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Efficient microprojectile bombardment-mediated transformation of rice using gene cassettes

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Abstract This study was aimed at determining whether gene cassettes (promoter-coding sequence-terminator) can be efficiently used in microprojectile acceleration-mediated co-transformation of rice in the place of whole plasmids, and to what extent their use influences the integration and expression of the co-transferred gene of interest. Two non-linked marker genes (*yfp* and *hph*) were co-introduced by microprojectile bombardment into cells of embryogenic calli in three separate experiments. Three different DNA structures were compared for their ability to transiently and stably transform rice cells: supercoiled or linearized whole-plasmid DNA, gene cassette DNA and single-stranded gene cassette DNA coated with *Escherichia coli* single-stranded binding (SSB) proteins. Our results demonstrate that microprojectile bombardment-mediated transformation of rice using gene cassettes is possible without significantly reducing transformation efficiency in comparison to the use of whole-plasmid DNA. Furthermore, no obvious difference in transgene integration pattern and inheritance was observed among plants transformed with gene cassettes compared to those transformed with the whole plasmid, except that concatemerization of molecules prior to integration was rarely observed in gene cassette transformants.

Keywords Gene cassette · Microprojectile-mediated transformation · Rice · Single-stranded binding proteins

Introduction

The principle of microjectile bombardment-mediated genetic transformation is based on the forced penetration of DNA into the cell nucleus where it can be integrated into genomic DNA during replication. This technology is able to deliver foreign DNA into regenerable cells, tissues or organs, and appears to provide a good method for achieving truly genotype-independent transformation in bypassing most tissue culture-related regeneration problems (Christou 1997; Luthra et al. 1997). In rice, cell suspensions, embryogenic calli, and immature or mature embryos have proved to be suitable targets for particle gun-mediated transformation. The transgenic plants generally show a low frequency of somaclonal variation and 50 to 80% of the regenerated plants proved to be fertile (Li et al. 1993; Arencibia et al. 1998; Chen et al. 1998a). Furthermore, multiple plasmids can be used to integrate multigenes of interest in the genome of a transgenic plant at a single genetic locus (Hadi et al. 1996; Chen et al. 1998b) allowing, for instance, the stacking of genes determining a biosynthetic pathway and their further co-segregation. Large fragments of DNA, including those in yeast artificial chromosomes, can also be introduced though particle bombardment (Van Eck et al. 1995). Aside from these many advantages, microprojectile bombardment-mediated transformation presents two main drawbacks: first, the transgene(s) of interest and the selectable marker gene are generally co-integrated in multiple copies at a single genetic locus in the genome of microprojectile bombardment transformed plants (Pawlowski and Somers 1996; Kohli et al. 1998). Consequently, the gene(s) of interest cannot be separated from the selectable marker gene by Mendelian segregation in the progenies of transgenic plants (Qu et al. 1996; Christou 1997). In *Agrobacterium*-mediated transfer, several authors have taken advantage of the more-frequent multi-locus insertion of the T-DNAs, to independently integrate the transgene of interest and the selectable marker gene. The two genes are carried by two T-DNA borne by a single binary plasmid (Komari et al. 1996), two binary plasmids in the same bacterial strain (Daley et al. 1998), or two different bacterial strains (McKnight et al. 1987, DeBlock and Debrouwer 1991; DeNeve et al. 1997). Genetic separation, resulting in se-

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lectable marker gene-free transgenic plants, occurs through natural recombination in the progeny of a variable proportion of the T0 plants.

Second, the use of the whole plasmid leads to integration of vector backbone sequences into the genome along with the transgenes (Christou 1997; Kohli et al. 1999). Though anticipated to be negligible in *Agrobacterium*-mediated transformed plants, integration of the binary plasmid backbone has now been reported to occur frequently without species specificity (e.g. Kononov et al. 1997; Wenck et al. 1997; DeBuck et al. 2000; Yin and Wang 2000 for recent references). Long T-DNA transfer, sometimes with a greater than unit length binary plasmid, has been detected in *Arabidopsis* (Wenck et al. 1997) and rice (Yin and Wang 2000). However, this drawback can be circumvented by PCR or Southern screening of *Agrobacterium*-mediated transformation events.

In particle bombardment-mediated transformation, the plasmids are thought to stabilise and protect transgenes against cellular nucleases. The wound created by microprojectile penetration in the cell, leads to activation of the DNA repair mechanism, but also to activation of the exogenous DNA degradation system (Hunold et al. 1994). Consequently, it was anticipated that linear DNA introduced by bombardment into the cell nucleus would be rapidly degraded by nucleases which constitute the first defence mechanism of the cell against exogenous DNA (Finnegan et al. 1998; Kumpatla et al. 1998). Despite this beneficial protective role, integration of the vector backbone sequence may be the cause of major problems: first, extensive stretches of prokaryotic vector sequences are not well-tolerated by higher eukaryotic genomes (Matzke et al. 2000). When integrated into plant DNA, excess vector sequences often spontaneously acquire dense methylation that can spread into the neighbouring transgenes (Jakowitsch et al. 1999). Sequences of bacterial origin mostly contribute to mark transgenes as invasive DNA because of their unusual sequence composition and AT-rich sequences or to an inability to bind eucaryotic nuclear proteins, resulting in highly efficient gene silencing. It was therefore hypothesised that such a local discrepancy may disorganise chromatin structure and contribute to de-stabilising gene expression (Iglesias et al. 1997; Kumpatla et al. 1998;).

Second, sequences of bacterial origin may be the source of transgene rearrangements. The vector backbone sequences bear recombination hotspots that stimulate plasmid-plasmid illegitimate recombination. Specific sequences, like plasmid origins of replication or AT-rich sequences are known to be recombinogenic (Muller et al. 1999).

Finally, the vector backbone sequence bears a bacterial origin of replication and a selectable bacterial gene. Persistence of these sequences in field-released commercial transformants is now considered undesirable from the stand point of most biosafety regulation authorities.

To bypass problems related to the integration of vector backbone sequences in microprojectile bombardement-derived transgenic plants, Fu and collaborators have recently used isolated minimal gene cassettes for successfully transforming rice (Fu et al. 2000). Furthermore, they observed that transgenic rice plants transformed with gene cassettes exhibited predominantly simple integration events with a low frequency of transgene rearrangement compared to those observed in plants transformed with supercoiled plasmids. However, it was not clear whether the comparisons were made between plants derived from the same embryo batch, genotype and experiment, although all these parameters may affect the pattern of integration.

Here we established the transformation efficiencies in microprojectile acceleration-mediated co-transformation of rice embryogenic calli using gene cassettes (promoter-coding sequence-terminator) and whole plasmid DNA. Furthermore, as degradation of linear DNA can be expected during the transformation process, we also investigated whether *Escherichia coli* SSB proteins (Single-stranded DNA binding proteins), also called "DNA melting proteins", can be efficiently used to coat the gene cassettes and protect them from cellular nucleases. To further determine how DNA conformation influences the integration (integrity and number of copies and the integration locus), expression and transmission of the gene of interest (here represented by the *yfp* reporter gene encoding a yellow-shifted mutant protein of the green fluorescent protein), and of the selectable *hph* marker gene, we conducted the molecular characterisation of primary transformants and progenies, and monitored the expression of the *yfp* gene during transient then integrative transformation of rice cells observing incident-light fluorescence stereomicroscopy in vitro in real time. Transgene expression during T0 plant development was further monitored using this reporter gene system.

Materials and methods

Gene constructs

The 5.1-kbp plasmid pILTAB227, consisting of the CaMV 35 S promoter with a duplicated enhancer sequence (35 S) controlling the hygromycin phosphotransferase *hph* gene and followed by the *nos* 3′ terminator (kindly supplied by Dr. C. Fauquet, ILTAB, La Jolla, USA), was used as the selectable construct in the microprojectile bombardment experiments. The pYFP plasmid used for co-transformation was obtained by introducing into pRT103 the *yfp* coding sequence between the CaMV 35 S promoter and terminator from pEYFP (Clontech) in the *Xba*I-*Xho*I sites. The 1.3-kbp pE35S-*yfp*-t35 S and the 1.5-kbp p35S-*hph*-nos3′ *HindIII* fragments (encoding the yellow fluorescent protein and the hygromycin phosphotransferase enzyme respectively) were isolated from cesium chloride preparations of the corresponding pYFP and the pILTAB227 plasmids (Fig. 1), and were separated from the plasmid backbones on low-melting agarose gels. Two different purification procedures were used: in unpurified (UPC) and purified (PC) cassette preparation the band corresponding to the cassette was excised from ethydium bromide-stained and unstained tracks respectively before purification through affinity columns. In the PC procedure, the cassette preparation was further treated twice with phenol-chloroform and 70% ethanol before re-suspension in water and final DNA quantification by the gel and by spectrophotometry.

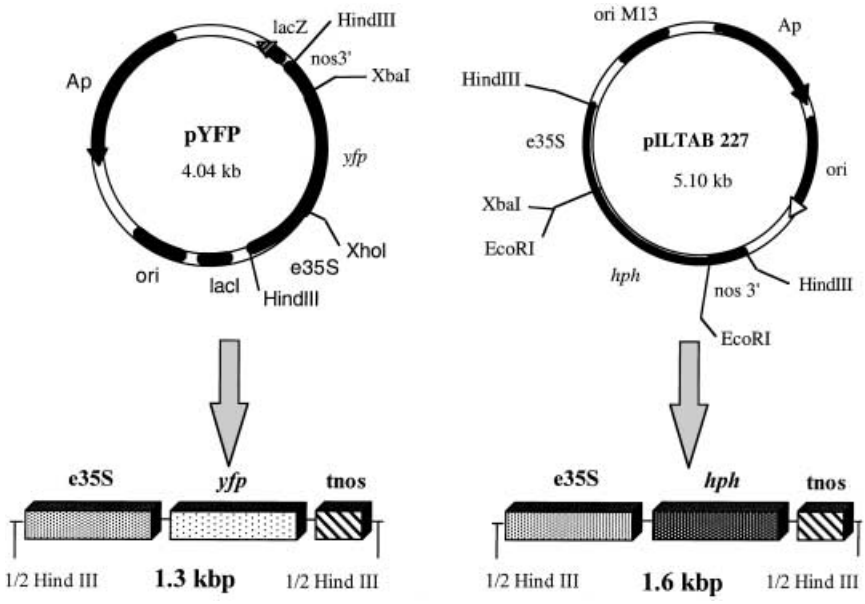

Fig. 1 Schematic representation of the plasmids and gene cassettes used for microprojectile-transformation of rice

Coating gene cassettes by SSB protein

The *yfp* and *hph* constructs of the UPC preparation were mixed according to either a 2:1 or a 1:1 ratio before thermal denaturation for 2.5 min at 100°C (resulting in SSB 1.1 and 1.2 preparations respectively). The 1:1 ratio representing an overall quantity of 2 µg of DNA was used to assess the influence of modifying this ratio on the chance of co-transformation and expression of the non-selected *yfp* gene. In the third transformation experiment, the two constructs of the PC preparation were mixed in a 2:1 ratio and referred as SSB3. Mixed single-strand gene cassettes were kept on ice for 5 min; 3.6 µg of SSB protein (Promega) sufficient for coating 1 µg of single-stranded DNA was added and the product used for coating the microprojectiles employed for bombardment.

Plant material and transformation

The two first transformation experiments and the third transformation experiment were carried out with varieties Taipei309 and Nipponbare respectively, both falling in the *japonica* subspecies (isozyme group VI according to Glaszmann 1987) of rice (*Oryza sativa* L.). Mature seed embryos were used to induce callus cultures that served as material for particle bombardment. The plasmids and the gene cassettes were introduced into embryogenic calli by microprojectile bombardment according to the procedure described in Chen et al. (1998a), and using the PDS1000/He particle gun device (BioRad laboratories, USA). In the second and third experiments, the quantity of DNA used for coating the microprojectiles was calculated so as to include the same number of *yfp* and *hph* gene molecules for bombardment with gene cassettes and whole plasmids (hereafter referred to as WP). The *yfp* and *hph* constructs of either PC, UPC or WP preparations were mixed according to a 2:1 ratio before precipitation on microprojectiles.

Southern-blot analysis

Total genomic DNA was extracted from leaf tissue of transgenic and control plants using the CTAB method (Hoisington 1992). Five micrograms of DNA were digested with appropriate restriction endonucleases, and DNA fragments were separated in 0.8% agarose gels and then transferred to nylon membranes (Hybond N+ Amersham) according to Southern. For hybridisation, the 0.7-, 1- or 3.34-kbp fragments respectively of the *yfp* and *hph* coding sequence or the pRT103 plasmid served as a template for synthesising [α-^{32}P] labelled probes through random priming. Following hybridisation, the membrane was washed twice for 10 min at 65°C (first in 2× SSC and 1% SDS and then in 0.1× SSC and 0.1% SDS) and analysed by autoradiography.

Detection of YFP activity

Tissues (callus or leaves) were observed under a Leica MZ FLIII fluorescence stereomicroscope fitted with a YFP-plant filter set (excitation spectrum 440–470 nm, barrier filter at 495 nm, and emission spectrum 525–550).

Results

Influence of SSB protein-mediated protective treatment and purification procedure of gene cassette DNA on the expression of the *yfp* gene in bombarded callus cultures

The influence of protecting gene cassette DNA with SSB proteins was first investigated by bombarding Taipei 309 embryogenic calli with microprojectiles coated respectively with whole plasmids (WP1), unpurified gene cassettes (UPC1) and single-stranded unpurified gene cassettes coated with SSB proteins (SSB1.1 and SSB1.2) (Table 1). Sectors of expression of the *yfp* gene were scored at the surface of embryogenic calli bombarded with the different DNA preparations over a 30–35 day time course during hygromycin-resistant cell-line development on selective medium. The number of transient and stable expression units observed at the surface of embryogenic calli bombarded with the WP1 preparation 24 h after bombardment was 2-, 4- and 10-fold higher than those observed for the SSB1.1, SSB1.2 and UPC1 preparations respectively (Fig. 2A). Four hundred and thirty eight expression units were counted at the surface of 60, randomly selected, embryogenic calli bombarded with the positive control WP1, which was attributed the

Table 1 Summary of the three microprojectile-bombardment transformation experiments of embryogenic calli of rice using unpurified cassettes (UPC), purified cassettes (PC) cassettes coated with SSB proteins (SSB), and whole-plasmid (WP) DNA preparations of the *yfp* and *hph* gene constructs

Experiment	Treatment	Total quantity of DNA (μg)	Ratio *yfp*:*hph*	Number of bombarded calli	Number (%) of Hyg resistant calli transferred to regeneration medium	% Of calli exhibiting YFP activity	Number of Hyg^+ calli regenerating plants (YFP positive plants)	Transformation efficiency[a] (%)
1	SSB1.1	3	2/1	187	12 (6.4)	3.7	2	1.1
	SSB1.2	2	1/1	192	26 (13.5)	5.7	5	2.6
	UPC1	3	2/1	199	45 (22.6)	17.5	10	5
	WP1	3	2/1	204	77 (37.7)	37.2	8	3.9
2	PC2	1.14	2/1	165	74 (44.8)	36.5	40 (11)	24.2
	UPC2	1.14	2/1	176	71 (40.3)	40.8	53 (11)	30.1
	WP2	3	2/1	166	96 (57.8)	58.3	51 (17)	30.7
3	SSB3	1.14	2/1	186	18 (9.6)	0.0	7 (0)	3.8
	PC3	1.14	2/1	188	45 (23.9)	10.1	12 (6)	6.4
	WP3	3	2/1	180	102 (56.6)	46.6	38 (19)	21.1

[a] Number of regenerated plants per bombarded callus

value 100. Five days following the bombardment, the number of expression units scored in the four treatments exhibited a dramatic decrease. Thirteen days after bombardment, a comparable number of fluorescent sectors was quantified at the surface of calli of SSB1.1, SSB1.2 and UPC1 treatments, which were both lower than that observed in calli of the WP1 treatment. Thirty five days after bombardment, the number of fluorescent sectors was approaching that of YFP-positive, hygromycin-resistant calli, and eventually transferred to the regeneration medium.

In the first transformation experiment, we used gene cassettes, which were separated from plasmid backbones on a low-melting gel and stained with ethydium bromide. Despite further affinity column purification, the gene cassettes used for coating microprojectiles were therefore prone to ethydium bromide contamination and of poor purity compared to that of the whole plasmid preparation obtained from cesium-chloride preparative centrifugation. In order to investigate the influence of these putative pollutants, we further refined the purification of the gene cassette DNA. In unpurified (UPC2) and purified (PC2) cassette preparations, the bands corresponding to the gene cassette size were excised respectively from an ethydium bromide-stained and ethydium bromide-unstained tracks run in parallel on the same gel, before purification through affinity columns. In the PC2 procedure, the cassette preparation was further treated twice with phenol-chloroform to remove proteins and agarose associated with the DNA after affinity column purification. (Table 1). The number of transient expression events were remarkably higher at the surface of embryogenic calli bombarded with WP2 and PC2 microprojectile preparations than in UPC2 preparations (Fig. 2B); 1640 expression units were counted at the surface of 60 randomly selected embryogenic calli bombarded with the positive control WP2, which was attributed the value 100. The number of fluorescent units scored in embryogenic calli bombarded with the PC2 preparation was two-thirds that in control WP2 treatment. Expression units observed in WP2 and PC2 calli exhibited a parallel dramatic decrease after the first 5 days of incubation, and the values of PC2 and UPC2 treatments stabilized at the same level 10 days after bombardment as already noted in the first experiment.

In a third experiment, embryogenic calli of cv Nipponbare were in turn bombarded with microprojectiles coated with whole plasmids (WP3), purified gene cassettes (PC3) and single-stranded purified gene cassettes coated with SSB proteins (SSB3) (Table 1). The results obtained in this experiment were fully consistent with those of the two previous experiments in ranking SSB3 and PC3 treatments similarly with regards to the WP3 treatment (Fig. 2C). Transient expression events were remarkably higher on the surface of embryogenic calli bombarded with PC3 microprojectile preparations than with SSB3 preparations.

Overall, these results indicate that the efficiency of transient transformation using purified gene cassettes reaches 70–80% of that obtained with whole-plasmid DNA, whereas transient expression events are reduced when gene cassettes are poorly purified or coated with SSB proteins.

Influence of SSB protein-mediated protective treatment and the purification procedure of gene cassette DNA on transformation efficiency

Results of the first bombardment experiment (Table 1) showed that variable transformation efficiencies (the number of hygromycin-resistant-calli per bombarded embryogenic callus), ranging from 6.4 to 37.7 were observed among treatments. Transformation efficiency observed using UPC1 preparations was 2–4-fold higher than that observed in the SSB 1.1 and 1.2 treatments, whereas it reached 60% of that of the positive WP1 control. However, despite an otherwise satisfactory produc-

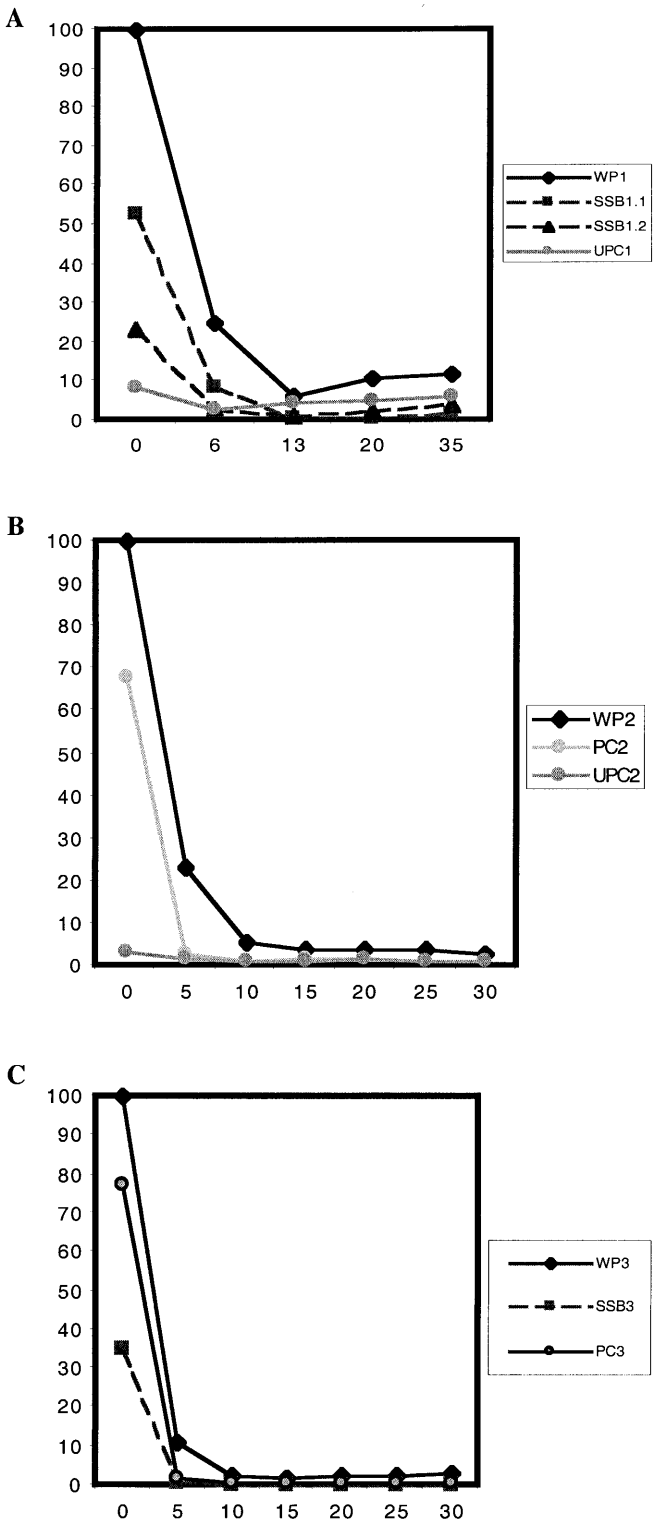

Fig. 2A–C Time course of transient and stable expression of the *yfp* gene over hygromycin-resistant callus development after bombardment of embryogenic calli with microprojectile preparations using either unpurified cassettes (*UPC*), purified cassettes (*PC*), cassettes coated with SSB proteins (*SSB*) and whole plasmids (*WP*). Fluorescent units were quantified at the surface of 60 calli per treatment

tion of resistant calli, consistent with that usually observed in cv Taipei 309, the number of transgenic plants regenerated was too low to make a further valuable comparison of the treatments.

Results of the second experiment showed that the efficiency of transformation was not dramatically different among treatments and fell within the range of efficiencies usually observed in cv Taipei 309 (Table 1). Efficiency of the UPC2 and PC2 treatments represented 70–77% that of the WP2 positive-control treatment. No difference in regeneration ability was noted among calli, despite the fact that resistant cell lines arising from calli bombarded with UPC2 preparations regenerated less-vigorous plants with a different texture and colour, and reduced growth, when compared to those generated by the other treatments.

Results of the third experiment were consistent with those of the two former bombardments: efficiency of the PC3 treatment represented 42% of that of the WP3 control treatment and was higher than observed in the SSB3 treatment.

Overall, the results of the three bombardment experiments indicate that efficient stable transformation of rice, approaching values observed with whole plasmid DNA, can be achieved by using gene cassette preparations. On the other hand, though the purification procedure of the gene cassette was found to dramatically influence transient transformation, conversely it had little influence on the stable bombardment-mediated transformation of rice cells.

Characterisation of transgene integration in plants regenerated from cultures bombarded with gene cassette and plasmid preparations

Genomic DNA of all the plants regenerated in the first experiment was isolated, digested with either *Xba*I-*Xho*I, which releases a 0.7-kpb fragment containing the *yfp* coding region, *Eco*RI, which released a 1-kpb fragment containing the *hph* coding region, or *BamH*I, which cut once in the plasmids (Fig. 1), then blotted and hybridised with the *yfp* or *hph* probe. DNA-blot analyses revealed that all the plants have stably co-integrated the *yfp* and the *hph* genes, and were independent transformation events exhibiting various levels of complexity in the hybridisation pattern (Fig. 3).

The complexity of *yfp* transgene integration among the plants regenerated following the four treatment patterns appeared to be quite different (Fig. 3A). Transformation events generated from embryogenic calli bombarded with the UPC1 preparation exhibited predominately simple integration patterns compared to those observed in plants regenerated from calli bombarded with WP1 and SSB1 preparations. All the UPC1 plants but two showed hybridisation signals corresponding to the 0.7-kbp diagnostic fragment of the *yfp* coding sequence. The faint intensity of the signal suggested that a low number of copies had integrated the genomes. Conversely, all the WP1,

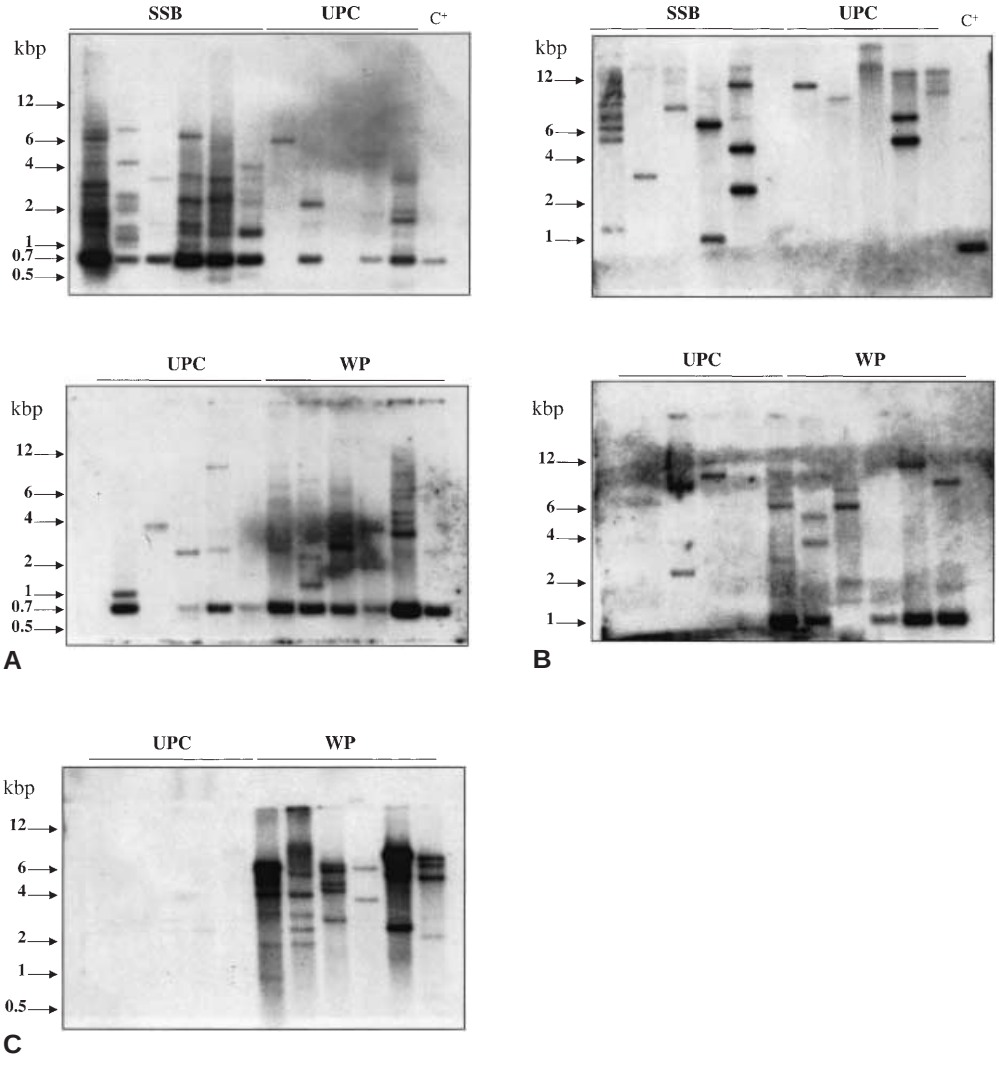

Fig. 3A–C Southern-blot analysis of T0 plants regenerated from calli bombarded with unpurified cassettes (UPC1), cassettes coated with SSB proteins (SSB1.1 and SSB1.2), or whole plasmid (WP1) DNA preparations. **A** The [α^{32}P]dCTP-labeled 0.7-kbp DNA fragment from the coding sequence of the *yfp* gene was used as a probe. Genomic DNA was digested with the restriction enzymes *Xba*I-*Xho*I. The expected size of the fragment corresponding to the *yfp* gene coding sequence was 0.7 kpb. **B** The [α^{32}P]dCTP-labeled 1-kbp DNA fragment from the coding sequence of the *hph* gene was used as a probe. Genomic DNA was digested with the restriction enzyme *EcoR*I. The expected size of the fragment corresponding to the *hph* gene-coding sequence was 1 kpb. **C** Genomic DNA was digested by the *BamH*I restriction enzyme and hybridised with labelled probes synthesised from the 3.34-kbp fragment of the pRT103 plasmid

SSB1.1 and SSB1.2 plants showed the 0.7-kbp diagnostic fragment generally associated with a very strong signal. Since equal amounts of DNA have been loaded for DNA gel-blot hybridisation, this result indicates that the latter events have integrated a higher transgene copy number than UPC1 plants. Hybridisation of *EcoR*1 blots with a probe consisting of the *hph* coding sequence revealed the 1-kbp diagnostic fragment only in WP1 plants, suggesting that extensive truncation of the selectable gene cassette occurred with other treatments (Fig. 3B). In order to ascertain the absence of plasmid DNA sequences in the genome of UPC1, SSB1.1 and SSB1.2 lines, genomic DNA digested by *BamH*I was hybridised with labelled probes synthesised from the 3.34-kbp fragment of the pRT103 plasmid (Fig. 3C). All but one of the WP1 plants showed multiple hybridisation bands each with strong signals, suggesting integration of numerous plasmid backbone sequences, whereas no band was detected in plants derived from other treatments.

Twenty T0 plants regenerated from each PC2, UPC2 and WP2 of the second experiment were similarly analysed by Southern blotting. Their genomic DNAs were digested with *Xba*I or *Hind*III for estimating the copy numbers and integrity of the transgenes respectively. Results of hybridisation to either the *yfp* or the *hph* probe showed that WP2 plants exhibited transgene integration patterns with the same degree of complexity as those observed in plants derived from the PC2 and UPC2 treatments When the *Hind*III restriction enzyme and the *yfp* probe were used, plants transformed with WP2 DNA were found to exhibit the expected 1.3-kbp diagnostic fragment corresponding to the full gene cassette (Fig. 4A). Most of the plants also harboured bands of higher- and lower-molecular-weight which most likely correspond to the rearranged or truncated *yfp* expression cassette. Southern-blot analysis of the 40 PC2 and 53 UPC2 lines showed only two and six lines, respectively, harbouring the expected 1.3-kpb diagnostic fragment. This suggests that reconstitution of *Hind*III sites or integration of multimers of the *yfp* gene cassette occurred at a low frequency (Fig. 4B). This also indicates that integration of multimers is not favoured by the use of gene cassettes.

We found that the co-integration frequency of the *yfp* and *hph* genes averaged 100% and 85.7% when the

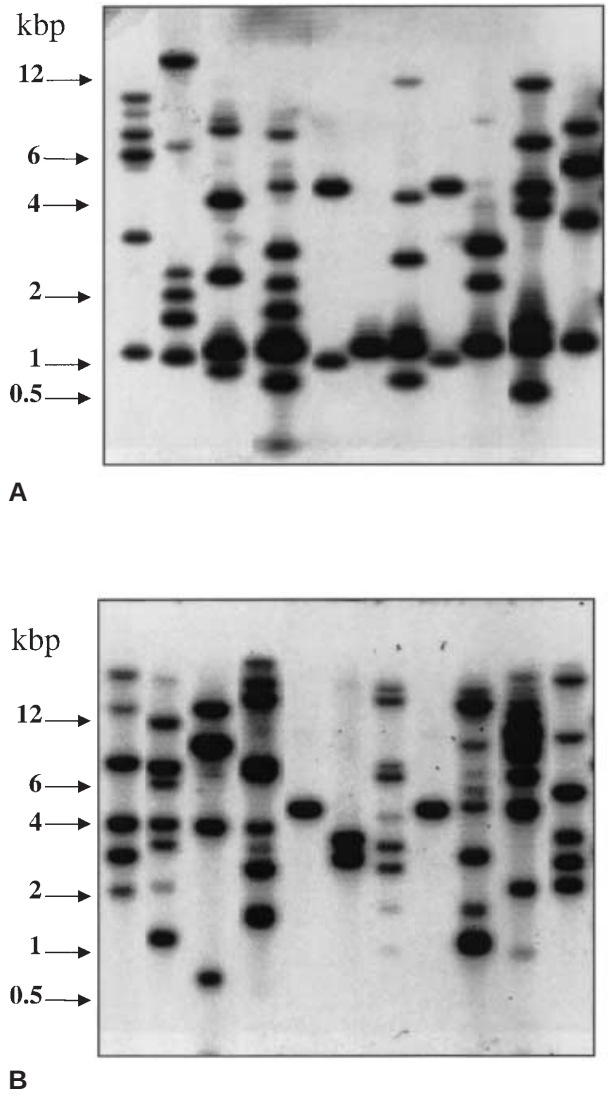

Fig. 4A, B Southern-blot analysis of DNA isolated from T0 regenerated plants of transformation experiment 2 probed with the [^{32}P]dCTP-labeled 0.7-kbp DNA fragment from the coding sequence of the *yfp* gene. **A** Genomic DNA of WP2 lines digested with the restriction enzyme *Hind*III **B** Genomic DNA of PC2 lines digested with the restriction enzyme *Hind*III

transgenes were carried by a plasmid and a gene cassette respectively. When the pRT103 plasmid probe was hybridised on stripped filters and analyzed by autoradiography, all the WP2 plants but one showed multiple high-intensity hybridisation bands, which indicates integration of the numerous plasmid backbone sequences in their genomes, whereas no PC2 and UPC2 DNA blots exhibited any hybridising fragments, confirming the absence of contaminating plasmid in the gene cassette preparations.

Genomic DNA of 63 T0 plants regenerated from PC3, SSB3 and WP3 treatments was similarly analysed. In contrast to what was observed in the second experiment, the plants regenerated from embryogenic calli bombarded with the PC3 preparation exhibited the most-complex transgene integration pattern (Fig. 5A). Eighty per cent of the 24 WP3 plants exhibited the expected 1.3-kbp diagnostic fragment along with a variable number of bands of diverse molecular weight (Fig. 5B). Southern-blot analysis of the PC3 plants showed that only two lines exhibited the expected 1.3-kbp diagnostic fragment, confirming the previous finding that reconstitution of *Hind*III sites or integration of multimers of the *yfp* gene cassette occurs only rarely (Fig. 5B). Co-integration of the *yfp* and *hph* genes was observed in 100% and 83.7% of the plants bombarded with plasmid or gene-cassette DNAs respectively. Similarly to what was observed in experiments 1 and 2, no PC3 and SSB3 plants exhibited hybridisation with the pRT103 plasmid whereas tracks corresponding to WP3 showed variable hybridisation patterns with the presence of multiple hybridisation bands.

Monitoring of *yfp* gene expression over plant development in events regenerated from cultures bombarded with gene cassette and plasmid preparations

To determine the relative frequencies of regenerated plants expressing the co-transferred *yfp* transgene in each treatment, and to determine the stability of *yfp* transgene expression over plant development, we further examined the vegetative organs of all the regenerated plants under a fluorescence stereomicroscope. These observations were performed at the in vitro stage, during adaptation in the greenhouse, and 20 and 35 days after transfer to the greenhouse (Table 2). Twenty five to thirty percent of the plants regenerated from the second transformation experiment, and expressing YFP at the in vitro stage, were found to loose this expression during adaptation to the greenhouse irrespective of the DNA preparation used for bombardment. When monitored over the course of plant development, a decrease in the frequency of *yfp* gene expression was observed only among plants transformed with the WP2 preparation. As for the plants generated in the third experiment, the loss of expression of the *yfp* transgene observed during adaptation to the greenhouse appeared less frequent than among the plants generated in the second bombardment, affecting 12.2% and 8.3% of the WP3 and PC3 plant populations respectively; 23.3 and 16.8% of the WP3 and PC3 plants exhibited putative *yfp* transgene silencing during further plant development in the greenhouse (Table 2). No SSB plants exhibited *yfp* transgene expression either at the in vitro stage or at a later developmental stage.

Transmission of the transgenes in T1 progeny of plants transformed with gene cassette and whole plasmid preparations

*Hind*III digests of genomic DNA of 20 seed progeny from four PC2, four UPC2 and four WP2 T0 plants, i.e. 240 T1 plants, were analysed for the segregation of *yfp* and *hph* transgenes through DNA-blot hybridisation. Ir-

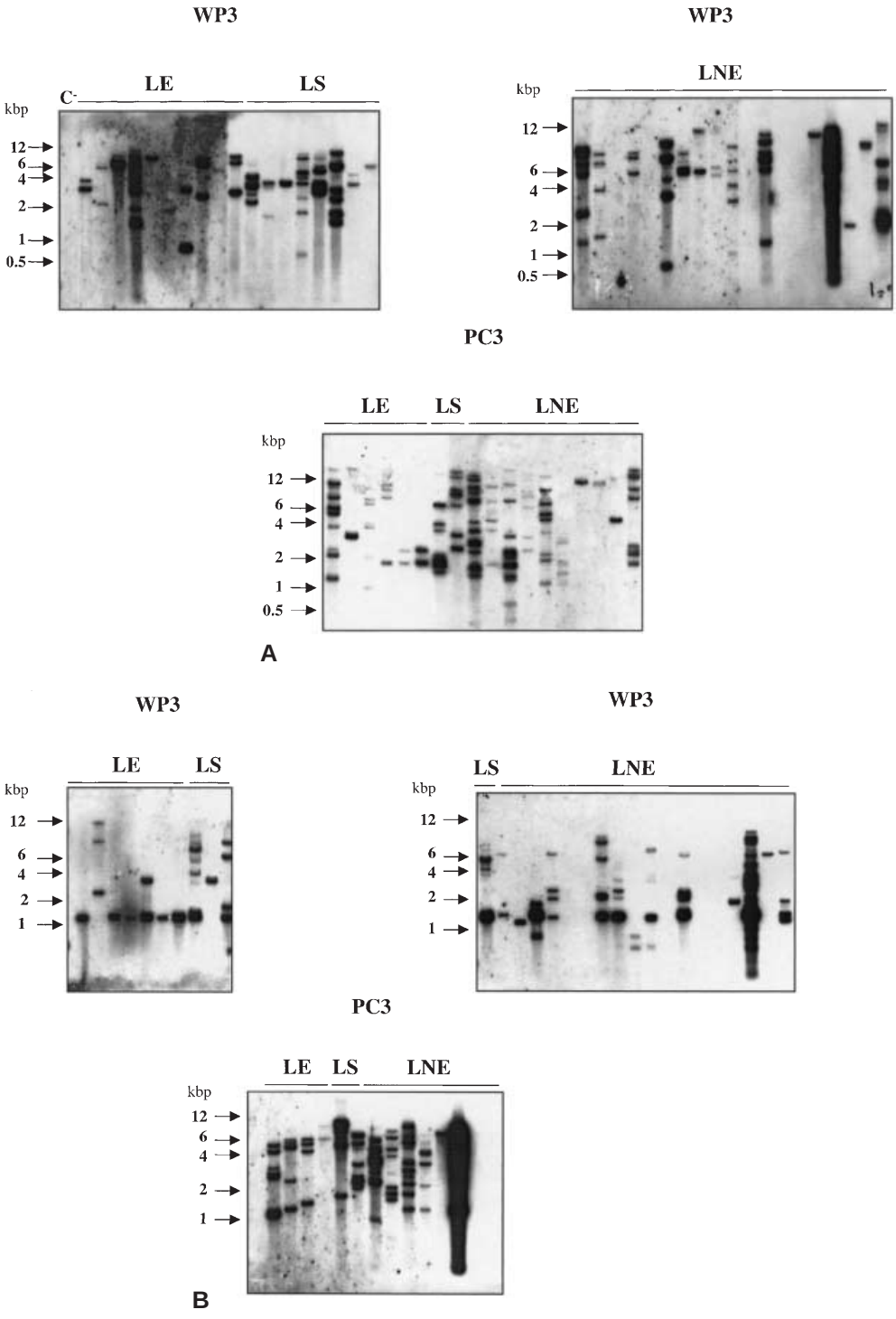

Fig. 5A, B Southern-blot analysis of DNA isolated from T0 regenerated plants of transformation experiment 3 probed with the [^{32}P]dCTP-labeled 0.7-kbp DNA fragment from the coding sequence of the *yfp* gene. **A** Genomic DNA of the WP3 and PC3 lines digested with the restriction enzyme *XbaI* **B** Genomic DNA of the WP3 and PC3 lines digested with the restriction enzyme *Hind*III. *LE*, *LS* and *LNE* correspond to plants, which either continuously expressed the YFP protein, less YFP activity or have never exhibited any YFP activity respectively

respective of the DNA preparation used for transforming the plants, the copies of the *yfp* and *hph* genes were found linked in the genome, and the hybridizing bands co-segregated according to the 3:1 ratio expected for Mendelian segregation at a single genetic locus (data not shown). This indicates that unselected and selectable gene cassette copies behave like their whole plasmid DNA counterparts, in integrating at linked insertion sites at a single genetic locus. This further suggest that transformation with gene cassettes does not favour unlinked integration of transgene copies.

Discussion

We have demonstrated that linear dsDNA can be used for coating microprojectiles in the biolistic transformation of rice. This result confirms the work of Uzé and co-

Table 2 The expression of the *yfp* reporter gene over T0 plant development among plants regenerated from calli bombarded with purified cassettes (PC), unpurified cassettes (UPC), and whole-plasmid (WP) DNA preparation

Experiment	Treatment	In vitro expression (%)	Expression during adaptation to greenhouse condition (%)	Expression 20 daysafter transfer to greenhouse (%)	Expression 35 days after transfer to greenhouse (%)	Frequency of events exhibiting an apparent loss of expression of the *yfp* gene
2	PC2	27.5	17.5	17.5	17.5	0
	UPC2	20.7	15.0	15.0	15.0	0
	WP2	33.3	21.5	17.6	13.7	58.8
3	PC3	58.3	50.0	41.6	41.6	28.6
	WP3	43.9	31.7	26.8	24.3	44.6

workers (1999) who reported that different linear or circular conformations of ss or ds DNA, are able to integrate into the wheat genome following microprojectile bombardment. In that report the use of dsDNA, linear or circular, yielded the highest numbers of transient expression units whereas linear DNA, either ss or ds, led to the highest conversion rates from transient to stable transformation events. These results are consistent with our observations, though linear dsDNA in our case was represented by minimal gene cassettes. ssDNA coated with SSB proteins also exhibited a low transient transformation efficiency but a comparatively high conversion rate from transient to stable. On the other hand, in wheat transformation, linear dsDNA treatments exhibited overall the highest efficiency whereas transformation with the ds gene cassette remained below the level observed with circular ds whole plasmid DNA in rice. These differences can be ascribed to extensive erosion of the isolated gene cassette by the cell nuclease as illustrated by DNA-blot hybridisation.

A microprojectile penetrating the cell nucleus is able to deliver several thousand gene cassettes. Competition between the DNA repair mechanism and the exogenous DNA degradation system can explain why some transgenes are eventually integrated into the genomic DNA before degradation, even though a majority of transgenes would be effectively eliminated. The dramatic decrease in *yfp* gene expression units over embryogenic callus development illustrates this degradation as well as the appearance of gene silencing mechanisms, which both reduce the number of observable stable expression events. The consistent parallel decrease observed in calli bombarded with PC and WP preparations in the three experiments presented here suggests that linear- and plasmid-DNA are equally susceptible to these phenomena.

The fact that the degree of gene cassette DNA purification influences transient expression of the transferred genes may be attributed to the persistence of interfering molecules in the unpurified DNA preparation. Such compounds may prevent the transient transcription in the nucleus before integration into the chromosomes. They may also reduce the efficacy of circularisation of the monomer or multimer of the insert fragments resulting from the presence of sticky *Hind*III ends. Circularisation of the fragments would result in greater protection from exonucleases. Poor DNA purification also appeared to have a negative effect on callus development which, though not preventing plant regeneration, further altered plant vigour.

Protection afforded by SSB proteins resulted in a higher rate of *yfp* gene expression during the 1st week after transformation compared to UPC treatment. Before transcription in the nucleus, the single-stranded gene cassette must be completely converted into dsDNA, which would not be, protected by the SSB proteins. Kohli et al. (1999) demonstrated that the transformation plasmid may undergo rearrangements prior to or during integration into the genome. De Groot et al. (1992) suggested that ssDNA recombines before the single-strand is completely converted into dsDNA. The *E.coli ssb* gene product, an essential component in DNA replication, recombination and repair, is a 75.6-kDa homo-tetrameric-helix de-stabilising protein that binds selectively and co-operatively to ss-DNA, and facilitates DNA unwinding by the DNA helicases (Ferrari et al. 1994) while protecting the DNA from the action of nucleases. SSB proteins hold the DNA in a conformation which facilitates the function of other replication, recombination or repair enzymes (Lohman and Ferrari 1994). We hypothesise that the latter properties could enhance transgene recombination and/or integration in genomic DNA. In this case, the higher number of YFP-expressing sectors observed during the 1st week following bombardment could be principally due to a higher frequency of stable expression events. Southern-blot analysis of plants regenerated from SSB treatments revealed very complex transgene integration patterns, which probably reflect the influence of SSB protein. Though SSB proteins seem not well suited for genetic transformation of rice, our results clearly indicate that an artificial DNA-protein complex can be delivered by bombardment and integrated into the target cells. A complete stochiometric complex mixture of ssDNA, VirD2 and VirE2 protein might be proved to be more efficient for rice transformation and other crops recalcitrant to *Agrobacterium*-mediated transformation. Moreover, biolistic transformation using different combination of ssDNA and Vir protein could help in the investigation of the function of these proteins.

Comparative analyses of integration patterns of plants regenerated from embryogenic calli bombarded with gene-cassette and whole-plasmid DNA preparations, which included plants derived from three transformation experiments and two genotypes, did not permit the establishment of a clear relationship between the use of one DNA form and a simplification of the pattern of integration. As with WP DNA, gene cassettes were prone to multiple-copy insertion and rearrangements before integration. The only clear influence of gene cassette DNA on the pattern of integration observed among transgenic plants was the low frequency of concatemerisation before integration, most likely due to rapid degradation of *Hind*III sticky ends or linear DNA re-circularisation. Southern-blot analyses of several progenies of T0 plants transformed with gene-cassette DNA suggest that integration occured at multiple insertion sites at a single genetic locus as seen in whole plasmid DNA. The fact that the observed complexity of integration patterns varies among experiments implies that a valid comparison of the respective influences of whole plasmid and gene-cassette DNA can only be made among plants generated in the same experiment, using the same embryo, callus batch and genotype. In our hands, complexity of the integration pattern was found to be positively correlated with the amenability to transform a cultivar, which in turn is largely determined by its overall tissue-culture ability and the physiological status of the cells used in the experiment.

Our results contrast with those of Fu and coworkers (2000) who recently reported an overall remarkable simplification of the integration patterns among transgenic rice plants transformed with gene cassettes compared to those transformed using whole plasmids. They concluded that the two substrates integrate into the genome in different ways, and that the vector backbone plays a significant role in the integration process due to the fact that minimal gene cassettes would provide shorter regions of homology and fewer recombination hotspots than whole plasmid DNA.

The frequency of expression of the co-transferred *yfp* gene in young regenerated plantlets was similar among treatments. When monitored over the course of plant development, however, a decline in the frequency of plants expressing the *yfp* gene was only observed among Taipei 309 plants transformed with WP preparations. In cv Nipponbare, loss of expression of the *yfp* transgene occurred two-fold more frequently among plants transformed with whole plasmid DNA than among those transformed with gene cassettes. This may suggest that integration of additional sequences of bacterial origin favour the recognition of foreign DNA intrusion and subsequent silencing of the integrated genes, which merits further investigation. These results appear consistent with previous findings recently reported by Fu et al. (2000).

Our results demonstrate that microprojectile bombardment-mediated transformation of rice using gene cassettes is possible without significantly reducing transformation efficiency, in comparison to the use of whole-plasmid DNA. Further experiments will be dedicated to test whether other artificial DNA-protein complexes lead to the repeatable production of transgenic plants exhibiting a low transgene copy number.

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