Rhizobia species: A Boon for “Plant Genetic Engineering”

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Abstract Since past three decades new discoveries in plant genetic engineering have shown remarkable potentials for crop improvement. *Agrobacterium* Ti plasmid based DNA transfer is no longer the only efficient way of introducing agronomically important genes into plants. Recent studies have explored a novel plant genetic engineering tool, *Rhizobia* sp., as an alternative to *Agrobacterium*, thereby expanding the choice of bacterial species in agricultural plant biotechnology. *Rhizobia* sp. serve as an open license source with no major restrictions in plant biotechnology and help broaden the spectrum for plant biotechnologists with respect to the use of gene transfer vehicles in plants. New efficient transgenic plants can be produced by transferring genes of interest using binary vector carrying *Rhizobia* sp. Studies focusing on the interactions of *Rhizobia* sp. with their hosts, for stable and transient transformation and expression of genes, could help in the development of an adequate gene transfer vehicle. Along with being biologically beneficial, it may also bring a new means for fast economic development of transgenic plants, thus giving rise to a new era in plant biotechnology, viz. “*Rhizobia mediated transformation technology*.”

Keywords Plant genetic engineering · *Agrobacterium* · *Rhizobia* · Binary vectors · Transgenic plants

Introduction

Before 1960, *Classical plant breeding* was the most commonly used method to produce new agriculturally efficient crops. However, the use of this method is limited only to a certain extent. It cannot be used for: (1) sexually incompatible plants [1] because classically breeding plants should be closely related and sexual incompatibility between plants creates taxonomic restriction for transfer of genes to crops [2], (2) tightly linked genes in plants [1], since tightly linked genes with desirable and undesirable traits are difficult to separate even after backcrossing. In addition to these limitations, this approach is time consuming and laborious for plant breeders. To overcome all these shortcomings, researchers tried manipulating genes of interest to create new plant varieties with enhanced economic and productive efficiency. They have used vectors or directly transferred the desired genes from fungi, plants, or bacteria to plants by recombinant DNA technology. Researchers made various efforts to transfer genes into a wide range of plants to study more about plant genetics and biochemistry. Along with the productive competence, new technologies have contributed to ecological issues through the development of biofuels [3, 4], fibers [4], biodegradable plastic [5], phytoremediation [6], edible vaccines [7], industrial enzymes like trypsin, α -amylase, cellulase, avidin [7] and monoclonal antibodies [8]. Clearly, the new plant genetic engineering techniques offer a significant advantage to the traditional plant breeding, both biologically and economically.

At present, chemical techniques, using polyethylene glycol (PEG) and many physical techniques, such as biolistic, electroporation, microprojectile and bacterial gene delivery through *Agrobacterium* and virus transduction have being employed for DNA transfer into plants. The

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PEG method includes extensive protoplast isolation process from the target tissue, culturing of the tissue, and regeneration of the tissue from transformed protoplast, which imposes certain limitations to its applicability [1]. The physical methods like biolistic, electroporation, and microprojectile require special instruments and are expensive. The bacteria-mediated transformation techniques use soil bacteria, such as *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* that cause crown gall disease and ‘hairy root syndrome’, respectively, through their natural interaction with plants. Due to its ease and low cost, bacteria-mediated transformation procedure is now commonly used for genetic transformation in plants as opposed to other chemical and physical approaches illustrated above.

Agrobacterium is generally considered as the only vehicle capable of transferring agronomically important genes into plants. *A. tumefaciens* is a ubiquitous soil bacterium with a unique natural ability of causing “crown gall disease” by transferring a section of its own DNA, known as ‘transfer DNA’ or T-DNA, to the plant cell nucleus [9]. Once the T-DNA integrates into the host genome, it expresses itself. This ability of *A. tumefaciens* allows researchers to manipulate the T-DNA by replacing the disease causing genes with the genes of interest [10]. Using this method, researchers have successfully implemented biotic stress resistance genes into plants for pest resistance, herbicide resistance, virus resistance, disease resistance and abiotic stress resistance genes for salt stress resistance, cold stress resistance, heat stress resistance [1, 7].

The *Agrobacterium* transforms plants commonly by binary vectors and cointegrate vectors. The binary vector system uses two plasmids: first, in which the “vir” gene that induces tumor is removed and is thus called “disarmed Ti plasmid” and second, which possesses vir gene and lacks T-DNA and is referred to as the “Helper plasmid”. The cointegrate vectors are constructed by a recombination between a vector carrying the gene of interest between the right and left T-DNA borders of the T-DNA region within Ti plasmid lacking tumor-causing genes (“disarmed” Ti plasmid) in *Agrobacterium*. The drawback of cointegrate vectors is that they are difficult to construct and dependent on site-specific recombination of plasmids in *Agrobacterium* [11]. As compared to cointegrate vector binary vectors have several advantages; they are easy to propagate in laboratory bacteria like *Escherichia coli* and are smaller in size than the normal Ti plasmid. Due to this ease in handling they are more commonly used as compared to cointegrate vectors. There are two methods for transformation of a plant by *Agrobacterium* carrying the gene of interest in cointegrate or binary vector, viz. 1) Leaf-disc method and 2) Floral dip method. In leaf-disc assay, the tissue explant, viz. the leaf is incubated with *Agrobacterium* from a few

hours to a few days and is then cultured in media [11], whereas in floral dip method, the floral bud is dipped in a solution containing *Agrobacterium* [12].

Although *Agrobacterium*-mediated transformation is the method of choice for plant genetic engineering, many economically important plant species are highly recalcitrant to this method [13]. The drawbacks associated with *Agrobacterium*-mediated transformation technique are: (1) generation of defense response in plants [10, 14, 15], (2) limited host range [10, 13] and (3) its association with complex patent issues. In 2005, driven by the complexity of patents for *Agrobacterium*-based technique, researchers from *pCambia* showed that *non-Agrobacterium* species (*Rhizobia* sp.) are capable of transferring genes to different plants. Their research showed that certain *Rhizobia* sp. are competent enough to transfer genes both in monocots and dicots [16]. The plants like *Arabidopsis*, *tobacco*, and *rice* were stably transformed with *non-Agrobacterium* sp. The use of *Rhizobia* sp. confers certain advantages, viz. its properties are well studied, a synthetic medium is easily available and the species exist in large numbers. Further studies on their different metabolic pathways and complex molecular interactions involved in processes like symbiosis may help in getting acquainted with the response generated by *Rhizobia* sp. in plants, which may increase transformation efficiency. This paper mainly focuses on the scope and use of *Rhizobia* sp. in plant genetic engineering.

Rhizobia sp. (Non-*Agrobacterium* sp.)

Rhizobia root nodule bacteria (RNB) show symbiotic association with leguminous plants. They are morphologically motile, gram negative, sporulating rods, possess poly β -hydroxybutyrate granules and are pleomorphic under adverse growth conditions [17]. Their unique ability of fixing atmospheric nitrogen in leguminous plants has earned them a valuable place in the field of agricultural plant biotechnology. Along with the ability of the bacteria to produce nodules on plant roots, a number of different physiological, morphological, cultural and biochemical qualities were assessed for species differentiation [18]. *Rhizobia* were first classified on the basis of cross-inoculation capability, i.e., the ability of *Rhizobia* to nodulate a specific group of hosts [18]. The seven cross-inoculation groups identified were: (1) *Sinorhizobium meliloti* which nodulate alfalfa group, (2) *Rhizobium trifolii* which nodulate clover group, (3) *Rhizobium leguminosarum* nodulate which nodulate pea, (4) *Rhizobium phaseoli* which nodulate bean, (5) *Rhizobium lupini* which nodulate lupine group, (6) *Bradyrhizobium japonicum* which nodulate soybean and (7) *Rhizobium* sp. which nodulate cowpea [18]. However, this method most variably failed in classifying different species [17].

Later, molecular systematics provided a breakthrough classification of *Rhizobia* sp and also helped to evaluate *Rhizobia* diversity in different environments. *Rhizobia* are phylogenetically diverse and consist of 62 species found in 12 genera [19]. Currently, RNB are classified into five alpha-proteobacteria families: Rhizobiaceae (*Rhizobium*, *Sinorhizobium* or *Ensifer*, *Allorhizobium*), Phyllobacteriaceae (*Mesorhizobium*), Bradyrhizobiaceae (genus *Bradyrhizobium*), Methylobacteriaceae (genus *Methylobacterium*) Hyphomicrobiaceae (genus *Azorhizobium*, *Devosia*) Methylocystaceae and two genera of beta-proteobacteria [20–22]: *Burkholderia* and *Ralstonia*, currently known as Cupriavididae. The other genera which have been recently included in *Rhizobia* are, *Herbaspirillum* and *Ochrobactrum* [19]. The ability of beta-proteobacteria to nodulate plants changed the view that bacteria of alpha-proteobacteria genera only are able to nodulate legumes. Beta-proteobacteria are free living nitrogen fixing *Rhizobia* and possess *nodA* and *nifH* (nitrogen fixation) genes similar to that of alpha-rhizobia [21]. Further phylogenetic analysis from *nodA* and *nifH* genes from alpha and beta-proteobacteria suggests that beta-*Rhizobia* have evolved from diazotrophs through multiple gene transfers [21].

The Close Relation Between *Rhizobia* sp. and *Agrobacterium* sp.

In 2007, Fred et al. confirmed the close relation between *Agrobacterium* and *Rhizobium* on the basis of 16s rRNA analysis. Since past few decades the amalgamation of the two genera, *Agrobacterium* and *Rhizobium*, has often been suggested [23–25]. The phenotypic comparisons of *Agrobacterium* sp. and *Rhizobium* sp., subsequently supported by phylogenetic analysis of 16sRNA sequence, showed that the genera could not be distinguished as a separate monophyletic clade [23, 24, 27–35]. The intergeneric transmission of Ti plasmid and nodulating plasmid from nodulating *Rhizobium* sp. to tumorigenic *Agrobacterium* sp. and from tumorigenic *Agrobacterium* sp. to *Rhizobium* sp. has been demonstrated [36]. Further analysis of fatty acid profiles showed that *Rhizobia* sp. and *Agrobacterium* are closely related [37]. Based on phenotypic data and phylogenetic studies it has been proposed that all the species be amalgamated into a single genus, *Rhizobium*, consisting of pathogenic, nitrogen fixing soil bacteria and *A. tumefaciens* be reclassified as *Rhizobium radiobacter* [23]. It is still unclear whether *Agrobacterium*, *Sinorhizobium* and *Rhizobium* are phylogenetically distinct and different in their genomic organization [38, 39]. However, the transformation of *S. meliloti* with Ti- or Ri-plasmid from *Agrobacterium* for *vir* gene expression showed no tumor generation [40].

Methods Used for Transformation of *Rhizobia* sp.

Many techniques have been developed to transfer plasmid DNA into various gram negative bacteria like *Agrobacterium* and *E. coli*, but fewer methods have been implemented for *Rhizobia* sp. Several approaches, such as conjugation, electroporation, transformation, and transduction, are used to transfer DNA into *Rhizobia* sp. [41]. Although conjugation is the most commonly used method with *Rhizobia* sp., this procedure is complex, time consuming and is confined to the strain carrying the *mob* gene in plasmid [42]. The other method used is Electroporation, which has shown reliable transformation efficiency in *Rhizobium* [43, 44], *Bradyrhizobium* [45], *Mesorhizobium* *haukii* [43] and *Sinorhizobium* [43]. However, this method is costly and requires special instruments and handling. Though transduction transfers large plasmids via phage yet it is not widely used because phage are strain specific and cannot be used for other *Rhizobia* sp. [41, 42]. Selvaraj et al. (1981) used the simple and easy, modified CaCl_2 method of *E. coli* and *Agrobacterium* to transfer plasmid DNA into *Rhizobium meliloti* [46]. But this method does not work for *B. Japonicum*, which is transformed efficiently by electroporation and conjugal transfer with *E. coli* [45]. In 2005, the freeze–thaw method was modified to develop a simple and rapid way of transforming *Rhizobia* sp. using broad host range plasmids. This method showed efficient transformation of following *Rhizobia* sp.: *S. meliloti* 1021 *S. meliloti* 2011, *Sinorhizobium fredii* USDA205, *Bradyrhizobium lupini* 2257 and *Mesorhizobium loti* 40 by binary vectors and the size of vectors showed no correlation with the transformation efficiency [42].

Rhizobia Mediated Plant Transformation

Although numerous experiments, involving the genetic engineering of the plants or the *Rhizobia* sp., have been conducted to improve the nitrogen fixing abilities of *Rhizobia* sp. in leguminous and non-leguminous plants [47] yet not much work has been done to explore their ability to transfer genes into plants. The genes like *nif* and *nod* have been transferred into plants using different transformation techniques, described earlier, to increase their N_2 fixing capabilities and one such method includes the transformation of plants through *Agrobacterium* sp. [47]. *Rhizobia* sp may be used in the same experiments as transformation vehicles and if found successful could become an alternative to *Agrobacterium* sp.; by finding answers to certain questions viz:

- Whether N_2 fixing capabilities will increase, decrease or remain unaffected.

- Whether transformation efficiency will increase or decrease.
- Whether symbiotic process is involved in the transformation of the plant.
- The similarities and/or differences between nodule formation by *Rhizobia* sp and tumor generation by *Agrobacterium* sp.

This could give an insight into the biochemistry and genetics of plants and *Rhizobia* sp. and such studies could help in the exploration of the physical and biochemical factors affecting the process of symbiosis, which may indirectly affect the transformation of plants.

The ability of *Rhizobia* sp. to transfer genes into plants is a newly developed idea and, therefore, presents a novel perspective in plant genetic engineering [16]. The close genetic relation between *Agrobacterium* and *Rhizobium* led Vincze et al., to transform non-*Agrobacterium* sp. by binary vectors. Vincze et al. (2006) showed the stable transformation of three binary vectors pPZP211 (10.1 Kb), pSoup (9.3 Kb), pART27 (10.9 Kb) into *Mesorhizobium loti*. The transformation efficiency of *M. loti* with pPZP211 was 160×10^3 CFU/ μ g DNA, with pSoup was 5.3×10^3 CFU/ μ g DNA, and with pART27 was 61×10^3 CFU/ μ g DNA. The above research work indicated that the transformation efficiency was sufficient enough to transform genetically engineered vectors into *Rhizobia* sp., which could further be transformed into plants. It has been demonstrated by pCambia research organization that *Rhizobia* sp., viz. *Rhizobium* WR234 and *S. meliloti* from *Rhizobiaceae* family and *M. loti* from *Phyllobacteriaceae* family are capable of transferring genes into tobacco, rice and arabidopsis plants via a binary vector and a disarmed Ti plasmid [16]. These transformations were carried out by modifying the leaf disk and floral dip methods, respectively. Later the transformed plants were checked by expression of hygromycin resistance and GUS activity. In addition to this, *Rhizobia* sp. are able to transfer genes into plants that are incompatible with *Agrobacterium* sp., for example, *M. loti* could transfer genes into rice plants [16]. The gene transformation in rice plants through *S. meliloti* was 25% of that of *Agrobacterium*; however, the transformation rate of *M. loti* was approximately one-third of *S. meliloti*. Among all these *Rhizobia* sp., *S. meliloti* was more proficient in transferring genes to both monocots and dicots [16]. Thus, non-*Agrobacterium* sp., when transformed with a disarmed Ti plasmid and binary vector or co-integrate vector, are readily able to transfer T-DNA to plants. Vincze et al. 2006 also carried out the transformation of binary vector pPZP211 in *S. meliloti*1021, *S. meliloti*2011, *S. fredii* USDA205, *B. lupini*2257 [42].

Rhizobia sp. vs *Agrobacterium* sp.

Many economically significant plant species like soybeans, cotton, cereal grains, legumes, wheat, corn, certain species of horticulture, and industrially important plants are recalcitrant to *Agrobacterium* and their transformation is still problematic [13]. As indicated above, *S. meliloti* is competent to transfer genes into both monocots and dicots, which provides the basis for further research to prove that other *Rhizobia* sp. may also be able to mediate such transformation. The pCambia research organization have also constructed transbacter strains of bacterial species like *Rhizobium leguminosarum*, *S. meliloti* with unitary vectors and *Mesorhizobium loti*, *Rhizobium* sp. NGR and *S. meliloti* with binary vectors.

Agrobacterium is also considered to be a rare and opportunistic human pathogen [48–50]. In the isolates of human patients affected by bacteremia, peritonitis, and endocarditis, *Agrobacterium radiobacter*, a virulent *Agrobacterium* sp. was found to be the causative agent of the disease [49]. In addition, two other *Agrobacterium* sp., *A. tumefaciens* and *Agrobacterium* *vilis* were isolated from hospitalized patients [50], but no measures were undertaken to determine *Agrobacterium* as the causative agent. In 2007, *Agrobacterium* was associated with Morgellons syndrome [51]. Five Morgellons patients' skin fibres contained cellulose produced by *Agrobacterium* at the site of infections, which also possessed *Agrobacterium* DNA [51]. Under laboratory conditions, *Agrobacterium* has been found to attach and transfer T-DNA into several types of human cells [52]. The persistence of *Agrobacterium* is even found in transformed plants [53]. This ever expanding host range of virulent *Agrobacterium* in non-plant species raises the concern of accidental release of genetically modified *Agrobacterium* sp. to the environment. On the other hand, *Rhizobia* sp display minimal survival in ground water and sewage; they are inadequately found in desiccation and sunlight and exhibit less dispersal through soil and water [54]. These findings support the fact that *Rhizobia* are safer tools for environment and may be less pathogenic or toxic [54]. The agricultural products require protective measures when used by workers, whereas *Rhizobia* mediated plant field tests conducted, to date, have shown that the workers need no such precaution [54]. Thus, *Rhizobia* RNB happen to be a better, safer, and more environmental friendly option in the emerging field of plant genetic engineering.

One of the many factors involved in genetic transformation of plants is the receptiveness of plant tissue to the microbe [55]. The sensitivity of plant to microbe depends on the defense response generated by the plant and the counter strategies of the microbe, which further results in resistance or susceptibility to disease. Root nodule bacteria, like symbionts, could provoke different responses on

interaction with plants as compared to the plant pathogen *A. tumefaciens* [55, 56], which may have a potential to enhance the regeneration ability of the tissue, thereby increasing transformation efficiency [16]. Ditt et al. (2001), on the basis of their work, suggested that the *Agrobacterium* and *Rhizobium* may produce compounds that are recognized by plants in a similar manner, even though the final outcome of the two interactions is strikingly different [56]. The induction of nodule formation following infection of plants by *Rhizobia* sp. is different from plant hormone triggered tumor formation by *Agrobacterium*. *Agrobacterium tumefaciens* induces plant defense responses that lead to necrosis and apoptosis of plant cells [10, 14, 57–60], thereby resulting in decreased transformation efficiency. It has been found that *A. tumefaciens* causes hypersensitive reaction (HR) at the infection site generating reactive oxygen radicals, such as superoxide, hydrogen peroxide, and hydroxyl, peroxy, which leads to the cell death in grape tissue [61] and apoptosis in maize [58]. In order to check apoptosis of cell in maize, Hansen (2000) assayed each of the cultures for the presence of DNA laddering, which indicated apoptotic cell death of maize tissue and it was also found that the DNA laddering increased during the course of time [58]. *Agrobacterium vitilis* was also seen to cause HR response in tobacco and necrosis in grape tissues [59]. More elaborate studies on the molecular and cellular factors involved in *Rhizobia*-plant interaction may help to improve the transformation efficiency.

Currently, most basic research, related to *Agrobacterium*-transformation, is carried out in public institutions. But the private sectors now hold patents and the complexity of patents has led to restrictions on the use of *Agrobacterium*-mediated transformation technology by other private and public institutions. *Rhizobia* sp., on the other hand, are available as biological open license source (BIOS) tools with no major restrictions (www.bios.net). In future, this may help to overcome supremacy of a few multinational companies working in the area of plant transformation and may lead to the formation of a highly co-operative technology (www.bios.net).

Conclusion

Agrobacterium-mediated transformation of plants is mostly carried out by binary vectors [62]. *Rhizobia* sp. have also been found capable of transferring genes into plants through binary vectors [16]. By modifying and using different binary vectors or cointegrate vectors, the transformation efficiency in *Rhizobia* can be studied and this may later aid in studying transformation in plants. Research

done using *Rhizobia* may also help in a better understanding of their symbiotic relation with plants [16]. More studies should be conducted on biochemistry, genetics, and genomics of *Rhizobia* sp. to create an efficient *Rhizobia* sp. mediated transformation system in the field of plant genetic engineering. Studies on transformation could further focus on different *Rhizobia* sp. that are genetically related to previous effectively transformed species, like *S. meliloti*, *M. loti*, *Rhizobium* sp. NGR234, which could further aid this emerging technique. Many factors including trans-acting factors (genes on host chromosomes) and cis-acting factors (Ti plasmid) are involved in the stable integration of T-DNA in plant genes [63–66]. More studies may be carried out to determine the effect of these factors to improve the transformation ability of *Rhizobia* sp. Although the transformation efficiency by *Rhizobia* sp in plants is less as compared to *Agrobacterium*, the gene transfer may be optimized and transformation efficiency may be improved by introducing techniques that could alter the genetics and physiology of the host strain and plant species [16]. As a remarkable progress has been made in the gene transfer techniques in plants, the development of *Rhizobia* as gene delivery vehicles, which are easier and safer to handle, shall provide an impetus to public and private research institutions to begin a new era of “*Rhizobia mediated genetic transformation of plants*”.

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