In summary, the discovery that the sensitivity of N_2 fixation to soil drying is ameliorated at increased CO_2 has substantial significance for both natural and agricultural ecosystems. The anticipated advantage under global climate change of legumes is likely to be greater than originally presumed. Certainly, in natural ecosystems, it appears that legumes could be at a substantial competitive advantage over non-nitrogen-fixing species, and this could alter species distribution in favour of legumes in many ecosystems. In agricultural systems, amelioration of the sensitivity of N_2 fixation to soil drying under increased CO_2, combined with the inherent stimulation of growth by CO_2, is likely to result in substantial increases in the yielding capability of legumes, especially soybean.

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Enhanced transformation of plant cells following co-bombardment of VirE2 protein of *Agrobacterium tumefaciens* with DNA substrate

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Agrobacterium tumefaciens **transfers a segment of its Ti plasmid (T-DNA) to the nucleus of the host plant cell in single-stranded form. Of the two specific proteins, VirE2, that contains a nuclear localization signal plays a major role to mediate transport of target DNA into the nucleus of the plant cell. Here we report a DNA delivery method termed 'Vir-biolistic' that utilizes this property of the VirE2 protein for delivery of DNA target into the plant cell nucleus through microprojectile bombardment. Transient expression assay using *gus* reporter gene in immature wheat and maize embryos indicated higher number of transformants with Vir-biolistic compared to the popular 'biolistic' procedure of DNA delivery used for the production of transgenic plants. We also found that in wheat calluses co-bombarded with VirE2 protein, the stable integration frequencies of *gus* genes were threefold higher compared to the biolistic method. Thus, the Vir-biolistic method has the potential to increase the transformation efficiency in plants that are not amenable to *Agrobacterium*-mediated transformation.**

AGROBACTERIUM prepares and transfers DNA complex into the plant cell. The process is triggered by the activation of a series of *vir* genes residing on the Ti plasmid, by signals obtained from the host plant cell[1]. The activation of *vir* genes results in the generation of site-specific nicks within the T-DNA borders and production of linear single-stranded DNA (ssDNA) molecules (T-strands) which arrive in the plant cell as a single-stranded intermediate[2,3]. During transit, the T-strand is not naked, but is associated (T-complex) with two Vir proteins, VirD2 and VirE2. Both VirD2 and VirE2 possess functional nuclear localization signals that guide the T-DNA to the plant cell nucleus. The VirE2 is a ssDNA-binding protein that binds tightly and cooperatively without sequence specificity, coating the entire length of the T-strand[4–6]. After entry into the plant cell, the T-complex is targetted to the nucleus by a mechanism not fully understood as yet. It has been demonstrated that the VirE2 protein mediates nuclear uptake of ssDNA in plant cells[7].

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Agrobacterium-mediated transformation has several advantages over direct DNA delivery techniques like electroporation and 'biolistic' particle bombardment. These include introduction of a single or a few copies[8] of the desired gene into the plant genome, linked transfer of all the genes in the T-DNA, and easy manipulation *in vitro*. However, this method is not routinely used to transform monocotyledonous plant species. The biolistic procedure on the other hand, often leads to integration of multiple copies of the introduced gene at more than one locus. Further, the transformation efficiency with the biolistic procedure is quite low, i.e. 0.01–2% (refs 9 and 10). A research group has combined the advantages of *Agrobacterium* and the direct, biolistic gene delivery systems in a transformation technique termed 'agrolistic'[11]. The approach uses plant expression cassettes for *virD1* and *virD2* genes co-delivered with a vector containing T-DNA border sequences flanking a gene of interest, and demonstrate that *virD* gene products cleave T-DNA border sequences *in planta* and produce transformants with T-DNA-type insertion events (agrolistic events) with a frequency 20% that of biolistic events. The same group showed that the addition of *vir*E2 doubled the transformation efficiency. Targetting of the microinjected DNA into the cell nucleus by the VirE2 protein has been demonstrated in *Xenopus* oocytes, maize leaf cells, stamen hair cells of *Tradescantia virginiana*, and also mammalian cells[7,12–14]. These studies underline the role of VirE2 protein in transport of ssDNA into the nucleus. This created a foundation for testing our hypothesis that purified VirE2 protein if complexed with target ssDNA *in vitro* and delivered into plant cells using microprojectile bombardment, would increase the integration frequency.

Immature embryos of wheat and maize were used for transformation studies. The excised embryos were placed on solid BEG2 culture medium[15] with the scutellum side facing up; callus induction and selection were done as per the published reports[16]. Immature embryos of maize were also cultured in the BEG2 medium with the epiblast side (flat surface) up. The vector used in the transformation experiments was obtained from Richard Brettel (CSIRO Division of Plant Industry, Canberra, Australia). It contains a chimeric *bar* gene from *Streptomyces hygroscopicus* under control of maize *Ubi*1 promoter and *Agrobacterium tumefaciens* nopaline synthase (*nos*) terminator and the *E. coli uidA* (β-glucuronidase) gene under control of the rice *Act*1 promoter and rice *rbc*S terminator (Figure 1). The bar gene encodes for the enzyme phosphinothricin, which was used as the selective agent in the stable transformation experiments. For protein purification, a construct pET3b :: *vir*E2 containing the *Vir*E2 gene, driven by a T7 promoter[4], was overexpressed in *E. coli*, as described earlier with some modifications[17]. The entire chromatographic procedure was carried out at 4°C, and the eluted fractions containing VirE2 protein were stored at – 20°C till further use. The chromatography fractions were analysed by SDS–PAGE for the presence of VirE2 protein, and the amount of protein was estimated using standard Bradford dye-binding assay[18]. For plant transformation, 20 µg of the plasmid pDM803 was linearized with the restriction enzyme *Not*I and the digested DNA was ethanol-precipitated and redissolved in 40 µl of TE solution. The linearized pDM803 DNA was denatured by boiling for 5 min and then chilled on ice for 5 min. Denatured samples were either stored at – 20°C for later use or kept on ice till use on the same day. The linear ssDNA was mixed with the purified VirE2 protein in a 1 : 10 ratio (wt/wt) and incubated at 4°C for 10 min for protein–DNA complexing. The binding was confirmed by ethidium bromide fluorescence in a gel retardation electrophoresis[7].

The microcarrier (3 mg of 1 µm gold particles in 50 µl of 50% glycerol) was vortexed for 5 min to resuspend the agglomerated particles. Half of this was taken into a sterile eppendorf tube on ice and after vigorously vortexing, 105 µl of VirE2–ssDNA preparation (50 µg VirE2 + 5 µg DNA) was added to it. The vortexing was continued for another 3 min. Then 550 µl of absolute ethanol was added and vortexed for another 1 min, and kept for 5–10 min at 4°C with repeated vortexing to keep the gold particle suspended. The mixture was then pelleted by centrifugation (one pulse) and the supernatant discarded. The DNA–protein gold precipitate was washed with 150 µl of 70% ethanol without disturbing the pellet. Finally, it was resuspended in 50 µl of absolute ethanol for particle bombardment. Then 10 µl of this suspension was transferred to the centre of the macrocarrier disc, vortexing each time before taking out the aliquots. After air-drying, the ssDNA–VirE2 protein-coated gold particles were delivered into the cultured embryos using biolistic particle delivery system (PDS-1000 He, Bio-Rad), at a vacuum pressure of 26 inch Hg and a rupture disk strength of 1100 psi. Forty-eight hours after the bombardment, embryos were analysed for GUS expression by histochemical staining with X-Gluc[19]. Blue spots on the scutellar surface of the embryos were scored after 24 h of staining. Representative photo-

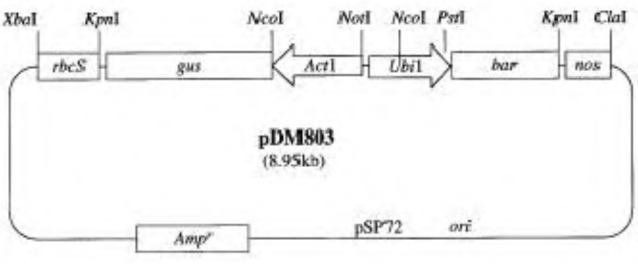

Figure 1. Plant transformation vector pDM803 (ref. 15). *Act*1, Promoter, first exon and intron of rice actin1 gene; *gus*, Coding region of the *E. coli* β-glucuronidase *uidA* gene; *rbc*S, 3′ transcript termination region of rice Rubisco gene; *Ubi*1, Promoter, first exon and first intron of maize ubiquitin 1; bar, Coding region of *S. hygroscopicus* phosphinothricin acetyltransferase gene; *nos*, 3′ transcript termination region of *A. tumefaciens* nopaline synthase gene; Amp^r, Ampicillin resistance gene of pSP72.

graphs of the transformed tissues and controls were taken using a stereo dissection microscope (Olympus). The data were analysed for mean and standard errors of means on Power Macintosh computer using Statview™ software.

Genomic DNA from the transformed and control calluses was extracted by the CTAB method[20]; 6 µg DNA was digested with restriction enzyme *Not*I, electrophoresed in 1% agarose gel, and capillary-blotted onto a nylon membrane (Hybond N^+). Labelling and detection were performed using ECL direct nucleic acid labelling and detection system, according to the manufacturer's instructions for the ECL kit. Gus amplicon fragment 850 bp in length amplified using PCR, was used as probe to hybridize the *gus* gene.

Initial Vir-biolistic transformation experiments were conducted with immature embryos of wheat. Different forms of the target vector pDM803 that contained *gus* gene were made with or without $CaCl_2$. Routinely used biolistic methods include $CaCl_2$ and spermidine during the coating of DNA to gold particle prior to bombardment. The treatments were (A) standard biolistic method with circular dsDNA, (B) VirE2-coated circular ssDNA without $CaCl_2$, (C) VirE2-coated circular ssDNA complex with $CaCl_2$, (D) standard biolistic method with linear dsDNA, (E) VirE2-coated linear ssDNA without $CaCl_2$, and (F) VirE2-coated linear ssDNA with $CaCl_2$. The first batch of sixty embryos per treatment was scored 24 h after the bombardment. Maximum number of blue spots was observed in treatment E followed by F, both using VirE2-coated ssDNA, but precipitated with or without $CaCl_2$, respectively. Number of GUS expressing spots was much lower with the biolistic treatment A. A second batch of sixty embryos per treatment was scored for GUS expression 48 h after bombardment. Interestingly, after 48 h the GUS expression improved significantly in treatments B, E and F, all employing Vir-biolistic transformation (data not shown). Initial results suggested that (i) presence of VirE2 in combination with target substrate can increase transient expression, (ii) presence of $CaCl_2$ is not essential for Vir-biolistic, while in biolistic $CaCl_2$ is normally used at the time of preparation of DNA substrate for bombardment, and (iii) linear form of ssDNA gives higher frequency of transient expression than circular ssDNA.

Based on the preliminary results, two best Vir-biolistic treatments (E and F) were compared with the biolistic method (A), in subsequent detailed studies. In the first experiment, wheat embryos were bombarded with the above three preparations of pDM803 vector DNA. The highest level of transient GUS expression (after 48 h) was observed in treatment E (80 ± 8 spots per embryo), where linear ssDNA was coated with the VirE2 protein without spermidine and $CaCl_2$. Inclusion of $CaCl_2$ (treatment (F)) produced marginal increase (55 ± 7 spots) than the biolistic (50 ± 5 spots; Table 1, Figure 2). The number of blue spots showing transient expression of *gus* gene in treatment E was almost double that of the standard biolistic treatment A, indicating that VirE2 protein can play an important role in improving the transformation efficiency in plants. Similar experiments were performed with immature embryos of maize. Maize embryos have a flat surface on the epiblast side and therefore are better explants for transient expression studies of the *gus* reporter gene. The number of transient blue spots in the treatment E was 165 ± 5 per embryo, which was once again almost double to that of control (80 ± 10) spots (Table 1, Figure 3).

The experiments conducted demonstrate that VirE2 protein does play an important role in increasing the number of transformed cells compared to the biolistic method.

Table 1. Transient expression assay for β-glucuronidase activity after 48 h following bombardment of immature embryos with DNA substrate

Crop	Treatment	Number of embryos scored for GUS spots	Number of GUS spots per embryo (Mean ± SEm)
Wheat	A	64	50 ± 5
	E	64	80 ± 8
	F	65	55 ± 7
Maize	A	38	80 ± 10
	E	40	165 ± 5
	F	37	45 ± 15

A, Biolistic treatment method (circular dsDNA + spermidine + $CaCl_2$);
E, Vir-biolistic treatment (linear ssDNA + VirE2 – $CaCl_2$);
F, Vir-biolistic treatment (linear ssDNA + VirE2 + $CaCl_2$).

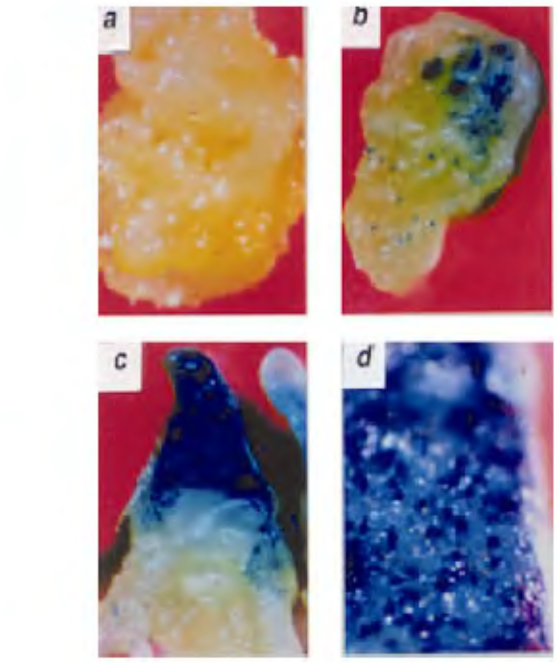

Figure 2. Transient GUS expression in wheat immature embryos 48 h after bombardment with pDM803 using different methods of gene delivery. ***a***, Untransformed control; ***b***, Transformed using biolistic method of DNA delivery; ***c*** and ***d***, Vir-biolistic treatment E (ssDNA + VirE2 – $CaCl_2$).

The higher number of cells showing transient GUS expression with Vir-biolistic method is attributed to the fact that in case of biolistic procedures, only those DNA molecules reaching the nucleus are capable of expression, and cells where DNA remains in the cytoplasm fail to express. This is most likely due to the absence of any mechanism to transport the target DNA from the cytoplasm into the nucleus. In the Vir-biolistic approach it is proposed that ssDNA is complexed with the *Agrobacterium* VirE2 protein carrying NLS motif. The ssDNA–VirE2 complex entering the cytoplasm quickly moves towards the surface of the nucleus with the help of VirE2–NLS and crosses the nuclear membrane using the host nuclear import machinery[13]. Besides, the VirE2-coated ssDNA will remain stable in the cell environment for a longer period due to physical protection against the cellular nucleases[17]. Presence of $CaCl_2$ during the coating of ssDNA–VirE2 complex to the gold particle did not enhance the transient expression in Vir-biolistic, suggesting that the complex effectively can bind to the gold particle by weak interactions. The data generated so far provide evidence of higher transient expression of target gene in the presence of VirE2 protein. In order to address the question of whether the increase in transient expression level due to VirE2 protein leads to increase in integration frequency of gene in question, we conducted stable transformation experiments using wheat immature embryos. A total of 745 and 750 embryos each for biolistic and Vir-biolistic methods, from two experimental events were used to analyse the integration frequency (Table 2). The bombarded embryos were subjected to selection procedures for four weeks for both biolistic and Vir-biolistic events. A total of twenty-two calluses for biolistic and thirty-four calluses for Vir-biolistic were recovered from the two experimental events.

To determine the pattern of integration in the resistant calluses Southern blotting was performed using 850 bp *gus* amplicon as probe. We observed that in the biolistic events of the calluses analysed, only three tested positive compared to Vir-biolistic events where fourteen calluses hybridized to *gus* probe out of thirty-four resistant calluses analysed (Table 2). We observed multiple integration patterns both in the biolistic and Vir-biolistic experiments. Integration of insert at different sites is a common phenomenon in targets, that is delivered through a gene gun. Integration frequency was calculated by the ratio of resistant calluses that showed Southern positive to the total number of the calluses surviving selection. By this formula, the frequency of Vir-biolistic events was threefold higher (41%) than that of biolistic events (13%), thus confirming that the observed enhancement of gene expression is because of the VirE2 protein. The Vir-biolistic technique exploits the inherent power of an *Agrobacterium* protein, VirE2, provides direct application for creating transgenics in plants, with immediate advantage for high-priority crop plants where *Agrobacterium* is not routinely used for transformation. Another potential application of this technology would be to transfer DNA target to the mammalian cells with higher efficiency, for therapeutic studies.

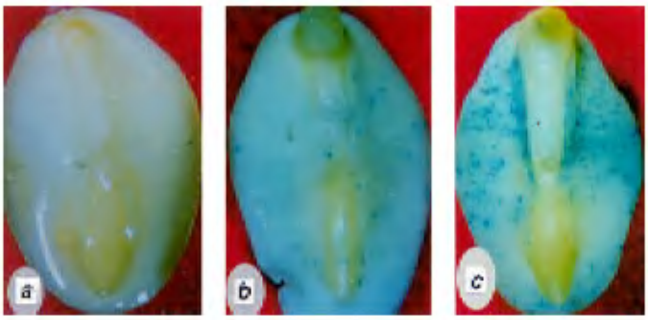

Figure 3. Transient GUS expression in immature embryos of maize 48 h after bombardment with plasmid pDM803. ***a***, Untransformed control; ***b***, Transformed using biolistic method of DNA delivery; ***c***, Vir-biolistic treatment E (linear ssDNA + VirE2 – $CaCl_2$).

Table 2. Summary of Southern blot analysis of transformed callus lines analysed after four weeks in selection media

Treatment*	Number of embryos bombarded	Number of calluses surviving selection	Number of Southern positive calluses	Integration frequency (%)
Biolistic (A)	745	22	3	13
Vir-biolistic (E)	750	34	14	41

*Values from two experiments. Integration frequency is calculated as number of Southern positive calluses to the number of calluses surviving selection × 100.

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Peroxidase isozyme polymorphism in popular sugarcane cultivars

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Peroxidase isozyme was used to identify the variation among popular sugarcane cultivars of South India including the somaclones developed against biotic and abiotic stress tolerance. Electrophoretic polymorphism was surveyed for 12 diverse sugarcane cultivars and 11 somaclones. A total of 145 bands were scored with an average of 6.3 bands per cultivar. Presence of more number of isozymes reflected the genetic complexity of the genus *Saccharum*, a polyploidy of the tribe Andropogoneae. Variation in some of the somaclones was observed, but it was less polymorphic compared to other sugarcane cultivars studied. Peroxidase isozyme diversity among these variants in terms of similarity indices may be useful in identifying diverse cross-combinations for deriving hybrids of sugarcane.

SUGARCANE is an important commercial crop of India. The rapid progress in the sugar-industry sector is largely due to the release of high-yielding, early-maturing and promising cultivars by sugarcane breeders. However, in recent years the productivity has declined due to increase in stress conditions and other environmental factors associated with sugarcane agriculture[1]. New genetic approaches like molecular marker technology have been adopted to map the sugarcane genome, in order to select better cross-combinations to develop popular hybrids. Isoenzyme markers are the oldest among the molecular markers. Isoenzyme markers have been successfully used in several crop-improvement programmes[2–4]. Isoenzymes have proven to be reliable genetic markers in breeding and genetic studies of plant species[5], due to consistency in their expression, irrespective of environmental factors.

In sugarcane, isozymes were first discussed in 1969 (ref. 6). Earlier studies have revealed variation among the relatives of sugarcane for peroxidases and other isozymes[3,7,8]. In this communication an attempt has been made to study diversity in peroxidase for understanding the species interrelationship and variation among sugarcane somaclones for their biotic and abiotic stress tolerance, to provide further indications about their genetic relations.

The experimental material consisted of 12 diverse popular sugarcane cultivars of South India and 11 somaclones (GSBT, Gulbarga Selection Through Biotechnology), selected against biotic and abiotic stress[9–11]. Leaf extracts of young shoots were homogenated in 0.1 N phosphate buffer (pH 7.2) and centrifuged at 12000 rpm for 15 min. The supernatant was subjected to electrophoresis as described by Laemli[12] on a non-denaturing polyacrylamide gel.

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