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Molecular and genetic characterization of elite transgenic rice plants produced by electric-discharge particle acceleration

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Abstract The recovery of transgenic rice plants expressing a number of exogenous genes was reported previously. Using immature embryo explants as the target tissue, plasmids containing both selectable and screenable marker genes were introduced into elite rice varieties via electric-discharge particle acceleration. Co-integration, copy number, expression, and inheritance of these genes were analyzed. A 100% co-integration frequency was confirmed by Southern-blot analyses of R0 plants. The majority of transgenic plants contained between one and ten copies of exogenous DNA and molecular and genetic analyses of progeny indicated that all copies in almost all R0 plants were inherited as a single dominant hemizygous locus. Co-expression of unselected genes ranged from 30–66% for *gus/hmr* constructs, depending on the promotor used, and up to 90% for *bar/hmr* constructs. The integrative structures of two unlinked transgenic loci of a rare R0 plant were analyzed in detail by Southern-blot analysis of its progeny.

Key words Transgenic rice · *Oryza sativa* · Co-expression · Particle bombardment · Herbicide resistance

Introduction

Rice is one of the world's most important agronomic crops. Accordingly, great effort has been directed toward its improvement through conventional breeding and genetic engineering. Previously, we reported the recovery of transgenic rice plants using direct gene transfer of exogenous DNA into immature zygotic embryos by electric-discharge particle acceleration (Christou et al. 1991). The method by-passes traditional variety-dependent, tissue-culture procedures involving protoplast and embryogenic suspension cultures (Datta et al. 1990; Peng et al. 1990; Tada et al. 1990) thus enabling the rapid recovery of desirable transgenic phenotypes in agronomically important cultivars.

Once an efficient transformation system has been developed, it is necessary to assess the fate and function of the exogenous genes in the host organism and its progeny. Of particular interest are the structure and expression of unselected genes, which often encode the desired transgenic phenotype. By extensive Southern blots analysis Goto et al. (1993) demonstrated co-integration and co-inheritance of selected and unselected exogenous genes transformed on separate plasmids. In the present study, we report co-integration and co-inheritance as well as stable co-expression of multiple genes transferred on a single plasmid into rice by electric-discharge particle acceleration.

Fifty-six R0 transgenic plants transformed with a single plasmid containing the selectable gene hygromycin phosphotransferase (*hmr*) and one or two unselected genes (β-glucuronidase; *gus* or phosphinothricin acetyltransferase; *bar*) were analyzed by Southern-blot analyses to determine copy number and co-integration frequencies. Co-expression frequencies for selected and unselected genes in these plants were also determined. The physical linkage of transgenes in multi-copy integration events was confirmed by co-segregation of transgenic phenotypes and Southern-blot patterns in R1 and R2 generations.

Materials and methods

Vector construction

All plasmids have the pUC19 backbone (Yanisch-Perron et al. 1985) in common (See Fig. 1). Plasmid WRG4517 contains both *bar* (De Block et al. 1987) and *hmr* (Van den Elzen et al. 1985) genes fused to 35 CaMV promoters (nucleotides 7013–7440 of the CaMV sequence; Gardner et al. 1981) and 304 bp of the polyA addition site from the soy *ssu* (Berry-Lowe et al. 1982) gene. The *bar* gene has the AMV

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leader and the *hmr* gene has the leader from CaMV 35*s* mRNA. The genes are immediately adjacent and are transcribed toward each other. Additional plasmids are derivatives of pWRG4517 and were constructed by replacemnt of the 35*S*–*bar*-soy poly A cassette with *gus* fused to four different promoters. pWRG4515, pWRG4520, pWRG4522 and pWRG4524 have *gus* (Jefferson et. al. 1987) genes driven by rice *Gt3* (Leisy et al. 1989), soy *ssu*, *Arabidopsis ssu* (Timko et al. 1988) and rice *ssu* (de Pater et al. 1990) promoters, respectively. All four *gus* genes end with the *nos* transcription termination site and transcription of the *gus* and *hmr* genes proceeds toward each other. Plasmid WRG2426 contains the *gus*, *bar* and *hmr* genes all driven by 35*S* promoters. The *gus* and *hmr* genes contain the AMV 5′ untranslated leader sequence. The *bar* gene has the maize *adh* 5′ untranslated leader with intron (Callis et al. 1987). The *bar* and *hmr* genes have 271 bp of the *nos* (nopaline synthase; Depicker et al. 1982) poly A addition site from *Agrobacterium tumefaciens*. The *gus* gene has the soy *ssu* transcription terminator. The three genes are immediately adjacent to each other with the *hmr* gene positioned between the *bar* and *gus* genes. Both *bar* and *hmr* are transcribed in a clockwise direction whereas *gus* is transcribed counter-clockwise.

Explants and transformation procedures

Rice varieties used in this particular study were: I72, IR54, Gulfmont and Koshihikari. DNA preparation, isolation and preparation of immature embryos, particle bombardment and plant regeneration were performed as described elsewhere (Christou et al. 1991). Transformants were selected on hygromycin at 50 mg/l. Putative transformants were subjected to molecular and genetic analyses to identify independent transformation events and confirm stable integration and inheritance of all exogenous genes.

Southern-blot analysis

DNA was prepared from leaf tissue harvested from primary transformants and their progeny (Dellaporta et al. 1984). The DNA was digested with restriction endonucleases and then fractionated by electrophoresis on a 0.7% agarose gel. Transfer to nylon membranes (Biodyne membranes, Irvine, Calf.) was performed as described (Southern 1975). ^{32}P-labeled double-stranded DNA hybridization probes were prepared using the oligo-labelling kit (Pharmacia, Milwaukee, Wis.) with [a-^{32}P dCTP] (3 000 Ci/mol; Amersham, Boston, Mass.). Templates for probe synthesis spanned the full coding region of each gene (Fig. 1), excluding promoter, leader, and poly-adenylation sequences. Hybridization and washing conditions were performed as described (Church and Gilbert 1984). Filters were stripped for re-hybridization by washing twice in boiling water for 5 min and subsequently analyzed by autoradiography using X-Omat AR5 film (Kodak) at −80 °C.

Gene-expression assays

β-Glucuronidase (GUS) activity was assayed by incubation of appropriate tissues for 4–6h at 37 °C in GUS assay buffer (Jefferson et al. 1987). Phosphinothricin acetyltransferase (BAR) activity was indicated by resistance to Basta using commercial rates of application.

Results

Southern-blot analysis of R0 plants

Southern-blot analysis of primary transformants was used to identify 56 independent transformation events (Table 1). The analysis was designed to characterize independent events by comparing patterns of bands which include both the chimeric gene insert and flanking rice genomic DNA. Co-integration of selected and unselected marker genes was determined by probing blots for all chimeric genes of interest (*gus*, *bar* and *hmr*).

The transforming DNA in each of the 56 transgenic individuals consisted of one of six different plasmid constructs (Fig. 1). All plasmids contained the *hmr* gene as the selectable marker and one or two unselected genes of interest (*gus* and *bar*). Southern blots with gene-specific probes indicated that all genes on the transforming DNA were integrated at least once in all independent events for a 100% co-integration frequency of selected and unselected genes transferred on the same plasmid. For most transformants, the complexity of the Southern-band patterns was comparable from one gene-specific probe to another, indicating similar copy numbers of the transferred genes (Fig. 2). This suggests that the transforming plasmids generally integrate as a complete unit. Estimation of copy-number was based on the intensity and number of bands on Southerns blots, in which the genomic DNA was digested with an enzyme that cuts once in the transforming plasmid. Thirty-one percent of all plants analyzed had one or two copies integrated per haploid genome, 42% had between three and nine copies and 26% had ten or more copies.

Co-expression of selected and unselected genes in R0 plants

In order to assess the frequency of co-expression of selected and unselected genes on each transforming plasmid, *gus* expression in 43 hygromycin-resistant individuals containing *gus* genes driven by 35*S*, Gt3, and several *ssu* promoters was visualized by histochemical staining of appropriate tissues. Plants with strong blue staining were recorded as positive for *gus* expression and those with no staining, or only a few localized spots, as negative. Herbicide resistance in 21 plants transformed with *bar* constructs was subjectively ranked as high (no

Table 1 Number of independent transformation events identified by Southern-blot analysis

Rice cultivar	Transforming plasmid DNA[a]						
	WRG 2426	WRG 4517	WRG 4515	WRG 4520	WRG 4522	WRG 4524	Total
Gulfmont	1	10	–	8	9	5	33
IR72	4	3	2	–	–	–	9
IR54	–	–	–	–	–	7	7
Koshihikari	7	–	–	–	–	–	7
Total transformants for each plasmid	12	13	2	8	9	12	56

[a] See Fig. 1 for plasmid structure

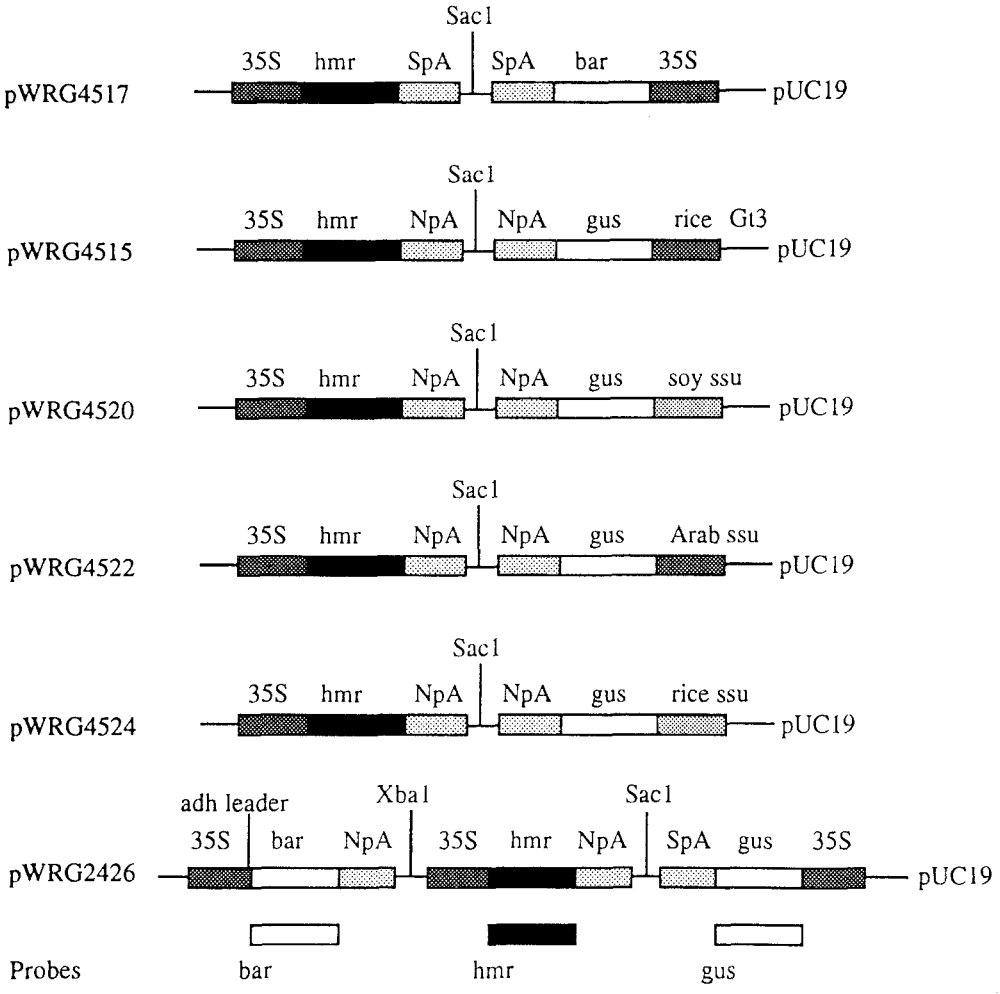

Fig. 1 Schematic representation of plasmids used in rice transformation (not to scale)

damage), low (some damage), or none (death) by the relative degree of necrosis observed five days after Basta spray. For the purposes of this analysis, both high and low Basta resistance were tallied as positive for *bar* expression. The results, outlined here, indicate that the overall co-expression frequency for all *gus/hmr* constructs was 30% (13 out of 43 transformants expressed *gus*) regardless of the promoter used to drive *gus*. Transformants containing plasmids with *gus* driven by soy *ssu*, rice *ssu* and *Gt3* promoters were all negative for *gus* expression. In plants containing *Arabidopsis ssu-gus* or *35S* constructs, co-expression rates were 33 (5 out of 15) and 66% (8 out of 12), respectively. Ninety percent (19 out of 21) of hygromycin-resistant plants containing *bar* constructs were resistant to Basta. Of 12 *bar*-expressing plants which hybridized to both the *bar* and *gus* gene probes, six expressed both *bar* and *gus*. All *bar* and *hmr* genes were driven by *35S* promoters.

To determine if copy number plays a role in the observed variations in co-expression, the number of functional copies of transgenic inserts per individual transformant was estimated. Results, shown in Table 2, indicated that as the number of *gus* transgenes exceeded ten, the frequency of expression of *gus* in hygromycin-resistant individuals decreased sharply from 44 to 13%. In the individuals with *gus* driven exclusively by *35s*, however, all low copy (1–2 copies) transformants expressed *gus* but none of the individuals with greater than ten integrated copies were *gus* positive. Conversely, *35S-bar/hmr* plants demonstrated the opposite relationship, with co-expression rate increasing with copy number from 80%, for one or two copies, to 100% for greater than ten copies. These results suggest that any positive correlation between gene-silencing and multi-copy integration events may be gene- or construct-specific.

Molecular and genetic analyses of transgenic progeny

We examined the patterns of unselected transgene inheritance in the R1 progeny of 19 *gus*- and/or *bar*-expressing transformants. Expression of *gus* and *bar* in transformed plants demonstrated 3:1 segregation consistent with Mendelian inheritance of a single dominant

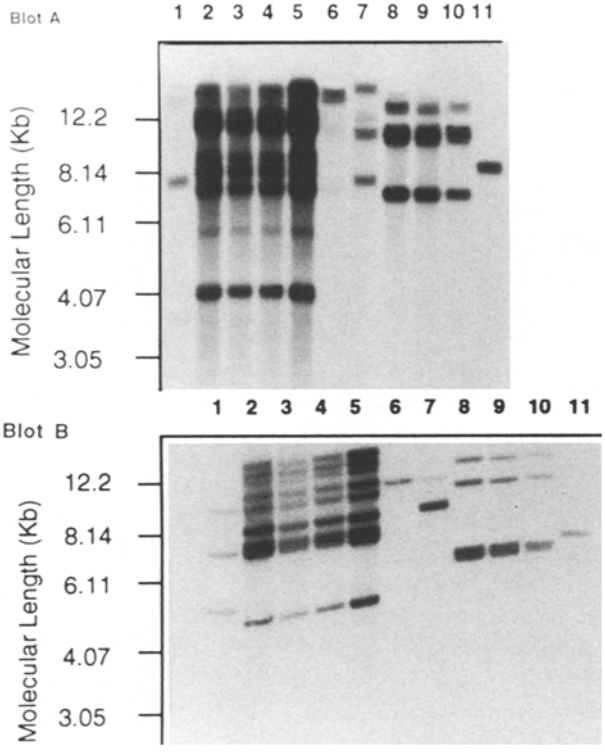

Fig. 2A, B Southern blots of rice transformed with pWRG2426. These blots identified five independent events out of ten primary regenerates. Some events (e.g., 496-1 and 496-4) produced multiple plants from the same callus. Genomic DNA from R0 transformants was digested with *Sac*1 (**Blot A**) or *Xba*1 (**Blot B**), transferrred to nylon membranes and probed with ^{32}P-labeled *gus* gene DNA (**Blot A**) or similarly labeled *bar* gene DNA (**Blot B**) **Blots A and B** have identical lane loadings of DNA from plants regenerated from hygromycin-resistant calli: 495-1 (*lane 1*), 496-1 (*lanes 2–5*), 496-2 (*lane 6*), 496-3 (*lane 7*), 496-4 (*lane 8–10*). *Lane 11* contains pWRG2426 DNA at one copy/genome digested with *Sac*1 (**Blot a**) or *Xba*1 (**Blot B**)

Table 2 Effect of transgene copy number on co-expression frequencies

Estimated gene copy number/genome	Co-expression frequencies[a]		
	GUS/HMR[b]	35SGUS/HMM[c]	BAR/HMR[d]
1 or 2 copies	44% (7 of 16)	100% (6 of 6)	80% (4 of 5)
3 to 10 copies	42% (8 of 19)	60% (3 of 5)	86% (6 of 7)
> 10 copies	13% (1 to 8)	0% (0 of 3)	100% (2 of 2)

[a] Co-expression frequency is defined as the number of transformants expressing both the selected and unselected genes per number of transformants shown to have those genes by Southern-blot analysis
[b] Plants in this group are all those that were transformed with a *gus* construct regardless of the promoter driving *gus*
[c] Plants in this group are a subset of *gus/hmr* plants that were transformed with pWRG2426 (*gus* driven exclusively by the 35S promoter)
[d] Plants in this group consist of those transformed with pWRG4517 and pWRG2426 (*bar* genes driven by the 35S promoter)

locus (Table 3) in all but two plants. One plant, 517-4, demonstrated a 1:1 segregation ratio for *bar* expression. This aberrant segregation can be explained by the passage of the transgene exclusively through one gamete. An insertion mutation of an essential gene required for pollen or ovum viability may account for this aberrant inheritance (Christou et al. 1989). A second plant, 495-1, produced a 15:1 segregation ratio for *bar* expression consistent with the presence of two unlinked genetic loci. However, analysis of *gus* expression resulted in a 3:1 ratio in the same group, indicating the presence of only one locus with a functional *gus* gene.

Southern-blot analysis of selected families demonstrated complete concordance betweeen the presence of

Table 3 Genetic analysis of R1 and R2 progeny of selected rice plants transformed with pWRG2426 (35S *bar*-35S *hmr*-35s*gus*)

Generation	Transformants	GUS+	BAR+	Total assayed	Expected ratio	χ^2	*P*
R1	495-1	41	51	53	3:1 GUS	0.039	0.80–0.95
					15:1 BAR	0.035	0.80–0.95
	496-1	0	74	101	3:1 BAR	0.040	0.80–0.95
	496-2	31	31	47	3:1 GUS	0.512	0.20–0.50
					3:1 BAR	0.512	0.20–0.50
	496-3	0	41	45	3:1 BAR	1.557	0.20–0.50
	496-4	57	57	86	3:1 GUS	0.870	0.20–0.50
					3:1 BAR	0.870	0.20–0.50
	496-5	0	31	46	3:1 BAR	0.355	0.20–0.50
	517-5	17	17	30	1:1 GUS	0.133	0.80–0.95
					1:1 BAR	0.133	0.80–0.95
R2	495-1-5	56	56	78	3:1GUS	0.107	0.50–0.80
					3:1 BAR	0.107	0.50–0.80
	495-1-12	27	44	46	3:1 GUS	1.630	0.20–0.50
					15:1 BAR	0.018	0.50–0.80
	495-1-13	0	33	46	3:1 BAR	0.065	0.50–0.80
	496-3-1	0	72	90	3:1 BAR	0.300	0.50–0.80
	496-4-1	48	48	59	3:1 GUS	0.318	0.50–0.80
					3:1 BAR	0.318	0.50–0.80

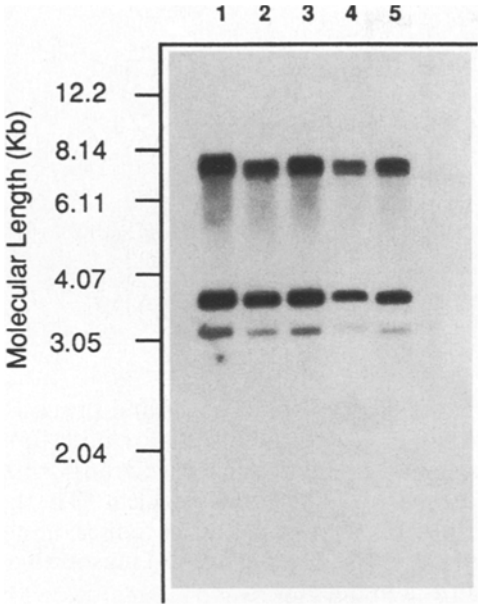

Fig. 3 Southern-blot analysis of DNA from a transgenic plan lineage (526-1) transformed with pWRG2426. DNA from R0, R1, and three R2 plants was digested with *Sac*1. The blot was hybridized with ^{32}P-labelled *gus* gene DNA. *Lane 1* is 526-1 (R0) DNA; *lane 2* is 526-1-1 (R1) DNA; *lanes 3–5* are 526-1-1-1 to 596-1-1-3 (R2) DNAs

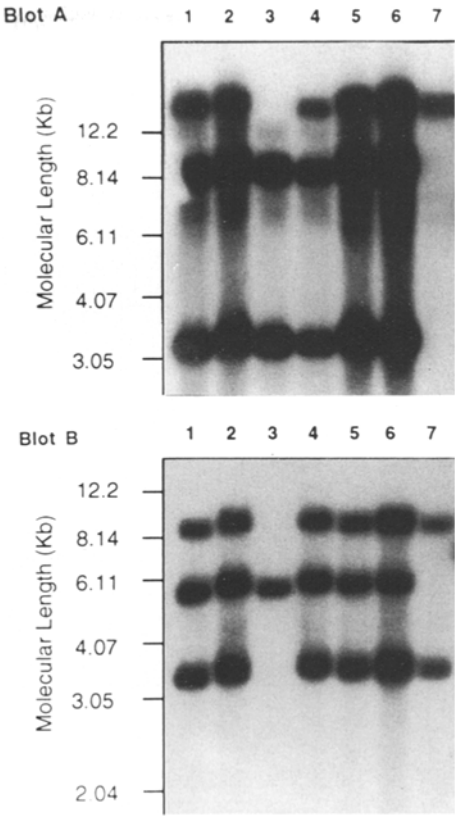

Fig. 4A,B Southern blots of transgenic rice family 495-1 containing multiple unlinked copies of pWRG2426. DNA from R1 progeny plants was digested with Sac1 (**Blot A**) or Xba1 (**Blot B**), transferred to nylon membranes, and probed with radioactively labeled *gus* gene DNA (**Blot A**) or similarly labeled *bar* gene DNA (**Blot B**). *Lane 1* contains DNA from the primary regenerant 495-1. *Lanes 2,4,5* and *6* are DNAs from individual progeny of plant 495-1 with both transgenic loci. *Lanes 3 and 7* are DNAs from individual progeny from the same transgenic parent plant with single but different loci. All plants with the single locus represented by *lane 7* were negative for *gus* expression

enzyme activity and the corresponding genes. Southern blots on R1 and R2 progeny resulted in the same banding pattern as the primary regenerate (Fig. 3). Segregation analysis from this and other systems indicated that the vast majority of loci involved in multiple integration events are genetically linked (Christou and Swain 1990; Karske et al. 1990; Saul and Potrykus1990). These Southern blots show that the majority of multiple inserts in transgenic plants are not tandem concatemeric arrays, but have multiple genomic DNA borders indicated by the numerous bands of similar intensity (Fig. 2). This suggests that the linked multiple inserts are significantly fragmented and/or separated by non-plasmid DNA. Only one of all individuals tested, plant 495-1, was shown to contain transgenic inserts at two different and segregating loci.

Analysis of a transgenic plant lineage with multiple genetic loci

It is the general rule that multiple transgenic insertions arising from direct DNA transfer segregate as a single unit (Christou and Swain 1990; Karske et al. 1990; Saul and Potrykus 1990). However, one of the pWRG2426 transformants, 495—1, demonstrated a 15:1 segregation for Basta resistance in the R1 generation, consistent with the presence of transgenic inserts at two different and segregating loci. Interestingly, *gus* expression in the same population segregated 3:1 (Table 3). Southern analyses using both *bar* and *gus* probes on multiple R1 seedlings from this individual indicated that *bar* and *gus* DNA were inherited in all progeny resulting in three distinct band patterns (Fig. 4). Two of the patterns had no common bands between them, while the third pattern exhibited a combination of the other two. These patterns represented progeny with either one or both loci, respectively. Subsequent Southern analysis (data not shown) demonstrated that at each locus there was one full size and one partial plasmid insert with non-plasmid DNA between the two inserts at both loci (Fig. 5). At one locus the partial plasmid copy lacks the *bar* gene while at the other locus the partial copy lacks the *gus* gene. The single *gus* gene at the latter locus is nonfunctional as determind by lack of GUS staining of progeny possessing only this locus. All three genotypes were resistant to Basta with no obvious difference in resistance betwen R1 plants that had either or both loci.

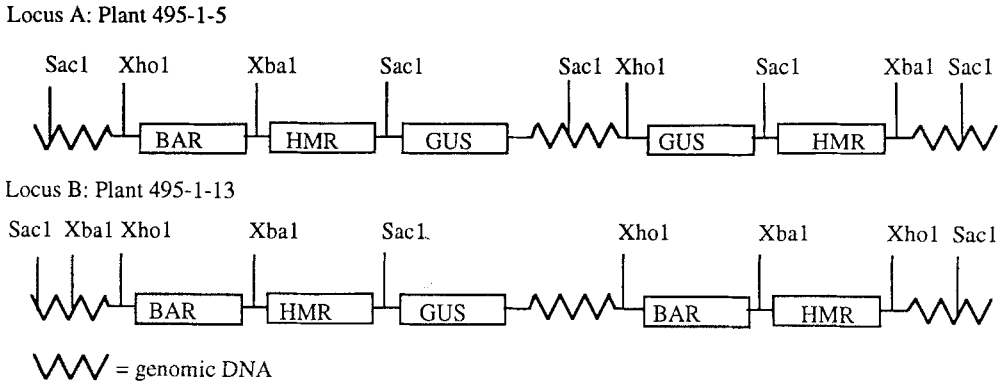

Fig. 5A, B Schematic representation of the integrative structure of two unlinked transgenic loci in plants derived from 495-1 (not to scale). The structures presented here were determined by Southern-blot analyses of progeny shown to have one of the two unlinked transgenic loci (see Fig. 4). **Locus A** (plant 495-1-5) and **locus B** (plant 495-1-13) both contain a full plasmid copy (pWRG2426) and a partial copy separated by genomic DNA. Plants containing **locus B** exclusively are negative for *gus* expression

Discussion

Southern-blot analysis of transgenic rice showed that 100% of 56 primary regenerates (R0) contained at least one copy of each input gene. Battraw and Hall (1992) also reported a 100% co-integration frequency when using linked genes to produce transgenic rice plants from protoplasts. This co-integration frequency is about 25% higher than other linked-gene transfer methods described for transgenic soybean callus (Christou and Swain 1990) and *Phaseolus vulgaris* (Russel et al. 1993). Schocher et al. (1986) reported a co-integration rate of 88% for genes co-transferred on separate plasmids into tobacco protoplasts. Similarly, all 12 transgenic rice plants described by Goto et al. (1993) contained both selected and unselected genes co-transferred on different plasmids. Others have reported lower co-integration frequencies (20–30%) using co-transformation with unlinked genes (Christou and Swain 1990; Peng et al. 1990; Rathore et al. 1993).

We observed a typical range of 1–10 copies of plasmid DNA per haploid genome. This is consistent with other reported methods of direct gene transfer (Schocher et al. 1986; Kartze et al. 1990; Linn et al. 1990; Battraw and Hall 1992; Peng et al. 1992; Goto et al. 1993). Our experiments have shown that in the vast majority of transformants, complex Southern patterns in R0, R1, and R2 generations are identical and reflect Mendelian inheritance of a single locus. The tightly linked nature of gene fragments associated with multi-copy integration events has also been reported elsewhere with both T-DNA integration (De Block and Debrouwer 1991) and direct transfer of linked (Christou et al. 1989; Battraw and Hall 1992) and unlinked genes (Kartzke et al. 1990; Saul and Potrykus 1990; Goto et al. 1993; Rathore et al. 1993). Given the higher incidence of co-integration with direct gene transfer of linked genes and the consistently tight linkage of transgenic inserts for both linked and unlinked genes, the rational for co-transformation of genes on separate plasmids is unclear. The fact that co-expression problems will further reduce the chance of obtaining useful phenotypes is good reason to optimize co-integration frequency.

Also typical of transgenic plants and plant tissues is the variation in the levels of expression of exogenous genes. These variations have been observed utilizing either T-DNA or direct DNA transfer methods and are largely attributed to a number of phenomena including, integrative fragmentation or rearrangement (Jongsma et al. 1987; Kartzke et al. 1990; Battraw and Hall 1992; Peng et al. 1992; Rathore et al. 1993) position effects (Shirstat et al. 1989; A1-Shawl 1990; Allen et al. 1993; Assad et al. 1993) and co-suppression (Jorgensen 1991; Hart et al. 1992; Allen et al. 1993; Assad et al. 1993; Rathore et al. 1993). In the present study, these variations are indiated by lack of GUS activity or suspectibility to Basta in R0 hygromycin-resistant plants.

Results from the present study suggest that the key variables affecting co-expression are inherent to the unselected gene and/or its promoter. All 35*S*-*gus* and 35*S*-*bar* constructs exhibited 66 and 90% co-expression, respectively. Plants transformed with pWRG2426 received both 35*S*-*gus* and 35*S*-*bar* as unselected transgenes on the same plasmid. However, while more than 90% of these plants expressed *bar*, only half of them expressed *gus*. These results indicate that *gus* expression may be more susceptible to truncation, position effects, and/or co-suppression than *bar* expression. The larger *gus* gene is twice as likely than *bar* to be truncated during integration based on gene size alone.

Our data indicate a relationship between copy number and gene silencing with *gus* constructs. Allen et al. (1993) also reported an inverse relationship between copy number and expression of 35*S*-*gus* transgenes in transformed tobacco NT cell lines. Linn et al. (1990) reported reduced gene expression with increased copy number in *Petunia* plants engineered with an unselected 35*S*-dihydrofoliate reductase (DFR) gene. Shirstat et al. (1989) and van der Krol et al. (1990) reported no correlation between expression and transgenic copy number of

transgenic leguminin and DFR genes, respectively, and Stockaus et al. (1987) reported an increase in gene expression with increased copy number. These apparent discrepancies support our hypothesis that factors affecting co-expression may be gene- or construct-specific.

The sensitivity of the assays used to measure and compare various unselected gene product activities and the level of gene expression required to produce detectable enzyme activity are additional factors that may affect apparent co-expression. It may be that significantly less protein is required to produce the Basta-resistant phenotype than is necessary to produce the GUS /phenotype. We have shown that choice of promoter is important in attaining detectable levels of *gus* expression. Of the tissue-specific promoters used, only the *Arabidopis ssu* promoter produced *gus* expression, albeit at a lower frequency (33%; 5 out of 15 plants examined). However, it is possible that these weak promoters would be sufficient to produce a positive BAR phenotype.

Only one multiple locus integration event was identified out of 56 independent events. The integrative stucture of both loci was determined (Fig. 5). These structures represent examples of the random nature of integration inherent to direct DNA transfer methods. If the desired transgenic phenotype is one that expresses the unselected but not the selectable marker gene, the structures we defined for the 495-1 lineage support the possibility of generating such phenotypes using linked-gene transformation. However, in our hands, production of transformants with multiple genetic loci occurred at a very low frequency.

Conclusions

The general conclusion from this study is that the molecular and genetic properties of transgenic rice plants derived through particle bombardment experiments were very smiliar to those of plants obtained through alternative direct DNA transfer procedures. The system described here, however, produced a 100% co-integration rate and 30–90% co-expression of the selectable marker gene and gene(s) of interest. The majority of transgenic rice plants recovered through particle bombardment using this method have 1–9, and less frequently ten or more, copies of the input gene per haploid genome. Transgenes were stably inherited and expression of the unselected gene(s) was consistent through subsequent generations. The results we present here confirm the utility of electric-discharge particle accelaration and cultivar-independent direct gene transfer into elite rice varieties.

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