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Silicon Carbide Whisker-mediated Plant Transformation

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Abstract

With the advancement in molecular biology, several metabolic and physiological processes have been elucidated at molecular levels discovering the involvement of different genes. Since the advent of plant transformation 33 years ago, use of plant transformation techniques sparked an interest in fundamental and applied research leading to the development of biological and physical methods of foreign DNA delivery into 130 plant species. Modern molecular biology tools have developed rich gene sources which are waiting to be transformed into plant species. But unavailability of efficient transformation methods is a major hurdle to expedite the delivery of these genes into plants.

Ever-expanding available gene pools in the era of third generation transgenic plants stressing the delivery of multiple genes for different traits; development and application of new transformation methods is the big need of the time to meet the future challenges for plant improvement. In recent years, silicon-carbide whiskers have proven valuable and effective alternative in which silicon carbide fibers are mixed with plant cells and plasmid DNA, followed by vortexing/oscillation. Cell penetration appears to occur thus whiskers function as numerous fine needles, facilitating DNA entry into cells during the mixing process. This technique is simple, easy and an inexpensive transformation method to deliver the DNA into monocot and dicot plant species. Whiskers, cells and plasmid DNA are combined in a small tube and mixed on a vortex or oscillating mixer. In this chapter we will discuss the use of silicon carbide fibers/whiskers to transform and produce different transgenic plants. This chapter will help the reader to know about emerging applications of silicon carbide and other fibers in the delivery of foreign DNA into plants, and critical parameters affecting DNA delivery efficiency will also be discussed.

1. Introduction

Plant Cell wall is commonly found as the non-living barrier in the ways of DNA deliver technologies being attempted for plant genetic engineering. In case of biological systems, the cell wall is dissolved by cell wall degrading enzymes secreted by donor host for contact of donor cell with recipient cell allowing exchange of biological materials along with net DNA delivery into recipient cells. But this limitation cannot be overcome in monocots which are

non-host for prokaryotic mediated deliveries. The cell wall problem in monocots was ruled out by use of protoplasts, ballistic particles penetration opening the way for DNA entry into plants whether monocots or dicots. Although protoplasts are attractive as a transformation target because a large number of treated cells receive, regeneration from protoplasts of many crops remains technically difficult. Particle bombardment is currently considered as the most efficient delivery system. However, it requires sophisticated, expensive equipment and supplies. The same problem also exists for microinjection in which the transformation operation is extremely labor-intensive and technically demanding particularly bacterial cells electro-transformation, but could not be widely applied in plant transformation. Electroporation can be applied to many tissue types, but the efficiency remains poor at this stage.

Genetic improvement of plants has been done by plant breeders for years with great success. Different schemes have been developed by plant breeders for crossing plants in order to transmit and concentrate desirable traits in specific lines. However, in the process of classical plant breeding, success rate of attempts are uncertain and slow due to complex quantitative nature of genes governing agronomic traits. To transmit a required gene of interest by classical methods requires a series of sexual crosses between two lines and then repeated back crossing between the hybrid offspring and one of the parents until a plant with the desired characteristic is obtained. Plant breeding is a lengthy process, taking ten to fifteen years to produce and to release a new variety. This process, however, is limited to those plants which can sexually hybridize, and undesired genes in addition to the desired gene will also be co-transferred.
Recombinant DNA technologies circumvent these limitations by enabling plant geneticists to identify and clone specific genes for desirable traits. Different recombinant techniques can be used to explore biological features like gene transfer by *Agrobacterium tumefaciens*, microprojectile bombardment, electroporation of protoplast, polyethylene glycol method, microinjection, silicon carbide mediated transformation, liposome mediated gene transfer and sonication assisted *Agrobacterium*-mediated transformation.
Among these techniques, silicon carbide mediated gene deliveries hold good promise to expedise the delivery of differet genes into plant species.

2. Silicon whiskers and Transformation mechanism

Silicon carbide (SiC) whiskers have been extensively studied for high tech applications because of several advantages such as high tensile strength, high elastic modulus, excellent shock and degradation resistance (Choi et al., 1997). SiC whiskers have great intrinsic hardness and fracture readily to give sharp cutting edges (Greenwood & Earnshaw, 1984). They are obtained by the thermal reduction of silica in a reducing atmosphere, one source of silica being rice husks (Mutsuddy, 1990). Industrially, silicon carbide whiskers are used as abrasives in the manufacture of cutting tools and in the production of composite materials. Silicon carbide and other whiskers from different sources have been utilized in the transformation of monocot and dicot plant species embryo and cell suspension cultures (Table 1).
The exact mechanism for whisker-mediated transformation is based on different mechanisms. Scanning electron microscopy work on whisker-treated BMS cells described by

Kaeppler et al. (1990) suggested that a SiC whisker may have penetrated the wall of a maize cell. Unlike asbestos fibers (Appel et al., 1988), the surface of SiC whiskers is negatively charged. This negative surface charge probably results in there being little affinity between DNA molecules (which are also negatively charged) and whiskers in neutral pH medium. In these experiments they observed that premixing whiskers and DNA, as reported by Kaeppler et al. (1990, 1992), was not required to achieve efficient DNA delivery. This could suggest that whiskers do not "carry" DNA into the treated cells but function as numerous needles facilitating DNA delivery by cell perforation and abrasion during the mixing process (Fig 1 a and b). We have also found that materials with characteristics similar to SiC whiskers, such as nitride whiskers, can deliver DNA into plant cells.

Whisker/fiber type	Plant species	Genes / Selection strategy	Reference
Silicon carbide	Tobbacco	GUS nptII and BAR gene	Kaepler et al., 1992
Silicon carbide	Maize	GUS nptII and BAR	Kaepler et al.,1992 Petolino et al., 1994 Frame et al., 1994 Frame et al., 2000
Silicon carbide Al-borate K-titanate	Rice	GUS nptII	Nagatani et al., 1993 Matsushita et al., 1999 Mizuno et al., 2004 Terakawa et al., 2005
Silicon carbide	Ryegrass	GUS nptII	Dalton et al., 1998
Silicon carbide	Wheat	GUS nptII	Omirulleha et al., 1996
Silicon carbide	Cotton	GUS, AVP1 nptII	Asad et al., 2008
K-titanate	Soya bean	GFP nptII	Khallafalla et al., 2006

Table 1. Whisker mediated Gene Delivery in different transgenic model and commercial plants

3. Critical parameters for silicon carbide whisker-mediated plant transformation

3.1 Explant types

Transgenic plants have been successfully regenerated from a variety of tissues including leaves, cotyledons, hypocotyls, shoot tips, seed embryos and embryonic meristem transformed by different techniques. However, somatic embryogenesis occurrence is most critical parameter in whisker mediated plant transformation. Plant cell calli with a high density of embryogenic cells are extremely important and remain by far, the best tissue for explants and should be prepared from healthy seedlings/seeds for optimal results.

Among the few reports showing the DNA delivery via whisker method, callus suspension cultures have been used as explants for tobacco, maize, rice, soyabean and cotton. In each

plant species, callus suspension cultures were derived from different plant parts. In rice, embryos for whisker treatment were prepared by trimming off of shoots and remaining scutellar tissues were used as explant (Matsushita et al., 1999). Immature maize zygotic embryos of A188 x B73 from greenhouse grown ears 10-12 days post pollination were aseptically excised and plated scutellum surface uppermost on modified N6 medium (Armstrong and Green, 1985; Chu et al., 1975; Mezuno et al., 2004). In case of wheat, mature embryos were used (Omirrulah et al., 1996). To initiate suspension culture, 3 g of proliferating type II callus from a single embryo and suspension cultures were maintained in medium in a 125 ml Erlenmeyer flask at 28°C in darkness on a rotary shaker at 125 r.p.m. Suspensions were subcultured every 3.5 days by addition of 3 ml packed volume of cells and 7 ml of conditioned culture medium to 20 ml of fresh culture medium. Cells on the filter paper disc were placed on solid N6 medium and subcultured weekly for 3 weeks after which the suspension cultures were reinitiated (Frame et al 1994). In cotton, embryogenic cell suspension cultures were prepared from two month old hypocotyls derived calli (Asad et al., 2008).

Unlike particle bombardment and PEG treatment of protoplasts, whiskers deliver plasmid DNA in a non-precipitated form, making it possible to control the quantity of DNA available for transformation. Stable transformation of BMS maize cells with as little as 0.1 pg of input plasmid DNA, the transformation efficiency being 50% as high as, that was achieved using 25 pg. Direct gene transfer tends to introduce multiple gene copies into cells and these gene copies often display complex integration patterns (Spencer et al., 1992). It remains to be shown definitively whether these integration characteristics have any functional significance but the ability to control input DNA quantity with whisker transformation may make it feasible to influence copy number.

3.2 Whisker types

Different types of whiskers have been used in the delivery of foreign DNA into the targert tissues. Transformation efficiency has been largely determined by type of whiskers and even their manufacturing processes. In most cases SiC whiskers have been used to transform different plant species but the drawback associated with them is their persistence and toxicity to lungs if inhaled. Up until now differet types of silicon carbide whiskers including Sc-9, A.A, TW(s), TW(m) and alfa Aesar have been reported with different efficiencies (Table 1).

The transformation efficiency of other materials (glass beads, carborundum a spherical form of SiC and silicon nitride) was much lower than that of silicon carbide. Even using SiC whiskers, transient GUS activities with different sources of whiskers varied significantly. In maize using A X B suspension cells, where transient numbers achieved from SC-9 whiskers (Advanced Composite Materials Co., Greer, SC) were at least 5 times higher than that from other sources, TWS100 (Tokai Carbon Co., Ltd., Tokyo, Japan) and Alfa Aesar (Johnson Matthey, Ward Hill, MA). In a separate experiment, SC-9 whiskers were compared to SiC whiskers supplied by Good fellow (Cambridge, England) using BMS maize suspension cells (Wang et al., 1995). Whiskers of potassium titanate fibers having average diameter 0.5um and length 5-30μm were used for rice transformation. PTW is a physically and chemically stable material and has similar characteristics to those of SCW (0.6μm diameter, 10-80μm length) and ABW with 0.5 μm diameter and length 25μm (Terakawa et al., 2005). They all require careful handling in the fumehood while wearing mask to prevent the mishap of inhaling while using. The difference in transformation efficiency between the two whisker-

types was again significant. Moreover other whiskers from other sources such as ABW (alborax Y; alboraxYS3A) and potassium titanate have been developed and utilized to deliver DNA into the crop plant species. Different concentrations of whiskers delivered DNA in different efficiencies.

3.3 Mixing method

In whisker mediated plant transformation, mixing is the most critical step in the successful DNA delivery. It is the mixing method, duration and speed which significantly affect the DNA deliveries in the tissues. In addition different mixing materials have been used to enhance the DNA delivery in silicon carbide mediated plant transformation. Initially most reports involved the use of vortex mixing (Asano et al., 1990; Dunahay et al., 1993; Kaepler et al., 1990; 92). Rapid oscillation of cell and whiskers can result in stable transformation provided that cells survive in sufficient number for adequate regrowth (Frame et al., 1994). DNA delivery in the whisker method is increased by increasing the duration of agitation, agitation speed and amount of whiskers. However cell damage occurs when agitation is severe, extra prolonged and too large an amount of whiskers resulting in severe damage losing cell viability (Petolino et al., 2000; Mizuno et al., 2004). GUS expression units were maximum when mixing speed was 8 for 30 min using 20mg of ABW where as most of silicon carbide whisker preparations were made at the rate of 5% (Asad et al., 2008).

3.4 Pretreatment of explant

Explant serves key role in the efficient transformation of plants by whisker methods. Pretreatment of plant cells with osmotic agents has markedly increased the recovery of stable clones in maize. AXB suspension of cells 1 day after sub-culture were transferred in 1.5ml eppendorf tubes and incubated for 30 min with 1ml of liquid N6 medium containing equal molarities of sorbitol and manitol. 0.5M S/M increased 3-5 fold DNA delivery compared with non-treated control (Vain et al., 1993). No obvious effect on subsequent cell growth was seen following incubation in this medium. Similarly, calli subcultured at different intervals behaved differently for getting kanamycin resistant calli colonies when embryogenic calli were utilized as explants in cotton (Asad et al., 2008). Different transformation results have been obtained for calli from different plants while in case of cotton embryogenic calli, calli at 14 days after subculture were found to be the best explant for silicon carbide whisker mediated plant transformation. Asano et al. (1991) used the fiber technique to transform a suspension culture of *Agrostis alba* L. (Redtop). The genus *Agrostis* includes economically important temperate grass species used for forage and tuff. The frequency of GUS expression units tended to be the highest when plasmid DNA, was delivered to cells 6 days rather than 3 or 10 days after subculture. In Chlaymydomonas *(C. reinhardtii)* High-frequency, stable nuclear transformation can be achieved by agitating wall-less cells in the presence of plasmid DNA, glass beads and PEG (Kindle, 1990). In the whisker-mediated transformation protocol (Dunahay, 1993), plasmid DNA whiskers and PEG were added to -10^8 cells. The mixture was agitated by vortexing for 1 to 3 min. Cell viability after whisker treatment was greater than 80% whereas the viability of cells treated with glass beads was less than 10%. Typically, 10 to 100 stable transformants/10^7 cells were obtained using the whisker protocol.

3.5 Selection strategy

The production of transgenic plants requires efficient selection systems to isolate transformed cells, calluses, embryos, shoots from explants subjected to transformation. The existing selection systems can be divided into two basically different groups, where the conventional systems constitute by far the largest group. They are based on a selective agent (herbicide or antibiotic) being converted to a less toxic substance (detoxification) by the selective gene product. The most widely used selectable marker gene is the neomycin phosphotransferase gene which confers resistance to aminoglycoside type antibiotics such as kanamycin, neomycin and G-418 (geneticin). A number of other selection systems have been developed based on resistance to bleomycin, bromoxynil, chlorsulfuron, 2,4-dichlorophenoxy-acetic acid, glyphosate, hygromycin or phosphinothricin. The other group comprises the so-called positive selection systems where the selective agent is converted into a fully metabolisable compound by the selective gene product, providing the transgenic shoots with a metabolic advantage over non-transgenic shoots which are retarded on such selection media (Penna et al., 2002).

A gene for antibiotic resistance is usually fused with the reporter gene and gene of interest. Antibiotic resistance genes are typically chimeric bacterial genes that can be inserted into the T-DNA portion of the plant transformation plasmids. The resistance gene usually codes for an enzyme that inactivates the antibiotic using phosphorylation. Two frequently used selective markers that employ antibiotics as the selective agent are neomycin phosphotransferase II (*NPT* II) and hygromycin phosphotransferase *(HPT).* When antibiotics such as kanamycin or hygromycin are present in the tissue culture media, transformed cells will continue to divide while untransformed cells will slowly die. The *npt II and hpt* gens are a good selectable marker to use in dicot and monocot transformation work with efficient selection of putative transgenic tissues. The antibiotic hygromycin is very toxic to plant cells and expression of the *hpt* gene effectively causes insensivity to hygromycin in transformed cells (Larkin, 2001). Aragão et al. (2000) reported a system to select transgenic meristematic cells after the physical introduction of a mutant *ahas* gene by using a selection system based on the use of imazapy, a herbicidal molecule of the imidazolinone class capable of systemically translocating and concentrating in the apical meristematic region of the plant. The mechanism of action of imazapyr is the inhibition of the enzymatic activity of acetohydroxyacid synthase [AHAS; acetolactate synthase, acetolactate pyruvate-lyase (carboxilating)], which catalyses the initial step in the biosynthesis of isoleucine, leucine and valine (Shaner et al. 1984). This selectable marker system, combined with an improved multiple-shooting induction protocol, can result in a significant increase in the recovery of fertile, transgenic material after gene delivery process. Zhang et al. (2000) reported other herbicide resistance marker gene for soyabean; gene *bar* encodes a phosphinothricin acetyl-transferase enzyme (PAT), which inactivates the herbicide phosphinothricin (PPT). Joersbo et al., (2003) reported galactose as selective agent and a UDP-glucose: galactose-1-phosphate uridyltransferase gene as selective gene for efficient transformation of potato.

The better selection strategy used in transformation experiments allows rapid growth of transformed tissue as a result of selective growth of transgenic tissues. A fair efficient selection system allows very few non-transformed callus lines to grow through the reasonable selection scheme. Although, in most of the cases, hygromycin and kanamycin have been used as plant tissue selection marker, phosphoinithricin have also been used in

the transformation of plant tissues. Kanamycin antibiotic concentration was optimized for cotton embryogenic calli transformation with minimum escapes. Different selection agents have been employed for the whisker-mediated transformed plant calli (Table 1). As the new and efficient plant transformation methods are being developed, use of alternative and more efficient selection methods are required.

3.6 Use of reporter genes

Optimization and success of transformation can be facilitated and checked out by the use of selectable marker and reporter genes, which are excellent for tracking transformation events and are frequently used in transformation protocols. Using genetic engineering, these genes can be placed under regulatory control of specific promoters, making their expression associated to the transformation process.

Gene expression dynamics can often be monitored through the use of reporter genes. These genes are designed to visually reveal the expression of transgenes through destructive or non-destructive procedures. The *Escherichia coli lacZ* gene was one of the first to be used as a reporter. The *lacZ* gene encodes a galactosidase protein, which has been widely characterized genetically and biochemically. This reporter gene was initially used to analyze gene expression in yeast and later introduced into tobacco. The main problem with this reporter gene is the background level of the ®-galactosidase enzyme in plants. Another gene that was widely used for the analysis of transgene expression encodes the chloramphenicol acetyltransferase (CAT) protein. The *cat* gene was successfully introduced into different plants, such as tobacco and several *Brassica* species. The CAT reporter system was a suitable marker for some but not all plant species. It was found that some *Brassica* species had high levels of endogenous CAT activity and the response of unknown inhibitors, drastically reduced the levels of expression of the bacterial CAT in transgenic plants. These problems and the lack of a histochemical detection method limited the applications of *cat* as a reporter gene for plant transformation. Some of the problems encountered with the *cat* and *lacZ* genes were solved by the reporter gene system GUS. The GUS reporter system utilizes a bacterial gene from *Escherichia coli* (*uidA*) coding for a ®-glucuronidase (GUS) and consists in placing this gene in the Ti-plasmid, which is transferred to plant cells during gene delivey. When the plant tissue is assayed, transformation events are indicated by blue spots, which are a result of the enzymatic cleaving of an artificial substrate to give a blue product. The addition of a plant intron to the GUS gene directs expression only in plant cells with no problem of background expression in *Agrobacterium*. Although the GUS system is commonly used, the GUS histochemical assay utilizes cyanide, which kills the tissue, and requires overnight incubation for visualization. Due to the low content or absence of endogenous GUS activity in most plants, the *uidA* gene became one of the most widely-used reporter genes in eukaryotic systems. Unlike the *lacZ* and *cat* reporter genes, the *uidA* gene expression could be quantified relatively easily since it is an inexpensive and sensitive method to assay gene expression; however, the detection assay is destructive, making it impossible to analyze the same piece of tissue over time. For all these reasons the GUS reporter system was successfully used with different plant species (Jefferson et al., 1987; Higuchi et al., 2001). Transformation experiments with soybean mostly use the GUS reporter system for determining gene expression and integration.

A better reporter system would not require killing the tissue and would permit rapid or immediate evaluation of gene expression directly in living tissues. So a new and simpler

reporter system was developed which uses the green fluorescent protein (GFP) from jellyfish (*Aquorea victoria*). This reporter gene does not require a destructive staining procedure and allows direct viewing of gene expression in living plant tissue. Similar to the GUS reporter system, *gfp* can be introduced into plants using the Ti-plasmid. Following T-DNA transfer, GFP can be viewed directly in living tissues with blue light excitation. The GFP reporter system permits detection of labeled protein within cells and monitoring plant cells expressing *gfp* directly within growing plant tissue (Haseloff & Siemering, 1998). Since the *gfp* gene was first reported as a useful marker for gene expression in *Escherichia coli* and *Caenorhabditis elegans*, it has been modified by several laboratories to suit different purposes to include elimination of a cryptic intron, alteration in codon usage, changes in the chromophore leading to different excitation and emission spectra, targeting to the endoplasmic reticulum (ER) and mitochondria and understanding the morphology and dynamics of the plant secretory pathway (Brandizzi et al., 2004). GFP has been used as a reporter system for identifying transformation events in *Arabidopsis thaliana,* apple, rice, sugarcane, maize, lettuce, tobacco, soybean, oat, onion, wheat, leek and garlic (Eady et al., 2005). The GFP reporter system has also been used for identifying successful plastid transformation events in potato. The *gfp* gene has successfully been used as a scorable marker to evaluate plant transformation efficiency using *Agrobacterium tumefaciens,* particle bombardment and whisker mediated gene transfer. The gene could be expressed as early as 1.5 h following introduction and, since its detection is nondestructive, *gfp* expression could be followed over extended periods of time. GFP has also been used as a reporter to analyze the compartmentalization and movement of proteins over time in living plant cells using confocal microscopy (Benichou et al., 2003).

As the original *gfp* gene comes from the jellyfish, the coding region was modified to permit expression in plant cells. Codon usage of the gene was altered to stop splicing of a cryptic intron from the coding sequence. The unmodified *gfp* contains an 84 nucleotide sequence that plants recognize as an intron and is efficiently spliced from the RNA transcript, resulting in little or no expression of *gfp.* Using a modified *gfp*, *mgfp4*, expression problems resulting from cryptic intron processing were eliminated for many plants. Although the *mgfp4* gene is clearly an effective reporter gene, brightly fluorescing transformants containing high levels of GFP were difficult to regenerate into fertile plants. GFP in plants accumulates in the cytoplasm and nucleoplasm, while in jellyfish, GFP is compartmentalized in cytoplasmic granules. GFP in plants may have a mildly toxic effect due to fluorescent properties of the protein and accumulation in the nucleoplasm. In order to overcome this problem *mgfp5-ER* was produced, which has targeting peptides fused to GFP to direct the protein to endoplasmic reticulum (ER). With this modification, fertile plants have been regenerated more consistently (Haseloff & Siemering, 1998). Unlike *mgfp4*, *mgfp5-ER* lacks temperature sensitivity found in the wild-type GFP. Wild-type GFP must undergo proper folding with specific temperature requirements to maintain its fluorescent properties.. In addition to better protein folding, *mgfp5-ER* has excitation peaks of 395 and 473 nm. A broad excitation spectrum allows better GFP viewing with UV and blue light sources. The *mgfp5-ER* has shown to be an excellent reporter gene for lettuce and tobacco transformed by *Agrobacterium*. *Mgfp5-ER* has also been used with success for transient expression in soybean embryogenic suspension cultures via particle bombardment (Ponappa et al., 1999). The gene *gfp* has been modified numerous times and there are several *gfp* versions for plants.

Modified versions, other than *mgfp4* and *mgfp5-ER*, include: *SGFP-TYG* which produces a protein with a single excitation peak in blue light, *smgfp* which is a soluble modified *mgfp4, pgfp* which is a modified wild type GFP and *sGFP65T* which is a modified *pgfp* containing a Ser-to-Thr mutation at amino acid 65. Different versions of *gfp* have varying levels of fluorescence. These differences may be dependent upon the transformed species, promoter and termination sequences, or gene insertion sites. In the future, selective markers may not be needed, but while the intricacies of GFP expression need more understanding, selective markers are helpful in providing an advantage to identifying successful transformation event (Wachter, 2005).

In another reporter system, the luciferase reaction occurs in the peroxisomes of a specialized light organ in fireflies (*Photinus pyralis*). The luciferase reaction emits a yellow-green light (560nm) and requires the co-factors ATP, Mg^{2+}, O_2 and the substrate luciferin (Konz et al., 1997). The glow is widely used as an assay for luciferase activity to monitor regulatory elements that control its expression. *Luc* is particularly useful as a reporter gene since it can be introduced into living cells and into whole organisms such as plants, insects, and even mammals. *Luc* expression does not adversely affect the metabolism of transgenic cells or organisms. In addition, the *luc* substrate luciferin is not toxic to mammalian cells, but it is water-soluble and readily transported into cells. Since *luc* is not naturally present in target cells the assay is virtually background-free. Hence, the *luc* reporter gene is ideal for detecting low-level gene expression. A second reporter system based on luciferase expressed by the *ruc* gene from Renilla (*Renilla reniformis*) has also become available. The activities of firefly and *Renilla luciferase* can be combined into a dual reporter gene assay.
Despite the availability of a number of reporter genes, only two reporter genes (GUS and GFP) have been reported in transgenic plants developed through silicon carbide/whisker mediated plant transformation (Khalafalla et al., 2006; Asad et al., 2008).

3.7 Transgene integration and expression improvement

The perfect transformant resulting from any method of transgene delivery, would contain a single copy of the transgene that would segregate as a mendelian trait, with uniform expression from one generation to the next. Ideal transformants can be found with difficulty, depending upon the plant material to be transformed and to some extent on the nature and the transgene complexity. As gene integrations are essentially random in the genome, variability is often observed from one transgenic plant to another, a phenomenon ascribed to 'position effect variation' (Chitaranjan et al., 2010).The general strategy to 'fix' this situation is to generate, probably at a high cost, enough transgenic plants to find some with the desired level of expression.

Efforts are being directed toward achieving stable expression of the transgene with an expected level of expression rather than that imparted by the random site of integration. Scaffold Matrix Attachment Regions (MARs) could potentially eliminate such variability by shielding the transgene from surrounding influence. MARs are A/T rich elements that attach chromatin to the nuclear matrix and organize it into topologically isolated loops. A number of highly expressed endogenous plant genes have been shown to be flanked by matrix attachment regions and reduce the variability in transgene expression (Chitaranjan et al., 2010). Several experiments have been carried out in which a reporter gene like GUS has

been flanked by MARS and introduced into transgenic plants and compared to populations containing the same reporter gene without MARs (Mlynarora et al., 2003). Other ways to avoid variation in gene expression due to position effect are plastid transformation and minichromosome transformation. Some guidance might come from genome sequencing, which might provide the necessary DNA ingredients to control gene expression. The ability to target integration could also lead to some control of transgene expression . It is foreseen that site-specific recombinases could assist in this endeavor. All these areas of research, which are primed for breakthroughs, should be carefully monitored for immediate implementation in the design of suitable vectors equally useful for use in different plant transformation methods. In the longer term, it is less expensive and ultimately more desirable to produce higher quality and fewer quantities of transgenic plants.

Prospects

Currently most of the reports on gene deliveries by SCW are limited to model systems and few agronomic plants have been transformed which are largely concerned with transgene delivery and analyses of reporter genes. But no report is available describing the stability and pattern of inheritance in subsequent generations proving the authenticity of this relatively new physical method of plant transformation. So being an emerging transformation method, research on gene delivery with viable markers like GFP and luc genes having uniform integration and expression levels are worth pursuing future tasks. There is also a practical need for a method of transformation that will decrease the complexity of the pattern of transgene integration and expression. Presently, most commercial transgenics are altered in single gene traits. The challenge for the genetic engineers is to introduce large pieces of DNA-encoding pathways and to have these multigene traits function beneficially in the transgenic plants.

Although a clearer understanding of the events surrounding the integration and expression of foreign DNA is emerging, there are many questions that remain unanswered. Are there target cells or tissues not previously attempted that are more amenable to transformation? Is there a physiological stage that allows greater transformation? Can it be manipulated to achieve higher transformation efficiency? Does the tissue chosen as a target affect the level of expression? It is becoming increasingly clear that plants transformed by *Agrobacterium* express their transgene more frequently. Can this be partly attributed to the fact that T-DNAs frequently integrate in telomeric regions (Hoopen et al 1996)? Transformation technologies have advanced to the point of commercialization of transgenic crops. The introduction of transgenic varieties in the market is a multi-step process that begins with registration of the new varieties followed by field trials and ultimately delivery of the seed to the farmer. Technical improvements and employments of new efficient plant transformation methods that have the greatest opportunities for new approaches, probably in the realm of *in planta* transformation, will further increase transformation efficiency by extending transformation to elite commercial germplasm and lower transgenic production costs, ultimately leading to lower costs for the consumer.

4. Conclusion

It is quite clear that whisker-mediated transformation of any species where regenerable suspension cultures exist should be possible once DNA delivery parameters have been

established. Up until now most of the work has been focused on the demonstration of the viability of this method by use of reporter genes such as GUS and GFP. Routine transformation protocols are limited in most agriocultural plants. The low success has been attributed to poor regeneration ability (especially via callus) and lack of compatible gene delivery methods, although some success has been achieved by introducing innovative gene delivery technology like silicon/whisker mediated plant transformation. One of the limitations for efficient plant transformation is the lack of understanding of gene expression during the selection and regeneration processes. Therefore, optimization of the transformation efficiency and reproducibility in different laboratories still represents a major goal of investigators. We believe this is because transformation methods have not yet been properly quantified and established for each and every crop plants species. To improve the efficiency of transformation, more appropriate and precise methods need to be developed. For monitoring the efficiency of each step, the jellyfish green fluorescent protein (GFP) perfectly qualifies, because frequent evaluation of transgene expression could provide detailed information about regulation of gene expression *in vitro*. Nowadays, GFP is a useful reporter gene in plant transformation and is also used as a tool to study gene expression dynamics in stably transformed clones. GFP can play an important role in the evaluation of transformation systems and in the avoidance of gene silencing. Progress in soybean transformation suggests that some systems will achieve the transformation efficiency required for functional genomics applications in the near future.

Recently, we have obtained stably transformed lines from silicon carbide whisker treatment of embryogenic callus derived from cotton coker-312, indicating that the method can be extended to target tissues other than suspension cells. In addition to these genes, other genes of agronomic importance have been transformed into commercial crops like cotton and have obtained fertile transgenic AVP1 cotton with significant salt tolerance.

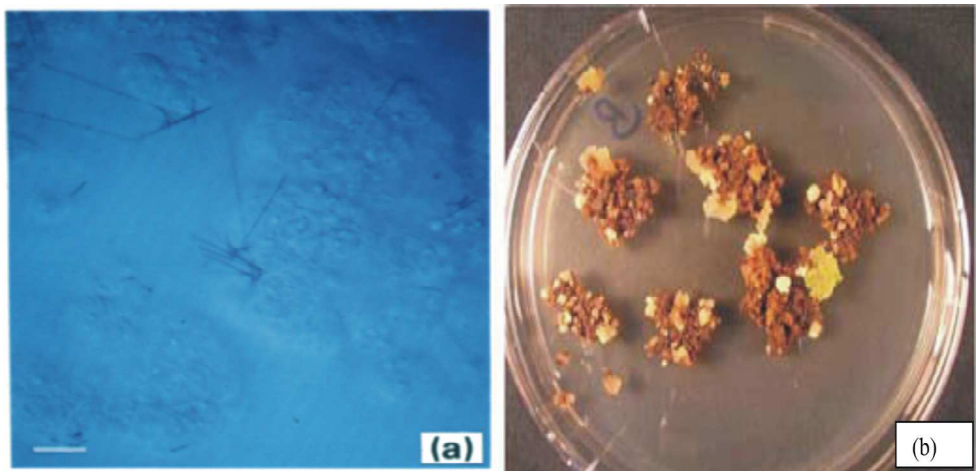

Fig. 1. a) Association of silicon carbide whiskers (needle-like material) with (a) A x B plant suspension cells visualized under light microscopy in maize (Frame et al., 1994); (b) induction of kanamycin resistant cotton calli from embryogenic calli transformed with silicon carbide whiskers (Asad et al., 2008)

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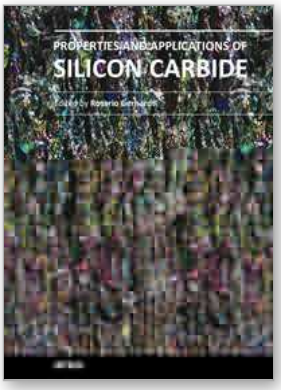

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In this book, we explore an eclectic mix of articles that highlight some new potential applications of SiC and different ways to achieve specific properties. Some articles describe well-established processing methods, while others highlight phase equilibria or machining methods. A resurgence of interest in the structural arena is evident, while new ways to utilize the interesting electromagnetic properties of SiC continue to increase.

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