Molecular Breeding (2005) 16: 247–260
DOI 10.1007/s11032-005-0239-5

Transformation of plants with multiple cassettes generates simple transgene integration patterns and high expression levels

Pawan K. Agrawal[1], Ajay Kohli[2], Richard M. Twyman[3] and Paul Christou[4,*]
*[1]Central Rice Research Institute, Cuttack-753006, Orissa, India; [2]Institute for Research on Environment and Sustainability, School of Biology, University of Newcastle-upon-Tyne, Newcastle-upon-Tyne, NE1 7RU, UK; [3]Department of Biology, University of York, Heslington, York YO10 5DD, UK; [4]ICREA, Universitat de Lleida, Department de Produccio Vegetal i Ciencia Forestal, Av. Alcalde Rovira Roure, 191, E-25198 LLEIDA, Spain; *Author for correspondence (e-mail: Paul.Christou@icrea.es)*

Received 8 March 2005; accepted in revised form 27 June 2005

Key words: Plant transformation, Cassette transformation, Multigene transgenics, Pathway engineering, Gene expression, Transgenic loci

Abstract

We transformed rice (*Oryza sativa* L.) simultaneously with five minimal cassettes, each containing a promoter, coding region and polyadenylation site but no vector backbone. We found that multi-transgene cotransformation was achieved with high efficiency using multiple cassettes, with all transgenic plants we generated containing at least two transgenes and 16% containing all five. About 75% of the plants had simple transgene integration patterns with a predominance of single-copy insertions. The expression levels for all transgenes, and the overall coexpression frequencies, were much higher than previously reported in whole plasmid transformants. Four of five lines analyzed for transgene expression stability in subsequent generations showed stable and high expression levels over generations. A simple model is proposed, which accounts for differences in the molecular make-up and the expression profile of transgenic plants generated using whole plasmid or minimal cassettes. We conclude that gene transfer using minimal cassettes is an efficient and rapid method for the production of transgenic plants containing and stably expressing several different transgenes. Our results facilitate effective manipulation of multi-gene pathways in plants in a single transformation step.

Introduction

Multiple gene transfer to plants is necessary for sophisticated genetic manipulation strategies, such as the stacking of transgenes specifying different agronomic traits, the expression of different polypeptide subunits making up a multimeric protein, the introduction of several enzymes acting sequentially in a metabolic pathway or the expression of a target protein and one or more enzymes required for specific types of post-translational modification (Slater et al. 2003). Although this can be achieved by single gene transformation followed by the crossing of plant lines carrying different transgenes, it is much quicker and more straightforward to introduce all the necessary genes simultaneously (Gelvin 1998).

Particle bombardment is the most convenient method for multiple gene transfer to plants, since microprojectiles can be coated with a DNA

mixture comprising any number of different transformation constructs (Twyman and Christou 2004). No complex cloning strategies are needed, multiple *Agrobacterium* strains do not have to be prepared and there is no sequential crossing of different transgenic lines. Many studies have been reported, in which two or three different transgenes have been introduced into plants by particle bombardment, and the maximum reported thus far is 13 although there is no reason to believe this represents an upper limit (Chen et al. 1998). A disadvantage of traditional particle bombardment methodology is that transformation is carried out with whole plasmid vectors, causing the vector backbone to be introduced into the plant genome along with the transgenes of interest (Kohli et al. 1999; Smith et al. 2001; Twyman et al. 2002). This superfluous DNA is undesirable, because it often contains sequences that may have unpredictable effects on the phenotypes of the resulting transgenic plants. Furthermore, since vector backbone sequences are prokaryotic in origin, they can act as strong triggers for *de novo* DNA methylation, often resulting in transgene silencing (Clark et al. 1997). Kohli et al. (1999) have shown that the plasmid backbone also contains a number of recombinogenic elements that are known to promote transgene rearrangement and concatemerization. This produces complex, multicopy transgenic loci that have the potential to form DNA secondary structures and/or to express hairpin RNAs, both of which also promote transgene silencing (Plasterk and Ketting 2000; Hammond et al. 2001). It is difficult to remove genes selectively and cleanly from such complex loci, therefore frustrating attempts to delete genes, such as selectable markers, from transgenic lines after regeneration.

A recent development that may help to address these issues is particle bombardment using minimal cassettes, an approach known as clean DNA transformation (Fu et al. 2000). The minimal cassette, comprising a promoter, coding region and polyadenylation site, is isolated from the cloning vector using restriction endonucleases and purified by agarose gel electrophoresis prior to the microprojectile coating procedure. Vector DNA transfer is prevented, because the plasmid backbone is not present during transformation. A comparison of transgenic rice lines generated by particle bombardment using whole plasmids and the minimal cassettes derived therefrom showed that the transgenic loci in plants transformed with minimal cassettes were smaller and much simpler in their organization than those in plants transformed with intact plasmids (Fu et al. 2000). Transgenic loci in the cassette transformants generally contained fewer transgene copies than those in the plasmid-transformed plants and transgene expression was shown to be stronger and more stable. The authors concluded that the absence of the backbone limited the amount of recombination that took place during transgene integration, therefore producing simpler and lower-copy-number loci that were less prone to silencing.

Fu et al. (2000) have also established that minimal cassette transformation was appropriate for the simultaneous transfer of two different transgenes with at least the efficiency of whole plasmid transformation. This has been confirmed in subsequent studies, in which up to three minimal cassettes have been used to coat the microprojectiles (Breitler et al. 2002; Loc et al. 2002). However, there has been no detailed investigation of transgene integration and behavior in plants simultaneously transformed with multiple cassettes. Important questions that need to be addressed include whether there is any bias for or against particular transgenes in multiple transformation experiments, whether the absence of significant homology between the cassettes promotes independent co-integration or the formation of concatemers, whether transgene segregation occurs in subsequent generations and whether the strategy is overall an efficient method for the production of transgenic plants containing and expressing several different transgenes.

We found that cassette transformation was very efficient for multiple gene transfer, and that most of the plants in our population showed simple integration patterns with single copies of the integrated transgenes. Further analysis showed that the transgenes were expressed in most of the plants and that, for all the transgenes, overall expression levels and coexpression frequencies were higher than previously reported for whole plasmid transformants. These data show that multiple cassette transformation is a valuable strategy for the production of multitransgenic plants showing high level and stable transgene co-expression over generations. A model of transgene integration that explains these results is discussed.

Materials and methods

Origin and construction of minimal cassettes

Minimal transgene expression cassettes were isolated from the appropriate source plasmids by digestion with restriction enzymes *Xho*I and *Xba*I (*bar*, phosphinothiricin acetyltransferase), *Not*I (*gus*A, β-glucuronidase), *Kpn*I and *Sal*I (*hpt*, hygromycin phosphotransferase), *Kpn*I (*luc*, firefly luciferase), and *Kpn*I and *Eco*RV (*as*, anthranilate synthase). The *bar*, *gus*A and *hpt* genes were isolated from the co-integrate vector pWRG2426 (Kohli et al. 1998), the *luc* gene was isolated from pAL52 (a kind gift from Dr. Wendy Harwood, John Innes Centre, Norwich UK) and the *as* gene was isolated from p35S-*as* (a kind gift from Dr. Johan Memenlink, University of Leiden, The Netherlands). In each case, the cassette was flanked by buffer sequences 2–20 bp in length for protection against exonuclease digestion in transformed plant cells. The structure of each expression cassette is shown in Table 1. After digestion, restriction products were separated on a preparative low-melting-point agarose gel and the gel slice containing the minimal cassette was isolated with a sterile razor blade. The DNA was extracted with phenol, phenol:chloroform and ethanol as described by Sambrook and Russel (2000).

Plant material and transformation

Mature seed-derived rice callus (*Oryza sativa* L.) was used for transformation. DNA preparation, precipitation of DNA onto gold particles, particle bombardment and recovery of transgenic plants on medium supplemented with hygromycin was carried out as described previously (Sudhakar et al. 1998). The five cassettes were mixed at a 1:3:3:3:3 mass ratio, with the selectable marker *hpt* present at one third of the concentration of each of the four non-selected genes.

DNA isolation, and transgene analysis by Southern blot hybridization and the polymerase chain reaction (PCR)

Transgene integration, organization and segregation were analyzed by Southern blot hybridization and PCR, following the isolation of DNA from rice leaves by phenol extraction (Edwards et al. 1991). For Southern blot hybridization, 5 μg aliquots of genomic DNA were digested overnight with restriction enzymes that had either a single recognition site in the cassette (single cutters) or no recognition sites in the cassette (non-cutters). Details of the enzymes used for each cassette are included in Table 1. The DNA was fractionated by 0.8% agarose gel electrophoresis and alkali-blotted onto Hybond-N^+ membranes (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Probe DNA (25 ng) was labeled by the random primer method, incorporating $\alpha - [^{32}P]$ dCTP (Feinberg and Vogelstein 1983). Pre-hybridization and hybridization were carried out at 65 °C in high-salt buffer (600 mM NaCl, 20 mM PIPES, 4 mM EDTA, pH 6.8) containing 5× Denhardt's solution (2% Ficoll-400, 2% polyvinylpyrrolidone-360), 10% sodium dodecylsulfate (SDS), 5% sodium pyrophosphate and 100 mg ml^{-1} salmon sperm DNA (Sigma). Filters were washed twice in 2× SSC (3 M NaCl, 0.5 M sodium citrate), 0.5% SDS and once in 0.2× SSC, 0.5% SDS at 65 °C. Autoradiography was carried out on Kodak Xo-Mat film for 6–8 days.

Standard PCR was carried out with Ampli-*Taq* DNA Polymerase (Promega) in the buffer supplied by the manufacturer using the primers listed in

Table 1. Structure of the minimal cassettes used for transformation. The table describes the size and structure of each cassette, and restriction enzymes with single recognition sites (single cutters) and no recognition sites (non-cutters) used for Southern blot analysis.

Transgene	Promoter	Poly(A)	Single cutter(s)	Non-cutter	Size (kbp)
bar	35S + *Adh*1 intron	*nos*	*Bam*HI, *Hin*dIII	*Nhe*I	1.9
*gus*A	35S	*ssu*	*Bam*HI, *Hin*dIII	*Nhe*I	2.7
hpt	35S	*nos*	*Bam*HI, *Hin*dIII, *Eco*RV	*Nhe*I	2.0
luc	*Ubi*1 + intron	*nos*	*Hin*dIII	*Eco*RV	4.1
as	Double 35S	CaMV	*Bam*HI,	*Nhe*I	3.5

Table 2. Primers for PCR analysis of integrated transgenes.

Transgene	Forward primer	Reverse primer	Amplicon size (kb)
hpt	5′-GATCTCCAATCTGCGGGATC-3′	5′-ACTCACCGCGACGTCTGTCG-3′	1.1
bar	5′-GCGGTCTGCACCATCGTCAA-3′	5′-GTCATGCCAGTTCCCGTGCT-3′	0.5
gusA	5′-ACGGCCTGTGGGCATTACGT-3′	5′-GTTCGGCGTGGTGTAGAGCA-3′	0.9
luc	5′-GCCGGTGTTGGGCGCGTT-3′	5′-GCGGGAAGTTCACCGGCG-3′	1.3
as	5′-GCTCTACGGTCACAGGTGAGTTGC-3′	5′-TCCTATCCGAGACTCAACTCCAC-3′	0.5

Table 2. The reaction parameters were optimized for each transgene, consisting of 32 cycles of 1 min denaturation at 94 °C (95 °C for *luc*, 1 min 30 s for *gusA*), 1 min annealing at 60 °C (62 °C for *bar* and *as*) and 1 min extension at 72 °C (2 min for *gusA*). After the final cycle, the reactions were maintained at 72 °C for 5 min (10 min for *gusA*) and then cooled to 4 °C. The PCR products were analyzed by 0.8% agarose gel electrophoresis. The transforming fragments were used as positive controls, whereas wild type rice plants and negative segregants were used as negative controls. Long PCR was carried out using Herculase (Stratagene) according to the manufacturer's recommendations. Copy numbers of transgenes were estimated through reconciliation of data from, single, double and non-cutter Southern hybridization results in combination with PCR and long PCR results as used by Kohli et al. (1998).

Reverse transcriptase polymerase chain reaction (RT-PCR)

RT-PCR was carried out to confirm transgene expression using the Access RT-PCR System kit (Promega) according to the manufacturer's recommendations. Primary amplifications were carried out for 32 cycles using the primers listed in Table 2. In some cases a secondary amplification for another 25 cycles was carried out on a 50 times diluted primary amplification product. The cycling parameters and positive and negative controls were as described above.

Enzyme assays

Quantitative assays for phosphinothricin acetyltransferase (PAT) activity were carried out by thin layer chromatography as described by de Block et al. (1987). Histochemical assays for β-glucuronidase (GUS) were performed as described by Jefferson et al. (1987). GUS activity was quantified using the substrate 4-methylumbelliferone glucuronide (MUG; Sigma) and the fluorescent product 4-methylumbelliferone was detected using a Perkin Elmer LS-50 luminescence spectrophotometer, with an extinction wavelength of 365 nm and an emission wavelength of 455 nm. Specific enzyme activity was expressed in pmol MUG min^{-1} mg^{-1} protein. Total protein concentrations were determined using the dye-binding method as described by Bradford (1976). Luciferase activity was detected in plant tissues using an LB 980 luminograph imaging system (EG and G Berthold). Leaves were washed with 5% Tween-20 followed by two rinses in water. The leaves were then coated with 1 mM beetle luciferin potassium salt (Promega) prior to examination. Luciferase activity was quantified according to the method of Ow et al. (1986) and expressed in relative light units (RLU) per mg of protein. The extraction buffer, assay buffer and the luciferin substrate were prepared as described by Baruah-Wolff et al. (1999). Anthranilate synthase activity was measured in leaf tissue collected at the booting stage of development according to the method of Widholm (1972). Quantification was carried out by fluorometric HPLC with an excitation wavelength of 340 nm and an emission wavelength of 400 nm.

Statistics

The significance of cotransformation and coexpression bias was determined using the students' *t*-test with a threshold value of $p = 0.01$.

Results

Relative transformation efficiencies with different cassettes

We analyzed 38 independent transgenic rice lines produced by bombarding mature seed-derived callus with microprojectiles coated with a mixture of five minimal cassettes (*hpt*, *bar*, *gus*A, *luc* and *as*; see Materials and methods). The first issue we wished to address was the efficiency of multiple gene transfer and the relative efficiency of transformation with different non-selected cassettes. As expected, all the transgenic lines carried *hpt*, because this was used as the selectable marker. All lines except one (line No. 181, 3%) also contained at least one additional (non-selected) transgene. With *hpt* included, five lines (13%) carried two transgenes, 15 (39%) carried three transgenes, 10 (26%) carried four transgenes and 7 (18%) carried all five transgenes (Table 3). The number of integrated, non-selected transgenes thus followed an approximately normal distribution in the population, and the average number of different transgenes per line was 3.5.

The efficiency of transformation with the non-selected cassettes varied from 40% to 90%, with *luc* showing the lowest efficiency and *bar* the highest. Since the *luc* transgene was approximately twice the size of the *bar* transgene, the molar ratio of the two constructs in the transformation mix would be approximately 1:2, since equal *total masses* of DNA were included for each construct. Therefore, with the molar ratio of the constructs taken into account, there was no significant difference in transformation efficiency and no significant cotransformation bias (tendency for any particular pair of non-selected transgenes to integrate together).

Transgene integration and organization

The next issue we wished to address was the organization of the transgenic loci. Specifically, we wished to ascertain whether cotransformation with multiple transgenes resulted in the simple integration patterns reported previously in plants containing two or three different transgenes, and whether the multiple cassettes integrated independently or as concatemers. The integration patterns in 25 randomly selected plants were studied by Southern blot hybridization after the digestion of genomic DNA with restriction enzymes that cut once in the respective transgene cassettes (Table 1). More than 80% of the lines showed simple integration patterns, as revealed by Southern blots with 1-4 hybridizing bands per lane, suggesting a low transgene copy number (Table 3, Figure 1). An interesting observation was that the complexity of transgene integration patterns followed a trend within each transgenic line, and was not a property that could be assigned to any particular transgene. That is, if there was a simple integration pattern for one transgene, then there tended to be simple integration patterns for all other transgenes in the same line. As an example, this phenomenon can be seen clearly if one compares lines 19 and 22 (the leftmost lanes on the Southern blots shown in Figure 1) with lines 108 and 124, located in the middle of each blot. All four lines carry at least four transgenes, but lines 19 and 22 show generally high copy numbers for all transgenes, whereas lines 108 and 124 show generally low copy numbers.

Southern blot analysis following the digestion of genomic DNA with restriction enzymes that do not cut in the relevant transformation cassette can help to distinguish between single copy transgenes and concatemers. In the case of single copy transgenes, Southern blots using single-cutter and non-cutter enzymes should reveal the same number of bands. However, in the case of concatemers, there should be fewer bands with non-cutter enzymes, because several transgenes would be expected to reside within the same restriction fragment. We compared the results of Southern blots displaying DNA prepared with single cutter and non-cutter enzymes in the same 25 transgenic lines as above, and found that, in the majority of cases, the number of hybridizing bands was similar (Figure 2). This indicated that most of the integration events involved single copy transgenes. Coincidently, results of digestion with non-cutter *Nhe*I probed with the *gusA* gene (Figure 2) revealed most of the transgenic lines containing a single band close to the 23 kb marker fragment. We believe that in all these cases the single or multiple transgene copies lie within genomic *Nhe*I fragments bigger than 23 kb – the resolving power of the 0.8% agarose gel we used. We have noted such results earlier with plasmid transformations

Table 3. Summary of transgene data for 38 independent T0 rice lines. We documented transgene integration and expression in all 38 lines, but copy number estimates were carried out only for the first 25 lines, which were studied in detail.

Plant line	Transgene integration (copy number)						Transgene expression					
	hpt	*bar*	*gusA*	*luc*	*as*	Summary	*hpt*	*bar*	*gusA*	*luc*	*as*	Summary
9	3	1	0	2	0	3(6)2	Y	Y	N	Y	N	3
19	6	2	3	6	6	5(23)5	Y	N	Y	Y	Y	4
22	7	2	0	10	7	4(26)7	Y	N	N	Y	N	2
24	3	2	3	4	3	5(15)3	Y	Y	Y	Y	Y	5
36	3	1	2	0	2	4(8)2	Y	Y	N	N	Y	3
39	1	1	1	0	0	3(3)1	Y	N	N	N	N	1
40	5	5	6	3	2	5(21)4	Y	N	Y	Y	N	3
72	1	0	1	0	0	2(2)1	Y	N	N	N	N	1
85	3	2	4	0	2	4(11)3	Y	Y	N	N	Y	3
88	1	1	1	0	0	3(3)1	Y	Y	N	N	N	2
97	1	2	1	0	1	4(5)1	Y	Y	Y	N	Y	4
108	2	1	0	1	4	4(8)2	Y	Y	N	N	Y	3
112	5	3	4	3	4	5(18)4	Y	Y	N	Y	N	3
118	1	0	0	1	0	2(2)1	Y	N	N	Y	N	2
121	1	1	0	0	0	2(2)1	Y	Y	N	N	N	2
124	2	3	6	1	4	5(16)3	Y	N	N	N	N	1
129	2	1	3	0	0	3(6)2	Y	Y	N	N	N	2
153	1	4	5	0	3	4(13)3	Y	Y	N	N	N	2
181	1	0	0	0	0	1(1)1	Y	N	N	N	N	1
197	4	0	1	0	2	3(7)2	Y	N	N	N	N	1
202	1	1	0	0	2	3(4)1	Y	N	N	N	N	1
219	4	4	4	6	5	5(23)5	Y	Y	Y	Y	N	4
226	3	1	3	1	0	4(8)2	Y	Y	Y	N	N	3
234	8	5	4	0	6	4(23)6	Y	Y	Y	N	Y	4
249	8	3	6	0	5	4(22)6	Y	Y	N	N	Y	3
17	Y	Y	Y	Y	N	4	Y	Y	Y	Y	N	4
83	Y	Y	Y	N	N	3	Y	Y	N	N	N	2
175	Y	Y	Y	N	Y	4	Y	Y	Y	N	Y	4
209	Y	Y	N	N	Y	3	Y	Y	N	N	N	2
215	Y	Y	N	N	Y	3	Y	Y	N	N	Y	3
221	Y	Y	Y	Y	Y	5	Y	N	Y	Y	Y	4
235	Y	Y	Y	Y	N	4	Y	Y	N	N	N	3
260	Y	Y	Y	N	Y	4	Y	Y	N	N	Y	3
265	Y	Y	N	N	Y	3	Y	Y	N	N	N	2
269	Y	Y	N	N	Y	3	Y	N	N	N	Y	2
273	Y	N	N	N	Y	2	Y	N	N	N	N	1
283	Y	N	N	N	Y	2	Y	N	N	N	Y	2
303	Y	N	N	N	Y	2	Y	N	N	N	N	1

For the first 25 lines, the number of integrated copies of each transgene is shown in the left half of the table and the summary column shows the total number of transgenes (out of a maximum of five) that are integrated in each line, followed in parentheses by the sum of all the copies of all integrated transgenes, followed by the average copy number per transgene. For the remainder of the lines, the presence (Y) or absence (N) of each transgene is indicated and the summary column shows the number of different integrated transgenes (out of a maximum of five). The right hand side of the table shows transgene expression as either present (Y) or absent (N) and the summary column shows the total number of transgenes (out of a maximum of five) that are expressed in each line.

as well (Kohli et al. 1998). We then scored each plant according to the number of *different* transgenes that had integrated, i.e. a maximum score of five, where all five transgenes had integrated, and a minimum score of two, representing *hpt* and one non-selected transgene. In our population of 38 transgenic lines, the total score was 91 out of a possible total of 190 (Table 3). By comparing Southern blots, we found that 67 (74%) of integration events were single transgenes, 13 (14%) were dimers (two copies in tandem), nine (10%) were trimers (three copies in tandem) and two (2%) were tetramers (four copies in tandem). Long PCR was then used with combinations of

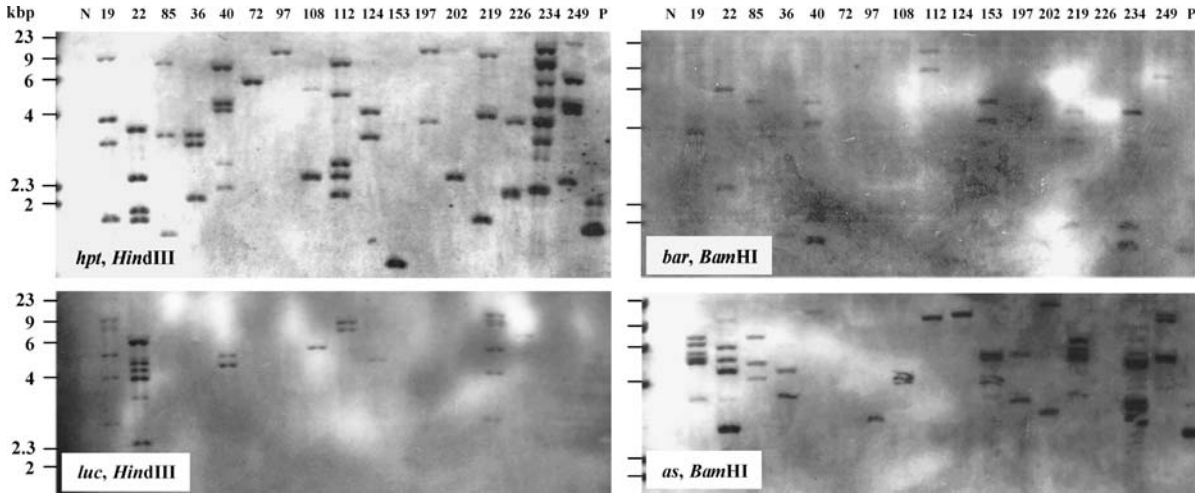

Figure 1. Southern blot analysis of R0 plants, using 5 μg of genomic DNA digested with the single-cutters *Hin*dIII (*hpt, luc*) or BamHI (*bar, as*). The numbers above each blot correspond to different plant lines, while N is the untransformed control plant and P is the positive control plasmid. The DNA markers are shown on the far left of each blot.

transgene-specific primers in 10 randomly chosen plants to establish whether the concatemers were homomultimeric (tandem copies of the same transgene) or heteromultimeric (mixed arrays of different transgenes). The absence of PCR products with discordant primer sets (primers for two different transgenes used for one amplification reaction) suggested that all concatemers in the transgenic plants were homomultimeric.

Transgene expression and co-expression

An important aspect of this study was the combined qualitative and quantitative analysis of the expression of all four non-selected transgenes, which was carried out in 25 selected transgenic lines. Expression analysis was performed at both the mRNA and protein levels. Transgene protein was detected wherever the corresponding mRNA was expressed. As expected, the *hpt* gene was expressed in all transgenic lines, since the product of this gene was essential for plant survival during regeneration. The expression frequency of the other transgenes ranged from 50% (*gusA)* to 71% (*bar*). Overall, 73% of integrated transgenes were expressed. We found that the levels of *bar*, *gusA* and *luc* expression were significantly higher in our transgenic plants than previously reported in the literature for plants transformed with plasmid DNA. The highest expression levels we observed for *bar*, *gusA* and *luc* respectively were $18{,}482 \times 10^{-6}$ enzyme units mg^{-1} protein, 46,873 pmols mg^{-1} min^{-1} and $63{,}962 \times 10^{3}$ RLU. There was no correlation between transgene copy number and expression level. For example, line 97 contained a single copy of *gusA* while lines 112 and 219 contained multiple copies. All three lines showed high level GUS activity with the single-copy line showing the highest level overall. Line 97 also contained a single copy of *bar* while line 85 contained multiple copies. Again, both lines showed high-level PAT activity with the multi-copy line showing the highest overall expression level in this case.

The analysis of coexpression frequencies showed that six of the 25 lines (24%) coexpressed two transgenes, eight lines (32%) coexpressed three transgenes, four lines (16%) coexpressed four transgenes and one line (4%) co-expressed all five transgenes. The remaining six lines (24%) expressed *hpt* alone. Overall, 12 of the 38 lines (32%) co-expressed all integrated transgenes (irrespective of the total number of transgenes that were present). There did not appear to be any significant bias in coexpression frequencies among the non-selected transgenes.

Transgene stability and segregation

In order to study the structural stability of integrated transgenes and the frequency of genetic

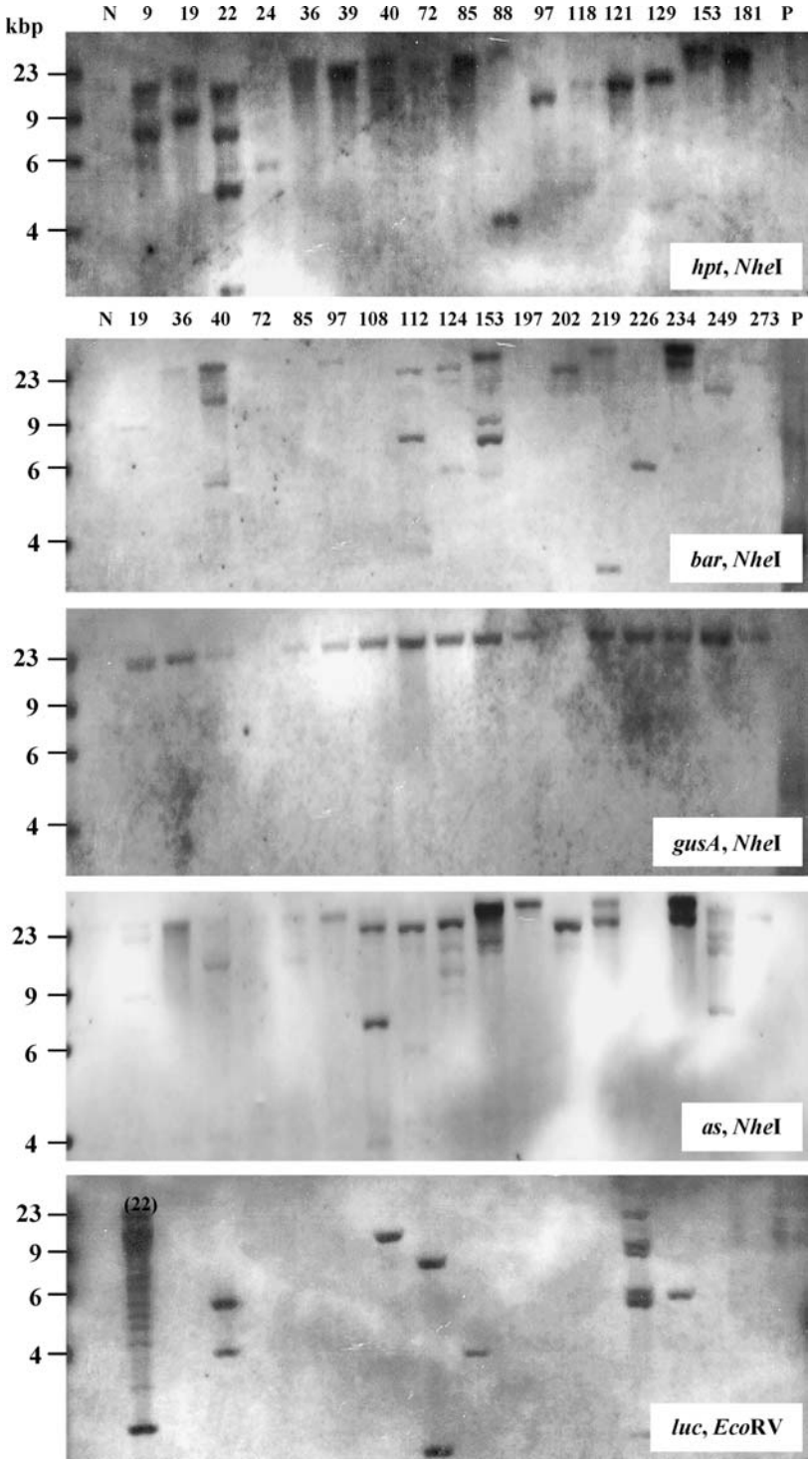

Figure 2. Southern blot analysis of R0 plants, using 5 μg of genomic DNA digested with the non-cutters *Nhe*I (*hpt, bar, gusA, as*) or *Eco*RV (*luc*). The numbers above each blot correspond to different plant lines (line 22 was substituted for line 19 in the *luc* blot because preliminary analysis with single cutter enzymes suggested a large number of copies of the transgene, and we were particularly interested to see how these genes were organized), while N is the untransformed control plant and P is the positive control plasmid. Note that no band can be seen in the positive control lane because the fragment ran beyond the extent of the cropped image. The DNA markers are shown on the far left of each blot.

segregation, we selected five lines for Southern blot analysis across three generations (lines 36, 97, 153, 226 and 234). There was a significant amount of segregation between different transgenes in the R1 and R2 plants, but with the exception of line 234, all the banding patterns for individual transgenes remained identical in all plants carrying them.

We analyzed seven R1 plants from line 97, which carried four transgenes (*hpt*, *bar*, *gusA* and *as*). Of these plants, six were positive for *bar* and *gusA*, four of these six were also positive for *as* and two of these four were also positive for *hpt*. The remaining plant carried no transgenes. Since, the plants were not grown under antibiotic selection, the *hpt* marker can be considered as a non-selected transgene from generation R1 onwards. The four transgenes therefore behaved as three segregating loci. Two of the R1 plants that carried *bar* and *gusA* were selected for further analysis. Eleven R2 progeny from these plants (97-1 and 97-3) were investigated further. Eight of these plants contained both transgenes, whereas three possessed none. Southern blot and long PCR experiments had already established that the *gusA* and *bar* transgenes in line 97 were single copy loci and represented independent integration events, but the segregation data suggested the genes were close enough together (1–10 Mb) to behave as a single genetic locus.

We analyzed 17 R1 progeny from line 226, which also carried four transgenes (in this case *hpt*, *bar*, *gusA* and *luc*). Six of these plants carried all four transgenes and one carried *gusA* and *hpt* but not the other two genes, while the remaining plants did not carry any of the transgenes. The locus structure in line 226 is therefore uncertain, although *gusA* and *hpt* appear to represent a separate locus (or loci) from the other transgenes. In contrast, the analysis of lines 36 and 153 identified R1 progeny carrying both *hpt* and *gusA*, plants carrying *hpt* alone, plants carrying *gusA* alone and plants carrying neither transgene, showing that the two genes represented distinct loci. Overall, these data indicated that the different transgenes had a tendency to integrate independently, resulting in segregation in later generations.

Stability of transgene expression

In the progeny of line 97, the expression of *bar* and *gusA* was stable over at least two generations. Indeed, while the expression levels in the R1 plants were comparable to those in the R0 primary transformant, there was an increase in expression in the R2 generation. The analysis included plants that contained the selectable marker and those in which the *hpt* gene had segregated. Most of the plants we analyzed showed stable transgene expression levels through to at least the R2 generation. An exception to the general rule was line 226. While all the R1 plants carrying *bar* and *gusA* expressed the transgenes, the expression levels were very much lower than observed in the primary transformant. It is not clear whether this reflects a structural change in the transgene locus structure that remained undetected in our experiments, or an epigenetic phenomenon.

Discussion

The simultaneous transfer of multiple genes to important crop plants such as rice is highly desirable, since this allows the rapid development of sophisticated strategies for trait modification, metabolic engineering and molecular farming. Although multiple gene transfer is possible using *Agrobacterium tumefaciens*, traditional approaches such as the use of multiple linked transgenes in single vectors, or multiple *Agrobacterium* strains carrying different plasmids, become progressively more labor intensive as the number of different transgenes increases. Recently, an *Agrobacterium*-based multigene assembly vector system has been described, which goes some way towards addressing these limitations (Lin et al. 2003). However, assembly relies on a rather complex process involving the Cre/*lox*P site-specific recombination system and homing endonucleases. In contrast, particle bombardment offers a very straightforward approach to multiple gene transfer, i.e. coating microprojectiles with a mixture of transformation constructs. This has been used simultaneously to introduce 12 different plasmids into soybean (Hadi et al. 1996) and 13 different plasmids into rice (Chen et al. 1998). The simultaneous introduction of up to five plasmids into rice can be considered a routine procedure (e.g. see Gahakwa et al. 2000; Tang et al. 1999).

When whole plasmids are used for particle bombardment, there is an overwhelming tendency for all the transgenes to integrate at a single locus

as a large concatemer, often containing 20 or more copies. Kohli et al. (1998) have suggested that this tendency reflects the fact that plasmids form concatemers prior to integration, due to recombination between the long stretches of homologous DNA in the vector backbone. The concatemers then integrate into the genome, often in clusters which might represent the localization of clustered repair complexes or replication forks (Kohli et al. 1998; Pawlowski and Somers 1998). The organization of plant transgenes into concatemers, and clusters interspersed with genomic DNA, has been observed not only in rice, but also in wheat, oat and maize, suggesting broadly conserved plasmid DNA integration mechanisms in the plant kingdom (Mehlo et al. 2000; Jackson et al. 2001; Svitashev and Somers 2001). Recently endogenous organelle DNA transfer to nuclear chromatin has also been shown to follow integration patterns similar to transgene integration (Huang et al. 2004). When the backbone is eliminated through the use of minimal cassettes, the substrate available for homologous recombination is limited. In effect, homologous recombination can only take place between identical copies of the transgenes themselves. This helps to explain the predominance of single-copy integration events and short homoconcatemers in our plants and the complete absence of heteroconcatemers.

As well as favoring homologous recombination, specific recombinogenic elements in the vector can also promote illegitimate recombination, ultimately causing transgene rearrangements and mutations (Kohli et al. 1999). The resulting transgenic loci are therefore complex in structure, containing rearranged plasmid fragments and interspersed segments of genomic DNA. This provides at least three independent triggers for transgene silencing: DNA secondary structures, aberrant RNA species and prokaryotic DNA that may be identified by genome scanning systems. Inverted repeats represent a particularly strong trigger, because these can form secondary structures at the DNA level and can result in the expression of hairpin RNAs that induce silencing by RNA interference (Voinnet 2002).

Non-replicating plasmid DNA introduced into animal cells is also rapidly concatemerized to form larger arrays, suggesting that animals and plants may have similar responses to the presence of exogenous DNA. The size and structure of the arrays in animal cells depends upon the amount, conformation and complexity of the source DNA. In techniques such as calcium phosphate transfection, a large amount of DNA enters the cell. In many cases, at least two independent transgenes are introduced (a selectable marker and one or more nonselected genes) and carrier DNA is used to bring the DNA concentration to optimal levels for coprecipitate formation. The carrier DNA is generally sheared or sonicated genomic DNA, and is thus linear and of high complexity. Shortly after transfection, the transgenes and carrier DNA form large, randomly arranged arrays reflecting the arbitrary ligation of DNA fragments. This structure, termed the transgenome, usually integrates at a single site. It is sometimes unstable and may be subject to full or partial excision, probably reflecting intrachromosomal recombination events (Perucho et al. 1980).

Carrier DNA is not used in other animal cell transfection methods. Less DNA therefore enters the cell and the DNA that does reach the nucleus is relatively simple (and hence repetitive) in its sequence. Even so, the use of plasmids for transformation can result in complex loci containing up to 30 transgene copies, and there may be several sites of integration. In certain transfection methods, the amount of DNA used in the procedure can be varied to favor the integration of single transgene copies. In mammals, linear DNA is integrated five times more efficiently than supercoiled plasmid DNA, because it provides a substrate for DNA ligase (Potter et al. 1984). It has been suggested that the excess of free DNA ends induces the expression of DNA ligase and other repair enzymes (Bishop and Smith 1989).

We propose a model in which the absence of plasmid backbone sequences in our transformation cassettes (a) reduces the amount of concatemerization prior to transgene integration, (b) limits the occurrence of transgene rearrangements, and (c) prevents homologous interactions between different transgenes during the integration event. These three factors working together therefore generate simple and intact transgenic loci represented by simple hybridization patterns (Figure 3). This model would also account for the high levels of transgene expression, the stability of expression over multiple generations and the high co-expression frequencies we observed, since many of the triggers for transgene silencing would be removed.

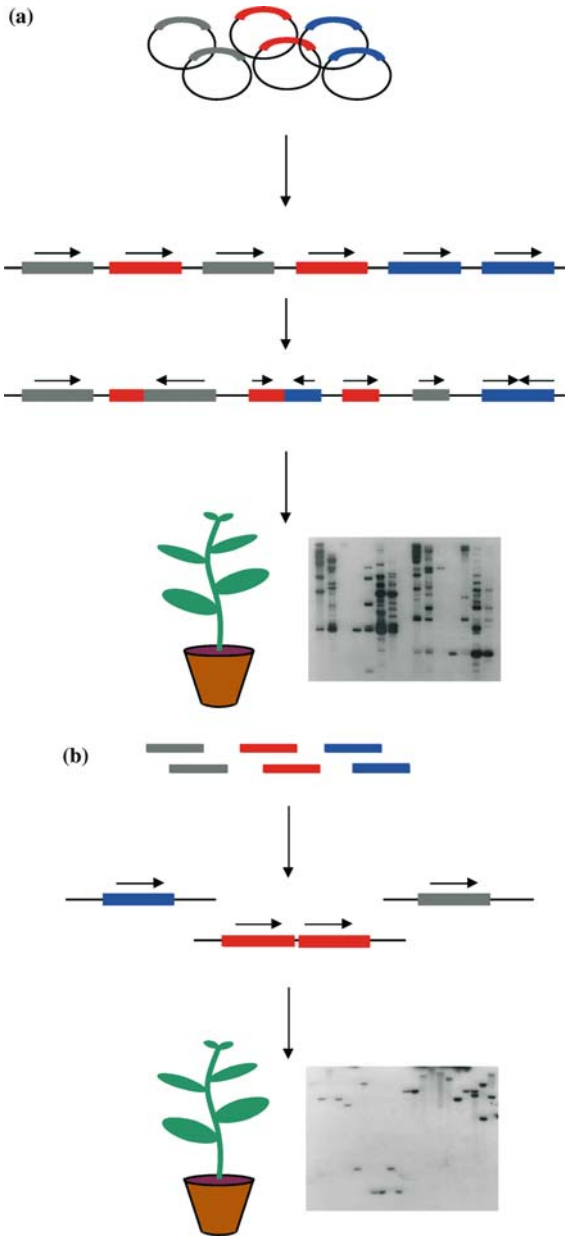

Figure 3. Model for transgene integration with multiple plasmids and multiple cassettes: (a) Multiple plasmids containing different transgenes but similar vector sequences undergo homologous recombination prior to integration, and recombinogenic sequences in the vector also promote illegitimate recombination (these are shown as separate events but are more likely to occur simultaneously). The result is a highly complex mixed concatemer, rich in prokaryotic sequences. Several concatemers may integrate into the plant genome, producing complex muti-copy loci that are prone to silencing and instability (Kohli et al. 2003). (b) Cassettes containing different transgenes do not undergo homologous recombination because there is no shared sequence. Therefore, only homoconcatemers are formed. The shorter sequences reduce the general level of homologous recombination, resulting in predominantly single copy loci or short concatemers. The absence of the vector backbone also reduces the incidence of illegitimate recombination. The resulting plants have simple transgenic loci that generally show strong and stable expression. The different transgenes may segregate in later generations, unlike plasmid-derived transgenic loci.

Minimal cassettes are generally not used for the transfection of animal cells, but are favored for the production of transgenic mammals. This is because linear DNA is a more efficient substrate for integration and prokaryotic vector sequences promote *de novo* methylation in mammals. While there have been no direct comparisons between plasmid transformants and cassette transformants in transgenic animals, the high frequency with which transgenic mice produced by pro-nuclear microinjection show strong and stable transgene expression confirms that linear cassettes are optimal substrates for gene transfer in animals as well as plants.

In the original description of multiple cassette transformation, Fu et al. (2000) provided only limited data concerning transgene organization. In one experiment involving simultaneous transformation with *hpt* and *gusA* cassettes, they indicated that the efficiency of cotransformation was at least as high as that for plasmid cotransformation, and that simple transgenic loci were still obtained. Similarly, in the study of Loc et al. (2002) using three cassettes, the authors provided little data concerning the structure and organization of the transgenes. Our analysis showed that all 38 lines in our population contained at least one non-selected transgene in addition to *hpt*, and that the number of cointegrated genes described a near-normal distribution in the population, with the largest number of plants containing three transgenes in total. There have been few studies involving large numbers of cotransformed transgenes, but our data agrees with previous reports that have addressed whole-plasmid transformation, since both Hadi et al. (1996) and Chen et al. (1998) noted a skewed normal distribution in the number of introduced transgenes. These previous studies also established that there was no preference for the integration of different plasmids. Similarly in our experiments, all the recovered plants carried the selectable marker due to the selection strategy

applied during regeneration, but with differences in molar loading ratios taken into account there appeared to be no transformation or cotransformation bias with any of the non-selected transgenes. These results suggest that competition for integration sites in the host plant is based on the number of available transgene ends rather than any particular sequence specificity of the transgene.

We also found that plants showing simple integration patterns for one transgene tended to have simple integration patterns for all the others, while plants with more complex patterns for one transgene tended to have more complex patterns for all the others. These data suggest that the complexity of a transgenic locus depends on factors intrinsic to the plant and not to the transgene. It has been shown that transgene integration occurs much more frequently during S-phase than at any other stage of the cell cycle (Kartzke et al. 1990), perhaps reflecting a tendency for transgenes to integrate at replication forks or repair sites near to them. Nucleases and ligases would be more abundant at this stage of the cell cycle, but would be unlikely to show preference for particular exogenous DNA sequences.

Although the above analysis provided adequate evidence for single copy integration events, it is still possible that all the transgenes could integrate – albeit independently – within close proximity to each other such that they behave as a single genetic locus. In the report by Fu et al. (2000), the *hpt* and *gusA* markers behaved as a single locus, but these investigators did not provide a detailed analysis of the locus structure so it was not possible to tell whether they had integrated as independent cassettes or concatemers. Abranches et al. (2001) have shown that integration sites dispersed over several megabases of DNA, and observable as discrete fluorescence *in situ* hybridization (FISH) signals on metaphase chromosome spreads, can still behave as a single locus in genetic segregation experiments. In order to determine whether the transgenes behaved as distinct loci, we carried out detailed segregation analysis in five lines and correlated the results with our Southern blot data. In line 97, which carried the genes *as*, *bar*, *gusA* and *hpt*, the four transgenes behaved as three genetic loci, with *bar* and *gusA* inherited together and the *hpt* and *as* loci segregating independently. Southern blot and long PCR experiments confirmed that the *gusA* and *hpt* loci were independent integration events and single copy transgenes, so it seems likely that these transgenes integrated by chance on the same chromosome, close enough together to be inherited as a unit.

Qualitative analysis of transgene expression showed that nearly three quarters of the transgenes in our rice lines were expressed. This figure includes the selected marker *hpt*, whose expression was required for plant survival during regeneration, but at least two genes were expressed in 76% of the lines and 32% of the lines expressed all their integrated transgenes. Multiple copy numbers or gene elements do not cause silencing if they are intact and not rearranged. Lack of vector backbone and physically the linear nature of the cassettes reduces such rearrangements. Use of similar elements like CaMV 35S for more than one cassette therefore does not cause silencing problems. The co-expression frequency fell as the number of transgenes increased, with only one of the lines containing all five transgenes also expressing all five genes. However, given the relatively small starting population, our data suggest that gene transfer with multiple cassettes still represents an efficient strategy for the production of transgenic lines expressing multiple transgenes. In previous experiments using whole plasmids, much lower coexpression frequencies have been observed even with relatively few transgenes (Schocher et al. 1986; Christou and Swain 1990; Li et al. 1993; Rathor et al. 1993; Cooley et al. 1995; Peng et al. 1995; Wakita et al. 1998). For example, Wakita et al. (1998) reported a 28% coexpression efficiency for two transgenes (*bar* and *NtFAD*) in a comparable population of 32 plants, which is less than half the efficiency demonstrated in this report for two transgenes. Fu et al. (2000) reported 88% coexpression for two cassettes (*hpt* and *gusA*). Unfortunately, there has been a tendency to undervalue the importance of coexpression frequencies in previous studies yet this will be perhaps one of the most important principles in the development of novel strategies for multigene engineering in crops.

Quantitative analysis of transgene expression levels showed that the level of expression from integrated cassettes was higher than reported in the literature for whole-plasmid transformants, and that there was no correlation between transgene copy number and expression level. The

highest level of luciferase expression previously reported in rice was $12{,}000 \times 10^3$ RLU in young rice leaves transformed with a *luc* plasmid (Baruah-Wolff et al. 1999) whereas the highest expression we observed was $63{,}962 \times 10^3$ RLU. Similarly, Gahakwa et al. (2000) reported 1350 pmol mg^{-1} protein min^{-1} GUS activity for rice plants transformed with a plasmid containing the *gusA* gene, whereas we observed a maximum of 46,873 pmol mg^{-1} protein min^{-1} in R0 plants rising to 333 nmol mg^{-1} protein min^{-1} in R2 line 97-1-1. The highest level of PAT activity reported in plasmid-transformed transgenic rice plants is 1240×10^{-6} units mg^{-1} protein (De Block et al. 1987) while we observed a maximum of $18{,}482 \times 10^{-6}$ units mg^{-1} protein in R0 plants rising to $97{,}426 \times 10^{-6}$ units mg^{-1} protein in R2 line 97-3-3. Loc et al. (2002) also observed 2–4-fold increases in expression levels compared to plasmid transformants when they introduced *gna* and *cry1Ac* gene cassettes into rice.

We conclude that particle bombardment with up to five minimal cassettes provides an improved method for the production of transgenic plants containing and stably expressing multiple transgenes. We propose a model in which the elimination of the vector backbone reduces the amount of substrate available for homologous recombination, prevents homologous interactions between dissimilar transgenes and removes many of the stimuli that promote illegitimate recombination. This favors the formation of intact, single-copy transgene loci that are not prone to silencing. Such loci were observed in more than 75% of the plants we studied, and these plants showed unprecedented high levels of transgene expression, stability of expression over several generations and high co-expression frequencies. These findings will facilitate the development of efficient methods for the production of plants expressing complex multisubunit proteins, surrogate metabolic pathways or recombinant proteins with authentic post-translational modifications.

References

Abranches R., Santos A.P., Wegel E., Williams S., Castillho A., Christou P., Shaw P. and Stoger E. 2001. Widely separated multiple transgene integration sites in wheat chromosomes are brought together at interphase. Plant J. 24: 713–723.

Baruah-Wolff J., Harwood W.A., Lonsdale D.A., Harvey A., Hull R. and Snape J.W. 1999. Luciferase as a reporter gene for transformation studies in rice (*Oryza sativa* L.). Plant Cell Rep. 18: 715–720.

Bishop J.O. and Smith P. 1989. Mechanism of chromosomal integration of microinjected DNA. Mol. Biol. Med. 6: 283–298.

Bradford M.M. 1976. A rapid and sensitive method for the quantification of microgram amounts of protein utilizing the principle of protein dye binding. Anal. Biochem. 7: 248–254.

Breitler J.C., Labeyrie A., Meynard D., Legavre T. and Guiderdoni E. 2002. Efficient microprojectile bombardment-mediated transformation of rice using gene cassettes. Theor. Appl. Genet. 104: 709–719.

Chen L., Marmey P., Taylor N.J., Brizard J., Espinoza C., D'Cruz P., Huet H., Zhang S., de Kochko A., Beachy R.N. and Fauquet C.M. 1998. Expression and inheritance of multiple transgenes in rice plants. Nat. Biotechnol. 16: 1060–1064.

Christou P. and Swain W.F. 1990. Cotransformation frequencies of foreign genes in soybean cell cultures. Theor. Appl. Genet. 79: 337–341.

Clark A.J., Harold G. and Yull F.E. 1997. Mammalian cDNA and prokaryotic reporter sequences silence adjacent transgenes in transgenic mice. Nucleic Acids Res. 25: 1009–1014.

Cooley J., Ford T. and Christou P. 1995. Molecular and genetic characterization of elite transgenic rice plants produced by electric-discharge particle acceleration. Theor. Appl. Genet. 90: 97–104.

De Block M., Botterman J., Vandewiele M., Dockx J., Thoen C., Gossel V., Rao M.N., Thomson C., Van Montagu M. and Leemans J. 1987. Engineering herbicide resistance in plants by expression of a detoxifying enzyme. EMBO J. 6: 2513–2518.

Edwards K., Johnstone C. and Thompson C. 1991. A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res. 19: 1349.

Feinberg A.P. and Vogelstein B. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132: 6–13.

Fu X., Duc L.T., Fontana S., Bong B.B., Tinjuangjun P., Sudhakar D., Twyman R.M., Christou P. and Kohli A. 2000. Linear transgene constructs lacking vector backbone sequences generate low-copy-number transgenic plants with simple integration patterns. Transgenic Res. 9: 11–19.

Gahakwa D., Bano Maqbool S., Fu X., Sudhakar D., Christou P. and Kohli A. 2000. Transgenic rice as a system to study the stability of transgene expression: multiple heterologous transgenes show similar behaviour in diverse genetic backgrounds. Theor. Appl. Genet. 101: 388–399.

Gelvin S.B. 1998. Multigene plant transformation: more is better!. Nat. Biotechnol. 16: 1009–1010.

Hadi M.Z., McMullen M.D. and Finger J.J. 1996. Transformation of 12 different plasmids into soybean via particle bombardment. Plant Cell Rep. 15: 500–505.

Hammond S.M., Caudy A.A. and Hannon G.J. 2001. Post-transcriptional silencing by double stranded RNA. Nature Rev. Genet. 2: 110–119.

Huang C.Y., Ayliffe M.A. and Timmis J.N. 2004. Simple and complex nuclear loci created by newly transferred chloroplast DNA in tobacco. Proc. Natl. Acad. Sci. USA 101: 9710–9715.

Jackson S.A., Zhang P., Chen W.P., Phillips R.L., Friebe B., Muthukrishnan S. and Gill B.S. 2001. High-resolution structural analysis of biolistic transgene integration into the genome of wheat. Theor. Appl. Genet. 103: 56–62.

Jefferson R.A., Kavanagh T.A. and Beven M.W. 1987. GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6: 3901–3907.

Kartzke S., Saedler H. and Meyer P. 1990. Molecular analysis of transgenic plants derived from transformations of protoplasts at various stages of the cell. Plant Sci. 67: 63–72.

Kohli A., Leech M., Vain P., Laurie D.A. and Christou P. 1998. Transgene organization in rice engineered through direct DNA transfer supports a two-phase integration mechanism mediated by the establishment of integration hot-spots. Proc. Natl. Acad. Sci. USA 95: 7203–7208.

Kohli A., Griffiths S., Palacios N., Twyman R.M., Vain P., Laurie D.A. and Christou P. 1999. Molecular characterization of transforming plasmid rearrangements in transgenic rice reveals a recombination hotspot in the CaMV 35S promoter and confirms the predominance of microhomology mediated recombination. Plant J. 17: 591–601.

Kohli A., Twyman R.M., Abranches R., Wegel E., Shaw P., Christou P. and Stoger E. 2003. Transgene integration, organization and interaction in plants. Plant Mol. Biol. 52: 247–258.

Li L., Qu R., Kochko A., Fauquet C. and Beachy R.N. 1993. An improved rice transformation system using the biolistic method. Plant Cell Rep. 12: 250–255.

Lin L., Liu Y.-G., Xu X. and Li B. 2003. Efficient linking and transfer of multiple genes by a multigene assembly and transformation vector system. Proc. Natl. Acad. Sci. USA 100: 5962–5967.

Loc N.T., Tinjuangjun P., Gatehouse A.M.R., Christou P. and Gatehouse J.A. 2002. Linear transgene constructs lacking vector backbone sequences generate transgenic rice plants which accumulate higher levels of proteins conferring insect resistance. Mol. Breeding 9: 231–244.

Mehlo L., Mazithulela G., Twyman R.M., Boulton M.I., Davies J.W. and Christou P. 2000. Structural analysis of transgene rearrangements and effects on expression in transgenic maize plants generated by particle bombardment. Maydica 45: 277–287.

Ow D.W., Wood K.V., Deluca M., Dewet J.R., Helinski D.R. and Howel S.H. 1986. Transient and stable expression of the firefly luciferase gene in plant cells and transgenic plants. Science 234: 856–859.

Pawlowski W.P. and Somers D.A. 1998. Transgenic DNA integrated into the oat genome is frequently interspersed by host DNA. Proc. Natl. Acad. Sci. USA 95: 12106–12110.

Peng J., Wen F., Lister R.L. and Hodges T.K. 1995. Inheritance of *gus*A and *neo* genes in transgenic rice. Plant Mol. Biol. 27: 91–104.

Perucho M., Hanahan D., Lipsich L. and Wigler M. 1980. Isolation of the chicken thymidine kinase gene by plasmid rescue. Nature 285: 207–210.

Plasterk R.H.A. and Ketting R.F. 2000. The silence of the genes. Curr. Opin. Genet. Dev. 10(5): 562–567.

Potter H., Weir L. and Leder P. 1984. Enhancer-dependent expression of human kappa immunoglobulin genes introduced into mouse pre-B lymphocytes by electroporation. Proc. Natl. Acad. Sci. USA 81: 7161–7165.

Rathor K.S., Chowdhury V.K. and Hodges T.K. 1993. Use of *bar*as a selectable marker gene and for the production of herbicide-resistant rice plants from protoplasts. Plant Mol. Biol. 21: 871–884.

Sambrook J. and Russell D.W. 2000. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schocher R.J., Shillito R.D., Saul M.W., Paszkowski J. and Potrykus I. 1986. Co-transformation of unlinked foreign genes into plants by direct gene transfer. Bio/Technology 4: 1093–1096.

Slater A., Scott N. and Fowler M. 2003. Plant Biotechnology: The Genetic Manipulation of Plants. Oxford University Press, Oxford, UK.

Smith N., Kilpatrick J.B. and Whitelam G.C. 2001. Superfluous transgene integration in plants. Crit. Rev. Plant Sci. 20: 215–249.

Sudhakar D., Duc L.T., Bong B.B., Tinjuangjun P., Maqbool S.B., Valdez M., Jefferson R. and Christou P. 1998. An efficient rice transformation system utilizing mature seed-derived explants and a portable, inexpensive particle bombardment device. Transgenic Res. 7: 289–294.

Svitashev S.K. and Somers D.A. 2001. Genomic interspersions determine the size and complexity of transgene loci in transgenic plants produced by microprojectile bombardment. Genome 44: 691–697.

Tang K., Tinjuangjun P., Xu Y., Sun X., Gatehouse J.A., Ronald P.C., Qi H., Lu X., Christou P. and Kohli A. 1999. Particle-bombardment-mediated co-transformation of elite Chinese rice cultivars with genes conferring resistance to bacterial blight and sap-sucking insect pests. Planta 208: 552–563.

Twyman R.M. and Christou P. 2004. Plant transformation technology – particle bombardment. In: Christou P. and Klee H. (eds), Handbook of Plant Biotechnology. Vol. 1. John Wiley & Sons, NY, pp. 263–290.

Twyman R.M., Stoger E., Kohli A. and Christou P. 2002. Foreign DNA: Integration and expression in transgenic plants. In: Setlow J.K. (ed.), Genetic Engineering Principles and Practice. Vol. 24. Plenum Press, NY, pp. 107–136.

Voinnet O. 2002. RNA silencing: small RNAs as ubiquitous regulators of gene expression. Curr. Opin. Plant. Biol. 5: 444–451.

Wakita Y., Otani M., Iba K. and Shimada T. 1998. Cointegration, coexpression and cosegregation of an unlinked selectable marker gene and *NtFAD3* gene in transgenic rice plants produced by particle bombardment. Genes Genet. Sys. 73: 219–226.

Widholm J.M. 1972. Anthranilate synthase from 5-methyltryptophan-susceptible and resistant cultured *Daucus carota* cells. Biochim. Biophys. Acta 279: 48–57.