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Review

Development of new and efficient transformation methods in plants using *Agrobacterium* remains

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Plant genetic engineering has become one of the most important molecular tools in modern crop breeding. Significant progress has been made in the development of new and efficient transformation methods in plants. *Agrobacterium* remains one of the predominant microbes employed in this approach. In particular, progress in *Agrobacterium* –mediated transformation of varieties of agricultural plants such as cereals, legumes and other crop plants has led to emergence of transgenic – enabling technologies, including generation of marker genes, gene targeting, and chromosomal engineering. This recent advancement has boosted agricultural biotechnology by augmenting the conventional plant breeding methods to achieve improved yield and quality of crops, hence production of economic crops in commercial quantity. Agricultural biotechnology has reduced crop losses from pest and diseases, improved the nutrient efficiency of food and animal feeds, reduce post harvest losses with increased shelf life of fruits and vegetables, and increased the stress tolerance of crop plants allowing them to tolerate various environmental extremes such as cold and drought.

Key words: *Agrobacterium*, transformation, agricultural biotechnology, transgenics.

INTRODUCTION

Agricultural biotechnology is any technique in which living organisms or parts of organisms are altered to make or modify agricultural merchandise, to boost crops, or develop microbes for specific uses in agricultural processes. Merely put, when the tools of biotechnology are applied to agriculture, it is termed as "agricultural biotechnology". Genetic engineering is additionally a half of agricultural biotechnology in today's world. It is currently a potential to hold out genetic manipulation and transformation on virtually all plant species, including all the planet's major crops (ArticleClick, 2011). Plant transformation is one in every of the tools involved in agricultural biotechnology, in which genes are inserted into the genetic structure or genome of plants. The two commonest methods of plant transformation are *Agrobacterium* Transformation - ways that use the naturally occurring bacterium; and Biolistic Transformation - involving the employment of mechanical means. Using any of these ways, the popular gene is inserted into a plant genome and traditional breeding methodology followed to transfer the new trait into different kinds of crops (ArticleClick, 2011).

Genetic transformation has been a powerful tool for enhancing the productivity of novel secondary metabolites; especially by *Agrobacterium* rhizogenes induced hairy roots (Leena and Jaindra, 2003). Genetic transformation is one of the biotechnological tools used to harness the production of secondary metabolites. The recent achievement and advancement of genetic transformation is the *in vitro* regeneration of medicinal plants from various explants to enhance the production of secondary metabolites which has lead to production of high-quality plant based medicine. The recent advances and developments in plant genetics and recombinant DNA technology have helped to improve and boost research into secondary metabolite biosynthesis. Emphasis has been paid on identifying enzymes of a metabolic pathway and then manipulating these enzymes to provide better control of that pathway. Transformation is currently used for genetic manipulation of more than 120 species of at least 35 families, including the major economic crops, vegetables, ornamental, medicinal, fruit, tree and pasture plants, using *Agrobacterium* mediated or direct transformation methods (Birch, 1997). However,

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Agrobacterium-mediated transformation offers several advantages over direct gene transfer methodologies (particle bombardment, electroporation, etc), such as the possibility to transfer only one or few copies of DNA fragments carrying the genes of interest at higher efficiencies with lower cost and the transfer of very large DNA fragments with minimal rearrangement (Shibata and Liu, 2000). The fundamental requirement in all this is a good yield of the compound, and reduced cost compared to the natural synthesis by the plants.

Research is on going for the application of plant transformation and genetic modification using *Agrobacterium rhizogenes*, in order to boost production of those secondary metabolites, which are naturally synthesized in the roots of the mother plant. Transformed hairy roots mimic the biochemical machinery present and active in the normal roots, and in many instances transformed hairy roots display higher product yields (Leena et al., 2003). Genetic transformation has been reported for various medicinal plants. Report of the successful regeneration of transgenic neem plants (*Azadirachta indica*) using *Agrobacterium tumefaciens* containing a recombinant derivative of the plasmid, the genetic transformation of *Atropa belladona* has been reported using *A. tumefaciens,* with an improved alkaloid composition (Yun et al., 1992). *Agrobacterium* mediated transformation of *Echinacea purpurea* has been demonstrated using leaf explants (Koroch et al., 2002).

Genetic transformation has been a powerful tool for enhancing the productivity of novel secondary metabolites of limited yield. Hairy roots, transformed with *A. rhizogenes*, have been found to be suitable for the production of secondary metabolites because of their stability and high productivity in hormone-free culture conditions. A number of plant species including many medicinal plants have been successfully transformed with *A. rhizogenes*. The hairy root culture system of the medical plant *Artemisia annua* L. was established by infection with *A. rhizogenes* and the optimum concentration of artimisin was 4.8 mg/L (Cai et al., 1995). Giri et al. (1997) induced the development of hairy roots in *Aconitum heterophyllum* using *A. rhizogenes.* Pradel et al. (1997) developed a system for producing transformed plants from root explants of *Digitalis lanata.* They evaluated different wild strains of *A. rhizogenes* for the productions of secondary products obtained from hairy roots and transgenic plants. They reported higher amounts of anthraquinones and flavonoids in the transformed hairy roots than in untransformed roots. An efficient protocol for the development of transgenic opium poppy (*Papaver somniferum* L.) and California poppy (*Eschscholzia californica* Cham.) root cultures using *A. rhizogenes* is reported (Park and Facchini 2000). Bonhomme et al. (2000) has reported the tropane alkaloid production by hairy roots of *A. belladonna* obtained after transformation with *A. rhizogenes*. Argolo et al. (2000) reported the regulation of solasodine production by *A. rhizogenes*-transformed roots of *Solanum aviculare*. Souret et al. (2002) have demonstrated that the transformed roots of *A. annua* are superior to whole plants in terms of yield of the sesquiterpene artemisinin. Shi and Kintzios (2003) have reported the genetic transformation of *Pueraria phaseoloides* with *A. rhizogenes* and puerarin production in hairy roots. The content of puerarin in hairy roots reached a level of 1.2 mg/g dry weight and was 1.067 times the content in the roots of untransformed plants. Thus, these transformed hairy roots have great potential as a commercially viable source of secondary metabolites (Leena et al., 2003).

A. tumefaciens is a widespread naturally occurring soil bacterium that causes crown gall, and has the ability to introduce new genetic material into the plant cell (Gelvin, 2003). The genetic material that is introduced is called T-DNA (transferred DNA) which is located on a Ti plasmid. Plasmid is a circular piece of DNA found in almost all bacteria (ABNE, 2010). This natural ability to alter the plant's genetic makeup was the foundation of plant transformation using *Agrobacterium*. Currently, *Agrobacterium*-mediated transformation is the most commonly used method for plant genetic engineering because of relatively high efficiency. Initially, it was believed that this *Agrobacterium* only infects dicotyledonous plants, but it was later established that it can also be used for transformation of monocotyledonous plants such as rice (ABNE, 2010). The closely related species, *A. rhizogenes*, induces root tumors, and carries the distinct Ri (root-inducing) plasmid. Although the taxonomy of *Agrobacterium* is currently under revision it can be generalised that 3 biovars exist within the genus, *A. tumefaciens*, *A. rhizogenes*, and *Agrobacterium vitis*. Strains within *A. tumefaciens* and *A. rhizogenes* are known to be able to harbour either a Ti or Ri-plasmid, whilst strains of *A. vitis*, generally restricted to grape-vines, can harbour a Ti-plasmid (Wikipedia, 2011).

However, only in the past two decades has the ability of *Agrobacterium* to transfer DNA to plant cells been harnessed for the purposes of plant genetic engineering. Since the initial reports in the early 1980s using *Agrobacterium* to generate transgenic plants, scientists have attempted to improve this "natural genetic engineer" for biotechnology purposes. Some of these modifications have resulted in extending the host range of the bacterium to economically important crop species. However, in most instances, major improvements involved alterations in plant tissue culture transformation and regeneration conditions rather than manipulation of bacterial or host genes (Stanton, 2003). Today, many agronomically and horticulturally important species are routinely transformed using this bacterium, and the list of species that is susceptible to *Agrobacterium*-mediated transformation seems to grow daily. In some developed countries, a high percentage of the acreage of such economically important crops as corn, soybeans, cotton, canola, potatoes, and tomatoes is transgenic; an

increasing number of these transgenic varieties are or will soon be generated by *Agrobacterium*-mediated, as opposed to particle bombardment-mediated transformation and regeneration conditions rather than manipulation of bacterial or host genes. *Agrobacterium*-mediated plant transformation is a highly complex and evolved process involving genetic determinants of both the bacterium and the host plant cell (Stanton, 2003). The overall advantages of using *Agrobacterium*-mediated transformation over other transformation methods are: reduction in transgene copy number, and intact and stable integration of the transgene (newly introduced gene) into the plant genome (Jones et al., 2005).

Agrobacteruim mediated plant transformation process

In this method, *A. tumefaciens* or *A. rhizogenes* is employed to introduce foreign genes into plant cells. *A. tumefaciens* is a soilborne gram-negative bacterium that causes crown-gall, a plant tumor. The tumor-inducing capability of this bacterium is due to the presence of a large Ti (tumor-inducing) plasmid in its virulent strains. Similarly, Ri (root-inducing) megaplasmids are found in virulent strains of *A. rhizogenes*, the causative agent of "hairy root" disease. Both Ti- and Ri-plamids contain a form of "T-DNA" (transferred DNA). The T-DNA contains two types of genes: oncogenic genes, encoding enzymes involved in the synthesis of auxins and cytokinins (causing tumor formation), and genes involved in opine production. The T-DNA element is flanked by two 25-bp direct repeats called the left border (LB) and right border (RB), respectively, which act as a *cis* element signal for the T-DNA transfer. Both oncogenic and opine catabolism genes are located inside the T-DNA of the Ti plasmid whereas the virulence (*vir*) genes are situated outside the T-DNA on the Ti plasmid and bacterial chromosome (Zupan et al., 2000). The *Agrobacterium*-mediated transformation process involves a number of steps:

1) Isolation of the genes of interest from the source organism;
2) Development of a functional transgenic construct including the gene of interest; promoters to drive expression; codon modification, if needed to increase successful protein production; and marker genes to facilitate tracking of the introduced genes in the host plant;
3) Insertion of the transgene into the Ti-plasmid;
4) Introduction of the T-DNA-containing-plasmid into *Agrobacterium*;
5) Mixture of the transformed *Agrobacterium* with plant cells to allow transfer of T-DNA into plant chromosome;
6) Regeneration of the transformed cells into genetically modified (GM) plants; and
7) Testing for trait performance or transgene expression at lab, greenhouse and field level (ABNE, 2010).

During transformation, several components of the Ti plasmid enable effective transfer of the genes of interest into the plant cells. These include:

1) T-DNA border sequences, which demarcate the DNA segment (T-DNA) to be transferred into the plant genome;
2) vir genes (virulence genes), which are required for transferring the T-DNA region to the plant but are not themselves transferred, and
3) Modified T-DNA region where the genes that cause crown gall formation are removed and replaced with the genes of interest.

The T-DNA carries genes for the biosynthetic enzymes for the production of unusual amino acids, typically octopine or nopaline. It also carries genes for the biosynthesis of the plant hormones, auxin and cytokinins. These plant hormones produce opines, providing a carbon and nitrogen source for the bacteria that most other micro-organisms cannot use, giving *Agrobacterium* a selective advantage. By altering the hormone balance in the plant cell, the division of those cells cannot be controlled by the plant, and tumors form. The ratio of auxin to cytokinin produced by the tumor genes determines the morphology of the tumor (root-like, disorganized or shoot-like) (Wikipedia, 2011).

Applications of *agrobacterium* to agricultural biotechnology

Plant transformation mediated by *Agrobacterium tumefaciens*, a soil plant pathogenic bacterium, has become the most used method for the introduction of foreign genes into plant cells and the subsequent regeneration of transgenic plants. *A. tumefaciens* naturally infects the wound sites in dicotyledonous plant causing the formation of the crown gall tumors. The first evidences indicating this bacterium as the causative agent of the crown gall goes back to more than ninety years. Since that moment, for different reasons a large number of researches have focused on the study of this neoplastic disease and its causative pathogen. During the first and extensive period, scientific effort was devoted to disclose the mechanisms of crown gall tumor induction hoping to understand the mechanisms of oncogenesis in general, and to eventually apply this knowledge to develop drug treatments for cancer disease in animals and humans. When this hypothesis was discarded, the interest on crown gall disease largely decreased until it was evident that this tumor formation may be a result of the gene transfer from *A. tumefaciens* to infected plant cells (Smith and Townsend, 1907).

A. tumefaciens infects plants by making a crown gall in region where the stem meets the roots. Researchers have discovered that the bacteria transfer part of their

DNA to the plant nucleus hence integrated into the plant genetic material. The transfer DNA or T-DNA is part of a large tumour inducing plasmid. The T-DNA carries an oncogenic region that code for the production of plant growth hormones which causes the proliferation of plant cells forming gall or tumour. It also codes for unusual derivatives of arginine which is a growth substance. This bacterium-plant interaction is known as genetic colonization. Scientist also discovered that the introduction of a foreign gene into the T-DNA would enable its transfer to the plant cell nucleus leading to the development of plant transformation using a disarmed, oncogenic, version of the Ti-plasmid that could transfer DNA into plants without causing the production of tumour. Having known that all that is required for gene to be introduced into a plant are the 25 bp repeated sequences at the borders of the oncogenic region (the left and right borders), the virulence gene of the Ti-plasmid. It was possible to separate these in a system of binary vectors (Steven and Andrew, 1998).

The earliest species to be transformed was tobacco, *Nicotiana tabacum* which rapidly became the model dicotyledonous plant. However, more recently, the workhorse has changed to *Arabidopsis thaliana* which has a very small genome of 120 megabases and is easier to transform. In other to transform tobacco and most other dicotyledonous plants, leaf disk are cut and placed in a Petri-dish containing liquid medium. The *A. tumefaciens* strain is placed on the surface of the disks and co-cultivation carried out for 2 to 3 days. The cutting of the leaf disks results in the plant producing wound-response compound, such as acetosyringone which induce the virulence genes. The leaf disks are then transfer to selection media, containing the herbicide or antibiotics of choice. This is often kanamycin as many binary vectors carry the Neomycin phosphotransferase gene which code for kanamycin resistance. Transformation occur along the cut edges of the disks, resulting in the formation of callus tissue which carries the DNA between the left and right border integrated at random into the plant genome. The callus tissue is then transfer into the regeneration medium also containing kanamycin, which only allow transgenic plants, expressing kanamycin resistance, to develop, the whole process takes about 3 to 4 months (Steven and Andrew, 1998).

Therefore, transformation with *Agrobacterium* can be achieved in two ways. Protoplasts, or leaf-discs can be incubated with the *Agrobacterium* and whole plants regenerated using plant tissue culture. A common transformation protocol for *Arabidopsis* is the floral-dip method: the flowers are dipped in an *Agrobacterium* culture, and the bacterium transforms the germline cells that make the female gametes. The seeds can then be screened for antibiotic resistance (or another marker of interest), and plants that have not integrated the plasmid DNA will die (Wikipedia, 2011). The initial results of the studies on T-DNA transfer process to plant cells demonstrate three important facts for the practical use of this process in plants transformation. Firstly, the tumor formation is a transformation process of plant cells resulted from transfer and integration of T-DNA and the subsequent expression of T-DNA genes. Secondly, the T-DNA genes are transcribed only in plant cells and do not play any role during the transfer process. Thirdly, any foreign DNA placed between the T-DNA borders can be transferred to plant cells, no matter where it comes from. These well-established facts, allowed the construction of the first vector and bacterial strain systems for plant transformation (Deblaere et al., 1985).

Agrobacterium-mediated transformation has currently become a very well-developed science. The use of artificial pesticides that will be harmful to man, and pollute groundwater and the atmosphere, has been considerably lessened with the introduction of genetically engineered insect-resistant cotton. Herbicide-tolerant soybeans and corn have conjointly enabled the use of reduced-risk herbicides that break down more quickly in soil. These are nontoxic to plants or animals, and herbicide-tolerant crops facilitate the preservation of topsoil from erosion since they thrive better in reduced tillage agriculture systems. Papayas proof against the ring spot virus were conjointly developed through genetic engineering, which saved the U.S. papaya industry. *Agrobacterium*-mediated transformation might additionally be useful in improving and enhancing the nutritious quality of some crops. As an example, enhancing the levels of beta-carotene in canola, soybean, and corn improves oil compositions, and reduces vitamin A deficiencies in rice. There also are researches going on in the field of biotechnology to produce crops that cannot be laid low with harsh climates or environments which can require less water, fertilizer, labor etc. This is able to greatly reduce the stress and pressures on land and wildlife (Article click, 2011). *Agrobacterium* is listed as being the original source of genetic material that was transferred to foods such as soybean, cotton, corn, sugar beet, Alfalfa, Wheat, Rapeseed Oil (Canola), Creeping bentgrass (for animal feed), and Rice (Golden rice)(Wikipedia, 2011).

Conclusion

Conventional breeding methods alone cannot feed the extra hungry mouths despite the successes of the green revolution with substantial strides in food grains production, as a result of world population explosion. Agricultural biotechnology has the potential to reduce crop losses from pest and diseases, to improve the nutrient efficiency of food and animal feeds; to extend post harvest losses with increased shelf life of fruits and vegetables; and to increase the stress tolerance of crop plants allowing them to tolerate various environmental extremes such as cold and drought. *Agrobacterium*-mediated transformation has

been used as a tool to transfer transgenic genes with desirable characteristics to varieties of crop plants, which has succeeded in boosting agricultural production in other to meet up with global population explosion.

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