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**REGENERATION OF SUGARCANE PLANTS
FROM PROTOPLASTS
AND APPLICATION OF
GENETIC TRANSFORMATION SYSTEMS
TO SUGARCANE**

by

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1.0 INTRODUCTION

Plant crop improvement through genetic engineering is one of the most promising fields of research in agriculture today. The introduction of desirable genes into promising cultivars may have the potential to produce superior cultivars without the major genetic reassortment that follows conventional crossing. Already, a bacterial gene conferring resistance to the herbicide glyphosphate has been cloned and introduced into tobacco plants, with the transformed plants showing greater tolerance to the herbicide than untransformed plants (Comai et al., 1985).

Desirable genes can be transferred into plant genomes by either Agrobacterium-mediated or direct gene transfer systems. The soil bacterium Agrobacterium is a natural plant genetic engineer which can introduce genes on a specialised segment of its DNA into genomes of intact plant cells. Direct gene transfer involves delivery of foreign genes into plant genomes without the need for Agrobacterium or viral vectors. In dicotyledonous plants, direct and Agrobacterium-mediated gene transfer have been used to achieve stable integration of foreign genes into the chloroplast and nuclear genomes (Fraley et al., 1986; Thomas and Hall, 1985).

In monocotyledonous plants, gene transfer using Agrobacterium vectors has had some success in certain members of the Liliaceae and Amaryllidaceae (Hooykaas-Van Slogteren et al., 1984; Hernalsteens et al., 1985) but efforts to transform other monocotyledons, particularly members of the Gramineae using Agrobacterium have not yet been successful. Using direct gene transfer methods, foreign genes have been stably integrated into protoplasts of Triticum monococcum (Lorz et al., 1985), Lolium multiflorum (Potrykus et al., 1985) and maize (Fromm et al., 1986).

The difficulty in regenerating transformed plants from protoplasts remains a major limitation to practical benefit from gene transfer into protoplasts of the Gramineae (Fraleigh et al., 1986). Reports of plant regeneration from protoplasts of specific cultivars of several species of the Gramineae (Lu et al., 1981; Coulibaly and Demarly, 1986; Vasil and Vasil, 1980; Yamada et al., 1986) including sugarcane (Srinivasan and Vasil, 1985) are therefore encouraging. Sugarcane (*Saccharum* spp. hybrid) is a major agricultural crop and has been routinely regenerated from callus cultures for many years (Heinz and Mee, 1969). Regenerated sugarcane plants may express variability and this somaclonal variation has been used for plant improvement (Heinz et al., 1977).

Recent advances in plant regeneration from protoplasts present the opportunity to adapt established gene transfer systems to sugarcane. This review outlines research relevant to the regeneration of sugarcane plants from protoplasts and summarises genetic transfer systems considered to be of potential use to the incorporation of genes into sugarcane.

2.0 PLANT TISSUE CULTURE

Plant cell walls are a physical barrier to DNA transfer, so that protoplasts produced by enzymatic digestion of the cell walls, are much more susceptible to direct gene transfer. Practical application of existing gene transfer systems for sugarcane crop improvement depends on the regeneration of transformed plants from protoplasts. Protoplasts of many different plant species can be cultured to form new cell walls and divide to form colonies. However, the induction of individual colonies to regenerate shoots or embryos that will produce plantlets has mainly been limited to dicotyledonous plants.

Protoplasts of graminaceous species have proved difficult to culture (Protrykus et al., 1985), however, induced plantlet formation from cereal protoplasts has recently been achieved for specific cultivars of pearl millet (Vasil and Vasil, 1980), rice (Yamada et al., 1986; Coulibaly and Demarly, 1986) and sugarcane (Srinivasan and Vasil, 1985). Previous attempts at sugarcane plant regeneration from protoplasts had been unsuccessful although

cell colonies and callus tissue were obtained from protoplast cultures (Maretzki and Nickell, 1973; Evans et al., 1980; Chen and Shih, 1983; Larkin, 1982). According to Vasil and Vasil (1984), in cereals and grasses only protoplasts derived from embryogenic cells are totipotent and capable of division and further organised development to form plants. Srinivasan and Vasil (1985) reported that protoplasts of the sugarcane cultivar B4362 derived from embryogenic cell suspension cultures regenerated cell walls, sustained cell division and eventually formed sugarcane plantlets. The cell suspension cultures were established from embryogenic callus cultures. In contrast, Larkin (1982) isolated protoplasts from embryogenic as well as non-embryogenic cell suspension cultures but failed to establish plant regeneration although cell cultures were re-established from both types of protoplasts.

Sugarcane embryogenic callus has best been induced on segments of young furred leaves obtained from immediately above the stalk apical meristem (Ho and Vasil, 1983a) and cultured on MS medium (Murashige and Skoog, 1962) supplemented with approximately 3.0 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 5-10% coconut water (Nadar et al., 1978; Ahloowalia and Maretzki, 1983; Ho and Vasil, 1983a). However, Larkin (1982) used CS medium (Gibson et al., 1976) supplemented with 3 mg L^{-1} 2,4-D and 0.25 mg L^{-1} kinetin to obtain embryogenic callus. Fitch and Moore (1984) reported that embryogenic calli of commercial sugarcane cultivars were capable of long term regeneration when grown on MS medium containing 0.5 mg L^{-1} picloram (2,3,5-trichloropicolinic acid) as a substitute for 2,4-D. According to Ho and Vasil (1983a) embryogenic callus tissue was morphologically distinguished from other types of callus tissue by being hard, compact, smooth and consisting of small, round, richly cytoplasmic cells that became white in later stages. Non-embryogenic callus tissue was usually friable, semi-translucent and consisted of loose, large and elongated cells.

Embryogenic cell suspension cultures have mainly been established by incubating embryogenic callus tissue with liquid MS medium containing 3 mg L^{-1} 2,4-D, 5-10% coconut water, $400\text{-}500 \text{ mg L}^{-1}$ casein hydrolysate (Liu and Hsieh, 1985; Ho and Vasil, 1983b) and 1.0 mg L^{-1} thiamine-HCl (Chen and Shih, 1983; Liu and Hsieh, 1985) on a gyratory shaker at 100 r min^{-1} at $27\text{-}28^{\circ}\text{C}$. Ahloowalia and Maretzki (1983) initiated cell suspension cultures on

MS medium containing 2,4-D and coconut water, and then established embryogenic cell suspension cultures using half strength MS liquid supplemented with 0.5 mg L^{-1} 2,4-D. Larkin (1982) used CS medium without arginine but supplemented with 2 mg L^{-1} 2,4-D and 0.05 mg L^{-1} kinetin. Therefore, to successfully culture embryogenic cell suspension cultures it is essential to incorporate an auxin such as 2,4-D and a source of cytokinin such as coconut water in the medium. Ho and Vasil (1983b) replaced varying amounts of the medium every 2-3 days for one month to minimise the accumulation of pigments secreted by the small pieces of callus. After several weeks, small clumps of embryogenic cells formed which were then cultured to establish embryogenic suspension cultures. Embryogenic cells were small, richly cytoplasmic and actively dividing with a prominent nucleus and conspicuous starch grains whereas, the non-embryogenic cells were large, vacuolated and often elongated with sparse cytoplasm and few starch grains. Two types of embryogenic cell suspensions were established. Type I suspensions were the initial embryogenic cell suspension cultures which grew slowly and had loose clumps of cells. Subculture of Type I suspensions by using 10-15 mL of inoculum every 2-3 days resulted in faster growing cells which formed tight cell clumps, these were designated Type II suspensions. These latter suspensions were maintained by subculturing 2.0-2.5 mL of inoculum into 25 mL of fresh medium every 4-5 days. Ho and Vasil (1983b) indicate that establishment of Type II embryogenic suspension cultures is an essential prerequisite for successful culturing of totipotent protoplasts which will eventually regenerate sugarcane plantlets.

Other important parameters for the successful isolation of totipotent protoplasts include the use of an appropriate cellulolytic enzyme mixture; optimising the duration of enzyme treatment, osmotic potentials, temperature and pH values; and the careful handling of protoplasts during washing, centrifugation and plating.

Srinivasan and Vasil (1985) isolated protoplasts by incubating cells from Type II embryogenic suspension cultures with an enzyme mixture of 2% RS cellulase, 0.2% pectinase, 0.1% driselase and 0.2% macerozyme for 5-6 hours at $25-26^{\circ}\text{C}$ at pH 5.9. Mannitol and sorbitol both at 0.2 M were also included in the enzyme mixture to control the osmotic potential. Sodium dihydrogen

phosphate (0.7 mM) and MES (3.0 mM) were added as pH buffers. After filtering to remove undigested cell material, the protoplasts were washed, resuspended at a density of $1-5 \times 10^5 \text{ mL}^{-1}$ and cultured in modified 8p medium (Kao and Michayluk, 1975) or CSK medium (Larkin, 1982) supplemented with coconut water, casein hydrolysate, 2,4-D and benzyladenine. Colony formation occurred after 2-3 weeks in the same media in liquid form or solidified with 0.3% agarose. Somatic embryos were produced on transfer to agar solidified MS medium containing 1% activated charcoal and 0.25-1.0 mg L^{-1} 2,4-D. Embryos matured on transfer to media containing 0.1-0.5 mg L^{-1} abscisic acid and germinated in media containing 0.5 mg L^{-1} benzyladenine to form plantlets. Larkin (1982) washed cells in 0.24 M calcium chloride (CaCl_2) prior to enzyme treatment and incubated cells for 5-20 hours at 22°C in a charcoal pretreated enzyme solution containing 2% driselase, 2% cellulysin, 1% hemicellulase and 0.1% pectolyase, 0.27 M sucrose, 0.12 M mannitol and 0.024 M CaCl_2 , at pH 5.8. Protoplasts were cultured in CSK medium at $24-27^\circ\text{C}$. Colonies were transferred to CS medium containing 0.2 mg L^{-1} 2,4-D and 0.05 mg L^{-1} kinetin.

Sugarcane protoplasts have also been cultured using a cell feeding technique (Chen and Shih, 1983) in which suspension cells were used as a feeder layer to support the division of the upper layer of cultured protoplasts. Evans et al. (1980) cultured protoplasts in Kao and Michayluk medium in either droplets or hanging droplets at 10^3 to 10^4 protoplasts mL^{-1} before plating colonies into regeneration medium.

Plant regeneration from sugarcane protoplasts has only been achieved by Srinivasan and Vasil (1985) and this was at a low frequency (Vasil, pers. com.). Practical application of established direct gene transfer systems to sugarcane depends on the regeneration of plants from protoplasts. It is therefore desirable to increase the frequency of regeneration from protoplasts and extend this technique to a range of cultivars.

It is proposed to confirm the reported results of Srinivasan and Vasil; increase the frequency and reliability of plant regeneration to a satisfactory level for sugarcane genetic engineering research; and then apply these techniques to a range of Queensland cultivars. For example, the isolation and regeneration of

protoplasts of Queensland commercial cultivars may depend on modifying the duration of enzyme treatment and the protoplast culture media. The concentration of plant hormones in the protoplast culture media may have to be altered until the optimum concentrations for cell wall regeneration and somatic embryo formation are found. Plating efficiency of protoplasts may also be improved by modifying the protoplast culture techniques. Ho and Vasil (1983b) cultured protoplasts in medium solidified with 0.3% agarose. However, Shillito et al. (1983) utilised the combination of agarose plating and bead culture to improve plating efficiencies of protoplasts in a wide range of dicotyledonous plant species. The bead culture consisted of incubating the agarose gel embedded protoplasts in large volumes of liquid medium. This technique eliminates many of the toxic properties of agar. By adopting such methods used for protoplast culture of dicotyledonous plants an improvement in plating efficiency of sugarcane protoplasts may be achieved.

3.0 GENETIC TRANSFORMATION SYSTEMS

3.1 Vector mediated gene transfer systems

Agrobacterium tumefaciens, a gram negative soil bacterium that causes crown gall disease of certain dicotyledonous plants has the capacity to genetically engineer plants in nature by using a tumor-inducing (Ti) plasmid as a natural gene vector (Gordon, 1981). A portion of the Ti-plasmid, called the T-DNA is transferred to and stably incorporated in the nuclear DNA of plant cells during the infection process (Fraley et al., 1986). In transformed plant cells, specific T-DNA genes are transcribed into mRNA which leads to the production of opines and the synthesis of specific phytohormones - an auxin and a cytokinin (Hooykaas and Schilperoort, 1985). Opines are utilised as a nutrient source by the bacteria and the phytohormones cause the proliferation and expansion of cells which results in the production of the tumor. The ends of the T-DNA have very short elements designated right and left T-border sequences, and any sequences between these elements are able to be integrated into plant nuclear DNA. A large region of the Ti-plasmid called the virulence region is responsible for the transfer of T-DNA (Fraley et al., 1986).

The agrobacteria penetrate a wound site and attach to walls of wounded cells. Lippincott and Lippincott (1969) have shown that attachment is an essential step in plant tumor induction. Adherence apparently depends on the interaction between the polysaccharide portion on the outer surface of the bacterium and the outer pectin-rich layers of specific host cell walls (Lippincott and Lippincott, 1977). Lippincott and Lippincott (1978) reported that grasses lack adherence sites in the cell walls but that after treatment with pectinesterase these cell walls may become susceptible to bacterial adherence. According to Stachel et al. (1985), the agrobacteria recognise susceptible plant cells by the presence of certain signal chemicals exuded from wounded but actively metabolising plant tissues. These signal chemicals apparently act as chemical attractants and/or as a nutrient source for the bacteria and stimulate the activation of the virulence region of the Ti-plasmid. This leads to the transfer of the T-DNA region of the Ti-plasmid into the plant cell genome.

The ability of Agrobacterium to transfer T-DNA to plant cells has been used to genetically manipulate plants for crop improvement (Gordon, 1981). The development of Ti-plasmids as vectors has involved insertion into the T-DNA of chimeric genes that function as dominant selectable markers, and the disarming of the T-DNA by removal of the phytohormone biosynthetic genes which interfere with regeneration of normal plants (Fraley et al., 1986). Zambryski et al. (1983) constructed a Ti-plasmid derivative (pGV3850) in which the oncogenic functions of the T-DNA were deleted and replaced by a fragment of pBR322 - a commonly used bacterial cloning vehicle. The T-DNA border sequences were retained along with the nopaline synthase (nos) gene as a T-DNA specific marker. Any gene cloned in a pBR-like plasmid can be easily inserted between the T-DNA borders by homologous recombination, thus making pGV3850 a versatile acceptor plasmid (Zambryski et al., 1984). Antibiotic resistance genes function as dominant selectable markers and have been used to select for the introduction of foreign DNA into host genomes (Comai et al., 1985). Bacteria contain a number of useful antibiotic resistance genes that function in plants. The amino glycoside phosphotransferase (APH(3')II) gene product inactivates a number of amino glycoside antibiotics such as kanamycin, neomycin and G418, and the

chloramphenicol acetyl transferase (CAT) gene product inactivates chloramphenicol. De Block et al. (1984) reported the construction of chimeric genes comprising of the nos promoter and genes specifying resistance to kanamycin, chloramphenicol and methotrexate into disarmed T-plasmid vector (pGV3850). Transformed plants expressing these genes are protected against the phytotoxic effects of the corresponding antibiotics (De Block et al., 1984, Herrera-Estrella et al., 1983). A new selectable marker which permits transformed cells to be selected in the presence of hygromycin B antibiotic has been created by fusing the bacterial gene for hygromycin phosphotransferase to an octopine synthase promoter gene (Waldron et al., 1985).

Transformed plant cells can be detected using several assay techniques. Phenotypic expression can be assayed by growing calli in tissue culture on selected media containing the appropriate antibiotic. The appropriate concentration of antibiotic can be determined by culturing untransformed calli on media containing a range of antibiotic concentrations and selecting the lowest concentration which suppresses callus growth. De Block et al. (1983) found that the chimeric gene APH(3')II containing the promoter signals of the nos gene allowed selection of transformed calli in the presence of kanamycin at 50-100 mg L⁻¹. Chloramphenicol resistance was also phenotypically expressed, although the resistant calli grew slowly on the antibiotic supplemented media. Phenotypically normal and fertile plants were regenerated from the resistant calli. Expression of antibiotic resistance genes by transformed cells can also be detected by assaying for the specific enzymatic activity. De Block et al. (1983) demonstrated CAT activity in stems, roots, leaves and callus tissue derived from transformed tobacco plants using the CAT assay developed by Gorman et al. (1982). The presence of enzymatic activity of APH(3')II in transformed tobacco plants has been demonstrated by Herrera-Estrella et al. (1983). Another useful assay for the detection of transformed plants after infection with a Ti-plasmid vector containing an intact opine gene is the assay for nopaline dehydrogenase activity (Otten and Schilperoort, 1978). Nopaline assays of tobacco and petunia plantlets inoculated with Agrobacterium containing pGV3850 all produced nopaline-positive tissue. Transformed plant tissues developed into normal fertile plants (Zambryski et al., 1983).

A most efficient method for transferring foreign DNA into the host genome is by a binary vector system (Bevan, 1984; Hoekema et al., 1985). In this system, foreign DNA is inserted directly between the T-DNA border sequences present in small plasmid vectors. The resulting clones are replicated in E. coli and then transferred to an Agrobacterium strain carrying the Ti-plasmid virulence region and a T region which is either totally or partially deleted. Binary systems have the advantage of being completely disarmed. Moreover, the relatively small size of the vector, which contains unique restriction sites between the border fragments, allows for easy manipulation of vector DNA. DNA cloned between the T-border sequences is then transferred to plant cells from Agrobacterium strains conferring virulence functions. In addition, binary vectors generally contain a dominant selectable marker to enable selection of transformed tissues (Bevan, 1984).

There are several techniques for the transformation of dicotyledonous plant cells by Agrobacterium. These include wounding and inoculation of intact plants or in vitro infection of explants. However, the two most useful techniques for genetic engineering are cocultivation of protoplasts with the bacteria, and the leaf disc transformation method (De Block et al., 1984; Zambryski et al., 1984; Horsch et al., 1985).

Marton et al. (1979) first reported the successful transformation of protoplasts of tobacco by cocultivation with virulent A. tumefaciens. In the cocultivation procedure, 3-4 day old regenerating protoplasts were incubated with A. tumefaciens for 24-36 hours. During this time bacteria bound to protoplasts and transferred one or more copies of their T-DNA to the plant genome. Subsequent washing and antibiotic treatment eliminated the bacteria. The protoplasts were grown into microcolonies before being transferred to selective medium (Zambryski et al., 1984). Using this method De Block et al. (1984) showed that genes conferring resistance to the antibiotics kanamycin and chloramphenicol could be transferred into protoplasts of Nicotiana sp. and that subsequently transformed plant tissues exhibited functional expression of the resistance traits. The transformation frequency ranged between 4-9% of the plated calli (Herrera-Estrella et al., 1983). Nevertheless, cocultivation of protoplasts is difficult to adapt to new

species and is limited to dicotyledonous plant species with well-developed protoplast regeneration procedures (Fraley et al., 1986). The procedure can take up to six months from protoplasts to plants and, according to Shepard et al. (1980), plants derived from protoplasts can be subject to more cell culture-induced mutations than plants derived from explant regeneration procedures. Fraley et al. (1984) have demonstrated that the cocultivation procedure could be extended to petunia protoplasts, and by the use of a feeder plate culture system the transformation frequency was increased to $>10^{-1}$. The time period for identifying transformants was reduced to less than three weeks following protoplast isolation.

The leaf disc transformation method involves the cocultivation of a leaf disc with a disarmed Agrobacterium strain containing selectable marker genes. Horsch et al. (1985) transformed petunia, tobacco, and tomato plants, and McCormick et al. (1986) transformed cultivated tomatoes using the leaf disc transformation method. Leaf discs were inoculated with a disarmed strain of Agrobacterium containing a modified Ti-plasmid with a kanamycin resistance gene and incubated for two days. Leaf discs were then transferred to a selective medium containing kanamycin where transformed plants formed shoots and roots. The advantages of the leaf disc transformation method are that a wide range of plant species have the capacity to regenerate whole plants from leaf cells through simple tissue culture manipulations (Vasil et al., 1979). Also, less time is required for the regeneration of plants from leaf discs rather than from protoplasts. This has the advantage of minimising somaclonal variation which occurs in plants after prolonged periods of time in callus (Scowcroft and Larkin, 1982).

Although the Ti-plasmid gene transfer system facilitated by Agrobacterium has been applied to dicotyledonous plants and to a lesser extent in certain monocotyledons, it has not been extended to the Gramineae which include the plants of greatest agricultural importance such as corn, rice, wheat and sugarcane. The reasons for the failure of Agrobacterium strains to transfer genes into plants of the Gramineae may include the inability of the bacteria to attach to the cell walls, the inability of the cells of Gramineae plants to induce the activation of the virulence region of the Ti-plasmid which is essential for T-DNA transfer, and non-responsiveness of Gramineae cells to the products derived from the T-DNA encoded genes.

Agrobacterium has been shown to attach to cell walls and transfer T-DNA into the host genome of three species of non-Graminaceae monocotyledon plants, even though tumors did not form in two of the species (Hooykaas-Van Slogteren et al., 1984; Hernalsteens et al., 1984). It appeared that the genetically transformed cells of Chlorophytum capense and Narcissus sp. did not respond to phytohormones derived from the T-DNA encoded genes responsible for tumor formation whereas transformed cells of Asparagus officinalis did respond to these phytohormones. Attachment of Agrobacterium to cell walls of the above monocotyledons may have been due to transient changes in the cell wall biochemistry which was triggered by the wounding process. Lippincott and Lippincott (1977, 1978) found that after pectinesterase treatment, cell wall preparations of barley, corn, oats and wheat were able to bind Agrobacterium. They concluded that cell walls of monocotyledons lack appropriate attachment sites as a result of methylation of pectic compounds and that pectinesterase removes these methyl esters thus allowing the polysaccharide portion of the outer surface of the bacterium to interact with the pectic-rich layers of the cell walls.

The inability of wounded monocotyledon cells to activate the virulence region of the Ti-plasmid necessary for gene transfer could be due to certain monocotyledon plant cells not producing, or only producing in low quantities, certain virulence inducing molecules. Stachel et al. (1985) showed that the activation of Agrobacterium virulence gene expression was determined by the presence of either of two plant signal molecules - acetosyringone and α -hydroxyacetosyringone in the exudates of wounded but actively metabolising plant tissue. These virulence inducers are believed to trigger formation by Agrobacterium of circular T-DNA intermediates which are transferred to adjacent plant cells (Koukolikova-Nicola et al., 1985).

Sugarcane has never been recorded as a host for Agrobacterium. Therefore, genetic transformation of sugarcane cells by Agrobacterium-mediated gene transfer is most unlikely to occur in nature. However, modification of the leaf-disc transformation method may predispose sugarcane cells to Agrobacterium-mediated transformation. Leaf explants from shoots of sugarcane containing only the inner furred leaves of the apical part of the

shoot would be treated with pectinesterase to form attachment sites for Agrobacterium. The treated leaf explants would then be cocultivated with an Agrobacterium strain containing a modified Ti-plasmid with a chimeric kanamycin resistance gene under the control of a eucaryote promoter. The two plant signal molecules reported to activate the virulence region of the Ti-plasmid necessary for gene transfer would also be included during the cocultivation. The Agrobacterium may recognise the wounded cells, attach to the treated cell walls and transfer T-DNA into the plant cells. Leaf explants would then be cultured on solidified MS medium containing the auxin 2,4-D, coconut water and an antibiotic to kill remaining Agrobacterium, until the formation of callus tissue. The callus leaf pieces would then be transferred to a medium without the auxin but supplemented with kanamycin whereby transformed plantlets would develop normally and non-transformed plantlets without the kanamycin resistance gene would die. To confirm that kanamycin resistance resulted from genetic transformation of plants, assays would be performed for enzymatic activity of the APH(3')II gene product, as well as nopaline dehydrogenase activity.

Agrobacterium plasmid based vector systems are the most widely used vector systems for introducing DNA into plants, however, other vector systems such as viral vectors have been developed for plants (Howell, 1981). Viral vectors developed for plant transformations are either DNA viral vectors such as Cauliflower mosaic virus (CaMV) (Brisson et al., 1984) and the gemini virus (Buck and Coutts, 1983) or RNA viral vectors such as brome mosaic virus (BMV) (French et al., 1986). The viral vector most studied is CaMV which has a genome composed of a circular double-stranded DNA. The advantage of viral vectors is the efficient infection process however, insertion of foreign genes into the CaMV DNA is restricted by the small number of regions that can be modified without destruction of the viral functions that are essential for infectivity.

Viral vectors have been used in the transformation of Graminaceae plant species. French et al. (1986) demonstrated the expression of CAT activity in barley protoplasts inoculated with brome mosaic virus RNA containing the CAT encoding sequences. BMV genome consists of three RNA components. RNA1 and RNA2 encode for viral RNA replication and RNA3 encodes for

protein, including the virion coat protein. Variants of BMV RNA3 were constructed in which the coat gene was replaced with sequences encoding the CAT gene.

3.2 Direct DNA delivery systems

Direct DNA delivery systems into plant cells may prove extremely useful in transformation of plant species, such as cereals, not presently amenable to Agrobacterium mediated transformations.

Most direct DNA delivery systems involve the transformation of plant protoplasts. Crossway et al. (1986) developed a microinjection technique for the direct integration of foreign DNA into tobacco mesophyll protoplasts. This technique involved the development of a holding pipette method for immobilising protoplasts during injection. Bacterial plasmid DNA was then transferred into the nucleus or cytoplasm of the protoplasts. After microculture into calli approximately 14% of the intranuclearly injected protoplasts and 6% of the cytoplasmic injected protoplasts contained foreign DNA.

Davey et al. (1980) incubated Petunia protoplasts with isolated Ti-plasmid DNA in the presence of poly-L-ornithine. T-DNA sequences were detected in some of the transformed calli tissue. A more efficient procedure for DNA transfer into plant cells was developed by Krens et al. (1982) in which tobacco protoplasts were incubated with Ti-plasmid in the presence of polyethylene glycol (PEG) and a post-incubation with high calcium concentrations. DNA analysis of transformed calli revealed that transformation with isolated Ti-plasmid DNA did not require T-DNA border sequences as in the Agrobacterium mediated delivery mechanism. Genetic transformation of tobacco protoplasts has also been achieved by incubating protoplasts with a plasmid DNA-calcium phosphate ($\text{Ca}^{2+}\text{-PO}_4$) coprecipitate, followed by polyvinyl alcohol and high pH treatments (Hain et al., 1985). The $\text{Ca}^{2+}\text{-PO}_4$ /PEG technique has been successfully used for direct gene transfer to cereal cells (Lorz et al., 1985; Potrykus et al. 1985). Lorz et al. (1985) transformed cells of Triticum monococcum by incubating protoplasts in the presence of PEG with plasmid DNA containing kanamycin resistance gene.

Transformed cells were selected on a medium containing kanamycin. Potrykus et al. (1985) demonstrated stable gene transfer into protoplasts of Lolium multiflorum using procedure of Krens et al. (1982). Protoplasts were subjected to a heat shock immediately before the Ca^{2+} - PO_4 /PEG coprecipitation transformation treatment. Since regeneration of cereals from protoplasts is possible only to the callus stage, the practical use of the Ca^{2+} - PO_4 /PEG technique for cereal plant improvement is limited.

Another method developed for direct gene transfer is electroporation, whereby gene transfer occurs when a high-voltage electric pulse is applied to a solution containing protoplasts and DNA. This results in transient pores being produced in the protoplast membrane (Zimmerman and Vienkin, 1982) and consequently DNA and other macromolecules enter the cell through these pores (Neumann et al., 1982). The efficacy of electroporation depends on buffer composition, DNA concentration and amplitude and duration of the electric pulse (Fraley et al., 1986; Fromm et al., 1985). Fromm et al. (1985) developed an electroporation method for transferring pNOSCAT plasmid DNA into carrot protoplasts which was also effective when applied to tobacco and maize protoplasts. The transformed cells produced detectable levels of CAT activity. In 1986, Fromm et al., used electroporation to transfer and stably incorporate foreign DNA containing a chimeric kanamycin resistance gene into maize cells. Electroporation has advantages over the Ti-plasmid transfer systems in that it is faster to integrate DNA into cell genomes, it does not require special constructs with T regions, and that it works equally well on monocotyledon and dicotyledon plant protoplasts (Fromm et al., 1985; Fromm et al., 1986). Nevertheless, the electroporation technique like the Ca^{2+} - PO_4 /PEG technique will have limited use in cereal and sugarcane crop improvement unless intact plants can be regenerated from protoplasts of these Gramineae plants.

Recently, Morikawa et al. (1986) introduced tobacco mosaic virus RNA directly into tobacco mesophyll cells using electroporation. The injected virus genome was expressed in the recipient cells. This method for introducing foreign genetic material into plant cells does not require protoplasts and thus if applicable to monocotyledons it may have the advantage of plant regeneration from transformed cells.

4.0 CONCLUSION

Current gene transfer systems developed for modifying plants include direct gene transfer to protoplasts and Agrobacterium mediated gene transfer.

Direct gene transfer systems have been applied to both monocotyledon and dicotyledon cells. For the practical application of available direct gene transfer techniques to crop improvement, intact plants have to be regenerated from protoplasts. Low frequency of regeneration of intact plants from sugarcane protoplasts has recently been reported. Improvement in the frequency of regeneration from sugarcane protoplasts will be attempted by systematic experimentation with the concentrations of growth factors and plant hormones in the protoplast culture medium, and with the protoplast culturing techniques.

The Agrobacterium mediated gene transfer system has the advantage that it does not require protoplasts but can be applied to callus tissue or leaf discs from which intact plants can be readily regenerated. Less time is then required in the callus stage, which results in reduced somaclonal variation of regenerated plants. However, Agrobacterium mediated gene transfer has limitations in that Agrobacterium can not readily infect monocotyledon cells. Recent results suggest that it may be possible to overcome this limitation in sugarcane plants by treating leaf tissue prior to infection with pectinesterase to expose infection sites and by adding virulence inducing chemicals which stimulate the transfer of T-DNA into host genome during co-cultivation of Agrobacterium and leaf tissue. Antibiotic resistance genes will be used to test for the introduction of foreign DNA into the genome of sugarcane plants. Transformed plants will be detected using assays for transient expression of the antibiotic resistance genes, and selection will be applied for phenotypically transformed plants.

These studies have important practical implications for application of genetic engineering technology to crop improvement, initially in sugarcane, and subsequently in other graminaceous crops for which sugarcane may serve as a model.

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