly those which are being

extracted or consumed from plants (Sloan, 2000). This attention has been mirrored by significant advances in cloning most of the carotenoid genes and in the genetic manipulation of crop plants with the intention of increasing their levels in the diet.

12.2.1 The Carotenoid Biosynthetic Pathway

During the past decade, a near-complete set of genes required for the synthesis of carotenoids in photosynthetic tissues has been identified, primarily as a result of molecular genetic- and biochemical genomics-based approaches in the model organisms such as *Arabidopsis* (*Arabidopsis thaliana*) and several agricultural crops such as the tomato. Mutant analysis and transgenic studies in these and other systems have provided important insights into the regulation, activities, integration, and evolution of individual enzymes and are already providing a knowledge base for breeding and transgenic approaches to modify the types and levels of these important compounds in agricultural crops (Dellapenna and Pogson, 2006).

In higher plants, carotenoids are synthesized from the plastidic isoprenoid biosynthetic pathway (Lichtenthaler, 1999; Fraser and Bramley, 2004, DellaPenna and Pogson, 2006). They are biosynthetically linked to other isoprenoids such as gibberellins, tocopherols, chlorophylls, and phylloquinones via the five-carbon compound isopetenyl pyrophosphate (IPP). Two distinct pathways exist for IPP production: the *cytosolic mevalonic acid pathway* and the *plastidic mevalonate-independent methylerythritol 4-phosphate (MEP) pathway*. The methylerythritol 4-phosphate pathway combines glyceraldehyde-3-phosphate and pyruvate to form deoxy-D-xylulose 5-phosphate, and a number of steps are then required to form IPP and dimethylallylpyrophosphate (DMAPP) (Lichtenthaler, 1999). IPP is subject to a sequential series of condensation reactions to form geranylgeranyl diphosphate (GGDP), a key intermediate in the synthesis of carotenoids, tocopherols, and many other plastidic isoprenoids (Fig. 12.1).

The initial steps of plant carotenoid synthesis and their chemical properties have been thoroughly discussed in several prior reviews (Cunningham and Gantt 1998; Hirschberg, 2001; Cunningham, 2002; Fraser and Bramley, 2004; Cuttriss and Pogson, 2006). Briefly, the first committed step in plant carotenoid synthesis is the condensation of two molecules of GGDP to produce phytoene (Fig. 12.1) by the enzyme *phytoene synthase* (PSY). Phytoene is produced as a 15-*cis* isomer, which is subsequently converted to all-*trans* isomer derivatives. Two plant desaturases, *phytoene desaturase* (PDS) and *ζ-carotene desaturase* (ZDS), catalyze similar dehydrogenation reactions by introducing four double bonds to form lycopene. Desaturation requires a plastid terminal oxidase and plastoquinone in photosynthetic tissues (Beyer, 1989; Norris et al., 1995; Carol et al., 1999). Bacterial desaturation differs from plants in that a single enzyme, crtI (*phytoene desaturase*), introduces four double bonds into phytoene to yield all-*trans*-lycopene (Cunningham and Gantt, 1998). This bacterial enzyme was therefore used as a target to increase lycopene and other carotenoids content in plant species as will be further outlined.

Until recently, the higher plant desaturases were assumed sufficient for the production of all-*trans*-lycopene. This conclusion was reached despite the accumulation of tetra-*cis*-lycopene in *tangerine* (*t*) tomato and algal mutants (Tomes et al., 1953; Cunningham and Schiff, 1985) and biochemical evidence to the contrary from daffodil (Beyer et al., 1991). Recently, the *carotenoid isomerase* gene, *CRTISO*, was identified in *Arabidopsis* and tomato, which catalyzes *cis–trans* isomerizations and resulting in all-*trans*-lycopene (Isaacson et al., 2002; Park et al., 2002).

In plants, the carotenoid biosynthetic pathway diverges into two main branches after lycopene, distinguished by different cyclic end-groups. Two beta rings lead to the β,β branch (β-carotene and its derivatives: zeaxanthin, violaxanthin, antheraxanthin, and neoxanthin), whereas one beta and one epsilon ring define the β,ε branch (α-carotene and its derivatives). These initial reactions are carried out by two enzymes: *β-lycopene cyclase* (βLCY) and *ε-lycopene cyclase* (εLCY) (Fig. 12.1). βLCY converts lycopene into β-carotene which is later converted to zeaxanthin by *β-carotene hydroxylase* (βOHase). An epoxide group is introduced into both rings of zeaxanthin by *zeaxanthin epoxidase* (ZE) to form violaxanthin. Conversion of violaxanthin to neoxanthin is performed by the enzyme *neoxanthin synthase* (NXS). Both the β- and ε-lycopene cyclase enzymes (βLCY and εLCY, respectively) are initially required to form α-carotene (Cunningham and Gantt, 1998; Pogson et al., 1996), which is being converted to lutein, via zeinoxanthin, by *β-carotene hydroxylase* (βOHase) and *ε-carotene hydroxylase* (εOHase) (Fig. 12.1).

Unlike the flavonoid pathway (see herein below), the regulation of carotenoid biosynthesis at the gene and enzyme level is poorly understood. No regulatory genes involved in carotenoid formation have been isolated thus far. It was reasoned that a heavily branched pathway such as that of carotenoids formation from isoprenoid precursors is unlikely to be controlled by a sole regulatory process (Fig. 12.1). Instead, it was suggested that control points, yet to be identified, are likely to exist at each branch point which probably involve both transcriptional and post-transcriptional regulation events (Fraser and Bramley, 2004). Despite this apparent complexity, several examples exist which resulted in an exceptional up-regulation of the carotenoid biosynthetic pathway by transgenic ("golden" rice) and non-transgenic approaches (the *Or* gene identified in cauliflower and the light-responsive hp mutations identified in tomato). These examples underlie the great potential of current knowledge to modulate levels of these important phytonutrients for the benefit of human health and will, therefore, be separately discussed in a later part of this chapter.

12.3 Flavonoids

Flavonoids comprise a group of plant polyphenols that provide much of the flavor and color to fruits and vegetables (Ross and Kasum, 2002). They are a large family of low-molecular-weight secondary metabolite compounds that are widespread throughout the plant kingdom, ranging from mosses to angiosperms (Koes et al., 1994). Their basic chemical structure, a $C_6-C_3-C_6$ configuration, consists of two

aromatic rings joined by a three-carbon link. This makes the flavonoids good hydrogen and electron donors. Based on their core structure, the aglycone, the flavonoids can be grouped into different classes, such as *flavones* (e.g., apigenin, luteolin), *flavonols* (e.g., quercetin, myricetin), *flavanones* (e.g., naringenin, hesperidin), *catechins* or *flavanols* (e.g., epicatechin, gallocatechin), *anthocyanidins* (e.g., cyanidin, pelargonidin), and *isoflavones* (e.g., genistein, daidzein) (Ross and Kasum, 2002). Within each group, single or combinatorial modifications of the aglycones, such as glycosylation, methylation and acylation, contribute to the formation of individual compounds.

Flavonoids are mainly responsible for the blue to purple, red, and yellowish colors in plants. *Proanthocyanidins* and their monomer units, catechins (Fig. 12.2), are the natural substrates of polyphenol oxidases and are, therefore, involved in the browning phenomenon of fruits.

To date, more than 6,000 flavonoids have been described and the number is still increasing. Notably, most of them are conjugated to sugar molecules and are commonly located in the upper epidermal layers of leaves and fruits as well as in seed coats (Stewart et al., 2000, Willits et al., 2005). In plants, flavonoids are involved in many aspects of growth and development, including pathogen resistance, pigmen-

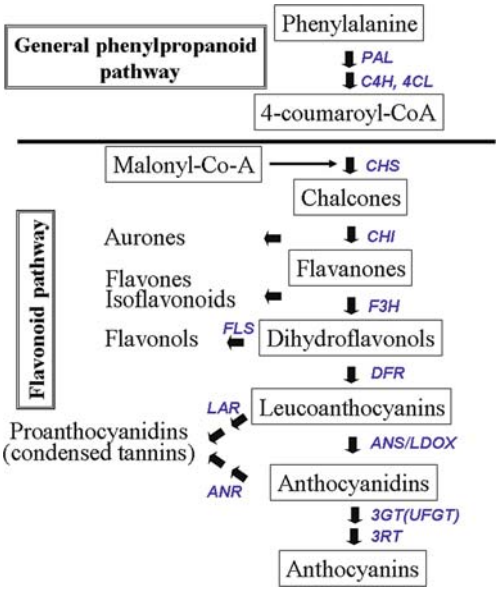

Fig. 12.2 A schematic presentation of the flavonoid biosynthetic pathway and its structural genes. Gene abbreviations: *ANR* = anthocyanidin reductase, *ANS/LDOX* = anthocyanidin synthase, *C4H* = cinnamate 4-hydroxylase, *4CL* = *4*-coumarate-*COA* ligase, *CHS* = chalcone synthase, *CHI* = chalcone isomerase, DFR = dihydroflavonol 4-reductase, *F3H* = flavanone 3-hydroxylase, *FLS* = flavonol synthase, *3GT (UFGT)* = *UDPG*-flavonoid-*3-O*-glucosyltransferase, *LAR* = leucoanthocyanidin reductase, *LDOX* = leucoanthocyanidin dioxygenase, *PAL* = phenylalanine ammonia lyase, *3RT* = anthocyanidin-3-glucoside rhamnosyl transferase

tation, and therefore attraction of pollinating insects, UV light protection, pollen tube growth, plant defense against pathogenic micro-organisms, plant fertility and germination of pollen, seed coat development, and in signaling for the initiation of symbiotic relationships (Harborne, 1986; Dooner et al., 1991; Koes et al., 1994; Dixon and Paiva, 1995; Parr and Bolwell, 2000; Schijlen et al., 2004).

Historically, flavonoids have been an attractive research subject mainly because of the colorful anthocyanins. These eye-catching pigments have been very useful in performing genetic experiments, including Gregor Mendel's study on the inheritance of genes responsible for pea seed coat color and the discovery of transposable elements interrupting maize pigment biosynthetic genes (McClintock, 1967; Lloyd et al., 1992; Koes et al., 1994).

The composition of flavonoids in different fruit species varies greatly (Macheix et al., 1990, Robards and Antolovich, 1997). The main anthocyanins in fruits are glycosides of six anthocyanidins that are widespread and commonly contribute to the pigmentation of fruits. Cyanidin is the most common anthocyanidin, the others being delphinidin, peonidin, pelargonidin, petunidin, and malvidin. Of the flavonols, quercetin, kaempferol, myricetin, and isorhamnetin are common in fruits, quercetin being the predominant flavonol. A third predominant flavonoid group in fruits is proanthocyanidins and their monomer units, catechins (procyanidin) or gallocatechins (prodelphinidins).

Delphinidin-derived anthocyanins are known to be responsible for the bluish colors, whereas cyanidin- and pelargonidin-derived anthocyanins are found in mauve and reddish tissues, respectively. Anthocyanins tend to form complexes with so-called co-pigments that can intensify and modify the initial color given by the pigment. Apparently, almost all polyphenols, as well as other molecules, such as purines, alkaloids, and metallic cations, have the ability to function as co-pigments. The final color of anthocyanins can also be affected by the temperature and pH of the vacuolar solution where they reside (Brouillard and Dangles, 1994; Brouillard et al., 1997; Mol et al., 1998; Cseke et al., 2006).

Because flavonoids impart much of the color and flavor of fruits, vegetables, nuts, and seeds, they form an integral part of the human diet (Parr and Bolwell, 2000). Rich dietary sources of flavonoids include soybean (isoflavones); citrus (flavanones); tea, apple, and cocoa (flavanols); celery (flavones); onion (flavonols); and berries (anthocyanins) (Table 12.1; Rice-Evans et al., 1996; Ross and Kasum, 2002; Le Gall et al., 2003).

12.3.1 The Flavonoid Biosynthetic Pathway

The flavonoid biosynthetic pathway has been almost completely elucidated and comprehensively reviewed (e.g., by Dooner et al., 1991; Koes et al., 1994; Holton and Cornish, 1995; Mol et al., 1998; Weisshaar and Jenkins, 1998; Winkel-Shirley, 2001). Many of the genes controlling this pathway have been cloned from several model plants including maize (*Zea mays*), snapdragon (*Antirrhinum majus*), petunia (*Petunia hybrida*), gerbera (*Gerbera hybrida*), and more recently, *Arabidopsis* (van

der Krol et al., 1988; Goff et al., 1990; Taylor and Briggs, 1990; Martin et al., 1991; Tonelli et al., 1991; Shirley et al., 1995; Elomaa et al., 1993, Helariutta et al., 1993, 1995; Holton and Cornish, 1995). These genes can be divided into two classes: (1) *structural genes* which encode enzymes that directly participate in the formation of flavonoids and (2) *regulatory genes* that control the expression of the structural genes.

An overview of the flavonoid pathway is presented in Fig. 12.2. Flavonoids are synthesized via the phenylpropanoid pathway, generating organic compounds that are biosynthesized from the amino acid phenylalanine. *Phenylalanine ammonia lyase* (PAL) catalyzes the conversion of phenylalanine to cinnamate. *PAL* also shows activity by converting tyrosine to *p*-coumarate, albeit with a lower efficiency. The *cinnamate 4-hydroxylase* (C4H) catalyzes the synthesis of *p*-hydroxycinnamate from cinnamate, and *4-coumarate:CoA ligase* (4CL) converts *p*-coumarate to its coenzyme-A ester, activating it for reaction with malonyl-CoA. The flavonoid biosynthetic pathway starts with the condensation of one molecule of 4-coumaroyl-CoA and three molecules of malonyl-CoA, resulting in the yellow-colored naringenin chalcone. This reaction is carried out by the enzyme, *chalcone synthase* (CHS), the key enzyme for flavonoid biosynthesis. In most plants chalcones are not the end-product, as the pathway proceeds with additional enzymatic steps to generate other classes of flavonoids, such as flavanones, dihydroflavonols, and finally, anthocyanins, the major water-soluble pigments in flowers and fruits and root crops like beets. Other flavonoid classes, i.e., isoflavones, aurones, flavones, proanthocyanidins, and flavonols, represent side branches of the flavonoid pathway and are derived from intermediates in anthocyanin formation (Fig. 12.2).

Naringenin chalcone is isomerized to the flavanone naringenin by the enzyme *chalcone isomerase* (CHI). Even in the absence of CHI, naringenin chalcone may spontaneously isomerize to form naringenin (Holton and Cornish, 1995). From these central intermediates, the pathway diverges into several side branches, each resulting in a different class of flavonoids. *Flavanone 3-hydroxylase* (F3H) catalyzes the stereospecific 3β-hydroxylation of flavanones to dihydroflavonols. For the biosynthesis of anthocyanins, *dihydroflavonol reductase* (DFR) catalyzes the reduction of dihydroflavonols to flavan-3,4-diols (leucoanthocyanins), which are converted to anthocyanidins by *anthocyanidin synthase* (ANS). The formation of glucosides is catalyzed by *UDP glucose-flavonoid 3-O-glucosyl transferase* (UFGT), which stabilizes the anthocyanidins by 3-*O*-glucosylation (Harborne, 1994; Bohm, 1998).

12.4 Health Benefits of Carotenoids and Flavonoids

Diet is believed to play an important role in the development of chronic human diseases (Willcox et al., 2003; Lila, 2007). It is now becoming recognized that certain fruits and vegetables can help prevent or treat chronic human diseases (Heber and Bowerman, 2001; Sloan, 2000; Lila, 2007). However, this recognition is primarily supported by in vitro and epidemiological studies, but by only a limited number of in vivo studies (Willcox et al., 2003). Nonetheless, it is currently believed that not

single components in plant-derived foods but rather complex mixtures of interacting natural chemicals are producing health-protective effects. These phytochemicals accumulate simultaneously in a plant, and they provide a multifaceted defensive strategy for both the plant and the human consumer (Heber and Bowerman, 2001; Lila, 2007).

Many phytochemicals are colorful, providing an easy way to communicate increased diversity of fruits and vegetables to the public (Joseph et al., 2003). These colors have provided various recommended color codes for plant-derived diet, advising consumers to ingest one serving of each color groups daily. For instance, a seven-color code was suggested by Heber and Bowerman (2001) which includes (1) red foods that contain lycopene, the pigment in tomatoes, which becomes localized in the prostate gland and may be involved in maintaining prostate health; (2) yellow-green vegetables, such as corn and leafy greens, that contain lutein and zeaxanthin, which become localized in the retina where age-related macular degeneration occurs; (3) red-purple foods containing anthocyanins, which are powerful antioxidants found in red apples, grapes, berries, and wine; (4) orange foods, including carrots, sweet potatoes, yams, mangos, apricots, pumpkin, and winter squash, which contain β-carotene; (5) orange-yellow foods, including oranges, tangerines, and lemons, which contain citrus flavonoids; (6) green foods, including broccoli, Brussels sprouts, and kale, which contain glucosinolates; and (7) white-green foods in the onion family that contain allyl sulfides. Interestingly five of the above color groups can be assigned to the carotenoid or flavonoid families of phytonutrients, underlying their importance for human nutrition.

Some members of the carotenoid family of compounds, such as β-carotene, are precursors (provitamins) of vitamin A. Following ingestion by humans and animals, β-carotene is being converted into vitamin A. Low dietary intake of fruits, vegetables, and preformed sources of vitamin A consumed from animals, can often lead to vitamin A deficiency that causes acute health disorders. Vitamin A deficiency is an endemic nutrition problem throughout much of the developing world, especially affecting the health and survival of infants, young children, and pregnant and lactating women. One of the earliest manifestations of vitamin A deficiency is impaired vision, particularly in reduced light (night blindness). Other health consequences of vitamin A deficiency include impaired immunity, xerophthalmia, keratomalacia, growth and developmental problems among children, and increased risk of mortality (Mayne, 1996; West, 2003; Wintergerst et al., 2007). Noteworthy, excessive intake of vitamin A, manifested as hypervitaminosis A, can also lead to health disorders such as birth defects, liver problems, and reduced bone mineral density. However, these toxicities are usually related to overconsumption of the preformed sources of vitamin A (i.e., retinyl esters from animal foods, fortified foods, and pharmaceutical supplements). Carotenoid forms, such as β-carotene as found in fruits and vegetables, usually give no such symptoms (Penniston and Tanumihardjo, 2006).

Studies carried out since 1970 displayed a correlation between high intake of carotenoids and health benefits. These studies have suggested that diets high in carotenoids reduce the risk of chronic diseases such as lung, breast, prostate, and colorectal cancers; cataract and macular degeneration; light-induced erythema; and

cardiovascular diseases (recently reviewed by Fraser and Bramley, 2004; Levin et al., 2006).

Recent studies have suggested that the consumption of tomatoes and tomato-based products reduces the risk of chronic diseases such as cancer and cardiovascular diseases. This protective effect has been associated with carotenoids, which are one of the major classes of phytochemicals in this fruit. The most abundant carotenoid in ripe-red tomato is lycopene, followed by phytoene, phytofluene, ζ-carotene, γ-carotene, β-carotene, neurosporene, and lutein (Khachik et al., 2002). Although the proposed health benefits of tomato and tomato-based products are usually related to lycopene, the possibility that other phytochemicals in the tomato fruit also contribute to these protective properties should not be ignored. A recent study, in which the effect of tomato lycopene on low-density lipoprotein (LDL) oxidation in vitro was compared with the effect of oleoresin (a lipid extract of tomato containing 6% lycopene, 0.1% β-carotene, and 1% vitamin E), provides evidence for a concerted and/or synergistic activity of phytochemicals. The tomato oleoresin exhibited higher capacity to inhibit LDL oxidation in comparison to pure lycopene, by up to fivefold. In addition, lycopene was shown to have a synergistic effect on LDL oxidation with vitamin E and, to a lesser extent, with β-carotene (Fuhrman et al., 2000). From a nutritional point of view, these findings reinforce the advantage of consuming tomato oleoresin rather than pure synthetic lycopene as a dietary supplement.

Lycopene, lutein, and zeaxanthin are the major carotenoids found in human blood and tissues and may be protective in degenerative eye diseases because they absorb damaging blue light. These carotenoids may also protect the skin from light-induced damage (Johnson, 2002; Sies and Stahl, 2003).

Carotenoids and flavonoids have been shown to play a role in preventing cardiovascular diseases due to their antioxidative property. These compounds may function individually, or in concert, to protect lipoproteins and vascular cells from oxidation, which is widely hypothesized to be one of the major causes of atherosclerosis. This hypothesis has been supported by studies that associate reduced cardiovascular risk with consumption of antioxidant-rich foods. Other cardioprotective functions provided by plant phytonutrients may include the reduction of LDL, homocysteine, platelet aggregation, and blood pressure (Willcox et al. 2003). Oxidation of the circulating LDL (LDL_{ox}) may play a key role in the pathogenesis of atherosclerosis and coronary heart disease. It is suggested that macrophages inside the arterial wall take up the LDL_{ox} and initiate the process of plaque formation. Dietary antioxidants such as vitamin E and β-carotene have been shown to prevent the formation of LDL_{ox} and their uptake by microphages in vitro (Rao, 2002). Healthy human subjects ingesting lycopene, in the form of tomato juice, tomato sauce, and oleoresin soft gel capsules, for 1 week had significantly lower levels of LDL compared with controls (Rao and Agarwal, 1998). At present, however, the role of lycopene in the prevention of coronary heart disease is strongly suggestive. Although the antioxidant property of lycopene may be one of the principal mechanisms for its effect, other mechanisms may also be involved. Lycopene was shown to inhibit the activity of an essential enzyme involved in

cholesterol synthesis both in vitro and in a small clinical study suggesting a hypocholesterolemic effect. Other possible mechanisms include enhanced LDL degradation, effect on LDL particle size and composition, plaque rupture, and altered endothelial functions (Rao, 2002).

Several studies focusing on dietary assessment suggest that the intake of tomatoes and tomato products may also be associated with a lower risk of prostate cancer. It is possible that lycopene is one of the compounds in raw and processed tomato products that may contribute to the lower risk of that type of cancer. However, this hypothesis remains to be further investigated. A recent study has also found an association between higher plasma lycopene concentrations and lower risk of prostate cancer, among older participants (>65 years of age) without a family history of prostate cancer (Wu et al., 2004). Several carotenoids have also been shown to have an effect on the immune response: β-carotene, lutein, canthaxanthin, lycopene, and astaxanthin are active in enhancing cell-mediated and humoral immune responses in animals and humans (Chew and Park, 2004).

There is an increasing evidence suggesting that flavonoids, in particular those belonging to the class of flavonols (such as kaempferol and quercetin), are potentially health-protecting components in the human diet as a result of their high antioxidant capacity (Rice-Evans et al., 1997; Lean et al., 1999; Sugihara et al., 1999; Dugas et al., 2000; Duthie and Crozier, 2000; Ng et al., 2000; Proteggente et al., 2002) and their ability, in vitro, to induce human protective enzyme systems (Cook and Samman, 1996; Manach et al., 1996; Janssen et al., 1998; Choi et al., 1999; Frankel, 1999; Hollman and Katan, 1999; Shih et al., 2000). Based on these findings, it was postulated that flavonoids may offer protection against major diseases such as coronary heart diseases and cancer (Hertog and Hollman, 1996; Steinmetz and Potter, 1996; Trevisanato and Kim, 2000; Singh and Agarwal, 2006). In addition, several epidemiological studies have suggested a direct relationship between cardioprotection and consumption of flavonols from dietary sources such as onion, apple, and tea (Hertog et al., 1993; Keli et al., 1996). In this respect, anthocyanins have received particular attention because of their very strong antioxidant activity as measured by the *oxygen radical absorbing capacity* (*ORAC*) assay. Antioxidants such as carotenoids and flavonoids are potentially useful agents in the management of human neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and schizophrenia because one of the factors increasing the incidence of those diseases is accumulation of oxidative damage in neurons (Levin et al., 2006).

The antioxidant activity of flavonoids is thought to slow the aging of cells and to protect against lipid peroxidation. In vitro studies have also shown that flavonoids can inhibit, and sometimes induce, enzymatic systems. They are thought to reduce the proliferation of certain types of tumor cells (Zava and Duwe, 1997; Kawaii et al., 1999) and to be involved in the apoptosis of HL-60 leukemia cells (Ogata et al., 2000).

The flavonoid quercetin, also present in the tomato fruit, was shown to have a strong inhibitory action against cholesterol oxidation, a process leading to the formation of oxysterols, a potentially cytotoxic, mutagenic, atherogenic, and possibly carcinogenic compound found in many commonly processed foods. A

supplementation with quercetin was shown to have a blood pressure lowering effect on spontaneously hypertensive rats (Duarte et al., 2001).

As outlined above, flavonoids comprise a group of plant polyphenols. Polyphenols are in general related to human health and were recently related to the *French paradox*. The *French paradox* refers to the observation that the French suffer relatively low incidence of coronary heart disease, despite having a diet relatively rich in saturated fats (Ferrières, 2004). This low incidence of coronary heart diseases was ascribed to consumption of red wine and was initially attributed to resveratrol, a non-flavonoid polyphenol naturally present in red wine. However, a recent study has identified a particular group of flavonoid polyphenols, known as oligomeric proanthocyanidins (condensed tannins) (Fig. 12.2), which are believed to offer the greatest degree of protection to human blood vessel cells and, therefore, to reduced coronary heart diseases (Corder et al., 2006).

In contrast to their suggestive positive effects, potential risks have been associated with excessive intake of carotenoids and flavonoids as supplements. For instance, harmful properties were found for β-carotene (provitamin A) in particular when given to smokers or to individuals exposed to environmental carcinogens. It was hypothesized that under these circumstances β-carotene was acting as a pro-oxidant rather than an antioxidant (Omenn, 1998). Flavonoids, at high doses, may also act as mutagens, pro-oxidants that generate free radicals, and as inhibitors of key enzymes involved in hormone metabolism. For example, although the protective effect of the flavonoid, quercetin, from oxidative stress has been strongly implied, its excessive intake is suggested to have an adverse effect on the body (Formica and Regelson, 1995; Skibola and Smith, 2000; Galati and O'Brien, 2004; Bando et al., 2007). It was further found that catechol-type compounds, including quercetin, are able to act as pro-oxidants by generating *reactive oxygen species* (*ROS*) and semiquinone radicals during the autocatalytic oxidation process (Guohua et al., 1997; Metodiewa et al., 1999; Kawanishi et al., 2005). Thus, the effect of dietary supplement of phytochemicals on human health should be further investigated, taking into account genetic and environmental factors, as well as specific sub-populations such as smokers. Nevertheless, a diet rich in fruits and vegetables as a natural source for those health-promoting phytochemicals is recommended (Heber and Bowerman, 2001; Riboli and Norat, 2003; Key et al., 2004; Srinath and Katan, 2004; Lila, 2007).

12.5 Approaches for Modification of Metabolite Biosynthesis

Strategies to increase and diversify the content of a certain metabolite in plants focused initially on (1) transgenic modulation of structural genes involved in its biosynthesis, (2) transgenic modulation of genes encoding transcription factors or other regulatory genes affecting its metabolic pathway, and (3) mutations (spontaneous or induced) in structural or regulatory genes and/or quantitative trait loci with pronounced effects on such metabolite levels. Recently, manipula-

tions of metabolic sink to efficiently sequester the end-products of the carotenoid biosynthetic pathway were also shown to be very effective in the accumulation of carotenoid compounds in fruits and vegetables (Lu et al., 2006; Diretto et al., 2007; Kolotilin, et al., 2007; Li and Van Eck, 2007; Simkin et al., 2007).

Modifying a biosynthetic pathway to increase the amount of a desirable compound may be further divided into (Davies, 2007) manipulation of its pathway flux within an organism or introduction of its biosynthetic genes from other organisms. The methods for increasing, preventing, or redirecting flux into or within the pathway include increasing levels of a rate-limiting biosynthetic enzyme, inhibition of the activity of a gene that competes for a limited substrate supply, and up- or down-regulation of the pathway using regulatory factors. For reducing production of undesirable compounds, the well-proven approach is to inhibit gene activity for one of the biosynthetic enzymes. *RNA interference* (*RNAi*) is an effective and reliable approach for preventing enzyme production, with examples of better performance than using antisense or sense-suppression constructs (Nakamura et al., 2006).

Successful genetic engineering of biosynthetic pathways requires knowledge of the production and accumulation of the metabolites of interest, the availability of DNA sequences encoding appropriate biosynthetic enzymes or regulatory factors, and gene transfer methods for the target species. Given a sufficient knowledge of the target system, predictive metabolic engineering approaches may be applied, in which data from metabolomics, transcriptomics, and proteomics are used to identify key targets, such as flux control points or regulatory proteins (Dixon, 2005). However, at present, the required information and tools are available only for a few pathways and crops. For most pathways, there is incomplete knowledge of the genes involved, key flux points, regulatory factors, and the impact of cellular compartmentalization or metabolic channeling. Thus, in many cases, a reiterative "trial and error" approach has usually been used to achieve a successful genetic engineering of biosynthetic pathways (Davies, 2007). A detailed checklist of tools and prior considerations needed to obtain a successful metabolic engineering of plant secondary metabolism has been lately presented which properly illustrates the complexity of this issue (Dixon 2005). This checklist includes understanding the target pathway, taking into account knowledge of pathway intermediates and the enzymes/genes associated with it, availability of precursors for an introduced pathway, the choice of the right gene to engineer in the case of multigene families, understanding of related competing pathways, prediction of spillover pathways, understanding the tissue or cell specificity of the pathway, availability of tissue-specific promoters, knowledge of the inter- and intra-cellular transport mechanisms for intermediates and end-products of the pathway, and knowledge of transcriptional regulators of the pathway and their targets.

Mutations (spontaneous or induced) in structural or regulatory genes of biosynthetic pathways as well as quantitative trait loci with pronounced effects on such phytonutrient levels have proven to be an excellent tool for both pathway engineering and gene identification (Table 12.2). Of particular interest are the

tomato light-responsive *high-pigment* (hp) mutations: hp-1, hp-1^{w}, hp-2, hp-2^{j}, and hp-2^{dg}. The identification of genes that cause these mutations has created a conceptual link between genes-related light signaling and overproduction of an array of fruit phytonutrients (Mustilli et al., 1999; Levin et al., 2003; 2004; 2006; Lieberman et al., 2004; Sapir et al., 2008). Due to their importance, these mutations and the transgenic modulation of light signaling genes to increase the functional properties of the tomato fruit will be separately discussed in this chapter.

Table 12.2 Gene identification and map location for selected mutants that increase or modulate carotenoid content in ripe tomato fruits

Mutation	Description	Gene	Map location	Reference
R	Yellow color of ripe fruit flesh	*PSY1*	Chromosome 3	Fray and Grierson, 1993
B	Orange color of fruits, fruit β-carotene highly increased	*βLCY*	Chromosome 6	Ronen et al., 2000
og, og^{c}	Corolla tawny orange, fruit β-carotene highly reduced, ∼15–20% increase in fruit lycopene content	*βLCY*	Chromosome 6	Ronen et al., 2000
DEL	Fruit color orange due to the accumulation of δ-carotene at the expense of lycopene	*εLCY*	Chromosome 12	Ronen et al., 1999
T	Fruit flesh and stamens orange colored. Fruits accumulate prolycopene (7Z,9Z,7′Z,9′Z-tetra-*cis*-lycopene) instead of the all-*trans*-lycopene	*CARTISO*	Chromosome 10	Isaacson et al., 2002
hp-1, hp-1^{w}	Fruit carotenoids, including lycopene, highly increased	*DDB1*	Chromosome 2	Lieberman et al., 2004; Liu et al., 2004
hp-2, hp-2^{j}, hp-2^{dg}	Fruit carotenoids, including lycopene, highly increased	*DET1*	Chromosome 1	Mustilli et al., 1999; Levin et al., 2003
hp-3	Fruits accumulate 30% more carotenoids	*ZE*	Chromosome 2	Galpaz et al., 2007
gh	Fruits milky white, fruit phytoene increased	*PTOX*	Chromosome 11	Josse et al., 2000; Mackinney et al., 956

12.5.1 Non-transgenic Approaches of Modulating the Carotenoid Biosynthetic Pathway in the Tomato Fruit

Fruit quality has been a major focus of most classical tomato breeding programs during the past century (recently reviewed by Foolad, 2007). Color and nutritional quality are among the major tomato fruit quality characteristics of interest. The attention to tomato fruit color has recently increased as the health benefits of lycopene, the major carotenoid in tomato that is responsible for the red fruit color, have become more obvious (Di Mascio et al., 1989; Levy et al., 1995; Stahl and Sies, 1996; Gerster, 1997; Kohlmeier et al., 1997). Several major genes with significant contribution to high contents of fruit lycopene (e.g., the genes encoding the hp and og^c mutant phenotypes) and other carotenoids (e.g., beta-carotene, B) were previously phenotypically identified and mapped onto the classical linkage map of tomato (Wann et al., 1985; Stevens and Rick, 1986). In addition, during the past two decades, numerous *QTLs* (*quantitative trait loci*) and candidate genes with significant effects on fruit color and/or lycopene content were identified in tomato wild accessions such as *S. pimpinellifolium*, *S. peruvianum*, *S. habrochaites*, *S. chmielewskii*, and *S. pennellii* and mapped onto tomato chromosomes along with the previously identified genes (Foolad, 2007). While some of the identified QTLs mapped to the chromosomal locations of many of the known genes of the carotenoid biosynthesis pathway, many mapped to other locations (Liu et al., 2003). It was therefore suggested that there might be more genes affecting fruit color in tomato than those known to affect the carotenoid biosynthesis pathway (Liu et al., 2003).

Tomato mutant accessions with divergent color phenotypes in their fruits were the subject of molecular genetic studies, leading to the identification of genes responsible for such phenotypes. A selection of such mutants, their gene identification, and map location are presented in Table 12.2, while their characteristic color is shown in Fig. 12.3. The sequence of these genes can now serve as recombination-free DNA markers to expedite breeding toward altering pigmentation and enhancing nutritional value of plant foods. Of particular interest are the light-responsive hp mutations that will be dealt with herein below. Another mutant that is becoming of special interest is *t* (*tangerine*), which produces orange-colored fruits accumulating prolycopene (7Z,9Z,7'Z,9'Z-tetra-*cis*-lycopene) instead of the all-*trans*-lycopene that accumulates in regular red-fruited tomatoes (Fig. 12.3; Isaacson et al., 2002). *cis* isomers of lycopene, thought to be powerful antioxidants, have been shown to be more bioavailable than the *trans* isomer, indicating that they are more efficiently absorbed and, therefore, deliver lycopene into the plasma more effectively. This might be interpreted to mean that *cis* isomers of lycopene are more beneficial and, therefore, more valuable to human health than the *trans* isomer (Ishida et al., 2007). Results recently published support the hypothesis that lycopene *cis* isomers are highly bioavailable and suggest that special tomato varieties can be utilized to increase both the intake and the bioavailability of health-beneficial carotenoids (Unlu et al., 2007). Because light-responsive hp mutants are characterized by higher total fruit carotenoids, hp-1/hp-1 *t/t* double mutant fruits share almost double the content of *cis* isomers of lycopene, in comparison to non-hp, +/+ *t/t*, mutant fruits,

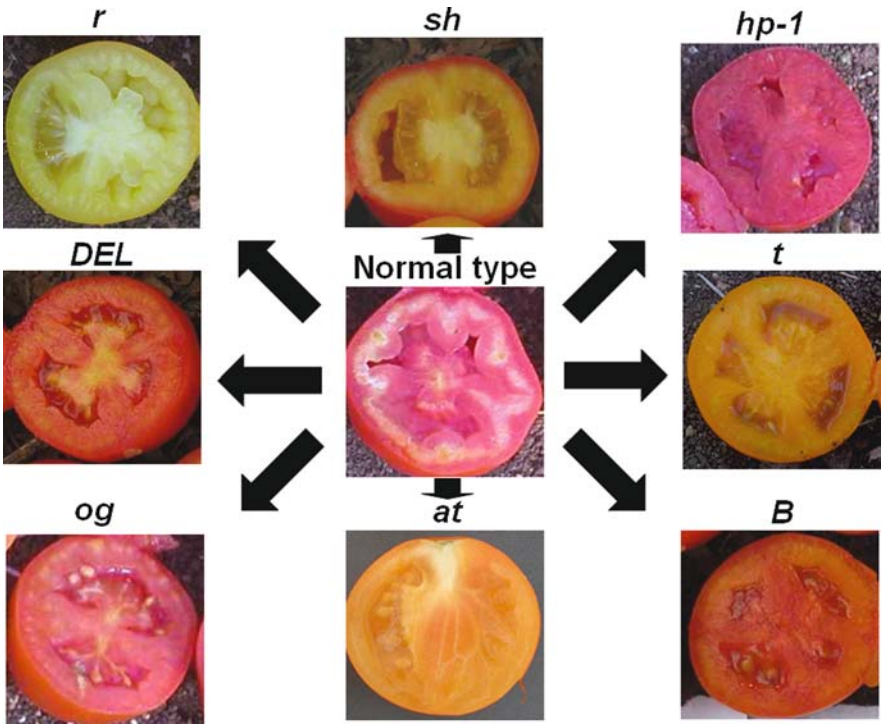

Fig. 12.3 Tomato fruit color mutants related to carotenoids biosynthesis. Abbreviations are as follows: *at* = *apricot*, *yellow-pink* color of fruit flesh; *B* = beta-carotene, high β-carotene, low lycopene in ripe fruit; *DEL* = delta, *Reddish-orange* mature fruit color, due to inhibition of lycopene, and increase of delta-carotene; *hp-1* = *high pigmen-1*, chlorophyll, carotenoids, ascorbic acid content of fruit intensified; *og* = *old gold*, increased fruit lycopene content; *r* = *yellow flesh*, yellow color of ripe fruit flesh; *sh* = *sherry*, fruit flesh yellow with reddish tinge; *t* = *tangerine*; fruit flesh and stamens orange colored

demonstrating the power of classical breeding to both modulate the profile and increase the content of selected carotenoids in the tomato fruit (Levin I, personal communication).

12.5.2 Non-transgenic Approaches of Modulating the Flavonoid Biosynthetic Pathway in the Tomato Fruit

Despite the relative success obtained in increasing flavonoid content in tomato fruits by transgenic modifications, there is an ongoing interest in breeding a high flavonoid tomato without genetic engineering (Willits et al. 2005). This interest is motivated by customers' reluctance to consume transgenic fruits and vegetables.

As recently summarized (Jones et al. 2003; Sapir et al., 2008), fruits of several tomato accessions, as well as species which are closely related to the cultivated

tomato, contain significantly higher amounts of anthocyanins (Giorgiev 1972; Rick 1964; Rick et al. 1994; Fig. 12.4). The Anthocyanin fruit (Aft, formerly Af) from *S. chilense*, Aubergine (ABG) from *S. lycopersicoides*, and the recessive *atroviolacium* (*atv*) mutation from *Lycopersicon cheesmaniae* cause anthocyanin expression in tomato fruit. We have also managed to introgress the trait of fruit anthocyanin expression from *S. peruvianum* accessions (Fig. 12.4), and recently, the wild species *S. pennellii* var. *puberulum* was shown to be a source for enriching tomato fruits with functional flavonoids (Willits et al., 2005).

Another approach to increase fruit flavonoids is through the introgression of the *high-pigment* (hp) mutations hp-1, hp-1^{w}, hp-2, hp-2^{j}, and hp-2^{dg}. These mutations are best known for their positive effect on carotenoid levels in ripe-red fruits (Levin et al., 2003; Mochizuki and Kamimura, 1984; van Tuinen et al., 2006; Wann et al., 1985). In addition, mature fruits of plants carrying the hp-1 mutation were also found to exhibit a 13-fold increase of the flavonol quercetin in tomato fruit pericarp relative to their isogenic counterparts (Yen et al., 1997). We have also shown similar increases in quercetin levels in fruits of the hp-2^{dg} mutant and in fruit skin of hp-2 and hp-2^{j} mutants (Bino et al., 2005; Levin et al., 2006).

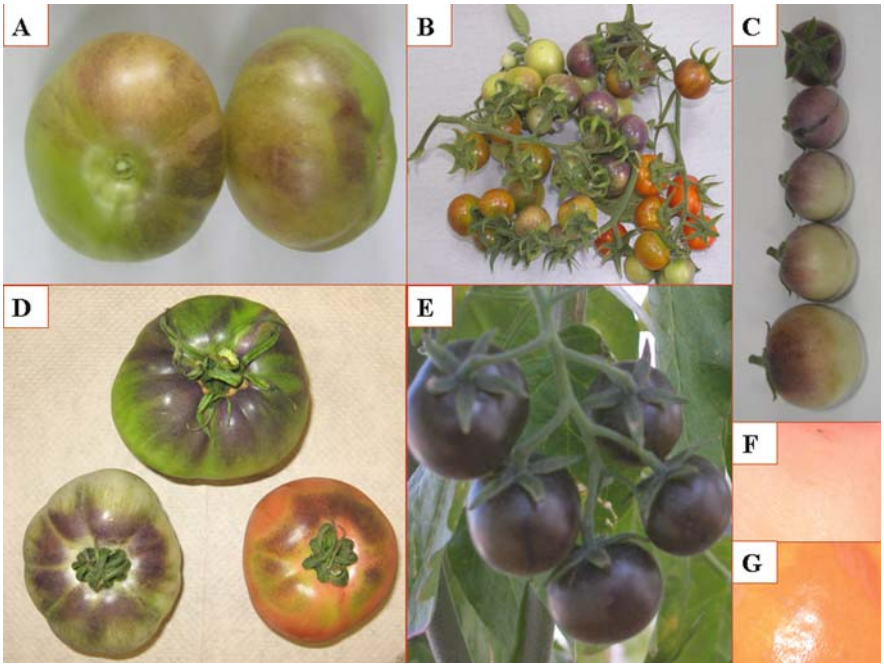

Fig. 12.4 Tomato fruit color phenotypes related to flavonoid biosynthesis. (**A**) Anthocyanin fruit (Aft) from *S. chilense*, (**B**) Aubergine (ABG) from *S. lycopersicoides*, (**C**) *S. peruvianum* (PI 128650), (**D**) Purple Smudge introgressed from *S. peruvianum*, (**E**) fruits of a double homozygous *AFT/AFT hp-1/hp-1* plant, (**F**) fruit skin from a tomato *y* mutant, and (**G**) fruit skin from regular tomato

Results recently presented in a textbook manuscript (van Tuinen et al., 2006), indicated that several phenolic compounds with high antioxidant capacity are new or increased in fruits of double mutant Aft/Aft hp-1^w/hp-1^w, as compared to fruits of single mutant parents. One of these compounds was identified as the flavonoid, rutin (van Tuinen et al., 2006). The hp-1^w mutation is as an extreme mutation (Lieberman et al., 2004), yielding plants with poor horticultural performances in comparison to its allelic hp-1 mutation. Thus, it has been of practical importance to also analyze the interaction between Aft and hp-1 mutants. We have recently shown that (Sapir et al., 2008) (1) Aft fruits are also characterized by significantly higher levels of the flavonols, quercetin and kaempferol, thus enhancing their functional value; (2) the tomato *ANT1* gene, encoding a MYB transcription factor, displayed nucleotide and amino acid polymorphisms between the Aft genotype, originating from *S. chilense*, and cultivated genotypes; (3) a DNA marker based on *ANT1* showed that the Aft trait is encoded by a single locus on chromosome 10 fully associated with *ANT1*; and (4) double homozygotes Aft/Aft hp-1/hp-1 plants displayed a more-than-additive (synergistic) effect on the production of fruit anthocyanidins and flavonols. This effect was manifested by ~5-, 19-, and 33-fold increases of petunidin, malvidin, and delphinidin, respectively, in the double mutants compared to the cumulative levels of their parental lines (demonstrated visually in Fig. 12.4).

Another important mutant related to the flavonoid biosynthetic pathway is the *y* mutant (Fig. 12.4). Fruits of this mutant are typified by colorless fruit epidermis, resulting in pinkish fruits that are preferred by consumers in most Asian countries. It is highly likely that the phenotype of the *y* mutant is attributed to major changes in the flavonoid pathway leading to the formation of naringenin chalcone, the yellow pigment accumulating in tomato fruit cuticle.

12.5.3 Metabolic Engineering of the Carotenoid Biosynthetic Pathway in Tomato

The economic value and health-promoting properties related to the tomato fruit make it an important target for increasing nutritional content either by traditional breeding or genetic manipulation. In view of the health-promoting properties of carotenoids and flavonoids, many attempts have been made to genetically modify the tomato fruit into overproduction of these phytochemicals. In most cases, this was achieved by modulating the expression of structural genes encoding biosynthetic enzymes of the dedicated pathway. In the carotenoid biosynthesis pathway, *phytoene synthase* (PSY), the enzyme that catalyzes the first committed step (Fig. 12.1), has been a preferred target for gene manipulation of the carotenoid biosynthetic pathway (Fraser and Bramley, 2004). The choice in PSY as such a target gene was also due to the fact that it exhibits the highest flux control coefficient among enzymes of the pathway, suggesting that it possess the greatest control over flux through the pathway (Fraser et al., 2002). Constitutive expression of

PSY in tomato plants resulted in earlier production of lycopene in the fruits of these transgenic plants, but the final concentration of lycopene was lower in these plants compared to their azygous controls. In addition, that manipulation has led to dwarfism, which is presumably caused by redirecting geranylgeranyl diphosphate (GGDP) from the gibberellin pathway into carotenoid synthesis (Fray et al., 1995; Fig. 12.1). In a later study, transformation of tomato plants with an additional PSY from *Erwinia uredovora* in a fruit-specific manner has led to a two to fourfold increase in total fruit carotenoids, whereas phytoene, lycopene, and β-carotene levels were increased 2.4-, 1.8-, and 2.2-fold, respectively. The transgene had no effect on the levels of related isoprenoids (tocopherols, plastoquinone, and ubiquinone), and the activities of other enzymes in the pathway were not significantly altered (Fraser et al., 2002).

Interestingly, increase in lycopene levels was also achieved by manipulation of the polyamines biosynthesis pathway. A two to threefold increase in lycopene was observed in tomato fruits expressing the yeast *S*-adenosylmethionine decarboxylase gene fused to a ripening-inducible *E8* promoter (Mehta et al., 2002).

Increase of β-carotene in tomato fruits has been achieved by various genetic manipulations. Constitutive expression of the bacterial phytoene desaturase (*PDS*) gene, which converts phytoene into lycopene, doubled the concentration of β-carotene in the fruit but halved the total carotenoid content. Interestingly, several endogenous carotenoid genes were up-regulated, except for PSY, which was repressed. These findings, coupled with the decrease observed in total carotenoids and the increase in β-carotene levels, suggest feedback inhibition within the pathway (Romer et al., 2000). Transgenic overexpression of the native lycopene β-cyclase gene in tomato fruit resulted in a 3.8-fold increase in the concentration of β-carotene, while the total carotene concentration was unchanged or slightly elevated. Transformation of an antisense construct of this same gene inhibited the enzyme expression by 50% and resulted in a slight increase in lycopene content (Rosati et al., 2000). Transgenic overexpression of the alternative lycopene β-cyclase has led to a greater increase in β-carotene concentration. That increase was accompanied by lower lycopene content in a manner that resembles the situation in the *B* mutant of tomato (Ronen et al., 2000). Recently, transgenic tomato lines containing a bacterial 1-deoxy-D-xylulose-5-phosphate synthase gene targeted to the plastid with the tomato DXS transit sequence resulted in increased carotenoid content (1.6-fold). Phytoene and β-carotene exhibited the greatest increases (2.4- and 2.2-fold, respectively). Extra-plastidic isoprenoids were unaffected in these lines (Enfissi et al., 2005).

Xanthophylls are an important class of target compounds, because of their antioxidant properties, their chemical stability, and the difficulties associated with their chemical synthesis. Metabolic engineering of xanthophyll content in tomato fruit was successfully achieved by overexpressing the *Arabidopsis* lycopene β-cyclase and the pepper β-carotene hydroxylase genes in a fruit-specific manner. This manipulation resulted in about tenfold increase in β-carotene level and accumulation of β-cryptoxanthin and zeaxanthin, two xanthophylls that were not detectable in the Moneymaker parental line (Dharmapuri et al., 2002).

12.5.4 Metabolic Engineering of the Flavonoid Biosynthetic Pathway in Tomato

There is a growing interest in producing food plants with increased amounts of flavonoids because of their potential health benefits. With several exceptions (Fig. 12.4), many tomato accessions contain only small amounts of flavonoids, which are usually produced in the peel of the fruit (Willits et al., 2005). Transformation of tomato plants with the chalcone isomerase (*CHI*) gene from *P. hybrida*, under the control of cauliflower mosaic virus (CaMV) 35S promoter, resulted in a dramatic increase in peel flavonoid levels. An up to 78-fold increase in flavonoids content was observed in transformed ripe-red fruits compared with the control, mainly due to an accumulation of rutin (Muir et al., 2001). In a different study, a significant accumulation of isoquercitrin, the immediate precursor to rutin, was achieved by ectopic expression of *CHI* (Olthof et al., 2000). Isoquercitrin is thought to be more bioavailable than rutin, the quercetin glycoside found in wild-type tomato peel and, thus, is considered to have a higher nutritional value. These studies show that ectopic expression of one gene, i.e., *P. hybrida CHI*, is sufficient to increase flavonol accumulation in tomato peel. However, no increase in flavonol levels were observed in leaves and in green, breaker, and turning tomato flesh from high flavonol transgenic plants, although relatively high levels of *CHI* transcripts were detected in these tissues. This indicates that, in tomato, flavonoid biosynthesis is subject to tissue-specific regulation and that in order to achieve a significant increase in flavonol accumulation in tomato flesh, a different approach is required (Willits et al., 2005). Indeed, flavonoid accumulation in tomato flesh, and hence an overall increase in flavonoid levels in tomato fruit, was achieved by simultaneous overexpression of the maize genes encoding the transcription factors LC and C1. LC and C1 are members of MYC- and MYB-type transcription factors families, respectively, that control the expression of several structural genes in the pathway leading to anthocyanins in maize (Dooner et al., 1991). Fruit-specific expression of both *LC* and *C1* genes had caused an up to 60-fold increase in kaempferol glycosides in tomato flesh tissue and an overall increase in total fruit flavonols of up to 20-fold (Bovy et al., 2002). In an alternative approach, genes encoding four key biosynthetic enzymes from *P. hybrida* leading to flavonols, chalcone synthase (CHS), CHI, flavanone-3-hydroxylase (F3H), and flavonol synthase (FLS), were ectopically and simultaneously expressed in tomato plants. About 75% of the primary transformants containing all four transgenes accumulated very high levels of quercetin glycosides in the peel and more modest but significantly increased levels of kaempferol- and naringenin-glycosides in columella tissue (Verhoeyen et al., 2002). That study has also shown that *CHS* and *FLS* appear to be the key genes leading to flavonol biosynthesis in tomato flesh (pericarp and columella) tissue. While ectopic expression of *CHS* alone resulted in increased levels of naringenin-glycosides, but no increase in flavonols, and the *FLS* transgene showed no significant effect by itself, expression of both *CHS* and *FLS* had a *synergistic effect* resulting in a significant accumulation of both naringenin glycosides and kaempferol glycosides (flavonols) in tomato flesh. Apparently, CHI is the key enzyme for flavonol accumulation in tomato peel,

while CHS and FLS enzymes are required for the production of flavonols in flesh tissue. Therefore, it was reasoned that, in order to achieve increased flavonol accumulation throughout the tomato fruit, ectopic expression of three genes encoding the biosynthetic enzymes CHS, CHI, and FLS would be needed. Indeed, cross harboring these three genes accumulates increased levels of quercetin glycosides in peel and kaempferol glycosides in flesh (Colliver, unpublished results). It is also noteworthy that a similar phenotype can be achieved by crossing tomatoes containing *LC* and *C1* transgenes with tomatoes containing the *CHI* transgene – the only structural gene for the production of kaempferol-type flavonols and pelargonidin-type anthocyanins that was not strongly induced by the LC/C1 transcription factors (Muir, unpublished results).

Further, T-DNA activation-tagging experiments in tomato identified a *MYB* transcriptional regulator of anthocyanin biosynthesis, termed *ANT1*, which shares high homology with *Petunia AN2* (Mathews et al., 2003). These ant1 mutant tomato

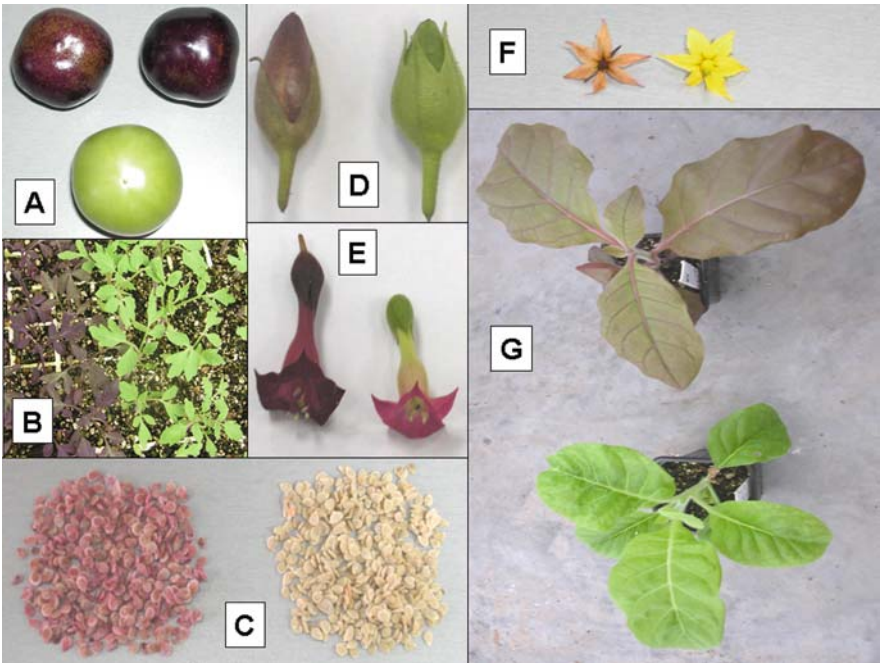

Fig. 12.5 Phenotypes obtained by overexpression of the *ANT1*gene in tomato and tobacco. (**A**) Transgenic tomato fruits (*upper two fruits*) in comparison to a non-transgenic fruit (*lower fruit*), (**B**) transgenic tomato seedlings (*left*) in comparison to their non-transgenic counterparts (*right*), (**C**) transgenic tomato seeds (*left*) in comparison to their non-transgenic counterparts (*right*), (**D**) a transgenic tobacco fruit (*left*) in comparison to its non-transgenic counterpart (*right*), (**E**) a transgenic tobacco flower (*left*) in comparison to its non-transgenic counterpart (*right*), (**F**) a transgenic tomato flower (*left*) in comparison to its non-transgenic counterpart (*right*), and (**G**) a transgenic tobacco seedling (*upper*) in comparison to its non-transgenic counterpart (*lower*)

plants yielded fruits with purple spotting on the fruit epidermis and anthocyanin-pigmented leaves and flowers. Overexpression of *ANT1*, controlled by the cassava vein mosaic promoter, generated similar phenotypes in Micro-Tom tomato plants and anthocyanin-pigmented leaves in tobacco plants. We have recently transformed the *ANT1* gene under the control of 35S promoter and obtained much stronger phenotypes in transformed Moneymaker tomato plants and similar phenotypes in tobacco plants. These results, which are presented in Fig. 12.5, visually demonstrate the immense potential of up-regulating the flavonoid biosynthetic pathway by transcription factors.

Recently, transgenic tomato plants accumulating new flavonoid compounds in their fruit peel were engineered using structural flavonoid genes from different plant sources (Schijlen et al., 2006). In this study, structural flavonoid genes (encoding *stilbene synthase*, *chalcone synthase*, *chalcone reductase*, *chalcone isomerase*, and *flavone synthase*) from different plant sources were used to produce transgenic tomatoes accumulating new phytochemicals. Biochemical analysis showed that the fruit peel contained high levels of stilbenes (resveratrol and piceid), deoxychalcones (butein and isoliquiritigenin), flavones (luteolin-7-glucoside and luteolin aglycon), and flavonols (quercetin glycosides and kaempferol glycosides). Using an online high-performance liquid chromatography (HPLC) antioxidant detection system, it was possible to demonstrate that, due to the presence of the novel flavonoids, the transgenic tomato fruits displayed altered antioxidant profiles. In addition, total antioxidant capacity of tomato fruit peel with high levels of flavones and flavonols increased more than threefold.

12.6 The Tomato Photomorphogenic Light-Responsive hp Mutants

Plants respond to light intensity, direction, duration, and spectral quality by modulating their developmental processes in an array of interactions that are referred to as *photomorphogenesis*. Photomorphogenic mutants have proven to be an excellent tool in the study of the complex interactions between light and plant development, and some of them have also been harnessed in several breeding programs of agricultural crops. Photomorphogenic mutants have been reported in a number of species, including *Arabidopsis*, *Sorghum*, *Brassica*, tobacco, tomato, and pea. In general, these mutants may be classified either as defective in photoreceptors or altered in some element of the light signal transduction pathway (Chory, 1993).

Several photomorphogenic mutants have been described in tomato (van Tuinen et al., 1997). Among these, mutants carrying the monogenic recessive *high-pigment* (hp-1, hp-1^{w}, hp-2, hp-2^{j}, and hp-2^{dg}) mutations are characterized by their exaggerated light responsiveness. These mutants display higher anthocyanin levels, shorter hypocotyls, darker foliage, and higher fruit pigmentation than their isogenic normal counterparts (Mochizuki and Kamimura, 1984; Wann et al., 1985, Peters et al., 1989, Mustilli et al., 1999; Levin et al., 2003). The high pigmentation of fruits of these mutants is due to significantly elevated levels of chlorophylls at most of their

pre-mature developmental stages. The mature ripe-red fruits of these mutants are characterized by an intense red color which is mainly due to increased levels of carotenoids, primarily lycopene. Because of their effect on fruit color, attributed to enhanced lycopene content, hp mutations have been introgressed into several commercial processing and fresh-market tomato cultivars that are currently marketed as lycopene-rich tomatoes (LRT) (Wann, 1997; Levin et al., 2006). The processing tomato varieties are primarily cultivated for the purpose of lycopene extraction, which is further used as food additive, food supplement, and food colorant in many processed products (http://www.lycored.com/). Current processing tomato cultivars harboring such mutations can reach a remarkable up to 3.5-fold increase in fruit lycopene content (from 80 to 280 $\mu g \cdot g^{-1}$ FW). Interestingly, this increase is higher than that reported thus far using the genetically modified alternatives discussed herein above.

The origins of hp-1, hp-1^{w}, hp-2, hp-2^{j}, and hp-2^{dg} mutations have been lately extensively summarized (Lieberman et al., 2004; Levin et al., 2006). Further, the hp-2, hp-2^{j}, and hp-2^{dg} mutations were mapped to the gene encoding the nuclear protein DEETIOLATED1 (DET1), a central negative regulator of photomorphogenesis (Mustilli et al., 1999, Levin et al., 2003). The gene encoding the hp-1 and hp-1^{w} mutant phenotypes has also been recently identified (Lieberman et al., 2004) and later independently confirmed by an additional laboratory (Liu et al., 2004). Results show that hp-1 and hp-1^{w} are alternative alleles at the tomato gene encoding UV DAMAGED DNA BINDING protein 1 (DDB1), recently shown to interact both biochemically and genetically with the DET1 protein (Schroeder et al., 2002, Liu et al., 2004). DDB1 is a protein evolutionally conserved from fission yeast to humans. It was initially identified, together with DDB2, as a subunit of a heterodimeric protein complex that recognizes the UV-induced DNA lesions in the nucleotide excision repair pathway. Mounting evidence has now established a major role of DDB1 as a substrate-recruiting subunit of the Cullin 4(CUL4)-based E3 ubiquitin ligase complexes that also contain RBX1 (also named ROC1), DET1 and, in the case of *Arabidopsis*, COP10 as well (Wertz et al., 2004; Hu et al., 2004; Bernhardt et al., 2006; Chen et al., 2006).

Tomato hp mutations (hp-1, hp-1^{w}, hp-2, hp-2^{j}, and hp-2^{dg}) are best known for their positive effect on carotenoid (lycopene and carotenes) levels in ripe-red fruits (Mochizuki and Kamimura, 1984; Wann et al., 1985, Levin et al., 2003). Interestingly, however, mature fruits of plants carrying the hp-1 mutation were also found to exhibit a 13-fold increase of the flavonoid, quercetin, in tomato fruit pericarp (Yen et al., 1997) and also some increase in ascorbic acid (vitamin C) (Mochizuki and Kamimura, 1984). In a study carried out during a summer season, similar increases were identified in quercetin levels in the fruit peel of the tomato mutants hp-2 and hp-2^{j} compared to their isogenic normal counterparts (Levin et al., 2006). These results suggest that other metabolites may be increased in tomato hp mutants. To validate this hypothesis, the overall metabolic modifications between hp-2^{dg} tomato mutant fruits and their isogenic non-mutant counterparts were compared (Bino et al., 2005). Targeted metabolite analyses, as well as large-scale non-targeted mass spectrometry (MS)-based metabolite profiling, were used to phenotype the differences

in fruit metabolite composition. Targeted high-performance liquid chromatography with photodiode array detection (HPLC–PDA) metabolite analyses showed higher levels of isoprenoids and phenolic compounds, as well as vitamin C, in hp-2^{dg} fruits. A selected list of such metabolites including their average levels in ripe-red fruits and their fold increase in hp-2^{dg} were presented in Levin et al. (2006). Non-targeted GC–MS profiling of red fruits produced 25 volatile compounds that showed a 1.5-fold difference between the genotypes (Bino et al., 2005). Analyses of red fruits using HPLC coupled to high-resolution quadruple time-of-flight mass spectrometry (LC–QTOF–MS) in both ESI-positive and ESI-negative modes generated, respectively, 6168 and 5401 mass signals, of which 142 and 303 showed a twofold difference between the genotypes. Of this total of 443 mass signals, 383 (∼86%) were up-regulated in the hp-2^{dg} genotype, while only 62 (∼14%) were found down-regulated in that mutant (Bino et al., 2005). Overall, these results show that the hp-2^{dg} fruits are more active metabolically and are characterized by overproduction of many metabolites, several of which are known for their antioxidant or photo-protective activities. It was hypothesized that these metabolites may serve as resources recruited by plants to respond to and manage light stress. Because hp-2^{dg} is highly iso-phenotypic to other tomato hp mutants, similar metabolic responses are also expected in these other mutants.

A transcriptional profiling study was also carried out on fruits harvested from hp-2^{dg} mutant plants in comparison to their isogenic counterparts using microarray technology (Kolotilin et al., 2007). Results show that a large portion of the genes that are affected by hp-2^{dg} mutation display a tendency for up- rather than down-regulation, indicating that this genotype is more active transcriptionally as well. Ontology assignment of these differentially regulated transcripts revealed a consistent up-regulation of transcripts related to chloroplast/chromoplast biogenesis and photosynthesis in hp-2^{dg} mutants throughout fruit ripening. A tendency of up-regulation was also observed in structural genes involved in phytonutrient biosynthesis. However, this up-regulation was not as consistent, positioning plastid biogenesis as a more important determinant of phytonutrient overproduction in hp-2^{dg} and possibly other hp mutant fruits. These results were linked to microscopic observations that revealed a highly significant increase in chloroplast/chromoplast size and number in pericarp cells of mature-green hp-2^{dg}/hp-2^{dg} and hp-2^{j}/hp-2^{j} fruits in comparison to their normal counterparts.

As noted herein, the identification of genes responsible for the light-responsive hp mutant phenotypes has created a conceptual link between light cues and overproduction of fruit phytonutrients, primarily those that accumulate in the plastids. It was further shown that in these mutants plastid biogenesis is the major determinant of the drive that increases these phytonutrients (Kolotilin et al., 2007). Interestingly, these concepts were also lately documented in the characterization of tomato plants mutated at the zeaxanthin epoxidase (*ZE*) gene (Galpaz et al., 2007) and its overexpression in an additional study (Wang et al., 2008). Fruits harvested from the mutant, termed hp-3, displayed 30% more carotenoids in the mature fruit compared to their isogenic normal counterparts. This increase in fruit carotenoids content was accompanied by at least a twofold increase in plastid compartment size (Galpaz et al.,

2007). In addition, constitutive overexpression of *ZE* in tomato plants characterized in a later study was found to display enhanced sensitivity of the tomato plants to photo-inhibition caused by high light stress (Wang et al., 2008).

12.6.1 Light Signal Transduction as a Target for Nutritional Enhancement

As indicated above, tomato hp mutants plants are characterized by overproduction of many metabolites, some of which possess antioxidant or photo-protective activities. The genes responsible for these mutations have been cloned and represent tomato homologs of light signal transduction regulatory genes, previously described in *Arabidopsis*. Therefore, targeting the light signaling pathway might be an effective approach to engineer fruit nutritional quality. Although carotenoid accumulation in edible plant tissues has been manipulated by altering corresponding biosynthetic enzymes (e.g., "golden" rice, Beyer et al., 2002), the outcome of such approaches has at times fallen short of expectations, as summarized above. This is probably because of a lack of understanding regarding endogenous mechanisms of regulation and accumulation of carotenoids and/or undesirable side effects on non-target metabolites derived from the altered pathway (Fray et al., 1995; Beyer et al., 2002; Liu et al., 2004). Engineering of an existing signal transduction network already capable of regulating flux through the carotenoid synthesis pathway in a biologically viable manner might represent an alternative to optimizing the carotenoid-associated nutritional benefit in plant tissues such as fruit (Liu et al., 2004). Indeed, recently it has been shown that manipulating tomato light signal transduction genes homologous to HY5 and COP1 from *Arabidopsis* can result in modified fruit carotenoid accumulation in tomatoes (Liu et al., 2004). Down-regulated LeHY5 plants exhibit defects in light responses, including inhibited seedling photomorphogenesis, loss of thylakoid organization, and reduced carotenoid accumulation. In contrast, repression of LeCOP1like expression results in plants with exaggerated photomorphogenesis, dark green leaves, and elevated fruit carotenoid levels. Manipulation of DET1 expression in tomato resulted in photomorphogenic phenotypes caused by post-transcriptional gene silencing and fruits with increased carotenoids (Davuluri et al., 2004). These results were later supplemented by fruit-specific RNAi-mediated suppression of DET1, resulting in increased fruit flavonoid content in addition to carotenoids (Davuluri et al., 2005).

Antisense tomato plants carrying the C-terminal portion of the tomato cryptochrome 1 (*TCRY1*) gene have also been characterized (Ninu et al., 1999). Synthesis of anthocyanins under blue light was reduced in antisense seedlings. In contrast, carotenoid and chlorophyll levels were essentially unaltered. Tomato cryptochrome 2 overexpression, on the other hand, resulted in a high-pigment phenotype, with overproduction of anthocyanins and chlorophyll in leaves and of flavonoids and lycopene in fruits. The accumulation of lycopene in fruits was accompanied by the decreased expression of lycopene β-cyclase genes (Giliberto et al., 2005). These results finally confirm the hypothesis that genes encoding

components of the light signal transduction machinery also influence fruit pigmentation and thus represent powerful tools for the manipulation of tomato fruit nutritional quality. Because light signaling genes are evolutionarily highly conserved, it seems reasonable that they may have an impact on the nutritional quality in plant species other than the tomato, including species that are distantly related to the tomato.

12.7 Outstanding Examples of Engineering Metabolic Pathways in Other Plant Species

Metabolic engineering of the carotenoid, flavonoid, and other metabolic pathways in the tomato and other species has been recently extensively reviewed (Galili et al., 2002; Fraser and Bramley, 2004; DellaPenna and Pogson, 2006; Yonekura-Sakakibara and Saito, 2006; Davies, 2007; Li and Van Eck, 2007). In addition to the tomato, efforts to up-regulate synthesis of carotenoids were also invested in agricultural species such as the potato (*Solanum tuberosum*) and rice (*Oryza sativa*), while synthesis of flavonoids was successfully up-regulated in potato and corn (*Z. mays*). In addition, major metabolic engineering efforts were carried out to modulate levels of other phytonutrients such as tocopherols, vitamin C, iron, selenium, and zinc (Davies, 2007; DellaPenna and Pogson, 2006; Li and Van Eck, 2007). Outstanding in this regard are the "golden" rice (Fig. 12.6), achieved by a transgenic approach, and the *Orange* (*Or*) gene mutation identified in cauliflower (*Brassica oleracea*, Fig. 12.6). In both cases, accumulation of high levels of β-carotene was conferred in tissues that are normally devoid or contain very low levels of carotenoids.

The apparent lack of high levels of carotenoid accumulation in low-pigmented tissues of crops such as rice endosperm and cauliflower curds could be due to (1) low metabolic flux into the carotenoid biosynthetic pathway, (2) high metabolic flux out of the carotenoid biosynthetic pathway into branching points and/or toward non-carotenoid end-products, (3) inactivation and absence of key genes in the biosynthetic pathway, and (4) lack of a deposition sink to efficiently sequester the end-products of the carotenoid biosynthetic pathway. While modulating metabolic flux by structural or regulatory genes of metabolic pathways was demonstrated above, and recently elsewhere (Davies, 2007; DellaPenna and Pogson, 2006), the "golden" rice and the *Or* gene mutation exemplify, respectively, the latter two possibilities.

The "golden" rice was named for its bright yellow endosperm due to the production and accumulation of β-carotene, a precursor of vitamin A, which is normally not produced in regular rice (Fig. 12.6). Engineering "golden" rice was designed to combat vitamin A deficiency in third-world Southeast Asian countries in which rice is a major nutritional commodity. The "golden" rice was first engineered with the insertion of the *PSY* gene from daffodil (*Narcissus pseudonarcissus*) and the bacterial *phytoene desaturase* (*CrtI*) gene from *E. uredovora*, which can catalyze three enzymatic steps from phytoene to all-*trans*-lycopene (Ye et al., 2000). The *PSY* gene was inserted under the control of an endosperm-specific glutelin

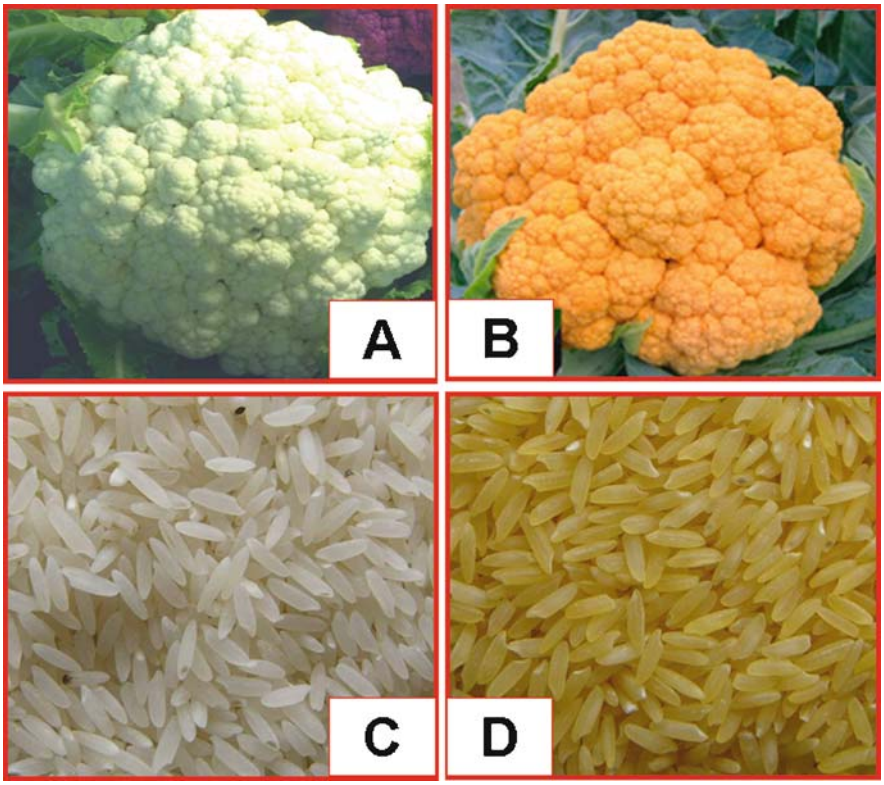

Fig. 12.6 Phenotypes of the *Or*mutant and the "golden" rice. (**A**) Regular cauliflower, (**B**) *Or* mutant cauliflower, (**C**) regular rice, and (D) "golden" rice

promoter, and in order to localize the gene product to the plastids (site of carotenoid biosynthesis), *CrtI* was designed as a fusion with the transit peptide of RUBISCO (ribulose-1,5-bisphosphate carboxylase/oxygenase) small subunit under the control of 35S promoter. An alternative construct was made by co-transformation with constructs carrying the *PSY/CrtI* gene, as described above, and the *LCY* gene under the control of a glutelin promoter. By the latter approach, the carotenoid content of edible rice endosperm was about 1.6 $\mu g \cdot g^{-1}$ dry weight (Ye et al., 2000).

In 2005, "golden" rice 2 was developed and the β-carotene content was increased up to 23-fold (about 37 $\mu g \cdot g^{-1}$ dry weight) compared to the original "golden" rice, a level adequate to provide the recommended dietary allowance of provitamin A for children in an average daily consumption of rice. The higher β-carotene content was achieved by choosing the maize *PSY* gene rather than the *PSY* genes from *Arabidopsis*, daffodil or the carotenoid-accumulating vegetables such as tomato, bell pepper, and carrot (Paine et al., 2005).

Recently, a similar approach has been employed to successfully produce "golden" potato tubers (Diretto et al., 2007). Earlier, seed-specific overexpression of a bacterial phytoene synthase gene (*crtB*) in a seed-specific manner produced

"golden" canola (*Brassica napus*) seeds containing up to 50-fold higher total carotenoids (Shewmaker et al., 1999).

Another novel alternative approach to increasing metabolites in plant tissues emerged from the recent work on isolation and functional characterization of the carotenoid gene mutation, denoted *Or,* in cauliflower (Fig.12.6; Lu et al., 2006). *Or* is a spontaneous semi-dominant mutation that confers the accumulation of high levels of β-carotene in various tissues normally devoid of carotenoids (Li and Van Eck, 2007).

The *Or* gene was found to encode a DnaJ cysteine-rich domain-containing protein. Rather than directly regulating carotenoid biosynthesis, the *Or* gene appears to mediate the differentiation of proplastids and/or non-colored plastids (leucoplasts) in apical shoot and inflorescence meristematic tissues of the curds into chromoplasts for the associated carotenoid accumulation (Lu et al., 2006; Li and Van Eck, 2007). Transformation of the *Or* gene into wild-type cauliflower converts the white color of curd tissue into distinct orange color with increased levels of β-carotene (Fig. 12.6). Examination of the cytological effects of the *Or* transgene revealed that expression of the *Or* transgene leads to the formation of large membranous chromoplasts in the cauliflower curd cells of the *Or* transformants (Lu et al., 2006). Interestingly, when the *Or* gene, under the control of a potato granule-bound starch synthase promoter, was introduced into potato, it resulted in the production of tubers with orange-yellow flesh (parenchymatous tissue). The total carotenoid levels in the *Or* transgenic potato lines were up to sixfold higher than in the non-transformed controls. Further examination of the cellular contents of these transgenic tubers by light microscopy showed that while the tubers in the controls contain exclusively various sizes of starch grains in amyloplasts, the *Or* transgenic tubers have additional orange bodies. These orange bodies include intact chromoplasts and a large number of more sharply outlined orange structures of helical sheets and fragments released from chromoplasts. These results and those of others have led to the conclusion that *Or* gene-associated carotenoid accumulation in these transgenic tubers is most likely due to the formation of carotenoid sequestering structures in chromoplasts, which provide a metabolic sink to facilitate accumulation of carotenoids. It was thus demonstrated that successful metabolic engineering of carotenoid accumulation can be also achieved by creating a metabolic sink (Li and Van Eck, 2007).

This conceptual approach was also recently tested in tomatoes following overexpression of fibrillin (Simkin et al., 2007). Fibrillin is involved in the formation of lipoprotein structures, such as plastoglobules and fibrils in certain chromoplast types, which have been implicated in the overproduction of pigments due to a sink effect. In order to examine its effect in differentiating chromoplasts of a non-fibrillar type, the pepper fibrillin gene was expressed in tomato fruits. Both the transcript and protein were found to accumulate during tomato fruit ripening from an early mature-green stage. However, formation of carotenoid deposition structures in tomato chromoplasts, such as fibrils, was not observed. Nevertheless, a twofold increase in carotenoid content and associated carotenoid-derived flavor volatiles (6-methyl-5-hepten-2-one, geranylacetone, β-ionone, and β-cyclocitral) was observed. The transgenic fruit displayed delayed loss of thylakoids in differentiating chromoplasts,

leading to the transient formation of plastids exhibiting a typical chromoplastic zone adjacent to a protected chloroplastic zone with preserved thylakoids. These results therefore suggest that fibrillin may protect plastids against degradation, thus extending their carotenoid production life span and leading to greater carotenoid accumulation. In this respect, the recent transcriptional profiling carried out on hp-2^{dg}fruits has underlined plastid number as the main contributor to plastid-accumulating phytonutrients (Kolotilin et al., 2007). This study has further shown that in mature-green fruits harvested from hp-2^{dg} mutant plants, the plastid compartment size is 8.4-fold higher as compared to its normal counterpart, suggesting a similar potential to increase fruit carotenoid content. However, upon ripening, a sharp decrease was observed in plastid compartment size in fruits of hp-2^{dg}, primarily attributed to a sharp decrease in plastid number, which was much more attenuated in their normal counterparts. Ripe-red fruits of the hp-2^{dg} mutant were characterized by only ~2.8-fold increase in chromoplast compartment compared to their normal counterpart. This increase corresponds to the 2.3-fold increase usually observed in total carotenoids between these genotypes at this ripening stage. These results cumulatively suggest that prevention of the enhanced plastid degradation observed upon ripening in hp-2^{dg} mutant fruits could potentially be a target to increase carotenoid accumulation in these mutant fruits. Such prevention of plastid degradation could be possibly achieved via overexpression of fibrillin in hp-2^{dg} mutant plants. An alternative approach to achieve higher carotenoid accumulation in hp-2^{dg} mutant plants could be via overexpression of DnaJ to create an alternative metabolic sink.

12.8 Concluding Remarks and Perspectives

It is now becoming recognized that consumption of fruits and vegetables can prevent or even be used to treat chronic human diseases. However, this recognition is mainly supported by in vitro and by epidemiological studies that seem to vary between sub-populations. There is therefore a need for more clinical in vivo trials to substantiate these effects on a whole organism basis and in different human sub-populations. There is also a need to formulate appropriate directives for recommended daily allowance for each metabolite in each sub-population.

It is predicated that the recent completion of the human genome sequence, the advances made in high-throughput technologies, and the emerging area of nutragenomics will uncover more precisely the possible relationship between human genetic makeup and the type and quantity of phytonutrients needed to maintain proper health. This may position phytonutrient consumption behavior in humans more at the level of pharma- rather than nutraceuticals with recommendations for a critical dosage rather than a daily allowance. In other words, food may become medicine and vice versa, in accordance with Hippocrates statement, “Let thy food be thy medicine and thy medicine be thy food”.

Meanwhile, transgenic genetic modifications (GMO) have already been exploited and found to be useful in enriching and diversifying the content of phytonutrient metabolites in a variety of plant species. As outlined in this chapter, these

modifications can be justified, but it is not entirely clear whether consumption of plant foods highly enriched with a certain phytonuterients will indeed contribute to maintenance of proper health and/or to treat chronic human diseases.

Despite the relative success obtained in increasing the phytonutrient content of plant foods by GMO modifications, consumers, in particular those that share higher health awareness, are reluctant to consume transgenic plant foods. Luckily, several genetic resources such as the tomato light-responsive hp mutants and the *Or* gene mutation identified in cauliflower show that there are efficient non-GMO alternatives to increase phytonutrient content in plant foods. Of particular interest are the tomato hp mutants characterized by higher levels of both carotenoids and flavonoids in the fruits. Moreover, ripe-red fruits, harvested from these mutants, also display increased levels of several other metabolites, including vitamins C and E. Thus, consumption of fruits of this type may maximize positive synergistic health effects that were already documented among several of these phytonutrients.

The genes that cause hp mutant phenotypes were cloned and identified as two evolutionarily conserved genes active in light signal transduction, known also as photomorphogenesis. The identification of the genes that encode the hp mutant phenotypes has therefore created a conceptual link between photomorphogenesis and biosynthesis of fruit phytonutrients and thus point to modulation of light signal transduction machinery as an effective approach toward practical manipulation of the kinds and amounts of fruit phytonutrients. The high-evolutionary conservation of these genes also suggests that similar effects may be obtained by manipulating these genes in plant species other than the tomato either by transgenic or non-transgenic methodologies.

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Part IV
Risks and Benefits Associated with Plant Biotechnology

Chapter 13
Risks and Benefits Associated with Genetically Modified (GM) Plants

Peter B. Kaufman, Soo Chul Chang, and Ara Kirakosyan

Abstract Genetically modified (GM) plants are those whose genomes have been modified by the introduction of foreign DNA constructs derived from bacteria, fungi, viruses, or animals. The most common genetically modified plants include soybeans, maize/corn, rapeseed mustard, potatoes, cotton, sugarcane, tomato, rice, and aspen/*Populus*.

In this chapter, we list 16 goals of genetic engineers in developing GM plants. These are plants that manifest frost hardiness; insect and herbicide tolerance; virus resistance; altered starch, cellulose, and lignin production; altered levels and kinds of oils and proteins in seed crops; higher levels of antioxidants in edible fruits, synthesis of new metabolites like beta-carotene in rice grains and vaccines in non-edible plants; and sequestration of hazardous wastes from polluted ("brown field") areas.

The next section of this chapter includes a discussion of the purported benefits and risks of GM plants. Our goal here is to present this information in as balanced a fashion as possible.

Lastly, we address important questions and answers concerning GM plants and food products.

13.1 Genetically Modified Plants: What Are They and Goals for Their Production

Genetically modified (GM) plants are those whose genomes have been modified by the introduction of foreign DNA constructs derived from bacteria, fungi, viruses, or animals. The most common genetically modified plants include soybeans, maize/corn, rapeseed mustard, potatoes, cotton, sugarcane, tomato, rice, and aspen/*Populus*. It is important to emphasize here that the generation of transgenic plants can modify natural selection-induced evolution of plants.

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The *goals of genetic engineers in developing genetically modified plants* include the following:

- To develop herbicide-resistant crop plants such as "Roundup Ready"/glyphosate-resistant soybeans with a gene derived from bacteria.
- To develop insect-resistant crop plants such *Bt* (*Bacillus thuringinensis* or milky spore bacterium) cotton (to kill the cotton boll weevil), potatoes (to kill the Colorado potato beetle), and corn/maize (to kill the corn ear worm). *Bt*-containing plants can cause emergence of *Bt*-resistant insects. In "Botany of Desire" by Machael Pollan, he reports that Monsanto Company designed a method to avoid this. They suggest that farmers allot a part of a field for wild-type plants, while most of the field is used for *Bt*-containing transgenic plants. This strategy can cause wild-type insects to outcompete *Bt*-resistant mutant insects.
- To develop viral disease-resistant plants such as papaya and squash that have virus coat protein gene inserted into their genomes. The virus coat protein gene that is inserted into these plants prevents the virus from reproducing because it cannot make coat protein. The mechanism here is called *co-suppression.*
- To develop plants with enhanced levels of an essential vitamin such as "golden rice" that produces significant amounts of B-carotene/vitamin A in the grains based on the introduction of two foreign genes from daffodil and one from a bacterium. "Golden rice" – enrichment with carotenoids (provitamin A): This project produced a rice cultivar with enhanced levels of beta-carotene and other carotenoids, which are metabolic precursors of vitamin A. Vitamin A can be absorbed with fat by the human body. Because rice naturally contains only a negligible amount of beta-carotene, vitamin A deficiency is widespread in regions of the world where rice is a staple food. "Golden rice" was developed for people of underdeveloped countries. However, the primary drawback here is that people who live in underdeveloped countries do not have chance to have such well-nourished foods such as "golden rice" primarily due to its high cost.
- To develop plants with enhanced frost resistance, GM-frost resistance has been achieved by research scientists (Professor German Spangenberg and Dr Ulrik John of the Victorian AgriBiosciences Centre at La Trobe University) in Victoria, Australia, through the use of a gene sequence from Antarctic hairgrass (*Deschampsia antarctica*, *Poaceae*, tribe *Aveneae* (Oats)). The same degree of frost tolerance is achieved in cereals and grasses. Frost tolerance is activated by a protective protein that is activated once the temperature drops below 5°C, and the plant then has the ability to inhibit ice crystal growth which gives the plant its freezing tolerance.
- To develop plants with altered composition of sugars or starch. GM potato plants (EH 92-527-1) have been engineered to have tubers with a higher ratio of branched starch (amylopectin) to straight-chain starch (amylose). These plants have not been approved for human consumption and were developed primarily

for production of starch for industrial purposes. Overexpression of the sucrose-6-phosphate synthase (SPS) gene from maize in tomato, and the same gene from spinach in tobacco and potato, led to significant increases in sucrose biosynthesis in the transgenic plants (see Chapter 4).

- To develop tree crops with altered composition of lignin and cellulose in their wood (xylem) tissues through alterations in lignin biosynthesis (e.g., reduction, augmentation and/or structural changes) and cellulose biosynthesis (e.g., augmentation, reduction, and/or quality including high degree of polymerization and crystallinity).
- To develop crop plants whose fruits have longer shelf life due to decreased action of cell wall hydrolases such as cellulases and polygalacturonases (pectinases). The FlavrSavr® tomato is the most famous example. These tomatoes were the first GM fruit sold in the United States and were sold as tomato purée in the UK. Apples, raspberries, and melons with delayed ripening have also been developed.
- To develop seeds with "Terminator" or other sterilizing traits in crops and ornamentals so as to prevent collection of seeds of these plants and thus protect the interests of the owners of patents for such seeds.
- To develop plants with modified oil content and composition (e.g., polyunsaturated fatty acids such as linoleic acid and laureic acid) for maize, soybeans, rapeseed, and other oil crops: These modified crops could be important in the fight against cardiovascular disease, obesity, and certain forms of cancer.
- To develop plants with higher content of protein or amino acids, or modified amino acid composition for enhanced nutritional value: For example, a GM potato was developed in India containing more than one-third of protein including essential, high-quality nutrients. The novel gene came from the protein-rich grain amaranth plant. Another example is LY038, a maize line with enhanced lysine content for improved animal feed quality. It is now awaiting authorization in the EU.
- To develop gluten-free wheat: Celiac sprue patients cannot tolerate the protein gluten (something similar to an allergy).
- To develop higher levels of beneficial antioxidant compounds (e.g., lycopene, flavinols found in tomato) to prevent cardiovascular diseases and certain forms of cancer.
- To eliminate or reduce undesirable substances like allergens or toxic substances (e.g., caffeine, nicotine).
- To develop transgenic "amylopectin potato" that contains almost exclusively amylopectin (an increase from 75 to 98%) rather than a mix of different starches (amylose and amylopectin). This starch will be used for paper, textiles, and adhesives.
- To develop GM rapeseed oil with high erucic acid content. This oil is used in plastics and in high-grade industrial lubricants.
- To develop plants with higher carbon fixation rates via photosynthesis.
- To introduce into C-3 plants C-4 photosynthesis capabilities.

13.2 Benefits and Risks of Genetically Modified Plants

A number of positive and negative issues are associated with plant biotechnology and its applications (Ellstrand, 2000; Gadgil, 2000). The positive issues concern the major benefits that plant biotechnology may contribute to industry, agriculture, and the environment. One of the significant issues is concerned with economically feasible production of crops, pharmaceuticals, and other industrial products (Arber, 2009). We are able to grow more tolerant crops with new traits or produce important vaccines using plant cell factories. Other positive impacts on the environment may involve clearance of contaminated soil by means of phytoremediation as described in Chapter 7. Plants have been found to break down or degrade organic contaminants (similar to microbes), while others are able to extract and stabilize toxic metal contaminants by acting as traps or filters.

Scientists engaged in plant biotechnology should follow all regulatory mandates as well as socially and ethically acceptable targets (Boulter, 1995). Thus, the risks must be evaluated in connection with workplace safety, environmental contamination, and public exposure. In conducting risk assessments, it is important to consider all normal and foreseeable abnormal operations, maintenance, cleaning, and security. Following completion of a risk assessment and risk characterization, development of effective risk management programs, such as implementation through training and selection of risk management tools, is needed.

The criteria for *risk assessment* are based on both genetically modified and original unmodified plants, their potential environmental interactions, as well as the possible effects of plants or their products on the human health (Magaña-Gómez and de la Barca, 2009). Characterization of the novel trait plays a central role in the assessment process and overlaps the assessment of the modified plant. The main criteria assigned pertain to information about the genetic construct (inserted genes, regulatory mechanisms, marker genes, and donors or recipients), the gene functions, complementary and breakdown products, affected metabolic pathways, and potential toxic and allergic effects of the plant product(s). Thus, the negative issues relative to plant biotechnology include potential harm to non-target organisms or potential introduction of regulatory molecules (such as transcriptional factors or hormones) into the same organism with subsequent effects on other genes. The main issue associated with gene biotechnology is that the foreign genes could escape into nature and this process could be uncontrolled. This can lead to loss of biodiversity or to the derivation of new plant organisms with unpredicted properties. The other most serious concern is that pollen from genetically engineered plants could contaminate natural populations due to pollination. In order to escape such difficulties, foreign gene(s) may be transferred to the special organelles, such as chloroplasts or mitochondria, for gene function and possible application, and therefore, the risk assessment with pollination is kept to the minimum.

With the entry of genetically modified (GM) crops into our food chain, consumers are demanding both satisfactory information and choice about GM crops (Parker and Kareiva, 1996; Krebs, 2000; Pimentel et al., 2000). To satisfy this demand, many countries are introducing legislation to control the circulation of GM

crops or to trace the use of approved GM crops. GM plants must go through a rigorous stepwise screening process involving both confined and unconfined field trails. In general, plants with novel traits are regulated on the basis of the characteristics of the product, not the specific process by which the product is made. More specifically, when the next novel plant is assessed, emphasis is placed on the insertion of the novel gene(s) into the plant genome; the number of sites of integration (loci); the copy numbers; presence of rearrangements; the stability; the expression; alterations of metabolic pathways; the activity of an inserted gene product in the plant; and the activity of the gene product in the environment. Potential altered interactions of the novel plants involve identifying changes to the relative phenotype with respect to stress adaptation, composition, toxins, and agronomic characteristics.

A list of potential ecological benefits (Daily, 1999; Dyson, 1999) and risks of selected GM crops is presented in Table 13.1. It provides a framework that makes

Table 13.1 Examples of the potential ecological benefits and risks of selected GM crops

GM modification	Benefits	Risks
Herbicide resistance in crops	Reduced herbicide use Increased opportunities for reduced tillage systems	Increased herbicide use Reduced in-field biodiversity that may reduce the ecological services provided by agricultural ecosystems
Crops with *Bt* toxin	Reduced pesticide use Kills fewer non-target organisms than alternatives such as broad-spectrum pesticides	Promotes development of *Bt* resistance, which will eliminate *Bt* as a relatively safe pesticide Kills non-target caterpillars and butterflies such as monarchs (Pimentel 2000)
Virus resistance in small grains due to coat proteins	Reduced insecticide use to control insect dispersers of pathogens (Hails 2000)	Facilitates the creation of new viruses (Hails 2000) Moves genes into nonagricultural ecosystems where the subsequent increase in fitness of weedy species could eliminate endangered species
Terminator or other sterilizing traits in crops and ornamentals	Prevents the movement of traits to non-target species Prevents the movement of introduced species to other ecosystems (Walker and Lonsdale 2000)	Prevents farmers from developing their own seed supplies adapted to local conditions (Conway 2000)
Synthesis of vitamin A or other nutrients	Improves nutrition of people who depend heavily on rice (Conway 2000)	Disrupts local ecosystems if an ecologically limiting nutrient or protein is produced
Nitrogen fixation by non-legumes	Reduces energy used in fertilizer production and application (Pimentel 2000)	Adds to excess nitrogen leaching from agricultural activities, degrading human health and reducing biodiversity

it easier to screen for the possible combinations of technology, crop, and ecological contexts that are likely to be relatively benign or hazardous. However, constructing such lists is only the first step in a risk assessment. These risks need to be quantitatively assessed for specific organisms in different contexts on a case-by-case basis. Various groups of ecologists have developed a methodology for evaluating the use of GM crops (Tiedje et al., 1989, Scientists' Working Group on Biosafety, 1998). They recommend an incremental, tiered approach to risk assessment that moves from the laboratory to greenhouse and field trials, and finally, to gradually increased, monitored use.

While field trials are a necessary step in evaluating GM crops on their own, they are insufficient. A more comprehensive analysis is required that includes an assessment of the relative benefits and risks of GM crops for other ecosystems and for people. To illustrate this approach, we provide a partial list of the questions for assessment in Table 13.2. Comprehensive risk assessments could allow people to reap substantial benefits from GM crops while avoiding or mitigating serious risks (Arber, 2009).

Table 13.2 Questions to assess the relative benefits and risks of a GM crop

Impacts	Benefit-related questions	Risk-related questions
Agricultural and industrial	Are alternatives available that provide greater agronomic, economic, social, and ecological benefits? Does the GM crop prevent some specific harm to humans or to ecosystems; e.g., Does it reduce pesticide use?	Are risks minimized through good design; e.g., Is it certain that genes inserted into chloroplast DNA cannot escape through pollen? Has the organism been examined in order to determine whether genetic modifications to produce a desired trait have not also inadvertently produced risky changes?
Ecological	Does the GM crop help to solve an existing environmental problem; e.g., Does it produce sterile feral animals to control pests (Walker and Lonsdale 2000)?	Does the modified trait have the potential to increase the fitness ofthe organism outside of the managed environment; e.g., Does it impart herbivore resistance or increase the reproductive rate? In the locale of release, can the trait spread to other species; i.e., Can the species hybridize with other species nearby?
Social	Will the benefits of this GM organism be widely shared? Does the GM crop provide some specific benefit to humans or ecosystems; e.g., Does it enhance human nutrition or help to restore degraded land?	Is a mechanism in place for surveying possible negative effects after widespread release of the GM crop has occurred? Do institutions exist that could mitigate the potential harmful impacts of GM crops?

13.3 Purported Advantages of GM Food Plants

The world population has now reached six billion people and is predicted to double in the next 50 years. Ensuring an adequate food supply for this booming population is going to be a major challenge in the years to come. GM foods have the potential to meet this need in a number of ways.

Insect Pest Resistance: Crop losses from insect pests can be immense, resulting in substantial financial loss for farmers and starvation of people in developing countries. Farmers typically use many millions of kilos of chemical pesticides annually. Consumers do not wish to eat food that has been treated with pesticides because of potential health hazards and because runoff of agricultural wastes from excessive use of pesticides and fertilizers can poison the water supply and cause harm to the environment. Growing GM foods such as *Bt* corn can help to eliminate the application of chemical pesticides and to reduce the cost of bringing a crop to market.

Herbicide Tolerance: For some crops, it is not cost-effective to remove weeds by physical means such as by tillage. So farmers will often spray large quantities of different herbicides to destroy weeds. This is a time-consuming and expensive process that requires care so that the herbicide does not harm the crop plant or the environment. Crop plants genetically engineered to be resistant to one very powerful herbicide could help prevent environmental damage by reducing the amount of herbicide needed. For example, Monsanto has created a strain of soybeans genetically modified to be not affected by their herbicide product Roundup®. A farmer grows these soybeans which then only require one application of herbicide instead of multiple applications, thus reducing production costs and limiting the dangers of agricultural waste runoff.

Disease Resistance: There are many viruses, fungi, and bacteria that cause plant diseases. Plant biologists are working to create plants with genetically engineered resistance to these diseases.

Frost Resistance: Unexpected frost can destroy sensitive crop seedlings. An anti-freeze gene from cold water fish has been introduced into plants such as tobacco and potato. With this anti-freeze gene, these plants are able to tolerate cold temperatures that normally would kill genetically unmodified seedlings.

Drought Tolerance/Salinity Tolerance: As the world population grows and more land is utilized for housing instead of food production, farmers will need to grow crops in locations previously poorly suited for plant cultivation. Consequently, creating plants that can withstand long periods of drought or high salt content in the soil and groundwater will help people to grow crops in formerly inhospitable places.

Human Nutrition: Malnutrition is common in African countries which face hunger and poverty where impoverished people rely on a single crop such as rice for the main staple of their diet. However, rice does not contain adequate amounts of all necessary nutrients to prevent malnutrition. If rice could be genetically engineered to contain additional vitamins and minerals, nutrient deficiencies could be alleviated. For example, blindness due to vitamin A deficiency is a common problem in third-world countries. Researchers at the Institute for Plant Sciences, Swiss Federal

Institute of Technology, have created a genetically modified strain of "golden" rice containing an unusually high content of beta-carotene (vitamin A).

Phytoremediation: Not all GM plants are grown as crops. Soil and groundwater pollution continues to be a problem in all parts of the world. Plants such as poplar trees have been genetically engineered in order to clean up heavy metal pollution from contaminated soil (see also Chapter 7).

13.4 Genetically Modified (GM) Plants and Foods Derived from GM Plants: Questions and Answers

The questions and answers cited below have been prepared by the *World Health Organization* (*WHO*) in response to questions and concerns by a number of (WHO) Member State Governments with regard to the nature and safety of genetically modified food. They have been modified substantially for presentation here.

Q1. What are genetically modified (GM) organisms and GM foods?
Genetically modified organisms (*GMOs*) can be defined as organisms in which the genetic material (DNA) has been altered in a way that does not occur naturally. The technology is often called "modern biotechnology" or "gene technology", sometimes also "recombinant DNA technology" or "genetic engineering". It allows selected individual genes to be transferred from one organism into another, and also, between non-related species.

Such methods are used to create GM plants – which are then used to grow GM food crops.

Q2. Why are GM foods produced?
GM foods are developed – and marketed – because there is some perceived advantage either to the producer or consumer of these foods. This is meant to translate into a product with a lower price, greater benefit (in terms of durability or nutritional value), or both. Initially GM seed developers wanted their products to be accepted by producers so have concentrated on innovations that farmers (and the food industry more generally) would appreciate.

The initial objective for developing plants based on GM organisms was to improve crop protection. The GM crops currently on the market are mainly aimed at an increased level of crop protection through the introduction of resistance against plant diseases caused by insects or viruses or through increased tolerance toward herbicides.

Insect resistance is achieved by incorporating into the food plant the gene for toxin production from the milky spore bacterium, *Bacillus thuringiensis* (*Bt*). This toxin is currently used as a conventional insecticide in agriculture and is safe for human consumption. GM crops that permanently produce this toxin have been shown to require lower quantities of insecticides in specific situations, e.g., where pest pressure is high.

Herbicide tolerance is achieved through the introduction of a gene from a bacterium conveying resistance to some herbicides. In situations where weed pressure is high, the use of such crops has resulted in a reduction in the quantity of the herbicides used.

In some cases, biotechnology can be used to make virus-resistant crops. The most common way of doing this is by giving a plant a viral gene encoding the virus coat protein. The plant can then produce this viral protein before the virus infects the plant. If the virus arrives, it is not able to reproduce. The explanation for this is called co-suppression. The plant has ways of knowing when the viral coat protein should not be produced, and it has ways of eventually shutting down the protein's expression. When the virus tries to infect the plant, the production of its essential coat protein is already blocked. All genetically modified virus-resistant plants on the market (e.g., papayas and squash) have coat protein-mediated resistance. It may also be possible to confer resistance by taking a resistance gene naturally found in one plant and then transferring it to an important crop.

Q3. Are GM foods assessed differently from traditional foods?

Generally consumers consider that traditional foods (that have often been eaten for thousands of years) are safe. When new foods are developed by natural methods, some of the existing characteristics of foods can be altered, either in a positive or a negative way. National food authorities may be called upon to examine traditional foods, but this is not always the case. Indeed, new plants developed through traditional breeding techniques may not be evaluated rigorously using risk assessment techniques.

With GM foods most national authorities consider that specific assessments are necessary. Specific systems have been set up for the rigorous evaluation of GM organisms and GM foods relative to both human health and the environment. Similar evaluations are generally not performed for traditional foods. Hence, there is a significant difference in the evaluation process prior to marketing for these two groups of foods.

One of the objectives of the WHO Food Safety Programme is to assist national authorities in the identification of foods that should be subject to risk assessment, including GM foods, and to recommend the correct assessments.

Q4. How are the potential risks to human health determined?

The safety assessment of GM foods generally investigates (a) direct health effects (toxicity); (b) tendencies to provoke allergic reaction (allergenicity); (c) specific components thought to have nutritional or toxic properties; (d) the stability of the inserted gene; (e) nutritional effects associated with genetic modification; and (f) any unintended effects which could result from the gene insertion.

Q5. What are the main issues of concern for human health?

While theoretical discussions have covered a broad range of aspects, the three main issues debated are tendencies to provoke allergic reaction (allergenicity), gene transfer, and outcrossing.

Allergenicity. As a matter of principle, the transfer of genes from commonly allergenic foods is discouraged unless it can be demonstrated that the protein product of the transferred gene is not allergenic. While traditionally developed foods are not generally tested for allergenicity, protocols for tests for GM foods have been evaluated by the Food and Agriculture Organization of the United Nations (FAO) and WHO. No allergic effects have been found relative to GM foods currently on the market.

Gene transfer. Gene transfer from GM foods to cells of the body or to bacteria in the gastrointestinal tract would cause concern if the transferred genetic material adversely affects human health. This would be particularly relevant if antibiotic resistance genes, used in creating GMOs, were to be transferred. Although the probability of transfer is low, the use of technology without antibiotic resistance genes has been encouraged by a recent FAO/WHO expert panel.

Outcrossing. The movement of genes from GM plants into conventional crops or related species in the wild (referred to as "outcrossing"), as well as the mixing of crops derived from conventional seeds with those grown using GM crops, may have an indirect effect on food safety and food security. This risk is real, as was shown when traces of a maize type which was only approved for feed use appeared in maize products for human consumption in the United States. Several countries have adopted strategies to reduce mixing, including a clear separation of the fields within which GM crops and conventional crops are grown.

Feasibility and methods for post-marketing monitoring of GM food products, for the continued surveillance of the safety of GM food products, are under discussion.

Q6. How is a risk assessment for the environment performed?

Environmental risk assessments cover both the GMO concerned and the potential receiving environment. The assessment process includes evaluation of the characteristics of the GMO and its effect and stability in the environment, combined with ecological characteristics of the environment in which the introduction will take place. The assessment also includes unintended effects which could result from the insertion of the new gene.

Q7. What are the issues of concern for the environment?

Issues of concern include the capability of the GMO to escape and potentially introduce the engineered genes into wild populations; the persistence of the gene after the GMO has been harvested; the susceptibility of non-target organisms (e.g., insects which are not pests) to the gene product; the stability of the gene; the reduction in the spectrum of other plants including loss of biodiversity; and increased use of chemicals in agriculture. The environmental safety aspects of GM crops vary considerably according to local conditions.

Current investigations focus on the potentially detrimental effect on beneficial insects or a faster induction of resistant insects; the potential generation of new plant pathogens; the potential detrimental consequences for plant biodiversity and wildlife; a decreased use of the important practice of crop rotation in certain local situations; and the movement of herbicide resistance genes to other plants.

Q8. Are GM foods safe?
Different GM organisms include different genes inserted in different ways. This means that individual GM foods and their safety should be assessed on a case-by-case basis and that it is not possible to make general statements on the safety of all GM foods.

GM foods currently available on the international market have passed risk assessments and are not likely to present risks for human health. In addition, no effects on human health have been shown as a result of the consumption of such foods by the general population in the countries where they have been approved. Continuous use of risk assessments based on the Codex principles and, where appropriate, including post market monitoring, should form the basis for evaluating the safety of GM foods.

Q9. How are GM foods regulated nationally?
The way governments have regulated GM foods varies. In some countries GM foods are not yet regulated. Countries which have legislation in place focus primarily on assessment of risks for consumer health. Countries which have provisions for GM foods usually also regulate GMOs in general, taking into account health and environmental risks, as well as control- and trade-related issues (such as potential testing and labeling regimes). In view of the dynamics of the debate on GM foods, legislation is likely to continue to evolve.

Q10. What kind of GM foods are on the market internationally?
All GM crops available on the international market today have been designed using one of three basic traits: resistance to insect damage; resistance to viral infections; and tolerance toward certain herbicides. All the genes used to modify crops are derived from microorganisms.

Q11. What happens when GM foods are traded internationally?
No specific international regulatory systems are currently in place. However, several international organizations are involved in developing protocols for GMOs.

The *Codex Alimentarius Commission* (*Codex*) is the joint FAO/WHO body responsible for compiling the standards, codes of practice, guidelines, and recommendations that constitute the Codex Alimentarius: the international food code. Codex is developing principles for the human health risk analysis of GM foods. The premise of these principles dictates a premarket assessment, performed on a case-by-case basis and including an evaluation of both direct effects (from the inserted gene) and unintended effects (that may arise as a consequence of insertion of the new gene). Codex principles do not have a binding effect on national legislation, but are referred to specifically in the *Sanitary and Phytosanitary Agreement of the World Trade Organization* (*SPS Agreement*), and can be used as a reference in case of trade disputes.

The *Cartagena Protocol on Biosafety* (*CPB*), an environmental treaty legally binding for its parties, regulates transboundary movements of living modified organisms (LMOs). GM foods are within the scope of the protocol only if they contain

LMOs that are capable of transferring or replicating genetic material. The cornerstone of the CPB is a requirement that exporters seek consent from importers before the first shipment of LMOs intended for release into the environment.

Q12. Have GM products on the international market passed a risk assessment?
The GM products that are currently on the international market have all passed risk assessments conducted by national authorities. These different assessments in general follow the same basic principles, including an assessment of environmental and human health risk. These assessments are thorough; they have not indicated any risk to human health.

Q13. Are there implications for the rights of farmers to own their crops?
Yes, intellectual property rights are likely to be an element in the debate on GM foods, with an impact on the rights of farmers. *Intellectual property rights* (*IPRs*), especially patenting obligations of the TRIPS Agreement (an agreement under the World Trade Organization concerning trade-related aspects of intellectual property rights), have been discussed in the light of their consequences on the further availability of a diversity of crops. In the context of the related subject of the use of gene technology in medicine, WHO has reviewed the conflict between IPRs and an equal access to genetic resources and the sharing of benefits. The review has considered potential problems of monopolization and doubts about new patent regulations in the field of genetic sequences in human medicine. Such considerations are likely to also affect the debate on GM foods.

Q14. What further developments can be expected in the area of GMOs?
Future GM organisms are likely to include plants with improved disease or drought resistance, crops with increased nutrient levels, fish species with enhanced growth characteristics, and plants or animals producing pharmaceutically important proteins such as vaccines. At the international level, the response to new developments can be found in the expert consultations organized by FAO and WHO in 2000 and 2001, and the subsequent work of the Codex ad hoc Task Force on Foods Derived from Biotechnology. This work has resulted in an improved and harmonized framework for the risk assessment of GM foods in general. Specific questions, such as the evaluation of allergenicity of GM foods or the safety of foods derived from GM microorganisms, have been covered and an expert consultation organized by FAO and WHO will focus on foods derived from GM animals in 2003.

Q15. What is WHO doing to improve the evaluation of GM foods?
WHO will take an active role in relation to GM foods, primarily for two reasons: (1) on the grounds that public health could benefit enormously from the potential of biotechnology, for example, from an increase in the nutrient content of foods, decreased allergenicity, and more efficient food production and (2) based on the

need to examine the potential negative effects on human health of the consumption of food produced through genetic modification, also at the global level. It is clear that modern technologies must be thoroughly evaluated if they are to constitute a true improvement in the way food is produced. Such evaluations must be holistic and all-inclusive and cannot stop at the previously separated, non-coherent systems of evaluation focusing solely on human health or environmental effects in isolation.

Work is therefore under way in WHO to present a broader view of the evaluation of GM foods in order to enable the consideration of other important factors. This more holistic evaluation of GM organisms and GM products will consider not only safety but also food security, social and ethical aspects, access, and capacity building. International work in this new direction presupposes the involvement of other key international organizations in this area.

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Chapter 14
Risks Involved in the Use of Herbal Products

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Abstract The use of different herbal products can involve several kinds of risks that include improper labeling or failure to provide the correct constituents; inadequate testing of the herbal product in clinical trials; failure to provide the stated amounts of active constituents; contraindications between known herbs and synthetic prescription drugs used to treat the same disease; overdosing or underdosing; contamination of herbal preparations with pathogens, pesticides, and heavy metals; expired shelf life; and problems with formulations that render them ineffective (e.g., ineffective dried preps in capsules versus effective formulations taken as tinctures). In this chapter, we shall address many of these issues. They are basically issues of quality control that involve the latest advances in plant biotechnology.

14.1 Compromised Quality in the Preparation of Herbal Medicines

Herbs to be grown for the preparation of herbal medicines can be compromised in their quality for the following reasons:

- Herbs obtained from different sources (countries, regions, and growers) are mixed in order to make commercial preparations.
- Herbs are not grown under uniform field or greenhouse conditions from year to year.
- Herbs are not collected at the optimum stage of development.
- Herbs collected are adulterated with other herbs, some of which may be toxic or devoid of the same biological activity.

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- Processing of herbs after collection (e.g., drying or freeze-drying) is not uniform. Herbs are often sun-dried in the field and, as a result, lose much of their potency.
- Processed herbs are not packaged or stored properly before commercial sale.
- Herbs are adulterated with other constituents (e.g., preservatives and fillers) when packaged for commercial sale.

In order to mitigate these problems, growers and processors need to use standard conditions and guidelines for growing, harvesting, formulating, and packaging of herbal preparations. Good sources for this kind of information are found in Ody (1993) and Moore (1995).

14.2 Inadequate Testing of Herbal Medicine Products

Many medicinal herbs have not yet been subjected to testing in human clinical trials. Instead, they are promulgated for use based on animal (nonhuman) models or based on oral tradition or practices of shamans.

Even if human clinical trials are conducted, they can suffer from improper design. For example, this can include the following:

- Failure to use a double-blind, placebo-controlled, randomized clinical trial protocol.
- Failure to include greater than a single biologically active dose. The best judgment here is to include half optimum, optimum, and twice optimum levels/doses of the herb.
- Failure to include a sufficient number of time points to do good kinetics or to obtain meaningful data.
- Failure to carry out the study for a sufficient length of time. This is especially critical for many herbal preparations, which often tend to be slow acting or require administration of prescribed doses over an extended period of time (not hours or days, but weeks).

14.3 Risks in the Use of Medicinal Herbs

The use of medicinal herbs to treat specific human disease can involve risks, especially when used in combination with different kinds of synthetically produced prescription drugs. Patients taking herbal medicines as well as prescription drugs to treat a specific ailment must consult their doctor before using such combinations.

Examples (cited in The Merck Manual of Medical Information, second home edition, 2004, by Mark H. Beers) of such adverse interactions are given in Table 14.1.

Table 14.1 Some possible medicinal herb–drug interactions

Medicinal herb	Affected drugs	Interaction
Chamomile	Anticoagulants (such as warfarin)	Chamomile taken with anticoagulants may increase the risk of bleeding
	Barbiturates (such as phenobarbital) and other sedatives	Chamomile may intensify or prolong the effects of sedatives
	Iron	Chamomile may reduce iron absorption
Echinacea	Drugs that can damage the liver (such as anabolic steroids, amiodarone, methotrexate, and ketoconazole)	Echinacea taken for more than 8 weeks may damage the liver. When echinacea is taken with another drug that can damage the liver, the risk of liver damage may be increased
	Immunosuppressants (such as corticosteroids and cyclosporine)	By stimulating the immune system, echinacea may negate the effects of immunosuppressants
Feverfew	Anticoagulants (such as warfarin)	Feverfew taken with anticoagulants may increase the risk of bleeding
	Iron	Feverfew may reduce iron absorption
	Drugs used to manage migraine headaches (such as ergotamine)	Feverfew may increase heart rate and blood pressure when it is taken with drugs used to manage migraine headaches
	Nonsteroidal anti-inflammatory drugs (NSAIDs)	NSAIDs reduce the effectiveness of feverfew in preventing and managing migraine headaches
Garlic	Anticoagulants (such as warfarin)	Garlic taken with anticoagulants may increase the risk of bleeding
	Drugs that decrease blood sugar levels (hypoglycemic drugs such as insulin and glipizide)	Garlic may intensify the effects of these drugs, causing an excessive decrease in blood sugar levels (hypoglycemia)
	Saquinavir (used to treat HIV infection)	Garlic decreases blood levels of saquinavir, making it less effective
Ginger	Anticoagulants (such as warfarin)	Ginger taken with anticoagulants may increase the risk of bleeding
Ginkgo	Anticoagulants (such as warfarin), aspirin, and other NSAIDs	Ginkgo taken with anticoagulants or with aspirin or other NSAIDs may increase the risk of bleeding
	Anticonvulsants (such as phenytoin)	Ginkgo may reduce the effectiveness of anticonvulsants in preventing seizures

Table 14.1 (continued)

Medicinal herb	Affected drugs	Interaction
	Monoamine oxidase inhibitors (MAOIs, a type of antidepressant)	Ginkgo may intensify the effects of these drugs and increase the risk of side effects, such as headache, tremors, and manic episodes
Ginseng	Anticoagulants (such as warfarin), aspirin, and other NSAIDs	Ginseng taken with anticoagulants or with aspirin or other NSAIDs may increase the risk of bleeding
	Drugs that decrease blood sugar levels (hypoglycemic drugs)	Ginseng may intensify the effects of these drugs, causing an excessive decrease in blood sugar levels (hypoglycemia)
	Corticosteroids	Ginseng may intensify the side effects of corticosteroids
	Digoxin	Ginseng may increase digoxin levels
	Estrogen replacement therapy	Ginseng may intensify the side effects of estrogen
	MAOIs	Ginseng can cause headache, tremors, and manic episodes when it is taken with MAOIs
	Opioids (narcotics)	Ginseng may reduce the effectiveness of opioids
Goldenseal	Anticoagulants (such as warfarin)	Goldenseal may oppose the effects of anticoagulants and may increase the risk of blood clots
Licorice	Antihypertensives	Licorice may increase salt and water retention and increase blood pressure, making antihypertensives less effective
	Antiarrhythmics	Licorice may increase the risk of an abnormal heart rhythm, making antiarrhythmic therapy less effective
	Digoxin	Because licorice increases urine formation, it can result in low levels of potassium, which is excreted in urine. When licorice is taken with digoxin, the low potassium levels increase the risk of digoxin toxicity
	Diuretics	Licorice may intensify the effects of most diuretics, causing increased, rapid loss of potassium. Licorice may interfere with the effectiveness of potassium-sparing diuretics, such as spironolactone, making these diuretics less effective
	MAOIs	Licorice may intensify the effects of these drugs and increase the risk of side effects, such as headache, tremors, and manic episodes

Table 14.1 (continued)

Medicinal herb	Affected drugs	Interaction
Milk thistle	Drugs that decrease blood sugar levels (hypoglycemic drugs)	Milk thistle may intensify the effects of these drugs, causing an excessive decrease in blood sugar levels
	Saquinavir	Milk thistle decreases blood levels of saquinavir, making it less effective
Saw palmetto	Estrogen replacement therapy and oral contraceptives	Saw palmetto may intensify the effects of these drugs
St. John's wort	Benzodiazepines	St. John's wort may reduce the effectiveness of these drugs in reducing anxiety and may increase drowsiness and the risk of side effects such as drowsiness
	Cyclosporine	St. John's wort may reduce blood levels of cyclosporine, making it less effective, with potentially dangerous results (such as rejection of an organ transplant)
	Digoxin	St. John's wort may reduce blood levels of digoxin, making it less effective, with potentially dangerous results
	Indinavir (a drug used to treat AIDS)	St. John's wort may reduce blood levels of indinavir, making it less effective
	Iron	St. John's wort may reduce iron absorption
	MAOIs	St. John's wort may intensify the effects of MAOIs, possibly causing very high blood pressure that requires emergency treatment
	Photosensitizing drugs (such as lansoprazole, omeprazole, piroxicam, and sulfonamide antibiotics)	When taken with these drugs, St. John's wort may increase the risk of sun sensitivity
	Selective serotonin reuptake inhibitors (such as fluoxetine, paroxetine, and sertraline)	St. John's wort may intensify the effects of these drugs
	Warfarin	St. John's wort may reduce blood levels of warfarin, making it less effective and clot formation more likely
Valerian	Anesthetics	Valerian may prolong sedation time
	Barbiturates	Valerian may intensify the effects of barbiturates, causing excessive sedation

*14.3.1 Medical Risks in the Use of Kava Kava (***Piper methysticum***): A Case Study*

Kava kava is a herbal ingredient derived from the plant *Piper methysticum* G. Forst., which is a member of the pepper family (Piperaceae). It is native to many Pacific Ocean islands. The leaves and the root of the plant are used in herbal food and medicinal products. In recent years it has become popular in Europe in herbal remedies used to treat anxiety, tension, and restlessness.

It is considered a sacred plant by many of the traditional Polynesian cultures and has been used in prayer and ritual as well as for a wide variety of ailments ranging from asthma and rheumatism to weary muscles and sleeplessness. The main active components in kava kava (kavalactones) are found in the root of the plant. Kavalactones are thought to affect levels of neurotransmitters in the blood, which can affect the body's fight-or-flight response. While kava root was traditionally chewed or made into a beverage, it is now primarily taken as a natural anxiety remedy in capsule, tablet, beverage, tea, and liquid extract forms.

Evidence has mounted that in rare cases the use of products containing kava kava (mostly in the form of herbal medicines) has been associated with severe liver damage. Research indicates that this may be largely due to the use of stems and leaves in dietary supplements, which were not used indigenously. The occurrence of liver damage is unpredictable and the mechanism is unclear. Some of the compounds found in Kava extracts block several subtypes of the enzyme cytochrome P450, which may result in adverse interactions with concomitant use of other drugs and alcohol (Mathews et al., 2002). Because of these reports, regulatory agencies in Europe and Canada now warn consumers of the potential risks associated with kava kava and even remove kava-containing products from the market. Based on these and other reports in the United States, the Food and Drug Administration (FDA) issued a consumer advisory in March of 2002 regarding the "rare" but potential risk of liver failure associated with kava-containing products.

*14.3.2 Medical Risks in the Use of Ephedra (***Ephedra sinica***): A Case Study (Modified from Data Provided by www.rand.org/health)*

The herb **ephedra**, also known as **ma huang** (*Ephedra sinica* Stapf.), is a small shrub native to Asia, where it has a long history of medicinal use, as documented in ancient medical treatises from India and China. In traditional Chinese and Indian medicine, branches of the herb are used to treat colds, and it is also used as a diuretic. Modern European practitioners of herbal medicine use ephedra only to treat symptoms of respiratory diseases, such as bronchial asthma.

In the United States, the active components of ephedra are known as **ephedrine alkaloids.** They include ephedrine, pseudoephedrine, and norephedrine (also known as phenylpropanolamine and norpseudoephedrine). These constituents are

commonly found in over-the-counter cold and allergy medications. The ephedrine alkaloids are stimulants similar to, but much weaker than, amphetamines. These ephedra stimulants can increase heart rate and blood pressure and relax bronchial tissue, easing shortness of breath. At low doses, they are reputed to decrease appetite, increase alertness and productivity, improve mood, and decrease fatigue; at higher doses, they may promote anxiety, restlessness, and insomnia.

The use of ephedra to promote weight loss and to enhance athletic performance began to gain popularity in the United States in the early 1990s. The increase in popularity of herbal products, and over-the-counter medications that seem to promote weight loss, is probably due to a combination of factors. These include the recent precipitous rise in obesity rates, the reluctance of many obese people to talk with their doctors about weight control, and the growing belief on the part of many people that natural substances such as herbs are safer to use than synthetic prescription medicines.

Products that contain the herb ephedra have been promoted and used in the United States since the 1980s in order to increase weight loss and to enhance athletic performance. Yet, despite manufacturers' claims, little research has been done to assess whether or not ephedra products are safe. Furthermore, the research studies that have been done have been too small to allow any firm conclusions to be drawn.

The questionable effectiveness of these products might not have raised public concern, had the **US Food and Drug Administration (FDA)** and major manufacturers of ephedra-containing products not become the targets of growing numbers of consumer complaints in the late 1990s. Reports of adverse events, including serious adverse side effects and even deaths, many in apparently healthy young people, began increasing during this time. Prominent among the victims have been several college and professional athletes. Thus, in recent years, several major consumer health groups have called on the FDA to ban sales of ephedra-containing products.

The FDA classifies products containing herbal ephedra as dietary supplements, which are regulated by the Dietary Supplement Health and Education Act of 1994 (DSHEA). Under the DSHEA, dietary supplements are generally "presumed safe." Thus, manufacturers are required only to notify the FDA of their intent to market new products. However, they are not required to establish the safety or the effectiveness of their products. Once a dietary supplement is on the market, the FDA can restrict its use or ban sales of the product only if it can demonstrate convincingly that the product is unsafe.

The studies that have been conducted (see Shekelle et al., 2003a and 2003b) suggest that ephedra- and ephedrine-containing products may be modestly effective in promoting weight loss, but the evidence on enhancing athletic performance is not definitive. However, the use of ephedra or ephedrine does cause an increase in jitteriness, mood changes, palpitations, nausea, and vomiting. Moreover, the adverse-event reports raise serious concerns about the safety of ephedra and ephedrine products.

In response to the reporting of these studies, the federal government quickly moved to propose stricter labeling of ephedra products and solicited public comment

on whether the safety evidence thus far warrants further restrictions. By itself, the existing evidence is insufficient to link these products conclusively with death and other serious health problems. However, an analysis of the existing studies and their shortcomings suggests that a more definitive answer to questions about ephedra's safety could be obtained by doing what is called a **"case–control" study**.

Such a study would compare ephedra use by individuals who suffered death or another illness with use by similar individuals who have not suffered severe health problems. A study of this type could also be used to compare the safety of ephedra-containing supplements and products containing ephedrine. Finally, a case-control study could help answer safety questions quickly, thus avoiding the expense and time that would be needed to conduct a large-scale randomized controlled trial and potentially saving lives.

14.3.3 Risks Associated with the Use of Vaccinium*: A Case Study*

The genus *Vaccinium* is composed of approximately 450 species, many of which have been important food and medicinal plants for cultures worldwide throughout the millennia. All are considered nontoxic, although palatability and composition across such a wide range of species are, understandably, diverse. Interest in *Vaccinium*-based dietary supplements has increased dramatically over the past decade as consumers have become aware through the media of the numerous health benefits of *V. corymbosum* (highbush blueberry) and *V. angustifolium* (lowbush or "wild" blueberry). In fact, these fruits have been categorized, among a select group of other fruits and vegetables, as "superfoods" in consumer-oriented marketing campaigns. Although this claim is arguably legitimate, based on their replete flavonoid content and generally recognized safety profile, some considerations must apply.

Consumers face a dizzying array of products based on *Vaccinium* in the form of capsules, powders, liquid formulas, sports drinks, energy bars and as an ingredient in dairy, grain, and other food matrices. Although consumers are familiar, conceptually, with antioxidants and free radicals, they are often misled by claims of superior antioxidant activity of different products, which are usually based only on testing of a limited spectrum of antioxidant activities. One group sought to compare directly and make example of various commercial fruit juices through (1) evaluation of the total polyphenol content [by gallic acid equivalents (GAEs)]; (2) four tests of antioxidant potency including Trolox equivalent antioxidant capacity (TEAC), total oxygen radical absorbance capacity (ORAC), free radical scavenging capacity by 2,2-diphenyl-1-picrylhydrazyl (DPPH), and ferric reducing antioxidant power (FRAP); and (3) a test of antioxidant functionality, that is, inhibition of low-density lipoprotein (LDL) oxidation by peroxides and malondialdehyde methods of polyphenol-rich beverages in the marketplace (Seeram et al., 2008). In this study, total polyphenol content, composite antioxidant potency, and ability to inhibit LDL oxidation were consistent in classifying the antioxidant capacity of the polyphenol-rich beverages in descending order: *Punica granatum* L. (pomegranate)

juice > red wine > *Vitis x labruscana* (Concord grape) juice > *V. corymbosum* (blueberry) juice > *Prunus serotina* Ehrh. (black cherry) juice, *Euterpe oleracea* Mart. (a,caí) juice, *V. macrocarpon* (cranberry) juice > *Citrus sinensis* (L.) Osbeck (orange) juice, iced tea beverages, *Malus domestica* Borkh. (apple) juice.

While these results are interesting and arguably legitimate, different sample brands could alter readily the order of observed antioxidant potency. In many products, the amount of *Vaccinium* included is often very low, although its contribution to the final product may be inflated through labeling in order to use it as the marketing "handle." Furthermore, the quality of *Vaccinium* preparations used in a finished product, whether in dried or extract forms, may be inconsistent. The fresh fruit source is of utmost importance, as notable differences have been documented not only between species but also between cultivars, growth conditions, harvest time, storage, and ultimate processing – even for the same species. Drying technologies differ in terms of temperature and time required for the process, and the amount of flavonoids retained, especially the anthocyanins, significantly decreases under harsher conditions. Similarly, the yield of bioactive flavonoids is dependent upon the method employed to produce an extract. In recent years, advanced analytical methods have become available to assess the authenticity and quality of *Vaccinium* compositions for research purposes and standardization of commercial products for dietary supplements and clinical applications (Zhang et al., 2004; Määttä-Riihinen et al., 2004; Tian et al., 2005; Burdulis et al., 2007; Cassinese et al., 2007; Harris et al., 2007; Lin and Harnly, 2007; Grant and Helleur, 2008). Whereas the quality control of herbal medicinal products used by health-care practitioners is regulated in detail (e.g., German Commission E), no uniform requirements for food-derived supplements currently exist. A **standardized preparation**, typically an extract, is one with a consistent and guaranteed percentage of a definable bioactive compound or group of compounds. For *Vaccinium* dietary supplements, standardization is a voluntary effort by manufacturers to offer a high-quality product. The principal components of interest from *Vaccinium*, anthocyanins and proanthocyanidins, are notoriously difficult among all flavonoids to analyze quantitatively with accuracy (Krenn et al., 2007). Yet other biotechnological methods have been developed to improve yield and composition and mitigate against detrimental effects of storage and processing on the stability of flavonoids from *Vaccinium* in foods, nutraceuticals, and phytopharmaceutical dosage forms (Kalt et al., 1999; Connor et al., 2002; Gunes et al., 2002; Wang and Stretch, 2002; Lyons et al., 2003; Zheng et al., 2003; Lohachoompol et al., 2004; Vattem et al., 2005; Song and Sink, 2006; Srivastava et al., 2007; Puupponen-Pimiä et al., 2008; Brambilla et al., 2008; Wang et al., 2008).

Since flavonoids are known to be potent antioxidants, and compartments such as plasma, tissues, and urine have been shown to increase in antioxidant capacity following consumption of flavonoid-rich substances, a reasonable assumption is that they are readily bioavailable (Cao and Prior, 1998; Prior and Cao, 1999; Prior and Cao, 2000; Vinson et al., 2008). In fact, flavonoids are relatively abundant micronutrients in the diet, but bioavailability differs greatly from one type to another. Thus,

the most abundant dietary flavonoids are not necessarily those leading to the highest concentrations of active metabolites in target compartments.

Employing data from 97 studies, based on a single ingestion of pure compound, extract, or whole food/beverage, one group of investigators calculated mean values for the maximal plasma concentration, the time to reach the maximal plasma concentration, the area under the plasma concentration–time curve, the elimination of half-life, and the relative urinary excretion for 18 major flavonoids (Manach et al., 2005). They found gallic acid and isoflavones to be the best absorbed flavonoids, followed by catechins, flavanones, and quercetin glucosides, but with different kinetics. The least well-absorbed polyphenols are proanthocyanidins, galloylated tea catechins, and anthocyanins. Data were too limited for the assessment of hydroxycinnamic acids and other polyphenols. As a result of digestive and hepatic activity, the metabolites present in blood usually differ from the parent compounds. Depending on the flavonoid, plasma concentrations of total metabolites ranged from 0 to 4 $\mu mol \cdot L^{-1}$ from an intake of 50 mg aglycone equivalents, and the relative urinary excretion ranged from 0.3 to 43% of the ingested dose.

Intervention studies have indicated the type and magnitude of effects among humans in vivo, on the basis of short-term changes in biomarkers. A review of 93 such studies led workers to conclude that flavonoids have varying physiological effects (Williamson and Manach, 2005). They propose that isoflavones (i.e., genistein and daidzein) have weak hormonal effects, but significant ones on processes affecting bone health in postmenopausal women. Monomeric catechins, which occur in exceptional amounts in tea, influence energy metabolism as well as plasma antioxidant biomarkers. Proanthocyanidins, which are widely distributed in many foods, red wine, and supplements such as Pycnogenol (http://www.pycnogenol.com), have pronounced effects on the vasculature that are not limited to antioxidant activity. Quercetin, the principal flavonol in plant-based foods, red wine, and Ginkgo supplements, appears to influence certain markers of carcinogenesis and exerts small effects in vivo on plasma antioxidant biomarkers; nonetheless, some studies failed to corroborate those findings. In fact, the largest randomized, double-blind, placebo-controlled clinical trial ever conducted on a botanical medicine failed to show that extracts of *Ginkgo biloba* L. prevented dementia. Five academic medical centers in the United States between 2000 and 2008 evaluated 3069 community volunteers aged 75 years or older with normal cognition ($n = 2587$) or MCI (mild cognitive impairment; $n = 482$) (Dekosky et al., 2008). Proponents of Ginkgo may argue that this study does not undermine what has already been observed with regard to the usefulness of Ginkgo extract in providing symptomatic relief in persons who already suffer from dementia or Alzheimer's disease or prevent progression in younger, middle-age subjects.

Other workers have found an apparent lack of correlation between the effectiveness of anthocyanins, such as those derived from *Vaccinium*, in laboratory model systems and in humans, especially as cancer chemopreventive agents, as evidenced by epidemiological studies, further illustrating the importance of study design (Wang and Stoner, 2008). A discrepancy exists in the antioxidant and other bioactivities of flavonoids, which are powerful in assays conducted in vitro; the

measured in vivo activities are far more subtle. The reasons for incongruity are cited as (1) lack of validated in vivo biomarkers, especially in the area of carcinogenesis; (2) lack of understanding or consideration of bioavailability and the inherent complexity of flavonoid interactions in the in vitro experiments, which are subsequently used for the design of in vivo studies; and (3) lack of long-term observations. In the design of in vitro and in vivo studies, these issues mandate careful consideration. The length of human intervention studies should be increased, particularly to more closely reflect the consequences of long-term dietary consumption of flavonoids.

The Physicians Desk Reference (PDR) for Herbal Medicines is an authoritative source for efficacy and safety guidelines regarding phytotherapeutics and plant-based dietary supplements (Gruenwald et al., 2007). Although PDR does not endorse specific brands, a select few have contributed indirectly to its content through academic and independent citations therein. PDR employs a consistent format for reporting data, and the categories mirror those for FDA-approved prescription pharmaceuticals. The basic data include a plant's common name and Latin binomial. A description section follows and specifies the medicinal part(s), botanical characteristics, and features of the flower and fruit, leaves, stems and roots, habitat, production, and alternative (common) names. Next is a section on actions and pharmacology that lists known compounds present in the medicinal parts, with a subsection on effects. Clinical trials, if available, comprise the third section, followed by six sections that encompass indications and usage (segregated by approved and unproven uses), contraindications, precautions and adverse reactions, drug interactions, dosage, and, lastly, supporting literature citations.

Three *Vaccinium* species presented in the PDR include *V. myrtillus* (European bilberry), *V. macrocarpon* (cranberry), and *V. uliginosum* (bog bilberry). The PDR cites their beneficial effects but, notably, these presumably innocuous fruits also possess clear risks. *V. myrtillus*is contraindicated during pregnancy and should not be used while breastfeeding, whereas *V. macrocarpon* is contraindicated for use in patients with aspirin allergy, atrophic gastritis, diabetes (when product, such as juice, is sweetened with sugar), hypochlorhydria, and kidney stones. Use of the latter during pregnancy has been reviewed, in light of possible mitigation against elevated risk of urinary tract infections associated with this condition, and no adverse events came to light in a survey of 400 women (Dugoua et al., 2008). Alternatively, no evidence is available for safety during lactation. Although contraindications are not cited for *V. uliginosum*, an overdosage warning is given for signs of poisoning after consumption of large quantities and includes nausea, vomiting, states of intoxication, feelings of weakness, and visual disorders. Presumably, these untoward effects may be traced back to natural contamination of the fruit by a fungus.

Precautions and adverse reactions for *V. myrtillus* include side effects relating to the skin, gastrointestinal tract, and nervous system. Digestive complaints, including nausea, are due to the substantive tannin content of the fruit. High doses and prolonged use may lead to chronic intoxication; chronic administration to animals ($1.5\ g{\cdot}kg^{-1}$ per day minimum) has been reported to be fatal. *V. macrocarpon*is generally well tolerated, but high doses may also cause gastrointestinal upset and diarrhea. Since scientific evidence is not available for use during pregnancy, precaution

rather than contraindication is considered prudent. Side effects of *V. uliginosum* have not been observed in conjunction with proper administration of designated therapeutic dosages.

Drug interactions are some of the most serious considerations associated with *Vaccinium* preparations and supplements. *V. myrtillus* is considered a "moderate risk" when used with anticoagulants, antiplatelet and antithrombotic agents, and low-molecular-weight heparins. Clinical management requires close monitoring for signs and symptoms of bleeding with adjustment of anticoagulant dose if the patient regularly takes a consistent and standardized product. *V. macrocarpon* is considered a "high risk" for bleeding when used in conjunction with warfarin and clinic management involves discouraging patients from excessive use of these products. *V. macrocarpon* is a moderate risk when used with histamine$_2$-receptor antagonist (H_2 blocker) medications that are used to treat heartburn, gastroesophageal reflux disease, and ulcers. H_2 blockers and concomitant use may reduce effectiveness of the drug. Patients taking H_2 blockers should avoid regular consumption of these extracts or juice, although occasional use is probably not harmful. Caution is also prudent for patients taking H_2 blockers because the effect of *V. macrocarpon* extracts on gastric acids is not known. Furthermore, *V. macrocarpon* supplements may result in reduced effectiveness of gastric proton pump inhibitors with concurrent use and requires similar clinical management as for H_2 blockers. *V. uliginosum* may be anticipated to have similar interactions with anticoagulants or gastrointestinal drugs at very high doses, but no report of such effects is published in the literature.

Health-conscious consumers and medical practitioners must be careful about excessive intake of *Vaccinium* extracts and other dosage forms for additional reasons cited in the scientific literature. Their proposed use as anticarcinogens, and cardioprotective and neuroprotective agents, has prompted a dramatic increase in their consumption as dietary supplements (Skibola and Smith, 2000). Despite the fact that flavonoid preparations, many of which derive from *Vaccinium*, are marketed as herbal medicines or dietary supplements for a variety of alleged nontoxic therapeutic effects, most have yet to pass controlled clinical trials for efficacy (Galati and O'Brien, 2004). Although most of the work done to date indicates a chemopreventive activity of these compounds, some studies show cancer-inducing or no effects. Current knowledge about flavonoid toxicity, albeit limited, relates to potential dietary flavonoid/phenolic-induced adverse events, including their pro-oxidant activity, mitochondrial toxicity, and interactions with drug-metabolizing enzymes. The chemopreventive activity observed in animal experiments may result from their ability to inhibit phase I and induce phase II carcinogen metabolizing enzymes that initiate carcinogenesis. They also inhibit the promotion stage of carcinogenesis by inhibiting oxygen radical-forming enzymes, those acting as ATP mimics and inhibitors of protein kinases that contribute to proliferative signal transduction enzymes, and others that contribute to DNA synthesis. Finally, they may prevent tumor development by inducing tumor cell apoptosis by inhibiting DNA topoisomerase II and p53 downregulation but, at the same time, potentially elicit mitochondrial DNA apoptosis. While most flavonoids/phenolics indeed are considered safe,

flavonoid/phenolic therapy or chemopreventive use needs to be assessed carefully, as there have been reports of toxic flavonoid–drug interactions, contact dermatitis, hemolytic anemia, liver failure, and estrogenic-related concerns such as breast cancer and male reproductive health associated with dietary flavonoid/phenolic exposures. At higher doses, flavonoids may act as mutagens, pro-oxidants that generate free radicals, and as inhibitors of key enzymes involved in hormone metabolism. Phenolic acids, anthocyanins, stilbenes, catechins, and other flavonoids have documented effects on the cytochrome P-450 system (Rodeiro et al., 2008). There are several common mechanisms by which these chemicals exert their effects that could be conducive to additive, synergistic, or antagonistic interactions (Nichenametla et al., 2006). Since flavonoids readily cross the placenta, the unborn fetus may be especially at risk. Thus, the adverse effects of flavonoids may outweigh their beneficial ones at high doses. These high levels are above those typically obtained from a balanced vegetarian diet.

There are many different kinds of risks associated with herbal preparations, as delineated in the chapter "Abstract." We document many of these risks in case studies on herbal preparations of ephedra, kava kava, and *Vaccinium*. It turns out that the most serious risks are those associated with adverse interactions that can occur between herbal preparations and pharmaceutical prescription drugs (Table 14.1).

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Chapter 15
Risks Associated with Overcollection of Medicinal Plants in Natural Habitats

Maureen McKenzie, Ara Kirakosyan, and Peter B. Kaufman

Abstract Human exploitation of fragile plant communities and ecosystems has been occurring in recent times at an accelerating pace. In general, worldwide loss of habitat has resulted from human overpopulation, global warming, resource extraction, creeping agricultural developments (especially on marginal lands), extensive use of herbicides (as in Vietnam), construction of highways, desertification, fire, flooding/tsunamis, alien invasive species, and disease/insect attacks. This is happening in tropical rain forests worldwide due, in particular, to habitat destruction from mining, removal of forest trees through cutting and the use of fire, livestock overgrazing, and farming. In temperate regions the predominant causes are clear-cutting of forests, collecting wood from trees and shrubs for fuel, overgrazing by livestock, mining, damming river systems, and allowing urban sprawl to replace forest ecosystems. In Arctic regions, ecosystem destruction is the result of massive clear-cuts of boreal forests for pulpwood for paper manufacture, lumber, and wood products.

The Worldwatch Institute in Washington, D.C. has successfully documented these calamities over the past two decades. Unfortunately, their prognosis is not good for the future regarding the Earth's natural resources. Humans, with their burgeoning populations, continue to be engaged, despite sufficient warning, in overly exploitive activities that squander natural products that occur in vast ecosystems. As a result, the population is living way beyond the carrying capacity in many regions of the planet.

The purpose of this chapter is to point out ways which might reverse this trend. Critical considerations involve preserving natural and wilderness areas; commitment to sustainable harvesting of plants in these ecosystems; saving rare, threatened, and endangered species of plants in "gene banks," seed banks, tissue culture banks, nurseries, botanical gardens and arboreta, and parks and shrines; and cultivating plants in an ecologically friendly way. Following these strategies, the supply of natural products of medicinal value obtained from plants will be available in perpetuity

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and, at the same time, help to provide a livelihood for many people who depend upon these products for their income.

15.1 Causes for Loss of Medicinal Plant Diversity

Plants are recognized universally as a vital part of the world's biological diversity and an essential resource for the planet. In addition to the relatively small number of crop plants developed for food, fuel, and fibers, many thousands of wild plants have enormous economic and cultural importance and potential, providing nutrition and medicine to populations throughout world.

Many species of plants, including those of medicinal value, are becoming threatened, endangered, rare, nearly extinct, or extinct because of misguided human activities (see further). The primary causes for loss of medicinal plant diversity are destruction and overcollection of medicinal plants in their natural habitats. The exact definitions for these different categories, as defined by the *IUCN* (International Union for Conservation of Nature), are as follows:

- *Extinct*: the last remaining member of the species had died or is presumed beyond reasonable doubt to have died.
- *Extinct in the wild*: captive individuals survive, but there is no free-living, natural population.
- *Critically endangered*: faces an extremely high risk of extinction in the immediate future.
- *Endangered*: faces a very high risk of extinction in the near future.
- *Vulnerable*: faces a high risk of extinction in the medium term.
- *Least concern*: no immediate threat to the survival of the species.

The Botanic Gardens Conservation International (BGCI), which represents botanic gardens in 120 countries, stated that "400 medicinal plants are at risk of extinction, from over-collection and deforestation, threatening the discovery of future cures for disease." (BGCI, January 18, 2008). The most notable are Yew trees (*Taxus* spp.) (from which the bark is used for the cancer drug, paclitaxel); *Hoodia gordonii* Sweet ex Decne. (a source of weight loss supplements from Namibia); half of *Magnolia* spp. (used as Chinese medicine for 5,000 years to fight cancer, dementia, and heart disease); and Autumn crocus (*Colchicum autumnale* L. prescribed for gout). The group also found that 5 billion people benefit from traditional plant-based medicine for health care.

Many medicinal plants have been overcollected almost to the point of extinction in their natural habitats. In the United States, notable examples include Pacific yew (*Taxus brevifolia* Nutt.), ginseng (*Panax ginseng* C.A. Mey.), goldenseal (*Hydrastis canadensis* L.), black cohosh (*Cimicifuga racemosa* (L.) Nutt. or *Caulophyllum thalictroides* (L.) Michx.), American ginseng (*Panax quinquefolius* L.), bloodroot (*Sanguinaria canadensis* L.), prairie coneflower or echinacea (*Echinacea* spp.), helonias root (*Chamaelirium luteum* (L.) A. Gray), kava kava

(*Piper methysticum* G. Forst.; Hawaii only), lady's slipper orchid (*Cypripedium* spp.), Lomatium (*Lomatium dissectum* (Nutt.) Mathias & Constance), osha (*Ligusticum porteri* J.M. Coult. & Rose), partridge berry (*Mitchella repens* L.), peyote or mescal button (*Lophophora williamsii* (Lem. ex Salm-Dyck) J.M. Coult.), slippery elm (*Ulmus rubra* Muhl.), sundew (*Drosera* spp.), trillium (*Trillium* spp.), true unicorn (*Aletris farinosa* L.), Venus' flytrap (*Dionaea muscipula* J. Ellis), and wild yam (*Dioscorea villosa* L.) (Source: United Plant Savers, www.unitedplantsavers.org).

15.2 Use of Biotechnology to Rescue Rare or Endangered Medicinal Plant Species That Are Rare or Threatened by Extinction in Their Natural Habitats

The primary expertise to bridge the gap between conservation and scientific research is in *plant systematics and floristics* – the primary collection, inventory, description, and assimilation of information about plants. Once this information is obtained, modern biotechnology techniques have many possible contributions to offer medicinal plant conservation efforts. The following sections delineate plant conservation strategies that are aimed at rescuing medicinal plant species that are rare or threatened by extinction in their natural habitats.

15.2.1 Preservation of Natural Habitats and Ecosystems

National Parks: Natural resource policies aim to provide people the opportunity to enjoy and benefit from natural environments evolving by natural processes with minimal influence by human actions. The *National Park Service* (*NPS*) will ensure that lands are protected within park boundaries. Where parks contain nonfederal lands, the NPS uses cost-effective protection methods. Preservation of character and resources of wilderness areas designated within a park, while providing for appropriate use, represent the primary management responsibility. The National Parks and Conservation Association is a national nonprofit membership organization dedicated to defending, promoting, and enhancing our national parks, and educating the public about the NPS. It was established in 1919 to protect parks and monuments against private interests and commercialism and to block inappropriate development within parks. Most recently, this organization has done a magnificent job of mobilizing citizen action to prevent clear-cutting of timber and mining within and adjacent to the national parks. They have also helped to protect these parks from undue human intrusion with recreational vehicles, helicopters, campers, and "vehicles" of all types (including boats, jeeps, motorcycles, mountain bikes, snowmobiles, and dune buggies). Limiting access to the national parks because of "people pressure" and consequently over-crowding has become the norm. Together, these efforts help, but citizen action groups, such as the *National Parks and Conservation Association*, the *Sierra Club*, the *Nature Conservancy*, the *Wilderness Society*, the *Natural*

Resources Defense Fund, and the many other organizations who operate in the individual states, must be ever vigilant and ready for concerted action.

Sustainable Biopreserves for Indigenous Peoples: Based on a recent United Nations Conference on Environment and Development (UNCED), the United States has placed forest management and protection as a priority of UNCED. Further, discussions by the US government agencies and nongovernmental organizations have concluded that a provision needs to be included on the needs of indigenous peoples who use the forests for their livelihood, social organization, or cultural identity, and who have an economic stake in sustainable forest use (Plotkin and Famolare, 1992). Actions include promoting means for indigenous peoples and members of local communities to actively participate in decision-making processes for any proposed forest-related actions where their interests are affected (Plotkin and Famolare, 1992). Other propositions are to identify ways to enhance the value of standing forests through policy reform, more accurately reflecting the costs and benefits of alternative forestry activities, in addition to identifying economically valuable forest species, including timber and nontimber species, and the development of improved and sustainable extraction methods (Moran, 1992).

Nabhan (1992) has indicated that the following criteria offer the best guidelines for ensuring that indigenous peoples and other peasant communities benefit from applied ethnobotanical development, and that projects sustain rather than deplete or destroy biodiversity.

- The project should attempt to improve the objective and subjective well-being of local communities rather than seeking cheap production sites and importing inexpensive labor.
- Cultivation in fields or agroforestry management should be considered if there are threats that wild harvests will deplete the resource.
- Wildland management and sensitive harvesting practices should be introduced in cases where the resource might sustain economic levels of extraction in the habitat.
- The plant(s) chosen should offer multiple products or be adapted to diversified production systems.
- When possible, programs should build on local familiarity, use, and conservation traditions for the plant being developed.
- If possible, these programs should be based on locally available genetic resources, technologies, and social organizations to enable local people to retain control over the future of the resource.

15.2.2 Organizations Involved in Conservation of Medicinal Plants and Their Ecosystems

The important topic of ethnobotany and the sustainable use of plant resources is based principally on the work of the *World Wildlife Fund* (*WWF*), *the United Nations Educational, Scientific and Cultural Organization* (*UNESCO*), and the

Royal Botanic Gardens at Kew, United Kingdom. The *People and Plants Initiative* is creating support for ethnobotanists from developing countries who work with local people on issues relating to conservation of plant resources and indigenous ecological knowledge. Rather than promoting the discovery and marketing of new products, emphasis is placed on subsistence use and small-scale commercialization of plants which benefit rural communities. In cases of large-scale commercialization of wild plants, emphasis is on improving harvesting methods and mechanisms which allow communities an increasing share of profits (The Royal Botanic Gardens, Kew, 1996a).

One example is provided by the Kuna Indians of Panama. They have successfully established the world's first internationally recognized forest park created by indigenous people. The reserve provides revenues directly to the Kuna from the sale of research rights, and from ecotourists who come to learn about the rainforest. Coupled with this, it helps protect and preserve their native heritage. Scientists conducting research in the park are required to hire the Kuna to assist and accompany them during their stay. The Kuna control access to sites and require reports on all research. These terms allow the Kuna to patrol and protect outlying areas while learning from the scientists.

Head and Heismann (1990) in *Lessons of the Rainforest*, tell about the organization called Environmental Restoration in Southern Colombia (CRIC). It is composed of 56 Indian communities that are organized to protect Indian lands, resources, culture, and rights in an area where the forest has been destroyed by mines and cattle ranches. CRIC began a forestry program with three tree nurseries which provided seedlings to those communities that agree to plant a minimum of 1000 trees of native species. To date, one community has completed nine reforestation programs.

15.2.2.1 The Nature Conservancy

The main objective of the *Nature Conservancy* is to protect plants, animals, and ecological communities that represent biodiversity. To do this, they rely on conservation science to guide its work. Conservation science programs encompass biological, ecological, and technological knowledge that are used to identify and protect sensitive biodiversity, and in management methods and practices used to ensure its survival. The *Natural Heritage Program* and the *Conservation Data Center Network* programs collectively track in their databases the protected status and locations or rare and endangered species and ecological communities. Over the last four decades, the Nature Conservancy has protected more than 8.1 million acres (3.28 million ha) of habitat based on information about the location, range, and status of rare species. This number is even higher for total acreage protected to date: it is 9.3 million acres (3.77 million ha) of land in the United States and 40 million acres (16.19 million ha) throughout Latin America, the Carribean, and the Asia/Pacific regions. Indeed, it operates the largest system of privately owned nature preserves in the world.

In carrying out its work, the Nature Conservancy addresses ecological function and influences of people and develop better conservation planning methods and tools that will allow planning across immense biologically defined regions and the

range of a particular ecological community. Stewardship of land and its resources are an important component of the work of the Conservancy. In protecting areas identified as critical for biodiversity protection, boundaries of those areas are carefully chosen to encompass important biological components and the ecological processes that sustain them. Its presence in local communities enables it to address ecosystem protection, find solutions to environmental problems, and form partnerships. An organization-wide network electronically links all the Nature Conservancy's offices to support the information systems plan which provides up-to-date information (The Nature Conservancy, 1996).

15.2.2.2 The World Wildlife Fund

The *World Wildlife Fund* (*WWF*) has several important objectives, including (1) halting global trade in endangered animals and plants; (2) creating and preserving parks and protected areas around the world; (3) working to create strongholds for thousands of irreplaceable plant and animal species as well as protecting those and other areas from threats beyond their boundaries; (4) working with local leaders, groups, governments, and international funding institutions to coordinate conservation and improve living standards to help alleviate development pressures that may put wildlands in danger; and (5) organizing, supporting, and strengthening conservation efforts around the world (World Wildlife Fund, 1995).

The WWF uses *Geographic Information Systems* (*GIS*) technology to identify priority areas with the greatest biological wealth and the greatest degree of threat, with a focus on conservation priorities. The WWF works closely with the North American Commission for Environmental Cooperation to help ensure that its work promotes conservation initiatives, such as the North American ecoregion mapping and planning project for biodiversity management. It follows the trade agreement's effect on commodities production and health of forests, wildlife, and natural resources in North America. It also supports the Forest Stewardship Council which has developed criteria for identifying timber companies that produce environmentally sound, economically viable products. This Council consists of social, environmental, and indigenous groups from more than 24 countries, as well as representatives from the timber industry whose mission is to promote ecologically sustainable forest management. In Madagascar, the WWF brokered a debt-for-nature swap which has trained more than 350 local conservation agents and created a network of locally managed tree plantations. It is also helping to develop alternatives to cattle production and slash-and-burn agriculture in order to protect native forests (World Wildlife Fund, 1995).

15.2.2.3 The Sierra Club

The Sierra Club was founded by John Muir in 1892 in San Francisco, California, to help preserve the pristine beauty of the Sierra Nevada mountain range in California. Today, it is a national organization with chapters throughout the United States. It continues to expand, stop abuse of wilderness lands, save endangered species,

and protect the global environment. It helps to create and enlarge national parks, preserve forests, designate wilderness areas, halt dams, and prevent destruction of priceless habitats. The Sierra Club helped save Alaska's Arctic National Wildlife Refuge from imprudent utilization by oil companies, establish National Park and Wilderness Preservation Systems, and safeguard more than 132 million acres of public land.

This organization launched the *Critical Ecosystems Program*, which is designed to protect and restore 21 regional ecosystems in the United States and Canada. This program is involved in designing protection for public and private lands that are the core habitats for native species. It established task forces for each ecoregion, drawing together activists with expertise in various areas to develop strategies to save those regions. What are these strategies for the different ecoregions?

- Atlantic Coast and Great Northern Forest – preserve biodiversity by restoring and sustaining habitat for the full array of native plants and animals, establish sound forestry policy, and preserve wilderness.
- Central Appalachia, Southern Appalachian Highlands, and American Southeast – saving from development, as much as possible, the shoreline stretching 2000 miles (3200 km) from Florida to the mouth of the Rio Grand River.
- Interior Highlands, Great Lakes, Great North American Prairie – establish a system of national parks, reform Forest Service policies on grazing, oil and gas development, and coal mining on grasslands.
- Mississippi Basin, Rocky Mountains, and Colorado Plateau – enact legislation to protect 5 million roadless acres in Utah, eliminate timber sales that threaten old-growth ponderosa pine stands, do away with subsidized timber sales in all national forests, and protect the Grand Canyon by restricting development on its boundaries.
- Southwest Deserts, Great Basin/High Desert, Sierra Nevada, Pacific Northwest, and Pacific Coast – permanently protect the remaining ancient forests on federal land.
- Alaska Rainforests (Tongass and Chugach National Forests), the Boreal Forest extending from Alaska to Newfoundland, Hudson Bay/James Bay Watershed, the Arctic, and Hawaii – prevent further destruction of endangered and threatened plant and animal habitats (Elder, 1994).

15.2.3 Growing Rare and Endangered Plants in Botanical Gardens and Arboreta

According to the *New York Botanical Garden*, of approximately 250,000 species of flowering plants, it is estimated that some 60,000 of these may become extinct by the year 2050, and more than 19,000 species of plants are considered to be threatened or endangered from around the world. More than 2,000 species of plants native to the United States are threatened or endangered, with as many as 700 species becoming extinct in the next 10 years (The New York Botanical Garden, 1995). The New

York Botanical Garden currently grows 10 species of plants in the Federal Endangered Species List. They are striving to preserve rare and endangered plants and participate with other institutions in doing this. The Garden is a Participating Institution in the *Center for Plant Conservation* (*CPC*), serving as a rescue center for six native plant species that are imminently threatened, which form part of the *National Collection of Endangered Plants*, and are grown and studied to be conserved (The New York Botanical Garden, 1995). The CPC is located at the *Missouri Botanical Garden* in St. Louis, MO. This center is dedicated to conserving rare plants native to the United States in an integrated plant conservation context through a collaborative program of *ex situ* plant conservation, research, and education. It is made up of a consortium of 25 botanical gardens and arboreta (Center for Plant Conservation, 1996). A national survey by the CPC in 1988 found that over three-quarters of the endangered flora of the United States is in six areas: Hawaii, California, Texas, Florida, Puerto Rico, and the Virgin Islands. It has designated these areas as conservation priority regions. The CPC Priority Regions Program addresses the need for conservation through programs of land conservation, management, offsite collection in seed banks, botanical gardens and other institutions, research, and site surveys (Center for Plant Conservation, 1996). The National Collection of Endangered Plants contains seeds, cuttings, and whole plants of 496 rare plant species native to the United States. The collection is stored at 25 gardens and arboreta that form part of the CPC.

The *Royal Botanic Gardens at Kew*, United Kingdom, support six ex situ and in situ conservation projects. The activities range from acting as the UK Scientific Authority for Plants for *CITES* (*Convention on International Trade in Endangered Species of Wild Fauna and Flora*), cooperating in the recovery and reintroduction of endangered species, and in production of management plans for sustainable development and protected areas (Royal Botanic Gardens, Kew, 1996b).

The *Wrigley Memorial and Botanical Gardens* at Catalina Island, CA, is still another example. The garden places its emphasis on California island endemic plants. Many of these plants are extremely rare, with some listed on the *Endangered Species List*.

15.2.4 Plant Tissue Culture as a Method to Clone and Rescue Rare and Endangered Plant Species

Plant tissue culture has been the primary method used to rescue rare and endangered plant species and to increase their numbers of genetically similar offspring. It is a practice used to propagate plants under sterile conditions, often to produce clones of a plant. The most useful plant tissue culture protocols involve shoot-tip culture (mericloning), embryo culture, and shoot multiplication using elite germplasm. Germplasm of vegetatively propagated plant material is cheaper to maintain in tissue culture (Akerele et al., 1991), is less expensive to ship, and has the potential to yield more plants more quickly. It is one of the preferred ways to preserve rare and endangered plant species and to distribute these species to other botanical gardens

and arboreta around the world. Where conditions allow, some tissue-cultured plant material can be used to reintroduce species that have become lost or extinct in the wild.

The different techniques of plant tissue culture offer certain advantages over traditional methods of plant propagation including

- the production of exact copies of plants that produce particularly good flowers, fruits, or have other desirable traits;
- to quickly produce mature plants;
- the production of multiples of plants in the absence of seeds or necessary pollinators to produce seeds;
- the regeneration of whole plants from plant cells that have been genetically modified;
- the production of plants in sterile containers that allows them to be moved with greatly reduced chances of transmitting diseases, pests, and pathogens;
- the production of plants from seeds that otherwise have very low chances of germinating and growing, i.e., orchids and nepenthes; and
- to clean particular plant of viral and other infections and to quickly multiply these plants as "cleaned stock" for horticulture and agriculture.

Plant tissue culture relies on the fact that many plant cells have the ability to regenerate a whole plant (totipotency). Single cells, plant cells without cell walls (protoplasts), pieces of leaves, or (less commonly) roots can often be used to generate a new plant on culture media given the required nutrients and plant hormones.

Plant tissue culture is performed under aseptic conditions under filtered air. Living plant materials from the environment are naturally contaminated on their surfaces (and sometimes interiors) with microorganisms, so surface sterilization in chemical solutions (usually alcohol or bleach) is required of starting materials. The tissue which is obtained from the plant to start the culture is called an *explant*. Explants are then usually placed on the surface of a solid culture medium, but are sometimes placed directly into a liquid medium, particularly when cell suspension cultures are desired. Solid and liquid media are generally composed of inorganic salts plus a few organic nutrients, vitamins, and plant hormones. Solid media are prepared from liquid media with the addition of a gelling agent, usually purified agar.

The composition of the medium, particularly the plant hormones and the nitrogen source (nitrate versus ammonium salts or amino acids), has profound effects on the morphology of the tissues that grow from the initial explant. For example, an excess of auxin will often result in a proliferation of roots, while an excess of cytokinin may yield shoots. A balance of both auxin and cytokinin will often produce an unorganized growth of cells or callus, but the morphology of the outgrowth will depend on the plant species as well as the medium composition. As cultures grow, pieces are typically sliced off and transferred to new media (subcultured) to allow for growth or to alter the morphology of the culture. As shoots emerge from a culture (Fig. 15.1), they may be sliced off and rooted with auxin to produce plantlets which,

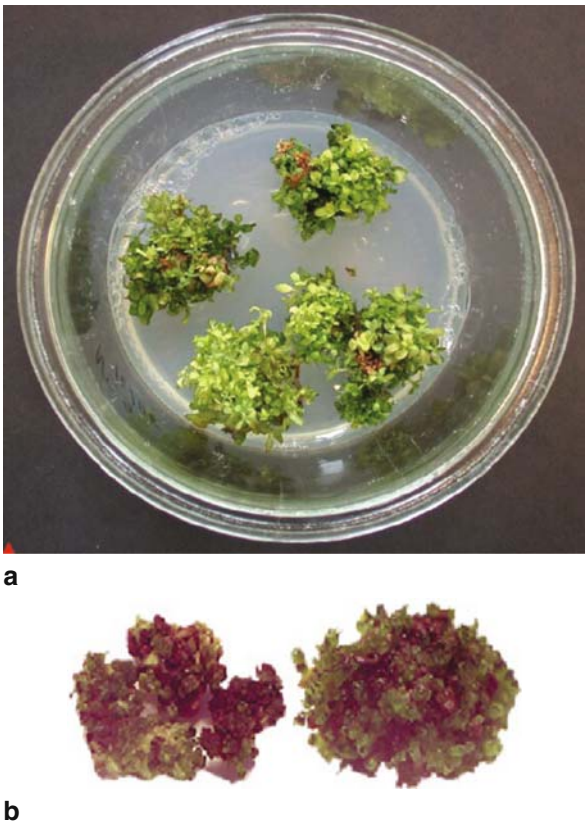

Fig. 15.1 *In vitro* shoot (**a**) and callus (**b**) cultures of *Hypericum perforatum* L.

when mature, can be transferred to potting soil for further growth as normal plants in the greenhouse.

The skill and experience of the tissue culturist are important in judging which pieces to culture and which to discard. Based on work with certain model systems, particularly tobacco, it has often been claimed that a totipotent explant can be grown from any part of the plant. However, this concept has been vitiated in practice. In many species, explants of various organs vary in their rates of growth and regeneration, while some do not grow at all. The choice of explant material also determines if the plantlets developed via tissue culture are haploid or diploid. Also the risk of microbial contamination is increased with inappropriate explants. Thus, an appropriate choice of explant made prior to tissue culture is very important.

The specific differences in the regeneration potential of different organs and explants have various explanations. The significant factors include differences in the stage of the cells in the cell cycle, the availability of or ability to transport endogenous growth regulators, and the metabolic capabilities of the cells. The most commonly used tissue explants are the meristematic ends of the plants like the stem tip, auxiliary bud tip, and root tip. These tissues have high rates of cell division

and either concentrate or produce required growth-regulating substances including auxins and cytokinins.

Some explants, like the root tip, are hard to isolate and are contaminated with soil microflora that become problematic during the tissue culture process. Certain soil microflora can form tight associations with the root systems or even grow within the root. Soil particles bound to roots are difficult to remove without injury to the roots, a circumstance that then allows microbial attack. These root-associated microflora generally will overgrow the tissue culture medium before there is significant growth of plant tissue.

Aerial (above soil) explants are also rich in undesirable microflora. However, they are more easily removed from the explant by gentle rinsing, and the remainder usually can be killed by surface sterilization with 10% sodium hypochlorite ($NaClO_4$). Most of the surface microflora do not form tight associations with the plant tissue. Such associations can usually be found by visual inspection as a mosaic, decolorization or localized necrosis (blackening or death of tissues) on the surface of the explant, and removed before culture.

An alternative for obtaining uncontaminated explants is to take explants from seedlings which are aseptically grown from surface-sterilized seeds. The hard surface of the seed is less permeable to penetration of harsh surface-sterilizing agents, such as hypochlorite, so the acceptable conditions of sterilization used for seeds can be much more stringent than for vegetative tissues.

One of the preferred methods of tissue culture is shoot-tip culture (*mericloning*). It is becoming the preferred tissue for exchange of clonal material. Tissue cultures produced from shoot-tip cultures can produce disease-free germplasm, particularly with respect to viruses. Shoot-tip explants, devoid of any vascular tissue, is typically free of any viral pathogens. This protocol was developed by George Morel in France as a way to rescue virus-infected orchid plants and rapidly propagate virus-free stock. This process is used for the micropropagation of virus-free stock of any plant species. Great success stories are seen in the shoot-tip propagation of virus-free potatoes, strawberries, cassava, pelargoniums, and orchids (Kyte and Kleyn, 1996).

In vitro ("in glass", microorganism-free cultures) disease elimination techniques help to ensure international exchange of germplasm, particularly since viral transmission through seed is known to occur (Akerele et al., 1991). It allows for a far greater number of plants to be produced in a given time than by conventional propagation methods. The Micropropagation Unit at Kew Botanic Gardens propagates plants which are rare, endangered, or difficult to propagate conventionally. Techniques include micropropagation from vegetative material and in vitro germination of seeds and spores. A large number of tropical *epiphytic* (growing on other plants) and *terrestrial* (growing in the soil) orchids are grown from seed in vitro under sterile conditions. Of these, many are members of island floras and are in jeopardy.

Plant tissue culture is used widely in plant science; it also has a number of commercial applications. These applications include the following:

- Micropropagation is widely used in forestry and in floriculture. Micropropagation can also be used to conserve rare or endangered plant species.

- A plant breeder may use tissue culture to screen cells rather than plants for advantageous characters, e.g., herbicide resistance/tolerance.
- Large-scale growth of plant cells in liquid culture inside bioreactors as a source of secondary products, like recombinant proteins used as biopharmaceuticals.
- To cross distantly related species by protoplast fusion and regeneration of the novel hybrid.
- To cross-pollinate distantly related species and then tissue culture the resulting embryo which would otherwise normally die (embryo rescue).
- For production of doubled monoploid plants from haploid cultures to achieve homozygous lines more rapidly in breeding programs, usually by treatment with colchicine which causes doubling of the chromosome number.
- As a tissue for transformation, followed by either short-term testing of genetic constructs or regeneration of transgenic plants.
- Certain techniques such as shoot apical meristem tip culture (mericloning) may be employed that can be used to produce clean plant material from virused stock, such as potatoes and many species of soft fruit.

15.2.5 Plant Seed Banks for Germplasm Preservation

15.2.5.1 Plant-Introduction Stations in the United States

Four regional plant-introduction stations in the United States occur in Pullman, WA, Ames, IA, Geneva, NY, and Griffin, GA. They are responsible for the management, regeneration, characterization, evaluation, and distribution of seeds of more than one-third of the accessions of the national system (i.e., nearly 197,000 accessions of almost 4,000 plant species). At Ames, approximately 40,079 accessions are held; the primary crops preserved include maize, grain amaranth, oilseed brassicas (e.g., rape, canola, mustard), sweet clover, cucumber, pumpkin, summer squash, acorn squash, zucchini squash, gourds, beets, carrots, sunflower, and millets. At Geneva, approximately 14,180 accessions are held; the primary crops preserved include tomato, birdsfoot trefoil, brassicas, and onion. At Griffin, approximately 82,277 accessions are held; the primary crops preserved here include sweet potato, sorghum, peanut, pigeon pea, forage grasses, forage legumes, cowpea, mung bean, pepper, okra, melons, sesame, and eggplant. At the Pullman station, approximately 60,277 accessions are held; the primary crops preserved there include common bean, onion, lupine, pea, safflower, chickpea, clovers, wild rye, lettuce, lentils, alfalfa, forage grasses, horsebean, common vetch, and milk vetch.

15.2.5.2 National Center for Genetic Resources Preservation in Fort Collins, CO

This center houses the base collection for long-term, backup storage of the National Plant Germplasm Storage active collections. It has recently expanded and remodeled its facilities, quadrupling the storage area, and adding modern research and

processing laboratories. It features quality cold-storage facilities for conventional seed storage and cryopreservation (low-temperature preservation, using liquid nitrogen at –196°C) storage capacity for seeds, pollen, and vegetatively propagated germplasm. The *National Seed Storage Laboratory* (*NSSL*) can store more than 1 million samples. The base collection of the NSSL is not duplicated in its entirety in any other gene bank. Furthermore, of the more than 268,000 accessions, about 60,628 are not duplicated at other sites.

15.2.5.3 International Rice Research Institute in Los Baños, Philippines

Rice (*Oryza sativa* L.) is the third best-represented crop in plant gene banks. This is most likely due to the fact that rice is a staple food crop in much of the Third World, particularly Asia. One of the main gene banks for tropical rice is at the *International Rice Research Institute* (*IRRI*). Japan and the United States maintain major collections of temperate rices and act as a backup for IRRI and the *International Institute for Tropical Agriculture* (*IITA*) materials. IRRI has assembled the world's largest rice collection. It represents the largest germplasm collection for any crop and is regarded as one of the best-managed gene banks. It has computerized rice collection data on samples that contain 45 morphological and agronomic characteristics for each entry. As many as 38 genetic evaluation and utilization traits are added, covering disease and pest resistance to tolerance to adverse soils and climates. Its germplasm collection is gradually regenerated and fresh seed is put in medium and long-term storage. Approximately 2000 rice varieties and much wild material still remain to be collected. The gene bank at IRRI is expected to continue growing until it reaches about 130,000 accessions (Plucknett et al., 1987).

15.2.5.4 International Potato Center in Lima, Peru

Potato (*Solanum tuberosum* L.) is the fourth leading world crop, exceeding all other in annual production of starch, protein, and several other important "nutrients" (Niederhauser, 1993). It is susceptible to many diseases and pests and is one of the heavy users of chemical inputs of any crop (Martin, 1988). Improved potato cultivars present a great potential benefit to the economic, environmental, and nutritional future of the world potato growers and consumers (Bamberg et al., 1995).

The *International Potato Center* (*CIP*) accepted the global mandate for potato genetic resources when it was founded. By 1980, more than 80% of total cultivated potato germplasm had been collected. Wild species of potato have also been systematically collected. The cultivated potato collection samples are grown annually at high altitudes and stored in conservation facilities. Duplicates of all lines are replaced by a new CIP harvest in each succeeding year (Reid et al., 1993).

Potato cultivars are distributed worldwide from CIP. Microtubers are more tolerant of physical and environmental disturbances and a few cultures are tolerant of delays in transit (Bamberg et al., 1995). They are now in use for distributing germplasm of potato from CIP and yam from the IITA. The CIP helped to initiate a joint database with potato gene banks around the world by sharing evaluation data

and technical procedures, professional exchanges, cooperation on prioritization and organization of collecting expeditions, duplicate storage of accessions, and cooperative research (Bamberg et al., 1995).

15.2.5.5 Crucifer Genetics Center in Madison, WI

The *Crucifer Genetics Center* (*CrGC*) has been established for the purpose of developing, acquiring, maintaining, and distributing information about seed stocks of various crucifers (members of the cabbage family, Brassicaceae) as well as crucifer-specific symbionts, namely, pathogens (organisms which cause disease in crucifers). It distributes seed from various genetic stocks of rapid-cycling brassicas (short life cycle from seed to seed), some wild crucifer species, a large number of mutants of *Brassica*, *Raphanus* (radish), and *Arabidopsis* (a cress), and pathogen symbiont cultures. The CrGC has been instrumental in introducing rapid-cycling brassicas into laboratory teaching experiments for students in elementary and high school and in colleges and universities for the study of plant genetics, development (flowering and fruiting), physiology (gravitropism, phototropism, and hormone action), and plant pathology. One of these plants, *Arabidopsis*, has been shown to develop from seed to seed in outer space on NASA's Space Shuttle. For humans, conservation of crucifer germplasm, as done at the CrGC, is important for humans; many of the brassicas are important in preventing cancer in humans (e.g., broccoli).

15.2.5.6 Commercial Seed Companies That Save and Sell Heirloom Seeds of Rare and Endangered Medicinal and Other Plants

Because of the loss of crop diversity with the advent of the green revolution and the breeding of crop varieties grown as monocultures, we have lost thousands of varieties of plants because they are no longer available for sale. This has happened with rice, wheat, and maize. Also witnessed with the loss of crop diversity, is a loss in disease and insect pest resistance, a loss or protein and essential nutrients in many of the grain crops, a loss in desirable flavor and texture in many vegetables, and an increase in the use of fertilizers, pesticides, and irrigation water. Many of the desirable cultivars of apples and roses, once grown very widely, almost completely disappeared from commercial seed or nursery catalogues.

The situation today is changing rapidly. Many of the "old-fashioned" rose cultivars or apple cultivars are now reappearing in the catalogues, primarily driven by consumer demand for more plant diversity and varieties that do not require so much in the way of fertilizer, pesticide, and water inputs. The same can be said for cucurbits (squash and melon), maize, legume crops (peas, beans, and their relatives), herbs, prairie plants, medicinal plants, woodland wild flowers, native trees and shrubs useful in landscaping and in forest restoration projects, aquatic plant species used in ponds to purify water polluted water from sewage treatment plants, and species of plants which are good scavengers of heavy metal pollutants in soils. Let us cite just a few examples of sources of seeds of rare and endangered plants.

- *Henry Doubleday Institute* at Ryton Gardens, Coventry, UK, has a heritage seed program whereby it distributes heirloom and rare varieties of seed plants which are generally not commercially available. The seed is not registered with the European Community, so, it cannot be sold, but it can be donated. We do not know if their seeds are exportable to the United States.
- The *Seed Guild* is an organization located in Lanark, UK, which buys seed from botanical gardens throughout the world, making them available to amateur gardeners and commercial outlets. The guild provides an opportunity to obtain unusual and rare seeds which are not generally on commercial seed lists. Their annual newsletter provides information on seed collecting expeditions and new sources of seed supply.
- Three commercial seed companies: Redwood Seed Company (PO Box 361, Redwood City, CA 94064) is an alternative seed company; Sandy's Exotic Plant Seed Company (7179A Nebraska, Fairchild, WA 99011) has available rare, exotic, and unusual seeds from around the world; and Prairie Moon Nursery (Route 3, Box 163 Winona, MN 55987) sells seeds of rare ferns, cacti, forbs (herbaceous plants), grasses, sedges, rushes, trees, shrubs, vines, and prairie mixtures.

15.2.5.7 Seed Banks in Botanical Gardens Established for International Seed Exchange

The Royal Botanic Gardens Kew Seed Bank, located at Wakehurst Place, UK, was founded in 1974. It provides storage for seeds of some 4,000 plant species from more than 100 countries. It is the most diverse collection anywhere in the world. It also holds a long-term collection of seeds sampled from wild populations within the United Kingdom and the world's arid and semi-arid lands. Their emphasis is placed on threatened plant populations and in the drylands, especially for plants of local economic value. Some 3,750 plant species are conserved according to internationally accepted standards for long-term conservation. When numbers permit, seed is offered for distribution. Samples are made available through a *List of Seeds* published every other year and distributed to organizations doing research work and subject to a commercialization agreement in the event of any commercial success, a policy of apportioning profits to the seeds' country of origin. This policy aims to abide with the spirit of the 1992 Rio Earth Summit and to keep pace with subsequent changes in national and international attitudes and legislation (The Royal Botanic Gardens, Kew, 1996a).

The CPC, located at the Missouri Botanic Garden in St. Louis, MO, maintains a Memorandum of Understanding (MOU) with the US Department of Agriculture NSSL in Ft. Collins, CO. Under this MOU, the NSSL stores seeds from rare US plants in the center's National Collection of Endangered Plants at no cost to the center or its participating institutions. The CPC's *National Collection of Endangered Plants* represents perhaps the most fundamental reserve of plant germplasm for many of the rarest plants in the United States.

15.2.6 Botanical Prospecting – Ethnobotanical Field Research

There is a correlation between plant genetic resources and the development of new pharmaceutical products. This correlation integrates biological, ecological, chemical, medical, legal, and economic aspects. The issues can involve property, resource and access right, reciprocity, technology transfer, export, and patent and royalty rights (Reid et al., 1993). The force behind biodiversity-prospecting is the demand for new genes and chemical compounds and to research the supply of these resources in wildland diversity. Interest has increased in the pharmaceutical industry. Development and improvement of screening techniques has increased the rate for chemical testing. *Ethnopharmacology* is another force. This field, which involves the use of plants and animals in traditional medicine, can greatly increase the probability of finding a valuable drug. Drug exploration based on indigenous knowledge may prove to be more cost- and time-effective than random screenings.

In the United States, approximately 25% of prescriptions are of drugs with ingredients that are derived from plant extracts or their derivatives. The demand for genetic resources in agriculture will grow as techniques for genetic manipulation improve and research investments show a return. Between 1985 and 1990, the number of biotechnology patent applications grew by 15% annually (Raines, 1991–1992). As an example, two cancer drugs derived from the rosy periwinkle (*Catharanthus roseus* (L.) G. Don), vincristine and vinblastine, alone earned $100 million per drug for Eli Lilly Company (Farnsworth, 1988). In addition, Paclitaxel (taxol) sold more than $600 million in 1996, and etoposide (from *Podophyllum peltatum* L. or mayapple) more than half that figure.

The stakes in drug development are high and payoff is uncertain. Finding a valuable compound has a high cost since the probability of locating one with a desired action is low. It is often necessary to test as many as 10,000 substances in order to find one that may reach the drug market (Reid et al., 1993). Developing a successful drug can require screening of some 1000 plant species. Research and development costs are generally high, at an average of $868 million per drug for those developed between 1989 and 2002, with nearly 12 years needed to go from discovery to market (DiMasi et al., 2003).

International laws directly affect biodiversity-prospecting. *Intellectual Property Rights* and *Human and Indigenous Rights* are measures to be used for the protection of traditional cultural manifestations, including cultivated plants, medicines, and knowledge of useful properties of plants (Akerele et al., 1991). These laws guarantee rights to participate in the use, management, access, and conservation of these resources and should involve *sharing in the benefits*. The objectives of such laws should include conservation of plant and animal diversity, sustainable development of genetic resources, and the fair and equitable sharing of the resultant benefits (Reid et al., 1993).

A Costa Rican private, nonprofit organization, Instituto Nacional de Biodiversidad (INBio), was established to facilitate conservation and sustainable use of biodiversity. Other private, nonprofit intermediaries are based in developed countries. In the United States, for example, the New York Botanical Garden, the Missouri

Botanical Garden, and the University of Chicago have all contracted with private pharmaceutical companies and public research organizations to provide samples of biodiversity for pharmaceutical development. It is important that pharmaceutical companies involved in such contracts return an equitable share of their profits from any plant-derived drugs they develop from such plants to the indigenous peoples from whom these plants and the knowledge about their medical uses are obtained. Good role models are provided by *DENALI BioTechnologies, LLC (DENALI)*, Kenai, Alaska 99611, and the former *Shaman Pharmaceuticals* from South San Francisco, CA.

15.2.7 Integration of Traditional Knowledge and Biotechnology to Address and Prevent Overcollection of Medicinal Plant Species in Their Natural Habitats

Overcollected medicinal plants in the United States (see above) were originally important to its Native American cultures. Settlers and immigrants to the land learned the healing values of these indigenous plants, but generally poor stewardship imposed through indifferent, thoughtless, and destructive practices has led to their demise. A similar lack of respect for traditional knowledge and habitat destruction has led to the loss of many species in biodiversity-rich tropical regions.

A little studied area of the world that is facing habitat change is the circumpolar Arctic. While presumably less diverse in number of species than temperate or tropical flora, the plants of this region are uniquely adapted to harsh conditions. Despite the vigor of individual species, plants in Arctic habitats are particularly interwoven and vulnerable to mismanagement, making their recovery from untoward events slow and often incomplete. Paradoxically, this environment is both robust and fragile.

The diverse Native cultures of Alaska have witnessed many changes to their traditional lifestyles in the last few decades. Since Alaska achieved statehood in 1959, and the Alaska Native Claims Settlement Act of 1971 extinguished the sovereign rights of Alaska Native tribes in exchange for $962 million (ANCSA, 1971), a rapid introduction of a currency-based economy, influx of new residents, aggressive natural resource exploration and development, and installation of military facilities and related technologies during the former Cold War transformed their lives forever. Current changes in the global climate further challenge the traditional, subsistence existence (defined as taking only what one needs from plants and animal resources to survive) that served them for thousands of years. Important medicinal and nutritional plants are regarded still with reverence and typically are guarded with ferocity.

DENALI, Alaska's only biotechnology company, is working with Native organizations throughout the state to develop medicinal plant resources through genetic analysis and modification, secondary metabolite identification, and bioactive compound quantification, with the intent to generate pharmaceutical agents. DENALI's collection program for research specimens follows a strict protocol for selection

and harvesting practices. Working with elders and healers, plants in the Alaska flora have been prioritized based on documentation of medicinal efficacy, abundance, sustainability of harvest, and potential economic value to the Native cultures who used them for subsistence purposes. Priority plants are harvested in small quantities (e.g., 1 quart to 2.5 gallon Zip-loc® bags) and specimen locations are marked by global positioning satellite (GPS) technology. Less than one-third of the biomass in any location is harvested at a given time in order to preserve material for future collection. Specimens are stored frozen prior to undergoing low-volume extraction, typically with 5–10 g of tissue per extraction and five solvents of highly different polarity. Subsequently, extracts are fingerprinted by high-performance liquid chromatography (HPLC) and mass spectrometry. If the occurrence of a specific compound is sought, HPLC analyses are conducted in the presence and absence of a reference standard (e.g., artemisinin in wormwood (*Artemisia* spp.) extracts).

Fractions containing novel compounds are pursued in assays that demonstrate bioactivity. In the event a bioactive(s) is identified, locations that were previously harvested are revisited; but, for large harvests (up to 1,000 kg), numerous locations are added in order to obtain additional biomass while minimizing the "footprint" of collection activity. The biomass is immediately dried by a rapid and gentle process known as *Refractance Window® Drying* (MCD Technologies, Inc., Tacoma, WA) and vacuum sealed so as to maintain stability for at least 3 years. This material is extracted on a larger scale with the preferred solvent and conditions delineated on a small scale (as above) to obtain the pure "lead" compound or "new chemical entity" (NCE) of interest. At this point, the compound is ready to undergo preclinical evaluations in animal models and early phases of human trials.

Today, Alaska Natives appreciate the need to integrate into the fabric of modern life, but they strive to maintain their resources and traditional knowledge of use and management. Examples of traditional medicinal plants under investigation and development include devil's club, also called Alaska ginseng (*Oplopanax horridus* C.A. Mey.), wormwood, also known by an alternative common name, stinkweed, (*Artemisia tilesii* Ledeb.), and blueberries (*Vaccinium* spp.) (Fortuine, 1988).

Devil's club is the panacea plant of the Tlingit, Haida, and Gitskan of Southeast Alaska and British Columbia (Fortuine, 1988; Moerman, 1998; Johnson, 2006). Traditionally, it is used for treatment of a variety of ailments including, but not limited to, wounds, infections, colds, cough, fever, arthritis, and diabetes. Due to protest over its special cultural significance, commercialization of the plant has not occurred on an industrial scale. Tribal elders and healers recognize that devil's club requires many years to reach maturity and medicinal value; thus, overcollection would be devastating to this species which currently grows abundantly in its natural habitat.

Modern science has demonstrated in vitro antioxidant, anti-proliferative, antibacterial, anti-tubercular, anti-fungal, anti-inflammatory, and anti-viral activity against respiratory syncytial virus; this is consistent with traditional uses (McCutcheon et al., 1995; Kobaisy et al., 1997; Tai et al. 2006; Inui et al., 2007). Notably, authentic ginsengs are used for very similar medicinal purposes. Devil's club constituents resemble those of authentic ginseng species and, as a result, it has been discussed as a substitute to overcollected ginseng species (*Panax* spp.)

for medicinal and nutritional supplement purposes. A partial list of bioactive compounds found in devil's club through HPLC profiling of extracts includes *trans*-nerolidol, sterols, including daucosterol and beta-sitosterol, syringin, a series of triterpenoid saponins, and a group of polyynes (Kobaisy et al., 1997; Gruber et al., 2004). Some of these compounds, known also to occur in *Panax* species, were identified in closely related *Oplopanax elatus* Nakai (as judged by sequences in ITS regions of nuclear rDNA), which is indigenous to the Russian Far East (Zhang et al., 1993; Wang et al., 1996; 1997; 2004; Artiukova et al., 2005).

The major bioactive ingredients of ginseng are ginsenosides, which are also classified as triterpene saponins. Because ginseng cultivation is relatively expensive, time-consuming, and labor-intensive, and because the productivity of ginseng cell and tissue culture is low, it has become important to provide alternatives to ginseng propagation through other biotechnological methods (Liang and Zhao, 2008).

Ribosomal and biosynthesis-related gene sequence analyses offer taxonomic positioning of devil's club in the family Araliaceae (Artiukova et al., 2005). Further taxonomic proximity may be established through comparison of conserved DNA sequences in the ribosomal ITS1-5.8S-ITS2 region between authentic ginseng, already characterized in six species, *Panax ginseng* C. A. Mey., *Panax quinquefolius* L., *Panax japonicus* C. A. Mey. (Japanese ginseng), *Panax notoginseng* (Burkill) F. H. Chen (Sanchi), *Panax trifolius* L., and *Panax major* Ting (Ngan et al. 1999). Once established as a ginseng, or close relative, the possibility for using devil's club (also referred to as *Panax horridus* C. A. Mey.) as a surrogate for ginsenoside production becomes viable.

Ongoing research strives to understand and improve ginsenoside levels in *Panax* by metabolic engineering that is based on the biosynthetic pathway of ginsenosides. Candidate enzymes include squalene synthase, oxidosqualene cyclase, involved in the cyclization reaction of 2,3-oxidosqualene, cytochrome P450 isoforms, various glycosyltransferses and, in particular, dammarenediol-II synthase, the first dedicated enzyme for dammarane-type ginsenoside biosynthesis, versus oleanane-type compounds that also occur in the genus *Panax* (Jung et al., 2003; Lee et al., 2004). DNA sequences that encode enzymes of ginsenoside biosynthesis should be compared between *Panax* species and devil's club. *Expressed sequence tags (ESTs)* provide a valuable tool that can be used to identify secondary metabolite-associated genes and help to predict the potential for ginsenoside production in devil's club (Jung et al., 2003). Because devil's club offers similarities to overcollected *Panax*, and is unthreatened in the wild, it may prove to be an acceptable substitute for metabolic engineering of a ginsenoside pathway in preparation of responsible cultivation and management in its natural habitat.

Wormwood is the most important medicinal plant of the Yu'pik Eskimos of the Yukon-Kuskokwim Delta region and the Athabascans of Interior Alaska. Wormwood has been used traditionally for many of the same purposes as devil's club, although it belongs to the family Asteraceae and is, taxonomically, quite dissimilar. As its common name would imply, some of the most interesting traditional uses were as an anti-parasitic and ethnoveterinary anti-helminthic for sled dogs. The closely related Chinese species, *Artemisia annua* L. (sweet wormwood),

has received extensive scientific and medical attention because of its compound, artemisinin, which is active against the malaria parasite, *Plasmodium* (Weina, 2008). These clues, derived from thousands of years of medicinal use, should trigger a similar investigative program on wormwood as that described above for devils' club. Recent research on *A. annua* is focused on secondary metabolite biosynthetic pathways for enhancement of artemisinin yield. As an unthreatened, prolific *Artemisia* species, Alaska wormwood may prove to be an alternative natural source of artemisinin, amenable to metabolite engineering for artemisinin production, or as a reservoir of other promising novel bioactive molecules, most of which await study and development.

15.2.8 The Concept of "Ranching" Wild Vaccinium Species with Superior Properties as a Nutraceutical and Potential Pharmaceutical

The beneficial properties of berries were understood instinctively by humans throughout the millennia and, of these, *Vaccinium* species have been revered by indigenous peoples for their food and medicinal values. Modern science now provides a biochemical basis for the health-promoting effects of *Vaccinium,* a staple wherever humans established a culture in cooler, higher latitude or altitude regions of the world and where a large variety of food plants were unavailable.

Several *Vaccinium* species of worldwide economic importance occur in the United States. The most widely cultivated is *Vaccinium corymbosum* L. (highbush blueberry), grown from the Mid-Atlantic to California, Oregon, and Washington, and from the Upper Midwest to the Mid-South, with Michigan and New Jersey leading production. *Vaccinium angustifolium* Ait. (lowbush, or "wild", blueberry) is adapted to the far North and is commercially important in Maine and Eastern Canada, as well as in parts of New Hampshire, Massachusetts, Michigan, and Wisconsin. *Vaccinium ashei* Reade (rabbiteye blueberry) is well-adapted to the warmer climates of the South. In addition to blueberries, the genus also includes *Vaccinium macrocarpon* Ait. (cranberry), another principal crop of more Northern locations.

During the past decade, demand for blueberries and cranberries has grown dramatically as a result of increasing awareness in the scientific community and by consumers of their healthful properties. In addition to greater amounts consumed annually as fresh, frozen, and processed fruit, these berries have become important components of *nutraceuticals* or dietary supplements. Cranberry extract, used primarily for maintenance of urinary tract health, was the 14th most popular dietary supplement, whereas *Vaccinium myrtillus* L. (European bilberry), a close relative of *V. angustifolium*, was placed no. 21 for its beneficial effects on retinal and vascular health (Nutrition Business Journal, 2004). Extracts of *V. myrtillus* are widely used in prescription and over-the-counter medications. Preparations derived from its fruit are recognized in "The Complete German Commission E Monograph: Therapeutic Guide to Herbal Medicines" (Blumenthal, 1998) and in the "PDR for Herbal Medicine", which also cites the bog bilberry, *Vaccinium uliginosum* L. (Gruenwald,

2004). There are currently over 180 *Vaccinium* phytopharmaceutical products available worldwide. As these medications become increasingly popular, European crops can no longer meet the global demand. In response to this shortfall, extracts of generally similar North American *V. angustifolium* are now being considered as an alternative to more expensive *V. myrtillus* extracts (Kalt and Dufour, 1997).

Three species that are not currently used in commerce, but stand out with regard to their recognized importance in the subsistence diets of Native Americans and Alaska Natives, are *Vaccinium ovatum* Pursh (evergreen huckleberry), *Vaccinium ovalifolium* Sm. (Alaska black huckleberry), and *V. uliginosum*. Although *V. ovatum* possesses a remarkable array of flavonoids with beneficial properties (Taruscio et al., 2004), its occurrence within its natural range and adaptability to cultivation is considered too limited to be commercially viable. *V. ovalifolium* is equally remarkable as *V. ovatum* in its profile of *flavonoid compounds* (Taruscio et al., 2004) and, like *V. uliginosum*, grows prolifically without cultivation of any sort, throughout Alaska. Some experts estimate that hundreds of millions of pounds of *Vaccinium* species are available each growing season (Matz, 1996).

V. ovalifolium forms dense thickets up to subalpine levels, and is the most common woodland and coastal forest berry, providing most of those picked in maritime, rainforest habitats of Alaska. *V. uliginosum* occurs on vast expanses of wet tundra habitat throughout the state, and is a ubiquitous, low spreading, dwarf, alpine species, and is the best-known and most-used berry in Alaska for food purposes (Hulten, 1968; Matz, 1996). Despite spectacular annual yield, these berries are cyclical in year-to-year productivity, and occur in remote areas with extremely rugged terrain that makes harvest of large quantities difficult and expensive. As a result, picking machinery cannot be employed and berries must be hand-gathered with claw-like implements. While these physical obstacles are considerable, there is the additional inherent danger of gathering berries where grizzly and black bears are eating voraciously in preparation for hibernation.

Irrespective of barriers to large-scale commercialization, DENALI has gathered sufficient quantities of *V. ovalifolium* (which in the wild extensively introgresses with the closely related species *Vaccinium alaskaense* How.) and *V. uliginosum*to formulate its first nutraceutical product, *AuroraBlue*®. Replete with flavonoids, including 15 prominent *anthocyanins*, a multitude of *polyphenolics*, high levels of monomeric, oligomeric, and, most importantly, high molecular weight *proanthocyanidin polymers*, AuroraBlue is comprised of biomass from >90% *V. ovalifolium/V. alaskaense*, <10% *V. uliginosum* and subspecies, and <1% of various other *Vaccinium* species that occur concomitantly in wild stands. The unique profile contributed to AuroraBlue by *V. ovalifolium* × *V. alaskaense* suggests a chemotaxonomic position for this species somewhere between the blueberry, bilberry, huckleberry, and cranberry, with the individual health-promoting characteristics of each bundled into one (DENALI BioTechnologies, 2008). Very recent studies reveal that preparations of wild *V. ovatum*and *V. ovalifolium* are the most effective *oxygen radical scavengers* in the Ericaceae and *V. ovalifolium* specimens growing at high latitudes (greater than the 55th parallel) have the highest *oxygen radical absorbing capacity* (*ORAC*) values of any *Vaccinium* species tested to date (Taruscio et al., 2004; DENALI BioTechnologies, 2008).

As demand for AuroraBlue has been increasing, DENALI embarked on a "*ranching*"*program* in 2002 with its Alaska Native collaborators to facilitate gathering and supply of commercial quantities of berries. Consistent with the truly wild features of the product, ranched plants are neither genetically manipulated nor supported with conventional fertilizers, pesticides, or herbicides. In ranching stands, soil pH, nutrient composition and other characteristics must mimic the original growth habitat. Should adjustment of nutrient composition of the soil be required, compost of wild salmon waste and chips of the white spruce *Picea glauca* (Moench) Voss constitute the fertilizer of choice. These efforts are directed not only to improving yield, but also to protecting and preserving the fragile and special ecosystems of the only temperate rainforests, as well as diverse tundra, in the world. The challenges DENALI faces are associated with successfully using nonintrusive approaches for greater productivity and preservation of the unique biochemical composition of ranched *V. ovalifolium* and *V. uliginosum*. Based on region-specific history and experience, DENALI's principal ranching approaches include the following:

Utilization of clear-cut stands. Left over from logging activities of the paper and pulp industry in Alaska's forested lands, clear-cut stands typically support vigorous growth of *Vaccinium* plants previously restricted by shade from the old-growth tree canopy. In most cases, *Vaccinium* is dependent upon the presence of associated plants of other genera that comprise its communities or complexes in the forest, and clear-cut stands retain these critical ecological relationships (Tirmentein, 1990). As a result of enhanced exposure to more intense sunlight, each bush grows taller with larger leaves, and fruit is more abundant, larger, and darker in epidermal pigmentation. The desirable flavonoid composition of plants in clear-cut stands is maintained, also, as the phenylpropanoid pathway from which flavonoids arise, is triggered by *UV-B light* (Dixon and Paiva, 1995). Thus, clear-cuts offer excellent areas for *Vaccinium* ranching.

Utilization of burned stands. Whereas controlled burning is a management technique employed in other areas, annual summertime wildfires are typical throughout Alaska. Subsequent to naturally occurring forest fires, some of the earliest and most prolific re-growth may be achieved by *Vaccinium*, but for *V. ovalifolium*, re-growth is variable (Tirmentein, 1990). Above ground portions of the plants are commonly killed by fire, but underground rhizomes survive wildfires or controlled burns. Survival typically decreases with higher fire intensity and severity, although some rhizomes survive even hot wildfires, as long as soil is sufficiently deep to offer some protection. Some plants may survive even after lethal heat penetration to depths of 3.5–4.7 inches (9–12 cm). If affected, rhizomes are typically most susceptible to heat damage during the period of active growth in the spring and early summer, which is prior to the most prevalent time for wildfires later in the season. The advantage of burning is freedom from competing genera that recover more slowly for some years following the burn.

Enrichment. In DENALI's experience over the past 5 years, stands that have been heavily picked by hand tend to produce more fruit over the following years, as a considerable percentage of berries removed from the bush fall to the ground for reseeding. That noted, individual, established plants within a stand appear to be

on a 2-year cycle for fruiting. The stands seem to display a modest subsequent gain in plant density, as well as in fruit density per plant. In general, *Vaccinium* seeds are thought to be poorly viable, although refrigerated and frozen fruit can be used to begin seedlings for over 10 years after being held in storage (Tirmentein, 1990). Interestingly, refrigerated berries appear as fresh as when picked for up to 6 months at 4°C. As a result of these observations, thinly populated stands are enriched with seeds from plants originating in nearby more heavily populated stands. Since these seeds are likely to be borne on plants emergent from the same rhizome system, they are considered to be genetically comparable.

Wild species are preferably relocated directly to ranching stands through hardwood cuttings, bare root specimens, or by container (USDA, NRCS, 2004). Specimens are transferred from their native habitat during dormancy, typically before the snow has melted in the spring and after leaves have dropped in the fall. Compared to cultivated highbush blueberries that thrive at a planting density of 1400–1700 per acre (Dailey, personal communication, 2005), wild stock prefers a higher planting density of 2700–4800 per acre. Like cultivated *Vaccinium* species, wild species can be propagated vegetatively through hardwood cuttings that are rooted and raised in a greenhouse for about 1 year before planting out, but will require at least 2–3 years of acclimation before fruiting. Although tissue-cultured plants also succeed in the field, they may have a different form than plants derived from hardwood cuttings, tending to spread at the base, making ultimate machine harvesting difficult, whereas hardwood cuttings will grow more upright. Tissue-cultured stock is no longer considered wild, and that distinction may lower the market value in certain segments of berries used for nutraceutical grade AuroraBlue. Similarly, genetically modified *Vaccinium* may not represent the most valuable source material for products based on totally natural marketing messages but, conversely, may serve as valuable sources of extractable flavonoids for pharmaceutical/phytopharmaceutical formulations.

Despite the target market for the above-said product, ranching will provide a more economical approach to provision of commercial quantities of truly wild Alaskan berries. As a result of these efforts, Alaska will be able to develop a sustainable economic sector based on a renewable resource that is far more environmental friendly than the traditional industries of oil drilling and extraction, logging, mining, fishing, and tourism. Valuable relationships are being formed between DENALI and state and borough economic development organizations, rural villages, native corporations and associations, and private landholders to create an emergent nutraceutical industry in Alaska. This industry will provide benefits to all who produce and consume wonderful *Vaccinium* fruits for their widely appreciated health-promoting properties.

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Chapter 16
The Potential of Biofumigants as Alternatives to Methyl Bromide for the Control of Pest Infestation in Grain and Dry Food Products

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Abstract Fumigation is still one of the most effective methods for the protection of stored grain and dry food from insect infestations. Phosphine and methyl bromide are the most widely used fumigants for the control of stored-product insects. Phosphine is mainly used today, but there are repeated reports that a number of storage pests have developed resistance to this fumigant. Methyl bromide has been identified as a contributor to ozone depletion by the United Nations World Meteorological Organization in 1995 and, thus, was phased out in most developed countries. Thus, there is an urgent need to develop alternatives with the potential to replace these fumigants.

The primary aims of the current study are to evaluate the potential use of essential oils obtained from aromatic plants as insect fumigants and to evaluate the toxicity of the known isothiocyanates (ITCs) as compared to a new ITC isolated from *Eruca sativa* (salad rocket) as fumigants for the control of stored-product insects. Also, the biological activity of carbon disulphide (CS_2), methyl iodide (CH_3I), and benzaldehyde (C_7H_6O) is evaluated.

The toxicity of the various fumigants was assessed against adults, larvae, and pupae of six major stored-product insects. Two essential oils isolated from *Lamiaceae* plants were found to be the most potent fumigants as compared with a large number of other essential oils. ITCs are also potential candidates, especially methylthio-butyl isothiocyanate, the main bioactive component in *E. sativa*, because of its low toxicity. Comparative studies with CH_3I, CS_2, and C_7H_6O showed that CH_3I was the most active compound against stored-product insects, followed by CS_2 and C_7H_6O. CH_3I was also found to be less sorptive and less penetrative in wheat than CS_2.

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16.1 Introduction

In developing countries, the post-harvest losses of cereals and other durable commodities caused by insect damage and other bio-agents range from 10 to 40% (Raja et al., 2001).

Fumigation with methyl bromide or phosphine is a quick and effective tool for the control of stored-product insect pests. In view of the scheduled phaseout of methyl bromide under the Montreal protocol, the role of phosphine in grain protection has increased and stands as the main alternative to methyl bromide. Lately, insect resistance to phosphine has become an important issue for effective grain treatment (Nakakita and Winks, 1981; Tyler et al., 1983; Rajendran and Karanth, 2000). A global survey of pesticide susceptibility demonstrated that 9.7% of the strains tested showed resistance to phosphine (Champ and Dyte, 1976). Another compound, 2,2 dichlorovinyl dimethyl phosphate, which is widely used as a fog fumigant for insect control in empty structures, is classified by the US Environmental Agency as a possible human carcinogen (Mueller, 1998). Therefore, there is an urgent need for new strategies. Thus, in recent years, research has focused on a search for alternative fumigants for the control of stored-product insects. In this chapter, we present a comprehensive laboratory and semi-field studies to evaluate the potential use of essential oils (EOs) obtained from aromatic plants and isothiocyanates (ITCs), methyl iodide (CH_3I), carbon disulfide (CS_2), and benzaldehyde (C_7H_6O) for the control of stored-product insects.

During previous centuries, traditional agriculture in developing countries has developed effective means for insect control using *botanicals*. Their efficiency and optimal use still need to be assessed in order to make these means of insect control cheap and simple for users. Lately, a new field has evolved which emphasizes the use of phytochemicals for insect pest management. The bioactivity of essential oils (EOs), the major volatile in aromatic plants, and their constituents, has been well documented against a large number of insect pests. An example is the EO obtained from the leaves of *Thugopsis dolabrata hondai* which was found to have high bioactivity against the cockroach (*Periplaneta fuliginosa*), the mite (*Dermatophagoids farinae*), and the termite (*Coptotermes farmosanus*) (Asada et al., 1989; Lee, 2004). Some EOs were found to exhibit repellent activity against various insects (Kalemba et al., 1991; Hassalani and Lwande, 1989; Mwangi et al., 1992). Others were found to be potent growth inhibitors and anti-feedants (Jermy et al., 1981; Koul et al., 1990). These essential oils were also found to be effective as nematicidal (Oka et al., 2000), anti-bacterial (Matasyoh et al., 2007), virucidal (Schuhmacher et al., 2003), and repellents against ectoparasites (Mumcuoglu et al., 1996).

The efficacy of essential oils as fumigants for the control of pest infestations in grain and dry food products was also evaluated. EOs and their constituents are known to possess insecticidal (Wilson and Shaaya, 1999; Shaaya et al., 1997) and insect repellent activity (Jilani et al., 1988) and to cause a reduction in progeny (Regnault-Roger and Hamraoui, 1995). For example, the fumigant toxic activity, anti-feedant, and reproduction inhibition induced by a number of EOs and their monoterpenoids were evaluated against the bean weevil *Acanthoscelides obtectus*

(Say) and *Callosobruchus maculatus* (F.) (Klingauf et al., 1983; Regnault-Roger and Hamraoui, 1995; Raja et al., 2001). EOs extracted from *Pogostemon heyneanus*, *Ocimum basilicum* (basal), and *Eucalyptus* showed insecticidal activity against *Sitophilus oryzae*, *Stegobium paniceum*, *Tribolium castaneum*, and *Callosobruchus chinensis* (Deshpande et al., 1974; Deshpande and Tipnis, 1977).

In our laboratory, in order to isolate active EOs, we screened a large number of EOs extracted from aromatic plants and isolated their main constituents. We have already isolated many such compounds from the EOs of a large number of aromatic plants (Shaaya et al., 1991, 1994, 1997). Using space fumigation (see Shaaya et al., 1997), two EOs obtained from Lamiaceae plants were found to be the most potent fumigants of all oils tested. The main component of one of the oils is pulegone. The other is not yet identified and it is called SEM76 (Shaaya and Kostyukovsky, 2006).

In our study of the mode of action of EOs, we could show that the target for EO's neurotoxicity is the octopaminergic system in insects. We can thus postulate that EOs may affect octopaminergic target sites (Kostyukovsky et al., 2002; Shaaya et al., 2002).

ITCs were chosen for this study because of the pesticidal properties of these chemicals (Fenwick at al., 1983) and because of the potential use of methyl ITC as fumigant for wheat (Ducom, 1994). In our study on the rates of sorption of homologous series of ITCs on wheat, we could show that the rate of sorption decreases with increasing molecular weight (Shaaya and Desmarchelier, 1995). In the case of methyl ITC, a withholding period over 1 week would be required before residues decayed to levels near the limit of detection (Shaaya and Desmarchelier, 1995).

Comparative studies with CH_3I, CS_2, and C_7H_6O showed that CH_3I was the most potent compound against stored-product insects, followed by CS_2 and C_7H_6O. CS_2, according to Winburn (1952), was one of the most effective grain fumigants as viewed from efficiency and low cost points of view. C_7H_6O occurs in kernels of bitter almonds, has low toxicity to mammals, and has widespread use in topical antiseptics.

16.2 The Materials and Methods

The *materials and methods* employed in the current study are described as follows.

The tested stored-product insects were laboratory strains of *S. oryzae, Rhyzopertha dominica, Oryzaephilus surinamensis, T. casteneum, Trogoderma granarium, Plodia interpunctella*, and *Ephestia cautella.*

The isothiocyanates (ITCs) are obtained by putting 100 g ground seeds into round bottom flask containing buffer solution (1% ascorbic acid). The flask is held in a water bath (temperature = 70°C) for 2 h to facilitate the hydrolysis of sinigrin to ITC by the enzyme myrosinase which is found inside the seeds. The second step is steam distillation with use of the Dean–Stark apparatus (Leoni et al., 1997). The yellow upper layer is then separated and extracted with petroleum ether. Finally, the petroleum ether is evaporated under a stream of air. The unknown ITC obtained

from the seeds of *E. sativa* was identified as methyl thio-butyl isothiocyanate by gas chromatography (GC), nuclear magnetic resonance (NMR), and infra-red (IR) spectroscopy. CS_2, CH_3I, and C_7H_6O were purchased from Sigma Chemical Company, St. Louis, MO, USA. The essential oils from the aromatic plants were obtained from freshly harvested leaves and stems by steam distillation.

Three types of *bioassays* were performed to evaluate the activity of the fumigants. The first screening of the compounds was space fumigation in glass chambers of 3.4-L capacity (for details see Shaaya et al., 1991). The highly active compounds were then assayed in 600-mL glass chambers, filled to 70% by volume with wheat (11% moisture content). Pilot tests were carried out in simulation glass columns of 10 cm in diameter × 120 cm in height, filled to 70% by volume with wheat (11% moisture content). The insects were introduced in cages, each holding 20 insects of the same species together with food. Groups of four cages were suspended by a steel wire at different heights from the bottom of the column. Percentage of insect mortality was then determined.

The *essential oils* (*EOs*) of aromatic plant families are volatiles that can be easily extracted by hot water vapors. The main components of the EOs are *monoterpenes* and, to a lesser extent, *sesquiterpenes* (Brielmann et al., 2006). The majority of EOs contain a limited number of main constituents, although minor compounds in the oil can also play an important role in the fragrance and biological activity.

In order to isolate bioactive EOs, we screened a large number of EOs extracted from aromatic plants and isolated their main constituents by methods cited in Shaaya et al. (1991, 1994, 1997). Using space fumigation methodology, the two EOs obtained from our experimental Lamiaceae plants were found to be the most potent fumigants as compared with all other essential oils obtained from a large number of aromatic plant species tested against stored-product insects (Table 16.1)
.

Table 16.1 List of aromatic plants whose essential oils were tested for bioactivity

Species	Family	Species	Family
Apium graveolens	*Apiaceae*	*Micromeria fruticosa*	*Lamiaceae*
Artemisia arborescens	*Compositae*	*O. basilicum*	*Lamiaceae*
A. judaica	*Compositae*	*O. gratissimum*	*Lamiaceae*
Carum carvi	*Apiaceae*	*Origanum vulgare*	*Lamiaceae*
Citrus limonum	*Rutaceae*	*Pelargonium graveoleus*	*Geraniaceae*
Coriandrum sativum	*Apiaceae*	*Petroselinum crispum*	*Apiaceae*
Cuminum cyminum	*Apiaceae*	*Pimpinella anisum*	*Apiaceae*
Cymbopogon citrates	*Poaceae*	*Rosmarinus officinalis*	*Lamiaceae*
Foeniculum vulgare	*Apiaceae*	*Ruta chalepensis*	*Rutaceae*
Laurus nobilis	*Lauraceae*	*Salvia dominica*	*Lamiaceae*
Lavandula officinalis	*Lamiaceae*	*Salvia fruticosa*	*Lamiaceae*
Majorana siriaca	*Lamiaceae*	*Salvia officinalis*	*Lamiaceae*
Matricaria camomilla	*Asteraceae*	*Salvia sclarea*	*Lamiaceae*
Mentha piperita	*Lamiaceae*	*Satureja thymbra*	*Lamiaceae*
M. rotundifolia	*Lamiaceae*	*Thymus vulgaris*	*Lamiaceae*

Table 16.2 Fumigant toxicity of SEM76 and pulegone on some stored-product insects (space fumigation)

Terpenoids	Concentration, $\mu L \cdot L^{-1}$	Stage	% Mortality (7 days after treatment)					
			Sitophilus	*Oryzaephilus*	*Rhyzopertha*	*Tribolium*	*Plodia*	*Trogoderma*
SEM76	0.5	Adult	100	100	100	87		
	1		100	100	100	100		
Pulegone	0.5		100	100	100	100		
Limonene	0.5		27	27	24	0		
SEM76	2	Larvae				60	90	55
	4					96	100	100
Pulegone	2					58	63	55
	4					100	100	100

Exposure time –24 h.
Third instar larvae and 3-day old pupae were used.

The main component of one of the oils was pulegone and of the other is not yet totally identified, and it is called SEM76. In space fumigation, these two volatiles caused total mortality of all adults tested at very low concentrations of 0.5 $\mu L \cdot L^{-1}$ air and exposure time of 24 h. A higher concentration of 4 $\mu L \cdot L^{-1}$ air was needed to kill larvae of *Tribolium, Trogoderma*, and *Plodia*. Limonene which is regarded as active monoterpene has much lower activity (Table 16.2).

Table 16.3 Fumigant toxicity of SEM76, with and without CO_2, against five stored-product insects on winter wheat, in columns 70% filling, in pilot tests

Stage	Concentration, $\mu L \cdot L^{-1}$	% Mortality (7 days after treatment)				
		Sitophilus	*Tribolium*	*Oryzaephilus*	*Rhyzopertha*	*Plodia*
Adults	70	100	66	100	70	–
	50 + 15% CO_2	100	96	100	100	–
	70 + 15% CO_2	100	100	100	100	–
Pupae	70 + 15% CO_2	–	75	–	–	100
Larvae	70	–	60	–	–	87
	70 + 15% CO_2	–	80	–	–	100

Exposure time – 7 days.

Pilot tests in simulation glass columns filled to 70% volume with wheat, under conditions similar to those present in large grain bins, showed that SEM76 at a concentration of 70 $\mu L \cdot L^{-1}$ air (equivalent to 70 $g \cdot m^{-3}$) and 7 days exposure time caused 100% kill of adults of *Sitophilus* and *Oryzaephilus*, but not of *Rhyzopertha* and *Tribolium* (Table 16.3). Supplementation of 15% CO_2 (200 $g \cdot m^{-3}$) caused reduction in the effective volatile concentration. A concentration of 50 $\mu L \cdot L^{-1}$ air was enough to cause 96–100% kill of all adult insects tested. For pupae and larvae of *Tribolium* and *Plodia*, a higher concentration is needed (Table 16.3).

16.3 Efficacy of Isothiocyanates (ITCs) as Fumigants for the Control of Pest Infestations in Grain and Dry Food Products

Mustard family (*Brassicaceae*) seeds contain ITCs, volatile essential oils that are known to possess insecticidal activity. By screening a number of various species of *Brassicaceae* seeds, namely, *Brassica nigra, B. carinata, B. tournefortii, Lepidium*

Table 16.4 The fumigant toxicity of four active isothiocyanates compared with methylthio-butyl ITC against adults of major stored grain insects. (Space fumigation)

Compound	Concentration, $\mu L \cdot L^{-1}$	Exposure time, h	% Mortality (7 days after treatment)			
			Sitophilus	*Rhyzopertha*	*Oryzaephilus*	*Tribolium*
Allyl ITC	1.0	1.5	–	100	100	0
	1.0	3.0	100	100	100	100
Methylthio-butyl ITC	1.0	3.0	100	89	100	0
	2.0	6.0	–	100	–	52
Methyl ITC	1.0	1.5	100	100	73	13
	1.0	2.5	100	100	100	100
Ethyl ITC	1.0	1.5	17	15	–	0
	1.0	3.0	100	100	–	0
	1.5	3.0	100	100	100	18
Butyl ITC	1.5	3.0	65	43	68	25
	3.0	3.0	100	100	100	100

Methylthio-butyl ITC was isolated from the plant *Eruca sativa*.

sativa, Sisymbrium irio, Sinapis alba, S. arvensis, E. sativa, and *Diplotaxis spp.*, only in the last three species was it possible to isolate from the seed oil an unknown ITC at concentrations of 98, 92, and 33%, respectively. Later, this compound was identified as methylthio-butyl ITC. In space fumigation, the biological activity of this compound was compared with four common ITCs, namely, allyl, methyl, butyl, and ethyl. Allyl and methyl ITCs were found to be the most active against adults of four stored-product insects. A concentration of 1 $\mu L \cdot L^{-1}$ air and exposure time of 3 h were enough to kill all the tested adult insects. The activity of methylthio-butyl ITC was comparable to that of allyl and methyl ITCs except for *Tribolium*, which was found to be much more susceptible to the two ITCs (Table 16.4).

In the case of *Plodia* larva also, a concentration of 1.5 $\mu L \cdot L^{-1}$ air of the three active ITCs and exposure time of 3 h were enough to get 100% kill. For larvae of *Tribolium* and *Trogoderma*, a higher concentration of 2.5 $\mu L \cdot L^{-1}$ air and exposure time of 3 h were needed. The pupae of these three insect species were the most resistant to the ITCs tested (Table 16.5).

Using high columns filled to 70% wheat to evaluate the toxicity of allyl ITC in grain, we could show that 20 $\mu L \cdot L^{-1}$ air (=20 g m^{-3}) and exposure time of 1 day were not effective in killing the insects at the bottom of the column when the fumigant was applied at the upper layer of the grain. Addition of CO_2 and circulation caused 100% kill at the different heights. Increasing the exposure time to 4 days and cycling was enough to obtain 100% kill (Table 16.6).

Table 16.5 The fumigant toxicity of four active isothiocyanates compared with methylthio-butyl ITC against larvae and pupae of major stored grain insects. (Space fumigation)

Compound	Concentration, $\mu L \cdot L^{-1}$	Exposure time, h	% Mortality (7 days after treatment)					
			Larvae			Pupae		
			Tribolium	*Trogoderma*	*Plodia*	*Tribolium*	*Trogoderma*	*Plodia*
Allyl ITC	1.5	3.0	23	84	100	52	65	–
	2.5	3.0	95	100	100	68	80	45
	5.0	3.0	100	100	100	100	98	100
Methyl ITC	2.5	1.5	65	77	100	50	7	97
	3.5	1.5	100	81	100	100	32	100
	5.0	1.5	–	100	–	–	–	–
Methylthio-butyl ITC	1.0	3.0	100	100	87	–	–	–
	2.0	3.0	100	100	90	–	–	–
Ethyl ITC	1.5	3.0	20	6	100	–	–	–
	1.5	4.5	49	23	100	–	–	–
	1.5	6.0	100	100	100	–	–	–
	2.5	3.0	–	–	–	2.5	3	27
Butyl ITC	1.5	3.0	5.5	23	7	–	–	–
	5.0	3.0	98	78	100	–	–	–

Methylthio-butyl ITC was isolated from the plant *Eruca sativa*.
Third instar larvae and 3-day old pupae were used

Table 16.6 Toxicity of allyl ITC against stored-product insects, using high columns filled with 70% wheat with and without CO_2

Insect	Insects' height, cm (top–bottom)	% Mortality (7 days after treatment)				
		Exposure time – 1 day			Exposure time – 4 days	
		20 μL·L^{-1} + 20% CO_2	20 μL·L^{-1} + cycling	20 μL·L^{-1} +cycling+20% CO_2	20 μL·L^{-1}	20 μL·L^{-1} + cycling
Tribolium	20	100	70	100	100	100
	120	0	17	90	0	100
Sitophilus	20	100	100	100	100	–
	120	35	100	100	94	–
Rhyzopertha	20	100	100	100	100	–
	120	5	85	100	78	–
Oryzaephilus	20	–	100	100	100	–
	120	–	100	100	100	–

16.4 Efficacy of CH_3I, CS_2, and C_7H_6O as Fumigants for the Control of Stored-Product Insects

In space fumigation, CH_3I was very effective against all insect stages tested. Exposure to a concentration of 3–5 μL·L^{-1} for 3 h was lethal and caused 100% mortality of all stages of the test insects, except for *Trogoderma* larvae (Table 16.7). Adults of *Tribolium* were found to be the most tolerant, followed by *Oryzaephilus*, *Rhyzopertha*, and *Sitophilus.* In the case of larvae and pupae, *Trogoderma* was the most tolerant, followed by *Tribolium* and *Plodia* (Table 16.7).

CS_2 was less effective than CH_3I and needed a concentration of 6–9 μL·L^{-1} air for 1 day to achieve total mortality of all the test insects except for *Trogoderma* larvae. In the case of CS_2, adults of *Tribolium* were found to be the most resistant, followed by *Sitophilus*, *Oryzaephilus*, and *Rhyzopertha.* The larvae of *Trogoderma* were more resistant than *Tribolium* (Table 16.8).

In experiments with 600-mL glass chambers filled to 70% volume with wheat, CH_3I also showed higher activity than CS_2. The concentration of CH_3I and exposure time needed to obtain a total mortality of the test insects were comparable to those in space fumigation tests (see Table 16.7). For CS_2, higher concentrations

Table 16.7 Toxicity of CH_3 I against stored-product insects, in space fumigation and in 600-mL chambers filled with 70% wheat

Test	Concentration, $\mu L_i L^{-}$	% Mortality (7 days after treatment)								
		Rhyzopertha	*Oryzaephilus*	*Sitophilus*	*Tribolium*			*Trogoderma*		*Plodia*
		Adults				Larvae	Pupae	Larvae	Pupae	Larvae
Space fumigation – 3 h	2.0	41	10	55	7	–	–	–	–	–
	3	94	85	100	65	40	–	–	–	100
	4	100	100	–	95	77	–	58	–	–
	5	–	–	–	100	100	100	70	0	–
	6	–	–	–	–	–	–	100	–	–
600-mL chambers with grain – 3 h	2.5	88	63	100	63	50	–	15	–	–
	3.5	100	88		80	60	–	40	–	–
	5	–	100		100	100	–	100	–	–

Specific gravity of CH3I –2.28
Third instar larvae and 3-day old pupae were used.

were needed (see Table 16.8). The large difference in the activity between the two compounds was probably due to higher sorption rate of CS_2 in wheat, as compared with that of CH_3I. In the pilot tests, in glass columns filled to 70% wheat, CH_3I again showed higher activity than CS_2, when circulation was applied. A concentration of 5 $\mu L \cdot L^{-1}$ air and exposure time of 3 h were enough to obtain 100% kill (Table 16.9) as compared with 20 $\mu L \cdot L^{-1}$ air CS_2 and 24 h exposure time (Table 16.10). In gravity applications, CS_2 penetrated better than CH_3I, but needed a higher concentration and exposure time to achieve total mortality (Tables 16.9 and 16.10). It should be mentioned that for methyl bromide fumigation the recommended concentration is 30–50 $g \cdot m^{-3}$.

16.5 Conclusions

Our findings, as well as those of other researchers, suggest that certain plant essential oils and their active constituents, mainly terpenoids, have potentially high bioactivity against a range of insects and mites. They are also highly selective to insects, since they are probably targeted to the insect-selective octopaminergic receptor, a

Table 16.8 Toxicity of CS_2 against stored-product insects, in space fumigation and in 600-mL chambers filled with 70% wheat

Test	Concentration, $\mu L \cdot L^{-1}$	Exposure time, days	% Mortality (7 days after treatment)					
			Rhyzopertha	*Oryzaephilus*	*Sitophilus*	*Tribolium*	*Trogoderma*	
			Adults				Larvae	
Space fumigation	5	1	72	53	23	0	–	–
	6		100	90	30	0	–	–
	7		–	100	74	10	–	–
	8		–	–	93	70	–	–
	9		–	–	100	100	100	60
600-mL chambers with grain	10	1	100	0	0	0	–	–
		2	–	17	32	0	–	–
		4	–	24	100	56	–	–
		5	–	100	–	100	–	–
	20	1	100	62	100	40	–	–
		2	100	100	100	100	–	–

Specific gravity of CS2– 1.26
Third instar larvae were used.

non-mammalian target. The worldwide availability of plant essential oils and their terpenoids, and their use in cosmetics and as flavoring agents in food and beverages, is a good indication of their relative safety to warm-blooded animals and humans. They are also classified as *generally recognized as safe* (*GRAS*). The ultimate goal is the introduction of these phytochemicals with low toxicity, which comply with health and environmental standards, as alternatives to methyl bromide and phosphine for the preservation of grain and dry food.

C_7H_6O was less active than CH_3I and CS_2 in space fumigation bioassays. A concentration of 3 $\mu L \cdot L^{-1}$ air and exposure time of 1 day caused 100% adult mortality of *Sitophilus, Rhyzopertha*, and *Oryzaephilus*. In the case of *Tribolium*, 65% mortality for adults and no effect on eggs and pupae were recorded (Table 16.11). In the case of *Ephestia,* this concentration caused 100% mortality of the eggs, but had no effect on pupae (data not shown). In studies with 600-mL fumigation chambers, a concentration of 50 $\mu L \cdot L^{-1}$ air and exposure time of 7 days caused 100% mortality of the adults tested except for *Tribolium*. Increasing the concentration to 100 $\mu L \cdot L^{-1}$ air yielded very low mortality of larvae, pupae, and adults of *Tribolium* (Table 16.11).

Table 16.9 Penetration of CH_3 I in 120-cm high columns filled with 70% wheat by gravity or circulation

Method used	Concentration, $\mu L \cdot L^{-1}$	Exposure Time, h	Insects' height, cm (top–bottom)	% Mortality (7 days after treatment)			
				Rhyzopertha	*Oryzaephilus*	*Sitophilus*	*Tribolium*
Gravity	5	24	Top 20 120 Bottom	100 10	100 10	100 30	100 0
	5	72	Top 20 120 Bottom	100 95	100 75	100 80	100 0
Circulation 3 × 45 min	5	3	Top to bottom	100% mortality of all insects			

ITCs are also potential candidates because only very low concentrations are needed for the control of stored-product insects. It should be mentioned that *E. sativa* (salad rocket) is used worldwide as a food supplement, and methyl thiobutyl ITC, the main bioactive component in this plant, has lower mammalian toxicity as compared to the other active ITCs tested. The lower toxicity makes this fumigant a promising candidate for the disinfestation of grain and dry food products.

Comparative studies with CH_3I, CS_2, and C_7H_6O showed that CH_3I was the most toxic compound to stored-product insects, followed by CS_2 and C_7H_6O. CH_3I was found to be less sorptive and less penetrative in wheat than CS_2. CH_3I is toxic to humans and its use in food as a fumigant is therefore limited. It should be mentioned that CS_2 is flammable and used mainly as a supplement to increase the activity of other fumigants. In fact, a mixture of trichloroethylene, carbon disulphide, and carbon tetrachloride (Calandrex^R) in a ratio of 64:26:10, respectively, was developed by us and was found to be effective against stored-product insects (Polachek et al., 1960). C_7H_6O has low toxicity to mammals, but it is less effective against stored-product insects than all other fumigants tested. CH_3I, CS_2, and C_7H_6O may play a role mainly as supplements to increase the activity of other fumigants.

In this context, we should keep in mind that a general consensus is very difficult to achieve in order to introduce broad-spectrum fumigants like methyl bromide or

Table 16.10 Penetration of CS_2 in 120-cm high columns filled with 70% wheat by gravity or circulation

Method used	Concentration, $\mu L \cdot L^{-1}$	Exposure time, h	Insects' height, cm (top–bottom)	% Mortality (7 days after treatment)			
				Rhyzopertha	*Oryzaephilus*	*Sitophilus*	*Tribolium*
Gravity	20	48	Top	100	100	100	100
			20	100	0	30	10
			120				
			Bottom				
	20	72	Top to bottom	100% mortality of all insects			
Circulation 3×45 min	20	24	Top to bottom	100% mortality of all insects			

Table 16.11 Fumigant toxicity of benzaldehyde against various developmental stages of stored-product insects using space fumigation and fumigation in 600-mL chambers filled with 70% wheat

Treatment	Concentration, $\mu L \cdot L^{-1}$	Exposure time, days	% Mortality (7 days after treatment)						
			Sitophilus	*Rhyzopertha*	*Oryzaephilus*	*Tribolium*			
			Adults				Pupae	Larvae	Eggs
Space fumigation	1.5	1	39	79	16	0	–		–
	3	1	100	100	100	65	0		0
Fumigation in 600-mL chambers	50	7	97	95	100	0	–		–
	100	7	100	100	100	19	–	0	–

Adult mortality in control was less than 5. Third instar larvae and 3-day old pupae were used.

phosphine. Because of this, alternative fumigants could be developed against particular species of insects or used for a specific food product commodity.

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