In Vitro Cell. Dev. Biol. 31:101–104, April 1995

1071-2690/95 $05.00 + 0.00

WHISKER-MEDIATED PLANT TRANSFORMATION: AN ALTERNATIVE TECHNOLOGY[1]

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(Received 18 October 1994; accepted 26 November 1994; editor T. A. Thorpe)

A number of different methods, involving direct DNA delivery are now available for plant transformation. Here we review the most recently developed technique which involves the mixing of silicon carbide whiskers with plant cells and plasmid DNA. Fertile transgenic plants have now been produced using whisker-mediated transformation, and this method can now be considered as a simple, inexpensive alternative for plant transformation. A brief review on transformation of animal cells and *Chlamydomonas* using whiskers technology is also included.

Key words: DNA delivery; genetic modification; silicon carbide; transformation.

INTRODUCTION

The possibility of incorporating biological substances into living cells has occupied biologists for some time. However, in many instances the membrane or cell wall forms a near impenetrable barrier, thereby frustrating attempts at genetic manipulation.

For plant biologists it is rather fortunate that there exists a natural gene delivery system—*Agrobacterium tumefaciens*—a soil bacterium that transfers a segment of its DNA to the plant nuclear genome (Klee et al., 1987; Hohn et al., 1989). Although most dicotyledonous plants can be readily manipulated using this system, many monocotyledonous species, including the cereal crops, are not amenable to *Agrobacterium* transformation (Hohn et al., 1989; Potrykus, 1990). Physical and chemical methods for directly introducing genetic material into plant cells have therefore been devised or adapted from animal research. Currently, the successful techniques for this kind of plant cell transformation include particle bombardment (Sanford et al., 1987), tissue electroporation (D'Halluin et al., 1992; Kloti et al., 1993), microinjection (Neuhaus and Spangenberg, 1990), and direct gene transfer to protoplasts (Paszkowski et al., 1989).

To deliver genes into the plant nucleus, one of the essential steps is to penetrate the cell wall and plasma membrane. In the case of protoplast transformation, the cell walls are enzymatically removed before polyethylene glycol (PEG) treatment or electroporation is used to introduce DNA into the cell. Particle bombardment and microinjection physically deliver DNA into plant cells, by high velocity microprojectiles and ultra-fine injection needles, respectively, so removal of cell walls is not required for these techniques. Total removal of cell walls is also not required in the electroporation of intact tissues where DNA transfer is mediated by a pulse of electric charge, although in some cases wounding or partial enzymatic degradation of cell walls will enhance transformation efficiency (D'Halluin et al., 1992).

[1] Presented in the Session-in-Depth The State of the Art for Transformation Systems: What is New; What Works? at 1994 Congress on Cell and Tissue Culture, Research Triangle Park, NC, June 4–7, 1994.

Each of the above transformation approaches is not without attendant disadvantages. Although protoplasts are attractive as a transformation target because a large number of treated cells receive DNA, 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. Electroporation can be applied to many tissue types, but the efficiency remains poor at this stage.

In this article we review an alternative transformation technology which has been developed recently in the plant field. Silicon carbide (SiC) whisker-mediated transformation, a simple, rapid, and inexpensive method, is becoming an attractive option for plant transformation.

Transformation of animal cells. Examples of living cells being transformed with fibrous materials were first documented in the mammalian field. Asbestos fibers were used to introduce plasmid DNA and viral RNA into monkey cells (Appel et al., 1988; Dubes and Mack, 1988). In the report of Appel et al., monkey COS-7 cell lines were mixed with a plasmid carrying the neomycin phosphotransferase gene and chrysotile fibers (~650 nm long and 45 nm in diameter). Transfection was achieved by incubating the mixture for 30 min without shaking. Transient assays were performed after 48 h. Stable G418 resistant colonies could be clearly defined 18 days after transfection with asbestos-treated plates containing ~3 times as many colonies as the standard calcium phosphate-treated plates. The authors also reported the observation of DNA binding to chrysotile fibers (which have a positively charged surface) under the electron microscope.

Dubes and Mack (1988) reported that chrysotile and amphibole asbestos fibers could mediate the transfection of deproteinized viral RNA into cultured monkey cells with an efficiency at least equivalent to that of calcium phosphate.

Transformation of non-regenerable plant tissues. The first report on plant tissue transformation utilizing SiC whiskers (fibers) was pub-

lished by Kaeppler et al. (1990). In their study, 250 µl packed cell volume of Black Mexican Sweet (BMS) maize suspension culture or tobacco suspension culture (designated TXD) was added to a premixed DNA/fiber suspension and vortexed for 60 s. The plasmid DNA carried the β-glucuronidase (GUS) gene, and analysis for transient GUS expression was by histochemical assays performed after 2 days to evaluate transformation efficiency. GUS activity was reproducibly observed in all samples of BMS and tobacco cultures that had been vortexed in the presence of culture medium, plasmid DNA, and fibers. The mean frequency of GUS expression units in samples of treated BMS cells was 139.5 (one unit = one blue cell or group of adjacent blue cells). It was estimated (from Smith et al., 1984) that the BMS cell density was 10^7 cells per gram fresh weight. The transient expression frequency in the 250 µl packed volume samples of BMS cells used in these experiments was estimated at 10^{-4} per treatment. Tobacco suspension cells treated with fibers and DNA exhibited GUS expression at a mean frequency of 373 GUS expression units per sample. GUS expression was not observed in control samples lacking either DNA, fibers, or vortex treatment.

The same group later reported the recovery of the stably transformed clones using the fiber technique (Kaeppler et al., 1992). Thirty-four BASTA-resistant BMS colonies and 23 kanamycin-resistant tobacco colonies were recovered after selection. Southern analysis indicated that these clones contained intact copies of the *bar* and *npt*II genes. Sixty-five percent of the resistant BMS colonies and 50% of the resistant tobacco colonies also expressed GUS activity. Inasmuch as both suspension cultures were non-regenerable, no plants were available for further analysis.

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 turf. The frequency of GUS expression units tended to be highest when DNA was delivered to cells 6 days rather than 3 or 10 days after subculture. No stable transformation results were described.

Transformation of Chlamydomonas. Dunahay (1993) reported that stable *Chlamydomonas* transformants were obtained using the whisker technique. *Chlamydomonas reinhardtii* is currently the only eukaryotic unicellular alga for which efficient transformation systems exist. Transformation of *C. reinhardtii* was previously facilitated by the use of wall-less cells, either genetic mutants or cells whose walls were degraded using autolysin, a species-specific cell wall-degrading enzyme produced during mating by *C. reinhardtii* (Kindle, 1990). 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). Transformation of intact (walled) *C. reinhardtii* cells can also be achieved with low frequencies by using microprojectile bombardment (Boynton et al., 1988; Kindle et al., 1989), the glass bead protocol, and by electroporation.

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.

Transformation of regenerable maize cell lines. In a recent paper (Frame et al., 1994) we presented the first report of transgenic plant production, in any higher plant species, using SiC whiskers as the DNA delivery system. Whisker-mediated transformation was carried out on regenerable suspension cultures of the hybrid A188 × B73 (A × B). Cells vortex-treated for 60 s with whiskers and plasmid DNA carrying the GUS gene (*uidA* or *gus*) and the *bar* gene exhibited transient GUS expression at a mean frequency of 150 GUS expression units. Low frequency transient GUS expression in a similar type of regenerable culture was reported previously (Kaeppler et al., 1990). Cells which we treated without DNA or whiskers did not show GUS activity, confirming the earlier finding (Kaeppler et al., 1990). We observed however, that when cells were mixed with DNA and whiskers solely by tapping a finger against the side of the Eppendorf tube, a few blue spots could be detected. Unlike the previous report (Kaeppler et al., 1990), we found that premixing of DNA with whiskers was not required to achieve transformation and did not enhance the efficiency of DNA delivery.

Our success in producing fertile transgenic plants with this approach is due in part to the use of treatments pre- and post-whisker transformation which enhance DNA delivery and the subsequent recovery of transformed tissue. Exposure of cells to a medium containing a high molarity of sorbitol and mannitol was previously found to be beneficial with microprojectile bombardment of maize cells (Russell et al., 1992; Vain et al., 1993). In our case, transient GUS expression of cells treated in medium containing 0.5 *M* sorbitol/mannitol before transformation was 3 to 8 times higher than that of non-treated cells (Frame et al., 1994). The length of time between subculture and treatment also influenced transient expression, with treatment 1 day post-subculture giving the highest number of GUS expression units.

The transformed status of the callus and plants produced was determined by a combination of polymerase chain reaction (PCR) and Southern analysis, together with assays demonstrating functional gene product (phosphin-othricin acetyl transferase [PAT] and GUS) activity. Transmission and expression of the introduced *bar* gene in the progeny of transgenic plants was as expected for a single dominant allele, providing further evidence of integrative transformation. The fate of genes introduced into maize cells via whisker transformation does not seem to differ from other direct DNA transfer approaches.

Transformation mechanism and whisker sources. The exact mechanism for whisker-mediated transformation is not known. Silicon carbide has great intrinsic hardness and fractures readily to give sharp cutting edges (Greenwood and Earnshaw, 1984). 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 our experiments we 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.

We have also found that materials with characteristics similar to SiC whiskers, such as silicon nitride whiskers, can deliver DNA into plant cells (Fig. 1). However, as can be seen in Fig. 1, the transformation efficiency of other materials (glass beads, carborundum—a spherical form of SiC and silicon nitride) was much lower than that

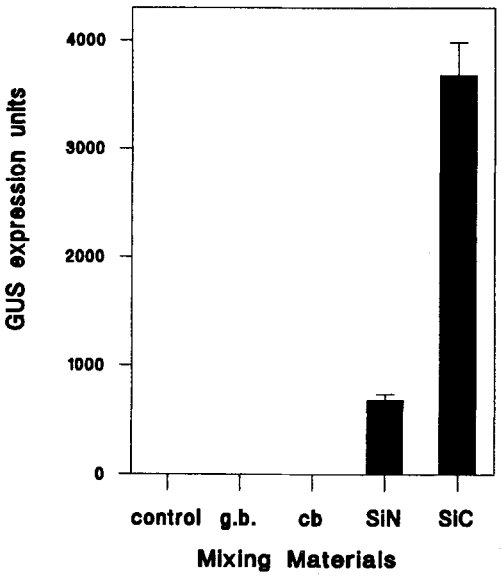

FIG. 1. Comparison of mixing materials on GUS expression in maize A × B suspension cells. Transformation process is as described earlier (Frame et al., 1994). In brief, 0.25 ml packed cell volume of maize A × B suspension cells were mixed with plasmid DNA carrying *gus* gene and various mixing materials in an Eppendorf tube. Mixtures were vortexed at full speed on a Vortex Genie II for 60 s. Transient GUS assay was performed after 2 days. *Control*: cells and plasmid DNA only; *g.b.*: glass bead (Microbeads AG, Woodley, Berks., UK); *cb*: carborundum (Carborundum Co., Manchester, England); *SiN*: silicon nitride whiskers (Goodfellow Metals, Cambridge, England); *SiC*: silicon carbide whiskers (Advanced Composite Materials Corp., Greer, SC). For each treatment, 40 µl of 5% of each material were added to cell suspension and DNA.

of silicon carbide. Even using SiC whiskers, transient GUS activities with different sources of whiskers varied significantly. Fig. 2 summarizes two separate experiments comparing whiskers from different sources on GUS transient expression. Experiment A (Fig. 2 *A*) was performed using maize A × 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 (Fig. 2 *B*), SC-9 whiskers were compared to SiC whiskers supplied by Goodfellow (Cambridge, England) using BMS maize suspension cells. The difference in transformation efficiency between the two whisker-types was again significant.

The reason for the variation in transient expression using different whiskers is not clear. Scanning electron microscopy showed that the SC-9 whiskers (0.6 × 10–80 µm) appeared more smooth and uniform than the Goodfellow whiskers (0.5 × 30 µm) with less clumping and debris apparent (P. Drayton and J. Thompson, unpublished data). Under the light microscope, whiskers from Tokai Carbon, TWS100(m) and TWS100(c) (very hydrophobic) and from Johnson Matthey seemed to be more homogeneous but shorter than the SC-9 whiskers (B. Frame, unpublished data). It is likely that the surface characteristics of whiskers and the possible presence of "toxic" by-products as a result of the manufacturing process may affect transformation efficiency. Such properties are currently being investigated.

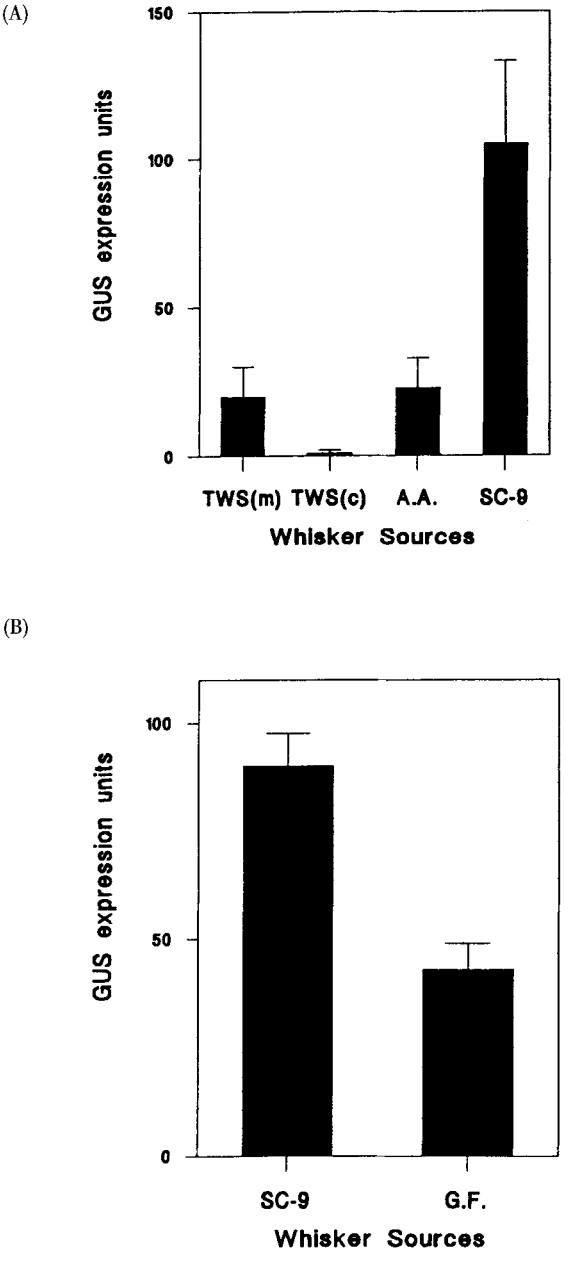

FIG. 2. Comparison of different SiC whiskers sources on transient GUS expression on maize suspension cells of A × B (*A*) and BMS (*B*). *TWS(m)* and *TWS(c)*: TWS100, m and c stand for metallic and ceramic material use; *A.A.*: Alfa Aesar; *SC-9*: Silar SC-9; *G.F.*: Goodfellow. For each treatment, 40 µl of 5% of each material were added to cell suspension and DNA.

In conclusion, whisker-mediated transformation offers a technically simple and inexpensive alternative for the genetic modification of plant cells. In contrast to the other methods that are in current use, whisker-mediated transformation does not require sophisticated equipment, expensive consumables, or a great deal of technical expertise. With a greater understanding of the process of DNA delivery, it is likely that the efficiency of the technique will be increased significantly and that a wider range of target tissues and species will be amenable to transformation with this approach.

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