Chapter 9
Role of Recombinant DNA Technology in Biofertilizer Production

Rohaya Ali

Abstract Nutrients present in plants are important for the maintenance of crops and production of healthy food for the constantly rising population of the world. For this efficient means of maintaining soil fertility are required. Soil maintenance approaches nowadays are largely reliant on chemical fertilizers, which may pose a severe threat to human well-being and the environment. In this backdrop, biofertilizers have been recognized as a substitute for elevating soil richness and hence crop productivity in sustainable agricultural practices. The utilization of advantageous microbial organisms as biofertilizers is extremely significant in agriculture as it aids in maintenance of food security and elevating crop produce. Furthermore, biofertilizers are highly significant in maintaining the quality of soil. Microbes which are frequently utilized as biofertilizers include potassium solubilizers, nitrogen fixers, mycorrhiza, cyanobacteria or blue green algae, plant growth-promoting *Rhizobacteria*. Biofertilizers aid in nutrient uptake by plants, offer forbearance to biotic and abiotic situations to plants and also maintain plant growth. Biofertilizers maintain nutrient richness by means of nitrogen fixation, solubilization of potassium, production of antibiotics, disintegration of organic substances and release of plant growth-promoting agents. Biofertilizers, when given in the form of seed or soil inoculants, contribute in nutrient cycling and lead to enhanced crop production. Biofertilizers, therefore, play a vital role in maintaining soil nutrients and hence agricultural produce. Furthermore, biofertilizer production by using the tools of molecular biotechnology like recombinant DNA technology can improve the metabolic pathways of production of important plant growth-promoting factors like phytohormones,, if recognized and transmitted to the useful plant growth-promoting microbes. Recombinant DNA technology offers numerous benefits, as explicit biological pathways can be controlled with high accuracy and entirely novel functions can be engineered into the microorganisms for producing efficient biofertilizers.

Keywords Biofertilizers · Solubilizers · Mycorrhiza · Phytohormones · Cyanobacteria

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K. R. Hakeem et al. (eds.), *Microbiota and Biofertilizers*,
https://doi.org/10.1007/978-3-030-48771-3_9

9.1 Introduction to Biotechnology

The term "biotechnology" was coined by Karl Ereky and is blend of the two words, i.e. biology and technology. This field is tremendously divergent, enormous and multidisciplinary. Thus, a clear-cut description of the subject is slightly hard. Biotechnology is fundamentally the utilization of biological substances like microbes or cellular constituents in controlled fashion for the advantage of mankind (Okeno et al. 2012). In other words, biotechnology is an integrated utilization of biochemistry, microbiology and engineering knowledge for the utilization of microbes, cultured cells or tissues to their best. Human beings have sustained their search for enhancing the natural potential of microbes and making them competent for novel methods. In past, people exploited microbes for the production of cheese, bread production or brewing alcohol, even if the process of fermentation was not tacit comprehensively (Carpenter et al. 2002). At present, application of biotechnology is highly complicated. Now, scientists can manipulate living organisms and transfer genetic matter among them, producing transgenic organisms. The present relevance of biotechnology is largely in the area of biomedicine and agriculture. Current methods allow the construction of novel and enhanced food products. In biomedicine area, novel vaccines, antibiotics, etc. have been produced against various diseases like AIDS, cancer and many hereditary diseases. Biotechnology is also used in the area of bio-fuel production, mining and pollution control. Genetically modified microbes and plants are utilized to remove toxic chemicals from oil spoil spills or industrial effluents (Chen et al. 2007). Besides, improved superiority of life and still there exists a countless exhilarating opportunities in the field of biotechnology (Figs. 9.1 and 9.2).

9.1.1 Subfields of Biotechnology

Generally, biotechnology is categorized in three major subtypes:

- Green biotechnology
- White biotechnology
- Red biotechnology

9.1.2 Green Biotechnology

Green biotechnology is a vital field of contemporary biotechnology. Its foundation is on the crop enhancement and manufacture of new crop products (McAllister et al. 2012). This is achieved by introducing foreign genes into the plants having huge economic importance. It comprises of three major areas which include:

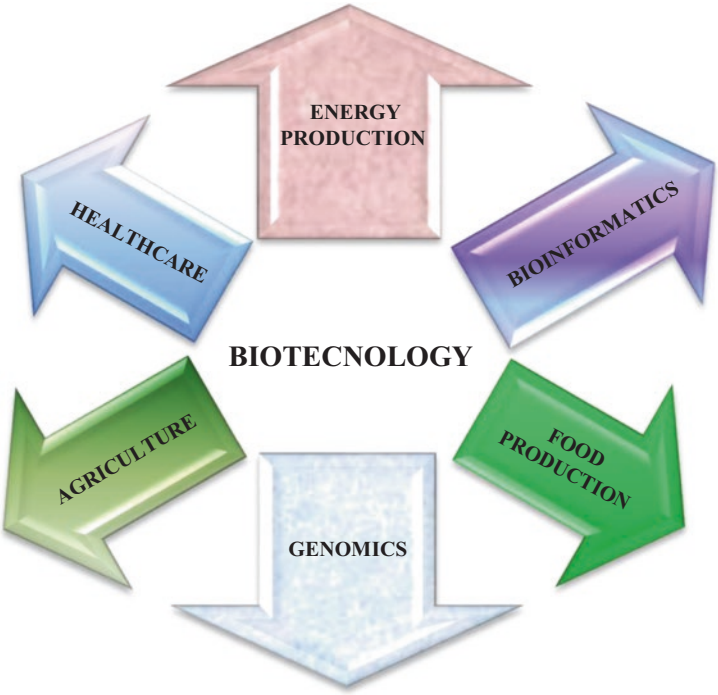

Fig. 9.1 Applications of biotechnology

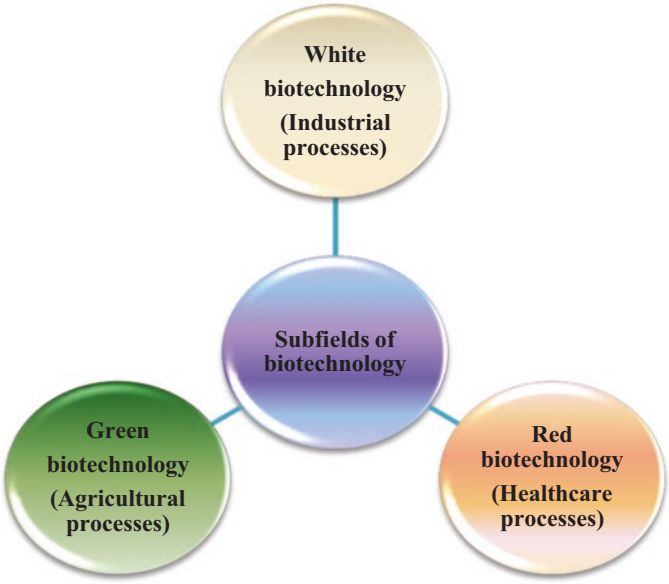

Fig. 9.2 Subfields of biotechnology

(a) Plant tissue culture
(b) Plant genetic engineering
(c) Plant molecular marker assisted breeding

Plant tissue culture involves the production of the whole plant or part of it under laboratory conditions. Its main advantage is the quick manufacture of plant materials like citrus fruits, banana, etc. On the other hand, plant genetic engineering involves the introduction of beneficial genes from one living organism to other. This generates improved varieties of crops with enhanced production (Brookes and Barfoot 2009).

In the case of plant molecular marker-assisted breeding, molecular markers (specific short sequences of DNA) are accountable for a preferred attribute. Thus, improved properties, like disease resistance, can be achieved (Horvath et al. 2012).

9.1.3 White Biotechnology

This area is concerned with industries. It utilizes enzymes, bacteria, yeast or moulds to produce valuable products. It results in the manufacture of wide range of bioproducts like vitamins, antibiotics, detergents, etc. (Bueno et al. 2016).

9.1.4 Red Biotechnology

It is concerned with medical biotechnology. It involves genetic manipulation of organisms to create antibiotics. Herein, the human body's own tools are utilized to eliminate the pathogens. It is of immense significance in the conventional drug discovery and also aids in improving the potential for cure, anticipation and analysis of diseases (Becker et al. 2008).

9.2 Recombinant DNA Technology

The growth and understanding of biological phenomena over the past few decades, both at molecular and cellular levels, is transfigured by the dawn of genetic engineering or recombinant DNA technology. This branch of biology is largely spawned under contemporary biotechnology that uses living organisms to generate enhanced and precious products for the betterment of human society. Chemical and biochemical engineering techniques are concerned with the production of recombinant DNA. Cultivation of microbes and their downstream processes rely on engineering techniques.

The history of recombinant DNA technology dates back to 1953, when the double helical structure of DNA was explicated by Watson and crick and the genetic code was cracked by Nirenberg. Afterwards, in 1973, the method of restriction digestion was invented by Cohen and Boyer which involve cut and paste of the DNA sequences (Ames and Martin 1964; Cohen et al. 1973).

Due to recombinant DNA technology, cloning of genes for production of polypeptides (growth factors, interferon, blood clotting factors, human insulin, viral coat proteins, etc.) has become achievable. Each of the polypeptide is unique in the context of its sequence or target. Now, with the advent of recombinant DNA technology, researchers can express a natural gene even in a very simple bacterium like *E. coli* (Brown et al. 2015).

Somatostatin was the first human protein produced in *E.coli* in 1977. Later in 1982, the first recombinant protein, i.e. human insulin, was available in the market. Kary Mullis in 1985 envisaged the idea of polymerase chain reaction (PCR) which revolutionized the field of recombinant biotechnology. Bimolecular archaeology, DNA fingerprinting, molecular ecology, forensics, etc. are novel branches that have become achievable due to PCR (Kakumanu et al. 2012; Huang et al. 2001).

9.3 Construction of the Recombinant DNA Molecule

To construct a recombinant DNA molecule, a DNA fragment (restriction fragment) is inserted in cloning vector at the corresponding restriction site. Then sticky ends of the vector and the foreign DNA are allowed to anneal. Then, by means of DNA ligase, they are joined covalently to create a chimeric DNA (Fig. 9.3).

9.4 Cloning of DNA by Recombinant DNA Technology

Comprehensive study of the working and construction of the gene at molecular stage needs a huge amount of individual gene in purest form. Recombinant DNA technology offers great advantage in cloning that permits researchers to create a huge quantity of matching DNA molecules. The DNA molecule so produced has sequences derived from diverse sources. In DNA cloning one of the important steps is to link the desired DNA fragment to a vector DNA that could duplicate within the host cell. As a result, the recombinant DNA molecule is produced that replicates together with the vector, producing a huge quantity of matching DNA molecules (Bonneau and Laarved 1999). The scheme of production of recombinant DNA is shown in the following diagram:

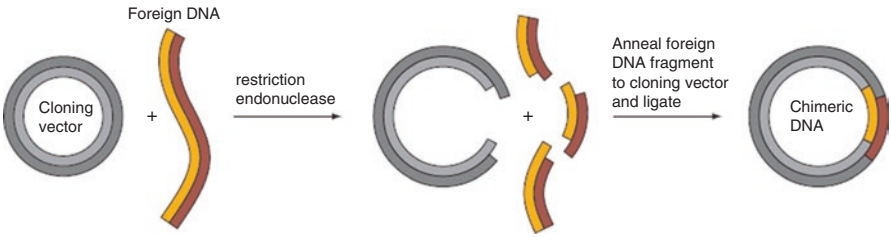

Fig. 9.3 Construction of recombinant DNA molecule. (Adapted from Biochemistry, 4th edition, Donald Voet and Judith G. Voet, 2011)

Vector DNA molecule + DNA molecule

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Recombinant DNA molecule

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Replication of Recombinant DNA

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Isolation and sequencing of the purified DNA molecule

9.5 Role of Restriction Enzymes in the Creation of Recombinant DNA Molecule

The main purpose of DNA cloning is to generate distinct, tiny regions of DNA molecule that comprises of definite genes. Practically very little amount of DNA molecules could be cloned in a vector. Thus, extremely lengthy DNA molecules must be cleaved into fragments that could be put easily into a vector DNA. To facilitate this process, restriction enzymes are used (Becker et al. 2008). Restriction enzymes are endonucleases that are obtained from bacteria and are characteristically able to distinguish and cleave specific four- to eight-base pair sequences, called "restriction site". These sites commonly are short palindromes (sequence same on each DNA strand when read in the 5′ → 3′ direction). One of the widely used restriction enzymes is from *E. coli* called *EcoR1* (Brown et al. 2015). This enzyme is able to make staggered cuts at the definite six-base pair (palindromic sequence) as shown in Fig. 9.4.

For every restriction endonuclease, bacteria also create a modification enzyme that protects the bacterium's own DNA from cleavage. This modification enzyme adds a methyl group to one or more bases, generally within the restriction site. This methyl group prevents endonuclease from cleaving the DNA. Methylating enzyme and the restriction endonuclease together form the "restriction modification system"

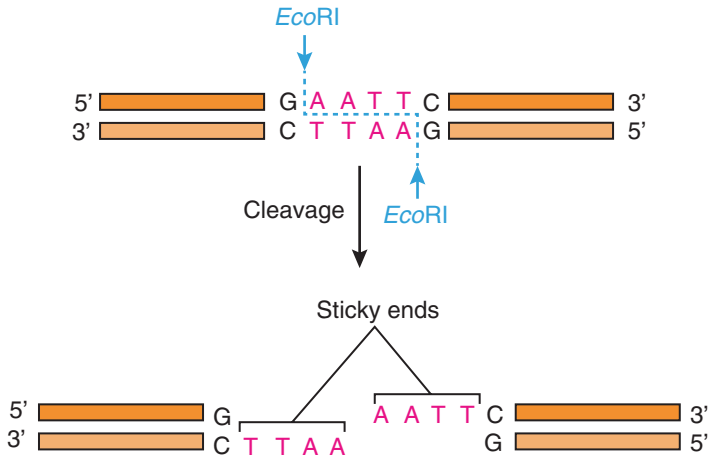

Fig. 9.4 Cleavage of DNA by *Eco*R1. (Adapted from Molecular Cell Biology, 4th edition, Lodish et al. 2002)

that protects the host DNA while it destroys incoming foreign DNA by cleaving it at all the restriction sites (Overton 2014).

9.6 Inserting DNA Fragments into Vectors

DNA ligases aid in the insertion of DNA fragments into the vector DNA. During normal DNA replication, these ligase take part in joining of short segments of DNA called *Okazaki* fragments. In case of DNA cloning purified DNA ligases covalently join the ends of restriction fragments and vector DNA that possesses complementary ends. This linkage is done through standard 3′ → 5′ phosphodiester bonds of DNA. The DNA ligase from the bacteriophage T4 can ligate both the blunt ends and the complementary sticky ends. Nevertheless, blunt-end ligation is intrinsically ineffective and needs huge amount of both DNA and DNA ligase (He et al. 2000).

9.7 Role of Recombinant DNA Technology in Agriculture

With the rise in demand for food, there is a huge demand to integrate biotechnology to enhance crop improvement strategies. The advent of biotechnology has revolutionized the whole crop improvement strategies by offering novel strains of crops, highly proficient, precise and selective pesticides and valuable fertilizers (Ewen and Pusztai 1999). Ancient communities maintained the crops by implementing selective breeding programs. By gathering seeds from the most advantageous crops, they

were able to generate crops that were adapted to the changing environment and could offer higher yield. In agriculture, biotechnology has resulted in enhancement of crop productivity by controlling diseases through improved genetic resistance programs. Thus, it offers efficient tools for enhancing and sustaining food security. It offers an outstanding scenario for improving health by engineering the nutritional quality of food, bioremediation and genetic conservation (Buikema and Haselkorn 2001).

The development of protein engineering and synthetic biology has revolutionized the exploitation of proficient microbial systems for the generation of value added products. Environmentally friendly routes of manufacture, creation of smaller amounts of by-products and enhanced output of the target compounds are advantages of microbial biosynthesis over the chemical synthesis.

Extensive optimization of growth conditions is required while obtaining compounds from their native host. However, with the help of recombinant DNA technology, several natural products, their derivatives, or even unnatural compounds have been created within established microbial hosts. Alkaloids, terpenoids, flavonoids, amino acids, peptides, antibiotics, organic acids and vitamins are some examples of microbially produced compounds through modern recombinant biotechnology (Chaurasia et al. 2008).

9.7.1 Soil Fertility

Soil is that portion of the earth on which plant grows. It comprises of three main layers (top soil, sub soil and parent soil). Uppermost layer, i.e. top soil, contains minerals, water, air and inorganic or organic matter. Minerals include silica, aluminium oxide, calcium, potassium, magnesium and extremely little quantity of nitrogen, sulphur, boron, zinc, molybdenum, etc. (Huang et al. 2004). Among all the minerals, only 14 are essential minerals. Essential nutrients are further categorized into micronutrients and macronutrients. The macronutrients are further classified into primary macronutrient and secondary macronutrients. Primary macronutrients are frequently limited in the soil, while secondary macronutrients are rarely limited (Dash et al. 2016). Soil quality decides the quality and quantity of agricultural production. Besides, it also offers niche to a wide range of living organisms. For this reason, proper management of soil is one of the key factors for enhanced crop productivity (Sanahuja et al. 2011).

9.8 Biofertilizers

Chemical fertilizers offer convenient technique to supplement soil with valuable nutrients and therefore help to overcome the growing requirements of food. Nevertheless, they are reasonably very costly and risky to human well-being. On the

other hand, they not only supply necessary nutrients to crops but also supply them in an easy accessible mode. Thus, chemical fertilizers can rapidly improve the development and yield of crops and are hence gaining fame around the world (Raja 2013). However, extensive employment of such fertilizers causes grave ecological problems. Nitrate leaching and contamination of groundwater are due to augmented exploitation of fertilizers. Inorganic fertilizers like calcium nitrate, ammonium chloride and sodium nitrate produce greenhouse gases that result in pollution. Elevated levels of greenhouse gases and heavy metal uptake by plants are major causes of environmental damage. Eutrophication of freshwater is also due to chemical fertilizers. Furthermore, chemical fertilizers can eradicate the advantageous microbial or insect community of the soil. Fortunately, nature has bestowed the soil with a variety of microbes with specific mechanisms to overcome this challenge. This mechanism besides maintaining soil quality also works in tandem with plants as an element of ecosystem. Such mechanism is what constitutes "biofertilizers" (Khosro and Yousef 2012). Biofertilizers constitute a central part of green agriculture. Biofertilizers contain proficient strains of microbes, organic products and departed and rotten parts of plants which supply nutrients to soil. It progressively elevates crop yield by means of enhancing soil fertility. They change the unavailable form of nutrients to the accessible form by escalating the population of microbes in the rhizosphere (Leonardo et al. 2006). Microbes are accountable for delivering soluble nutrients to crops (Chang and Yang 2009). These are helpful in a variety of ways that include solubilization of plant nutrients and fixing of atmospheric nitrogen. They also encourage the formation of growth-promoting phytohormones like cytokinins and auxins. They also defend the plant against various abiotic and biotic stresses (Mitragotri et al. 2014).

Biofertilizers aid plants in accessing the nutrient present in its surroundings. The microbes frequently employed as the biofertilizers include *Rhizobium*, *Azotobacter*, *Anabaena* (nitrogen fixers), *Pseudomonas putida*, mycorrhizal fungi, etc. (Liu and Golden 2002). Likewise, phytohormone-/auxin-producing bacteria could also be utilized as biofertilizer (Somasegaran and Springer 1994). All of these microbes enhance growth and development of plants (Table 9.1). The grievance from agriculturalists regarding the effectiveness of biofertilizer is their improper storage and the larger time period between field application and production. This restricts their employment due to compatibility and constancy issues under diverse soil environments. For this reason, improved shelf life is the basis for the popularization of biofertilizers (Adesemoye and Kloepper 2009).

Table 9.1 Plant growth-promoting substances associated with various microbes

Microbe	Plant growth promoting substance
Azotobacter	Vitamins, gibberellins
Azospirillum	Indole acetic acid, gibberellins, indole lactic acid
Cyanobacteria	Vitamins
Phosphate-solubilizing bacteria	Vitamins (thiamin, biotin, riboflavin)
Mycorrhizae	Cytokinin, gibberellins

Presently, a variety of marketable biofertilizers are obtainable and a variety of mechanisms have been formulated to guarantee maximum viability of the microbes used in such formulations (Bhattacharyya and Jha 2012). These strategies include:

- Optimization of biofertilizer formulation
- Usage of thermo-resistant or drought-resistant and genetically modified strains
- Employment of liquid biofertilizer

For dexterity, a carrier substance is utilized as a vehicle for the microbes which are to be used as biofertilizer. Carrier substances include clay, vermiculite, peat, seed, lignite powder, rice bran, charcoal, etc. For enhanced shelf life of biofertilizer formulation, a combination of these carriers is employed. Likewise, pre-sterilization of carriers is done to enhance the shelf life of microbes (Wani et al. 2013; Liddycoat et al. 2009). Liquid biofertilizer formulation is an important aspect to improve shelf life. These formulations enclose an adequate amount of cell protectants and nutrients that are responsible for the extended shelf life of biofertilizers. Besides, these formulations can endure huge temperature range (Santos et al. 2012; Ruiz-Sanchez et al. 2010).

Biofertilizers got commercialized with the launch of "nitrogen" by Hiltner and Nobbe. This preparation was for legumes. Later microbial inoculants for legumes were made like "Alnit". It proved advantageous for the development of non-leguminous plants. These bacteria were recognized to be local ammonifiers. Discovery of *Azotobacter* and *Clostridium* developed a new field for investigating economical bacterial fertilizers. The rhizosphere of these plants contains a range of species of soil bacteria that enhance plant growth by numerous ways. Such bacteria are jointly known as plant growth-promoting *Rhizobacteria* (PGPR). One of the ways is through fixing of atmospheric nitrogen which enhances the accessibility of exploitable form of nitrogen in the rhizosphere. They also promote symbiosis between plants and microorganisms (Mfilinge and Mtei 2014).

There are diverse modes of interactions between biofertilizers and plants, taking into account the extent of association between microbes with plant roots which are mentioned as follows (Youssef and Eissa 2014):

- Microbes living in the soil near the root, utilizing nitrogen and carbon metabolites leaking from the root (rhizosphere)
- Microbes colonizing the root surface (rhizoplane)
- Microbes colonizing the root tissue inhabiting intercellular spaces (endophytes)
- Microbes living inside cells in specialized root structures or nodules (symbionts)

9.8.1 Types of Biofertilizers

Biofertilizers are categorized into various types on the basis of microorganisms they contain (Chun-Li et al. 2014). The different types of biofertilizers are discussed below:

9.8.1.1 Symbiotic Biofertilizers

Symbiotic microbes infect root tissues and form new structures. In many cases, the application of molecular biology tools allows the discovery of the genes and signals involved in the beneficial interaction between the microorganism and the plant. The main symbiosis relating to agricultural application as biofertilizers is considered below.

9.8.1.2 Rhizobia

Rhizobium is an illustration of a symbiotic association colonizing legume roots and fixes the atmospheric nitrogen. It has a capability to fix atmospheric nitrogen in leguminous and non-leguminous plants. The different genus and species inhabiting legume root nodules are usually referred to as *Rhizobia*. These involve *Alphaproteobacterias*, e.g. *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Azorhizobium*, *Allorhizobium* and *Agrobacterium*, and *Betaproteobacteria*, e.g. *Burkholderia*. The best model describing the interaction between rhizobia and legume roots includes flavonoid/isoflavonoid molecules released by the plants which induce bacterial genes and consequently the synthesis of the lipochitin oligosaccharide (LCO) molecules, which in turn control infection and nodule growth in the root tissue (Rajaram and Apte 2008). Usually, it pierces the root hair and multiplies there in special root structures called root nodules. The quantity of nitrogen fixed depends on host, strain of *Rhizobium* and existing environmental conditions. They are very proficient biofertilizers for legumes as far as the magnitude of nitrogen fixation is concerned. The nod, nif and fix genes control the nodulation and nitrogen fixation by the bacterium (Lavakush et al. 2014).

9.8.1.3 Blue Green Algae

Blue green algae (BGA) are the most ancient organisms possibly the first among those that started evolving oxygen. These appear in numerous shapes (single celled, branched or unbranched with filaments). The majority of them possess special structure called heterocyst whose role is to fix nitrogen. The algae that are frequently applied in fields belong to *Anabaena*, *Nostoc*, *Scytonema*, *Tolypothrix*, etc. (Joseph and Meeks 1987). These are widely used in rice fields (Zhou et al. 1998). BGA secrete numerous growth-promoting substances like amino acids, vitamins, polysaccharides, sugars, etc. which boost the yield of crops (Schiefer et al. 2002; Hussain et al. 2002).

9.8.1.4 Mycorrhiza

Mycorrhiza is the best example of the symbiotic association between fungi and plant roots (higher plants). The fungi enhance the growth of plants and protect them from various stresses. These fungi colonize the root cortex and mycelia of the plants and help them to obtain nutrients from soil. These fungi are cosmopolitan in soil and are seen in the roots of thallophytes, gymnosperms, pteridophytes and angiosperms (Stewart 1980). Plants, on the other hand, protect fungi from root pathogens and also provide them with carbohydrates, hormones, nutrients, etc. The mycorrhizal plants have better forbearance to poisonous metals, salinity, elevated soil temperatures and unfavourable pH. Such plants also resist transplantation shocks. They play a significant task by enhancing growth and nutrient uptake in plants (Vessey 2003).

9.8.1.5 Free-Living or Non-symbiotic Biofertilizers

Since the description of PGPR by Kloepper and Schroth (1978), many different bacteria genera have been described as PGPR: *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Gluconacetobacter*, Herbaspirillum, *Bacillus*, *Burkholderia*, *Erwinia*, *Caulobacter*, *Azotobacter*, *Chromobacterium*, *Serratia*, *Micrococcus*, *Flavobacterium*, *Actinobacteria*, *Enterobacter*, *Arthrobacter*, *Agrobacterium* and *Hyphomicrobium* and fungus such as *Trichoderma*, among others (Gupta 2004).

Many PGPR have been described as endophytic bacteria. It is not clear if the plant growth promotion effects are a consequence of plant-microbe interaction in the external part of the rhizosphere or if an endophytic state is necessary (Hayat et al. 2010). Many different mechanisms have been claimed to be responsible for the plant growth promotion effect after in vitro experiments under controlled conditions. In some cases, the use of appropriate mutants helps in the definition of these mechanisms. But since different mechanisms are always present in a single strain, it is almost impossible to know which are the main mechanisms operating and driving the plant growth promotion. Irrespective of the real mechanisms operating in PGPR with a positive effect in the field, the use of these micro-organisms has dramatically increased in recent years and will probably continue to grow because biofertilizers appear as a valuable opportunity for future sustainable agriculture (Gonzalez et al. 2015). Many commercial products already exist which are based on *Pseudomonas* or *Azospirillum* strains in the market (Yang et al. 2009; Scalenghe et al. 2012). The different mechanisms operating in PGPR can be classified as N_2 (nitrogen) and P (phosphorus) nutrition effects and plant root development and fitness mediated by phytohormones **(**Fig. 9.5**).**

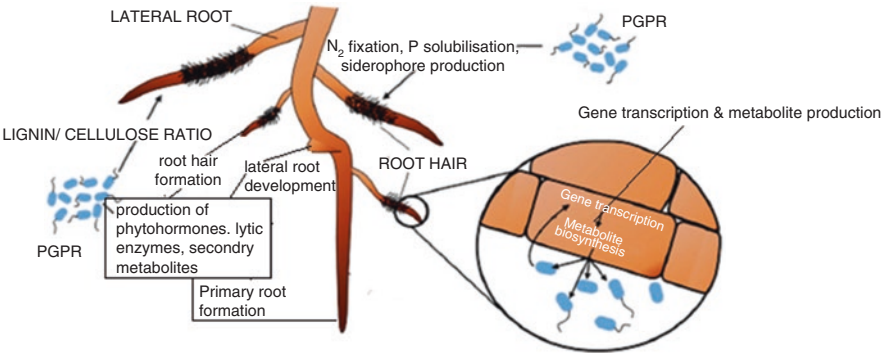

Fig. 9.5 Mode of action of PGPR

9.9 Phytohormone-Mediated Mechanism of Plant Growth-Promoting Microorganisms/Bacteria (PGPB)

One of the most visible effects on plants after inoculation with PGPB is the huge development – and sometimes changes in the architecture – of the root of the plant. This general improvement of root growth, including root-hair development, is one of the characteristic phenotypes of the interaction plant-PGPB.

It is likely that water and mineral uptake is consequently improved because of the increase in the root system, although the specific mechanism is not completely clear. Changes in hormone balance, enhancement of proton-efflux activity and modification in a wide range of related enzymatic activities would be part of the mechanisms behind this phenotype (Backman and Sikora 2008; Joo et al. 2005).

9.9.1 Auxins

The general root improvement phenotype can be reproduced by replacing phytohormones with PGPB. Auxin-related substances, such as indole acetic acid (IAA), appear to be involved in one of the most important mechanisms regarding the general root development improvement. Nevertheless, bacterial production of IAA in plants has not yet been demonstrated. There are no IAA completely deficient mutants, but IAA attenuated mutants were ineffective as PGPB, compared to parental strains (Ahmed and Hasnain 2010; Chen 2006).

9.9.2 Cytokinins

The role of cytokinins in the promotion of root development is not clear, but cytokinin-producing PGPB stimulate nodulation in legumes when co-inoculated with *Rhizobia*. Besides, it has been demonstrated recently that there is a Nod factor-independent mechanism for infection and nodulation, probably mediated by rhizobial cytokinin. This particular area deserves more attention in the future (Riefler et al. 2006; Sokolova et al. 2011).

9.9.3 Ethylene

Ethylene is related to general plant responses when a stress condition appears, even if it is a very low stress situation. When this happens, the plant synthesizes ethylene and stops its growth temporarily. This is because of the regulatory effects of ethylene on different cell functions. 1-aminocyclopropane-1-carboxylate is a precursor of ethylene synthesis. The enzyme ACC deaminase is present in some bacteria which can even use ACC as C (carbon) and N sources. When ACC deaminase is expressed by rhizospheric bacteria, root growth and development are enhanced. It is probably because of the elimination of the inhibitory concentrations of ethylene produced by the plant. This enzyme is not present in every bacteria, and its activity is codified by a single gene acdS. The introduction of this gene from *Pseudomonas putida* into other bacteria species confers plant growth-promoting functions to the recipient bacteria that are absent in the parental strain. This represents a potential biotechnology-based tool to improve microorganisms to be used as biofertilizers (Reid 1981).

9.9.4 Nitric Oxide

Nitric oxide (NO), a plant regulator volatile phytohormone, is also produced by some PGPB. Bacterial nitric oxide is a mediator in IAA-induced root development. NO can also mediate plant growth-promoting action in *Azospirillum brasilense* Sp245 inducing morphological alterations in tomato roots irrespective of the full bacterial capability for IAA biosynthesis (Butterbach-Bahl et al. 2013).

9.9.5 Helper Bacteria

In the studies of plant microbe interaction which induced some kind of plant growth promotion, there are other cases that do not fit into the previous definitions but which can be considered as another kind of biofertilizer. That is the case of bacteria

which improve a plant-microbe interaction as a third partner in the interaction. An example can be found in rhizospheric actinomycetes isolated from legumes or actinorhizal nitrogen-fixing nodules which are able to stimulate nodulation, consequently nitrogen fixation in the plant and finally plant growth. This tripartite plant-microbe interaction is not well known in terms of mechanism. However, it clearly shows that biofertilizers can be improved by the use of more than one microorganism at a time (Egamberdiyeva 2007).

9.10 Genetically Modified Microbes as Biofertilizers

Genetically modified organisms (GMOs) symbolize a genetic reserve. Such microorganisms may find a use as donor or recipient of desirable genes. Microorganisms play an important role in various sectors of agriculture, food processing, environmental management and pharmaceutical industries. Genes of microbes can be optimized or improved by means of various genetic modifications using recombinant DNA technology (Tabashnik et al. 2011). Usually, this is dependable on the recognition and selection of the mutants with favourable traits. In numerous cases, the usage of molecular biology tools or recombinant DNA technology allows the discovery of the genes and signals concerned in the advantageous interaction (endophytic, mycorrhizal and diazotrophic) between the microbe and plant **(**Fig. 9.6**).** These symbiotic interactions can assist plant growth and development through nitrogen uptake, siderophore production, phosphate solubilization, etc. (Ritika and Uptal 2014).

Recombinant biotechnology offers an advantage to decrease the employment of synthetic fertilizers. Biofertilizer technology has significantly developed in the market. The nature of multiple mechanisms discovered for PGPR actions and the option of genetically modifying a specific strain relating to a particular plant growth-promoting activity imply that the use of genetically modified organisms like biofertilizers will be an area of diverse potential in the coming times (Tabashnik et al. 2011). Further, the knowledge of microbial ecology and its dynamics will surely enhance the biofertilizer technology. Microbes are particularly targeted for genetic improvement since they are given huge importance in modern agriculture as they are used as biofertilizers. Biofertilizers represent an alternative to synthetic fertilizers which are facing lots of disparagement due to their negative impact on the ecology and human well-being. There is an important requirement to build up eco-friendly control using existing microbes. Such microbes would offer protection to plants against pathogens and would be economical, reliable and effective (Pishchik et al. 2002). To obtain this target, better-quality strains are needed. Thus, genetically modified microbes could be used for this purpose. Efforts are in progress to formulate proficient biofertilizers compatible with a broad choice of plants and soil by means of genetically engineered techniques. For example, biofertilizers have been formulated based on nitrogen-fixing rhizobial bacteria occurring naturally in the nodules of leguminous plants. Nevertheless, these microbes are not competent

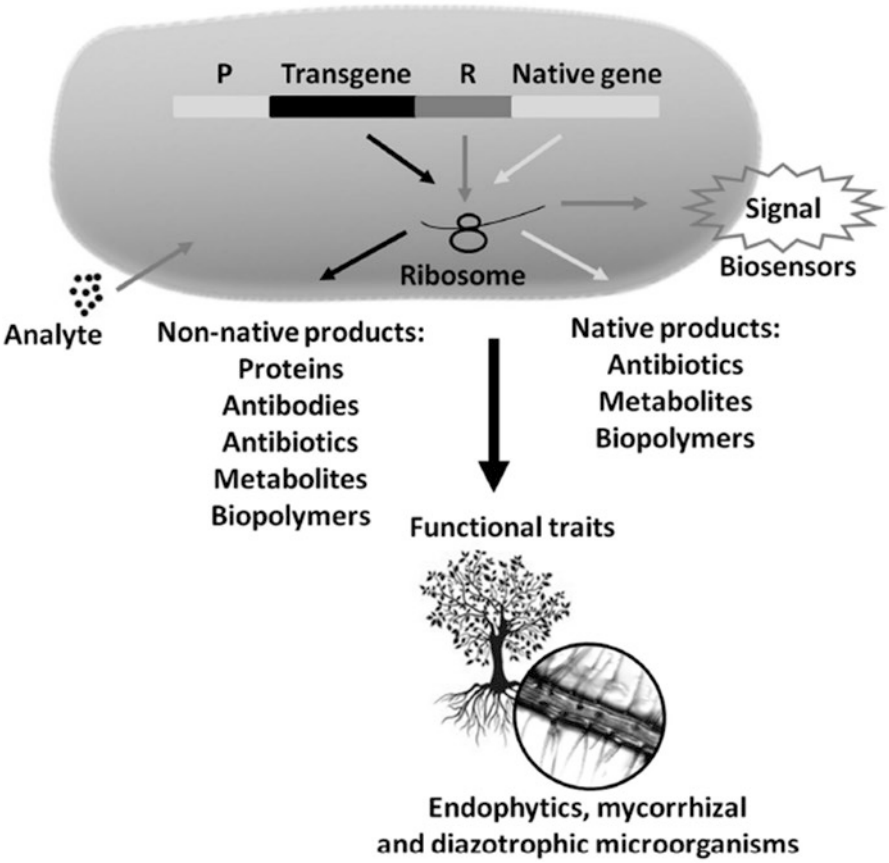

Fig. 9.6 The use of recombinant DNA technology in the generation of products assisting in the symbiotic interactions. (Adapted from Vitorino and Bessa 2017)

enough to supply nitrogen to non-legumes. In such cases, genetic engineering is of special importance, as it assists in the development of efficient delivery systems. In this way non-legumes could be grown together with symbiotic rhizobial root nodules devoid of externally applied nitrogen fertilizers (Aloni et al. 2006; Ruiz-Sanchez et al. 2010). The foreign genes used for transforming microbes could be integrated into the host genome or plasmid. To express a heterologous gene in bacteria and fungi, the regulatory area of this gene should be modified in promoter and terminator regions in order to optimize the function of the inserted gene in the new host. Adding specific genes which can bestow biocontrol ability could improve the biocontrol ability of microbes lacking such genes (Dash et al. 2016). For example, many *Rhizobacteria* with biocontrol activity produce chitinases. However, few *Rhizobacteria* like *Rhizobium meliloti* and *Pseudomonas putida*, both of which are outstanding root colonizers, are deficient in synthesizing chitinase (Bagwan et al.

2010). Incorporation of chitinase gene into their genome has enabled them to defend the plant against fungi.

Nitrogen-fixing property of *Rhizobium* inoculants could be augmented by means of genetic engineering tools. An additional way is by planting the crops that use nitrogen more proficiently. An example of such crops is genetically modified Canola which exhibits a noteworthy decline in the amount of nitrogen fertilizer that is leached into soil or lost into the atmosphere, and hence it improves the economies of farmers through the enhanced profitability. Moreover, biofertilizers when formulated by means of molecular biotechnology can improve the biological pathways of production of phytohormones like auxin, cytokinin, etc. which assist in plant growth and development (Nautiyal et al. 2008). Similarly, many pseudomonads in the rhizosphere manufacture siderophores which can chelate iron ions and thus escalate iron uptake by the plants. The genetically modified strain (RMBPC-2) of *Sinorhizobium meliloti* has added genes that control nitrogenase enzyme from the plant to the bacterium (Boccia and Sarnacchiaro 2015). Likewise, Trichoderma species are extensively found in the soil and are antagonistic to other fungi. *Trichoderma harzianum* is an efficient rhizosphere colonizer and is able to parasitize pathogenic fungi. Many extracellular enzymes like glucanases, chitinases, lipases and proteases synthesized by *Trichoderma* species have been improved with the transfer of chitinase genes, notably from *Serratia marcescens* (Awais et al. 2010). Thus, such genetically modified strains could act as efficient biofertilizers and will aid in crop improvement.

9.11 Conclusion

Our reliance on chemical fertilizers has encouraged the flourishing of factories or industries that are generating lethal chemicals that are not only dangerous for human utilization but can also perturb the normal environmental equilibrium. Now, attention is diverting towards consuming food grown with organic fertilizers than with chemical fertilizers (Leonardo et al. 2006). Biofertilizers can assist in solving the problem of food crises of the ever-rising worldwide population. It is essential to recognize the positive aspects of biofertilizers so as to apply it to modern agriculture. The employment of biofertilizers containing advantageous microbes improves the crop productivity to a larger extent (Kanchiswamy et al. 2015). Biofertilizers play an important role in maintenance of soil quality. This would in turn protect the environment and would require less expenditure. Besides, biofertilizers when formulated using the tools of molecular biotechnology can improve the biological pathways of production of plant growth-promoting substances, if identified and transferred to the useful plant growth-promoting microbes (Goswami et al. 2014). Recombinant biotechnology offers numerous advantages in this area, as particular metabolic processes could be tackled with additional accuracy, and entirely novel functions could be introduced in microbes.

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