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Transgene behaviour across two generations in a large random population of transgenic rice plants produced by particle bombardment

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Abstract The relationship between transgene copy number, rearrangement levels, inheritance patterns, expression levels, transgene stability and plant fertility was analysed in a random population of 95 independently transformed rice plant lines. This analysis has been conducted for both the selectable marker gene (*aphIV*) and the unselected reporter gene (*gusA*), in the presence or absence of flanking Matrix Attachment Regions (MARs) in order to develop a better understanding of transgene behaviour in a population of transgenic rice plants created by particle bombardment. In the first generation (T_0), all the independently transformed plant lines contained and expressed the *aphIV* gene conferring resistance to hygromycin, but only 87% of the lines were co-transformed with the unselected *gusA* marker gene. Both transgenes seemed to be expressed independently. Most lines exhibited complex transgene rearrangements as well as an intact transgene expression unit for both *aphIV* and *gusA* transgenes. Transgene copy number was proportional to the quantity of DNA used during bombardment. In T_0 plants, high *gusA* copy number significantly decreased GUS expression levels but there was no correlation between expression level and transgene copy number across the entire population of lines. Four main factors impaired transgene expression in primary transgenic plants (T_0) and their progeny (T_1): (1) absence of transgene expression in T_0 plants (41% of lines), (2) sterility of T_0 plants (28% of lines), (3) non-transmission of intact transgenes to some or all progenies (at least 14% of lines), and (4) silencing of transgene expression in progeny plants (10% of lines). Transgene stability was significantly related to differences in transgene structure and expression levels. The presence of Rb7 MARs flanking the *gusA* expression unit had no effect on plant fertility or non-transmission of transgenes, but provided copy number-dependent expression of the transgene and improved expression levels and stability over two generations. Overall, only 7% of the plant lines without MARs and 17% of the lines with MARs initially generated, exhibited stable transgene expression over two generations.

Keywords Transgene expression · *Oryza sativa* · Particle bombardment · Fertility · Matrix · Attachment Regions

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Introduction

In the past ten years there has been great progress in cereal transformation technologies. However, transgene expression in plants remains largely unpredictable, and there is considerable variation in expression levels and stability between independently transformed plants (Jones et al. 1985; Peach and Velten 1991; Walters et al. 1992). Different integration sites, copy numbers and transgenic locus configurations, as well as epigenetic silencing mechanisms, can all contribute to this variability (reviewed by Finnegan and McElroy 1994; Meyer 1995; Matzke and Matzke 1998; Iyer et al. 2000). Experimental procedures such as transformation systems (*Agrobacterium* vs direct transfer of DNA), construct configuration (Breyne et al. 1992), promoters (Mlynárová et al. 1995), coding sequences, terminators, selection strategy (Bhattacharyya et al. 1994), flanking Matrix Attachment Regions (MARs) (Mlynárová et al. 1994) or the plant tissue analysed (Ülker et al. 1999) have also been reported to influence transgene structure or expression in plants. The multiplicity of these factors, and their interactions, contribute strongly to the unpredictability, variability and instability of transgene expression in plants. This problem is particularly acute in plants generated by direct transfer of DNA (electroporation of cells and protoplasts, particle gun bombardment, silicon carbide fibres) due to the complex transgenic loci created in the plant genome. The numerous and uncontrolled transgene rearrangements, high gene copy number and systematic

transgene linkage (Lyznik et al. 1989; Gordon-Kamm et al. 1990; Wan and Lemaux 1994; Pawlowski and Somers 1996) can all favour variable and unstable transgene expression (Finnegan and McElroy 1994; Hansen and Chilton 1996; Matzke and Matzke 1998). Until recently most transgenic cereal crops were produced by technologies based on direct transfer of DNA, particularly particle gun bombardment (Christou 1996). In transgenic cereals, more than 50% of specific transgenes can be inactivated over successive generations (Pawlowski and Somers 1996; Iyer et al. 2000). High levels of transgene expression can also be hard to achieve in specific cereal tissues. These problems make molecular genetic studies difficult, and frustrate attempts at crop improvement through genetic engineering. Additionally, they create difficulties in predicting transgene behaviour when transgenes are transferred by conventional crossing, and in predicting gene flow by accidental out-crossing.

Numerous transgenic studies have been conducted in cereals; however, the relationship between transgene structure, expression level, inheritance pattern and stability in populations of transgenic plants over several generations often remains unclear. This is due to the difficulties associated with developing combined analysis of all these factors in large populations of transgenic plants over several generations. In theory, such a multi-factorial study would require most of the following: (1) production of large and random populations of independent transformation events (cell/callus lines), (2) regeneration, without phenotypic selection, of several primary plants (T_0) per transformation event, (3) study of all plants regenerated from each transformation event, expressing or not expressing the transgene(s), (4) detailed study of transgene structure (copy number, integration pattern, integration site), (5) quantification of transgene expression level during plant development using standardised conditions and protocols (controlled environment, defined plant developmental stages and tissue sampled), (6) assessment of plant development characteristics (growth, fertility), (7) study of transgene inheritance at the structural level, (8) study of transgene inheritance at the expression level, (9) study of transgene structure in the progeny, (10) quantification of transgene expression levels in the progeny, and (11) statistical analysis of these factors and their interactions. To-date, transgenic studies in cereals have addressed most of these aspects individually but rarely in combination. The absence of such multi-factorial analysis in a large and random population of transgenic plants, over generations, limits our understanding of which factors are the most significant for transgene behaviour and how these factors interact. It also limits the possibility of identifying sub-populations of plant lines with specific characteristics and behaviour. Most importantly it limits our ability to predict transgene behaviour across generations.

In the present study we have analysed the relationship, over two generations, between transgene copy number, rearrangement levels, inheritance patterns, expression level and stability for both the *aphIV* selectable marker gene and for the unselected *gusA* reporter gene, as well as plant fertility in a large random population of transgenic rice plants created by particle bombardment. This analysis has provided a better understanding of transgene behaviour in a population of transgenic plants as well as some understanding of how transgene structure and expression level can influence aspects of plant development and transgene stability.

Materials and methods

Rice transformation procedures

African elite rice (*Oryza sativa* L.) variety ITA212 was co-transformed by particle gun bombardment with the plasmid pJIC201 (ubi-5′ region :: *aphIV* :: SoyT) and one of the following plasmids: pGHNC12 (CaMV35S :: *gusA* :: nosT), or pGHNC11 (Rb7MAR :: CaMV35S :: *gusA* :: nosT :: Rb7MAR), or pGA984 (ARS1MAR :: CaMV35S :: *gusA* :: nosT :: ARS1MAR), as previously described (Vain et al. 1999). Independently transformed rice callus lines were selected for hygromycin resistance. Five transgenic T_0 plants were regenerated from each callus line (i.e. a transformation event) representing an independent plant line. Transgenic plants were transferred to a controlled environment room for growth to maturity. All transgenic plants produced were used in further experiments to ensure the study of randomised independent transformation events with the widest spectrum of expression for the non-selected *gus*A gene.

Analysis of GUS activity

Fluorometric analysis for β-glucuronidase activity was carried out on leaf tissue from rice plants at the five-leaf stage, following the method of Jefferson (1987). Fluorescence was measured using a Titertek Fluoroskan II after 0-, 30- and 60-min incubation. Each assay was performed in triplicate. Protein content was determined using a Bio-Rad protein assay kit. Data were expressed as pmol of 4-methylumbelliferone (MU) min^{-1} mg^{-1} of extracted protein. GUS activity was measured in five different T_0 plants and eight different T_1 plants for each independent line. The background activity (33 ± 4 pmol MU min^{-1} mg^{-1} protein) was subtracted from all fluorometric GUS measurements as previously described (Vain et al. 1999).

Detection of transgenic plants by the Polymerase Chain Reaction (PCR)

DNA was isolated from rice plants and PCR reactions were carried out as previously described (Vain et al. 1998). Three primer sets were used on each DNA sample: (1) one to amplify the 1,200-bp single-copy rice RFLP probe C213 (forward: 5′-AAAGGACCGGAATGACCACAA-3′; reverse: 5′-GAATGAACCACGCCCAAGAGT-3′) in order to ensure that each DNA sample was suitable for PCR amplification, (2) another to amplify a 1,271-bp fragment containing the *aphIV* gene (forward: 5′-ACTCACCGCGACG TCTGTCG-3′; reverse: 5′-GATCTCCAATCTGCGGGATC-3′), (3) the other to amplify a 2,038-bp fragment of the CaMV35S :: *gusA* expression cassette (forward: 5′-CCCACCCACGAGGAGCAT-3′; reverse: 5′-GCGCCAGGAGAGTTGTTGATT-3′). Additional PCR primers nested within the *aphIV* or *gusA* expression cassettes were used to amplify smaller fragments within these regions.

Gene copy number analysis

Genomic DNA extraction and Southern analyses were performed on primary transformed rice plants (T_0) as previously described

(Vain et al. 1999). Membranes were hybridised with probes generated by PCR amplification of 701 nt of the *gusA* gene or 981 nt of the *aphIV* gene, or with the R2272 rice RFLP probe (to control DNA loading). The filters were analysed by autoradiography followed by densitometry (Vain et al. 1999). Final copy number was calculated by linear regression analysis based upon hybridisation signals obtained from the reconstitution standards and normalisation by DNA loading (Vain et al. 1999).

Statistical analysis

Statistical analyses, following the requirements of each test, were performed using Minitab 13.1 and Genstat 5 software. The normality of distribution of each data set was evaluated employing the Anderson Darling test. Variances were compared using the Levene's test. Data sets were compared using ANOVA. Data sets not meeting ANOVA requirements were compared using the non-parametric Kruskal-Wallis and Mann-Whitney tests. Linear regression analysis was only performed on normally distributed data sets.

Transgene inheritance study

T_1 seeds were obtained by self-pollination of primary transformed rice (T_0) plants.

Segregation analysis at the structural level was conducted by germinating seeds on MSR6 medium (Vain et al. 1998) without hygromycin and PCR analyses were performed to test for the presence of the *aphIV* and *gusA* transgenes in up to 40 random T_1 seedlings from each T_0 plant. When PCR reactions were negative for the *aphIV* and *gusA* transgenes, PCR analysis was conducted for the presence of the C213 RFLP probe to confirm that DNA extractions were suitable for PCR amplification.

Segregation analysis at the expression level was assessed qualitatively by histochemical GUS staining (Jefferson et al. 1987) of the T_1 seed endosperm and germination of the corresponding isolated embryo on hygromycin-containing medium (MSR6H50, Vain et al. 1998). All plant lines exhibiting skewed segregation at the expression level were re-analysed at the structural level as described above.

Results and discussion

Transgene structure in T_0 rice plants

Transformed rice plants were regenerated from 95 independent transgenic callus lines co-bombarded with a plasmid containing the *aphIV* hygromycin resistance gene (PJIC201) and a plasmid containing the *gusA* reporter gene, either as a simple expression cassette (pGHNC12) or flanked by the Rb7 MARs from tobacco (pGHNC11) or by the ARS1 MARs from yeast (pGA984). PCR and Southern analyses showed that all the transgenic plant lines contained the *aphIV* gene, but that only 87% of the lines (83 out of 95 lines) contained the unselected *gusA* reporter gene (Table 1). Such a high co-transformation frequency is common in transgenic plants transformed by direct transfer of DNA (Lyznik et al. 1989; Gordon-Kamm et al. 1990). It may result from

Table 1 Transgene copy number and expression in T_0 and T_1 rice plants. *n*: number of independently transformed plant lines. *ne*: number of plant lines expressing the *gusA* gene. *nf*: number of fertile plant lines (expressing and not expressing the *gusA* gene). *nfh*: number of fertile plant lines which transmit intact transgenes to subsequent T_1 generation (expressing and not expressing the *gusA* gene). + in pmol of MU min^{-1} mg^{-1} of extracted protein. Mean: row entries followed by different letters (a/b) are significantly different at $P < 0.05$ by ANOVA. Data sets not meeting ANOVA requirements were analysed using Kruskal-Wallis and Mann-Whitney tests. CV (Coefficient of variation): row entries followed by the same letter are not significantly different at $P > 0.05$ using Levene's test performed on raw data expressed as a percentage of the mean

1st Plasmid (P1): 2nd Plasmid (P2):		PJIC201 (*aphIV*) pGHNC12 (*gusA*)	PJIC201 (*aphIV*) pGHNC11 (Rb7-*gusA*-Rb7)	PJIC201 (*aphIV*) pGA984 (ARS1-*gusA*-ARS1)
Lines with P1 only	*n*	3	3	6
Lines with P1 + P2	*n*	29	29	25
gusA copy number	*n*	29	29	25
	Mean	34 a	24 ab	15 b
	CV	84 a	95 a	74 a
aphIV copy number	*n*	29	29	25
	Mean	6 a	10 a	8 a
	CV	69 a	73 a	67 a
GUS expression levels (T_0) +	*n*	29	29	25
	Mean	1,158 a	2,935 b	3,548 b
	CV	141 a	93 a	104 a
	ne	17	24	18
	Mean	1,964 a	3,545 ab	4,924 b
	CV	88 a	73 a	71 a
Fertility (T_0)	*nf*	16	17	18
(# of seeds)	Mean	84 a	97 a	116 a
	nfh	12	11	11
	Mean	107 a	73 a	146 a
	CV	98 a	58 a	77 a
GUS expression levels (T_1) +	*n*	5	9	7
	Mean	1,711 a	2,801 a	3,829 a
	CV	96 a	88 a	82 a
Fertility (T_1)	*nfh*	12	11	11
(# of seeds)	Mean	144 a	167 a	174 a
	CV	97 a	65 a	77 a

either extra-chromosomal recombination before integration, duplication of the integrated transgenes or multiple adjacent integration sites. The random population of 83 co-transformed lines was composed of the following: (1) 29 independent plant lines without MARs (PGHNC12), (2) 29 independent plant lines containing Rb7 MARs (pGHNC11), and (3) 25 independent plant lines containing ARS1 MARs (pGA984) (Table 1).

Each independent line was assessed by Southern analysis to characterise the integration patterns and copy number of the *gusA* and *aphIV* genes (Fig. 1). Genomic DNA from the plant lines without MARs was digested using flanking restriction enzymes expected to release a transgene fragment of discrete size (Vain et al. 1999). The first probing was performed using the R2272 RFLP probe in order to normalise genomic DNA loading. The second probe used corresponded to the *gusA* gene (Fig. 1A). More than 80% of the lines exhibited complex banding patterns in addition to the expected intact 35S :: *gusA* :: nosT unit (overall 5.72 ± 1.25 bands per transformed line, $P < 0.05$). Many lines exhibited large variation in the relative intensity of bands composing their hybridization pattern. Such complex integration patterns are commonly observed after direct DNA transfer and reflect complex transgenic loci containing multiple intact and/or modified copies of the transgene as well as interspersed genomic DNA (Lyznik et al. 1989; Gordon-Kamm et al. 1990; Svitashev and Somers 2001). However banding patterns should be interpreted with caution as they rarely allow complete and true reconstitution of the transgenic locus configuration generated by particle bombardment. The CaMV35S promoter has been described as a possible hot spot for recombination in transgenic rice plants (Kohli et al. 1999). In our study, comparison between *Eco*RI/*Hin*dIII digests (releasing the entire 35S :: *gusA* :: nos expression unit) and *Eco*RI/*Xba*I digests (releasing the *gusA* :: nos sequence) suggested that 41% of the lines showed rearrangements compatible with recombination in the CaMV35S promoter region leading to head to head expression units (Fig. 1A). The third probe used corresponded to the *aphIV* gene (Fig. 1B). Most lines contained an intact 1.3-kbp fragment corresponding to the *aphIV* gene but also additional bands of higher and lower molecular weight (4.77 ± 1.24 bands per transformed line, $P < 0.05$).

The presence of flanking MARs appeared to generate less complex banding patterns for the *gusA* gene. However, no significant difference could be found in the number of hybridizing bands in the presence or in the absence of MARs, for either the *gusA* gene (flanked by MARs) or the *aphIV* gene (unflanked by MARs) (Kruskal-Wallis and Mann-Whitney tests on the number of bands in the presence vs the absence of MARs, $P < 0.05$).

Across the entire set of 83 independent lines, with or without MARs, the integration patterns of the *gusA* gene (4.49 ± 0.64 bands per transformed line, $P < 0.05$) were significantly more complex than those of the *aphIV* gene (3.55 ± 0.58 bands per transformed line, $P < 0.05$) (Mann-Whitney test, $P < 0.05$). However, there was no significant correlation between the complexity of the integration pattern (i.e. the number of bands) of the *aphIV* and the *gusA* genes across the independent transgenic lines (Fig. 2A).

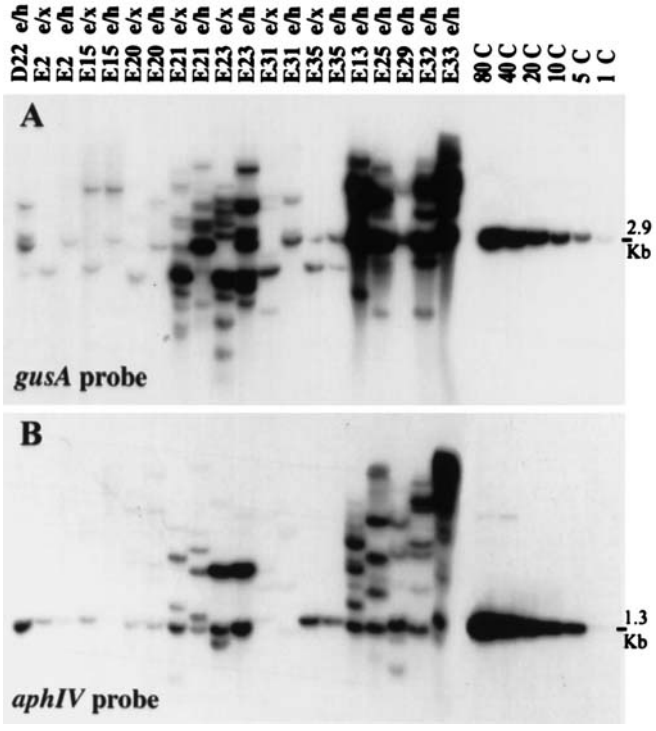

Fig. 1A, B Southern-blot analysis of transformed rice plants (T_0). Southern blots of independent transgenic plant lines co-transformed with pGHNC12 and PJIC201. Membrane **A** was probed with the *gusA* gene and membrane **B** was probed with the *aphIV* gene. Plant genomic DNA and reconstitution standards were digested using a combination of flanking restriction enzymes [*Eco*RI + *Hin*dIII (e/h) or *Eco*RI + *Xba*I (e/x)] to release a discrete size fragment from the *gusA* or *aphIV* expression unit (Vain et al. 1999). Reconstitution standards were prepared by serial dilution of pGHNC12 and pJIC201 plasmids into wild-type genomic DNA so as to introduce 1 to 80 *gusA* and *aphIV* gene copies per 2C equivalent. Expected discrete fragment sizes are indicated

Transgene copy number in T_0 rice plants

The *gusA* and *aphIV* gene copy numbers were determined for each transformed line by Southern analysis followed by densitometry. For most lines, genomic DNA of T_0 plants was analysed using two different restriction digests (Fig. 1). The error in copy number evaluation was estimated by the variation in a *gusA* positive control line present on each blot (10 ± 2.4 copies; $P < 0.05$, Vain et al. 1999). Previous studies showed that gene copy number-determination by Southern analysis plus densitometry and by quantitative PCR techniques were convergent and reliable in tobacco plants transformed with the pGHNC12 gene construct (Ülker et al. 1999). In the present study, the number of bands was not used to calculate the transgene copy number due to lack of reliability.

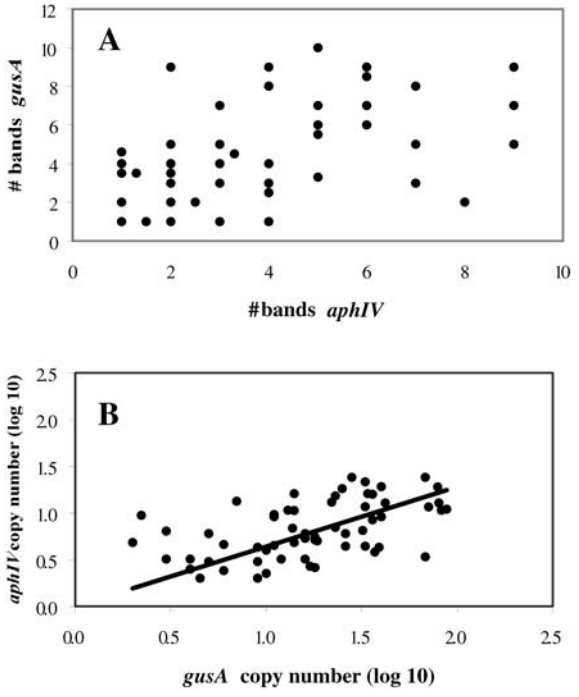

Fig. 2A, B Relationship between *gusA* and *aphIV* gene hybridisation pattern and copy number in T_0 rice plant lines. **A** Banding patterns were obtained from Southern analysis as described in Fig. 1 using restriction enzymes that are expected to release a transgene fragment of discrete size. **B** Linear regression analysis of *gusA* and *aphIV* gene copy numbers was performed using all lines with or without MARs containing both transgenes. Data were log10 transformed to ensure normality of distributions. Each data point represents one independent plant line

The *gusA* and *aphIV* gene copy numbers are shown in Table 1. In the population without MARs, the transformed plant lines contained on average 34 *gusA* and 6 *aphIV* gene copies. Forty one percent of these lines contained 1 to 20 *gusA* copies, 32% contained 20 to 40 *gusA* copies and 27% contained more than 40 *gusA* copies; 76% of the lines contained 1 to 10 *aphIV* copies and 24% of the lines contained 10 to 20 *aphIV* copies.

As previously reported (Vain et al. 1999), the presence of flanking MARs tended to reduce average *gusA* gene copy number (Table 1). However, it is difficult to draw conclusions from these average figures as very often the further relationship between copy number and other factors, such as transgene expression level, is not uniform across the entire population of transformants (Ülker et al. 1999; Vain et al. 1999). As expected, the *aphIV* gene copy number was not significantly different in the presence or absence of MARs (Table 1).

Across the entire set of 83 independent lines with or without MARs, there was a significant correlation (r = 0.33, $P < 0.01$) between the *aphIV* and *gusA* gene copy numbers (Fig. 2B). The rice plants also contained significantly more *gusA* gene copies (average of 25) than *aphIV* gene copies (average of 8) (Mann-Whitney test, $P < 0.05$). Interestingly, this ratio corresponded to the 3:1 molar ratio of *gusA*- and *aphIV*-containing plasmids used in the transformation experiments. This suggests that the number of gene copies integrated into the plant genome after particle bombardment may be directly proportional to the quantity of DNA used during the transformation process. This may provide a means of decreasing transgene copy number in transformation experiments by decreasing the quantity of DNA delivered during each shot.

Transgene expression in T_0 rice plants

Expression levels in transformed rice plants from each independent line were characterised by fluorometric GUS assay. To minimise the influence of environmental conditions, five different T_0 plants regenerated from each independent line were grown in a fully controlled growth room and analysed at the same developmental stage (five-leaf stage). Standardization of quantification of transgene expression was of particular importance as expression levels can vary during the plant life cycle and between leaves/tillers at different developmental stages (data not shown).

The average GUS expression level in the lines without MARs was 1,158 pmol MU min^{-1} mg^{-1} of protein, and varied from 0 to about 5,000 pmol MU min^{-1} mg^{-1} of protein (Fig. 3, Table 1). Despite the presence of the *gusA* transgene in all these plant lines, 41% of the transformed lines (12/29) did not exhibit GUS activity. The absence of transgene expression in primary transgenic plants is often the main factor impairing overall transgene expression in populations of plant lines generated by particle bombardment (Gordon-Kamm et al. 1990; Register et al. 1994; Vain et al. 1999). The absence of transgene expression can result from a combination of structural and epigenetic mechanisms (Finnegan and McElroy 1994; Meyer 1995; Matzke and Matzke 1998; Iyer et al. 2000). Transgene expression levels in the remaining population of expressing lines (17/29) did not exhibit obvious distribution discontinuity (Fig. 3). There was large variation in transgene expression levels between independent plant lines (coefficient of variation inter-expressing lines = 73%, Table 1). The overall expression profile of the population of independent plant lines without MARs was comparable to those previously published in other transgenic studies (Jones et al. 1985; Gordon-Kamm et al. 1990; Peach and Velten 1991). The variability of GUS activity among the five T_0 plants regenerated from each line was strongly dependent upon the mean (significant correlation of intra-line variance and mean expression level r = 0.82, $P < 0.001$) and therefore was assessed using the coefficient of variation (CV). The average CV among T_0 plants regenerated from the same line was 50% ± 15% ($P < 0.05$, Fig. 3). This variability could mostly be attributed to inter-experiment variations of GUS assay measurements, as similar CV values (40% to 60%) were obtained among 43 wild-type and 18 positive control plants, respectively, across different experiments.

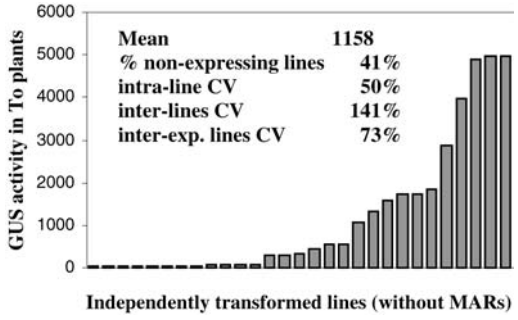

Fig. 3 Distribution of transgene expression levels in T_0 transgenic rice plant lines. Each data point represents the average *gusA* expression level of five T_0 rice plants from each independent line transformed with the pGHNC12 (35S :: *gusA* :: NOS) construct. GUS activity is expressed in pmol MU min^{-1} mg^{-1} of protein. *CV* = coefficient of variation (standard deviation/mean). *Exp* = expressing the *gusA* gene

In the population of plant lines without MARs, the *aphIV* and *gusA* genes appeared to be expressed independently since all lines exhibited strong hygromycin resistance but only 59% of the lines expressed the *gusA* gene. Independent expression of co-transformed transgenes is common to many transformed plants (Gordon-Kamm et al. 1990; Mlynárová et al. 1995; Vain et al. 1998). There was no significant difference in *aphIV* copy number or fertility between lines expressing and not expressing the *gusA* transgene ($P > 0.05$, ANOVA). However, non-expressing lines contained significantly more *gusA* gene copies (52 copies) than expressing lines (23 copies) ($P = 0.016$, ANOVA). This suggests that high gene copy number could affect transgene expression levels.

As previously reported (Vain et al. 1999), the presence of flanking MARs had a pronounced effect on transgene expression in T_0 rice plants and significantly altered the expression profile of the population of transgenic plant lines. Flanking MARs significantly reduced the occurrence of non-expressing lines from 41% (12/29) in the absence of MARs, to 17% (5/29) and 28% (7/25) for Rb7 and ARS1 MARs respectively (Table 1). Flanking MARs also significantly increased average GUS activity by 2.8-fold in the overall populations and by 2.1-fold among expressing lines (n and ne in Table 1). Flanking MARs also increased the maximal level of *gusA* expression by up to three-fold. Variation in GUS expression levels between independent lines was similar both in the presence and in the absence of MARs (CVs not significantly different, Table 1). This absence of normalisation of transgene expression at the population level is not surprising. As hypothesised by the loop model (Mirkovitch et al. 1984), flanking MARs should reduce the variability of transgene expression between independent transformants carrying the same number of active transgene copies. The expression levels of transgenes flanked by MARs should also vary in direct proportion to the active copy number. Such MAR effects should not necessarily lead to a decrease in the variability of transgene expression in the entire population of transgenic lines containing MARs. When transgenic plants are produced by direct transfer of DNA, the range of copy numbers is so broad that the copy number dependence of transgene expression provided by flanking MARs can increase the maximal expression level and is therefore not expected to (and generally doesn't) decrease the variability of transgene expression in the entire population of transgenics (Allen et al. 2000). Only when the range of transgene copy numbers is limited (e.g. after *Agrobacterium*-mediated transformation) can the copy number-dependent and position-independent transgene expression produced by MARs lead to an overall decrease of transgene expression variability in the entire population of transgenic plants (Mlynárová et al. 1994).

Among the entire set of 83 independent plant lines, with or without MARs, there was no significant difference in *aphIV* copy number or fertility between lines expressing and not expressing the *gusA* transgene ($P = 0.246$ and $P = 0.29$, respectively, ANOVA). However, non-expressing transgenic plant lines contained significantly ($P = 0.003$, ANOVA) higher *gusA* copy numbers (39 copies) than expressing ones (20 copies).

Transgene expression vs copy number in T_0 rice plants

Comprehensive analysis of the relationship between *gusA* gene copy number and expression levels was carried out for each independently transformed plant line (Vain et al. 1999). In the population of lines without MARs, despite the fact that non-expressing lines contained significantly more *gusA* gene copies than expressing lines (see previous section), there was no correlation between expression level and *gusA* gene copy number across the entire population of lines without MARs ($r = 0.3$, $P > 0.05$). This is comparable to previous studies of transgenic cereals transformed by direct transfer of DNA (Linn et al. 1990). Flanking Rb7 MARs provided copy number-dependent expression of the *gusA* transgene (up to 20 copies), but expression was generally reduced in lines carrying a higher copy number (Vain et al. 1999) probably due to silencing phenomena or transcriptional limitations (Mlynárová et al. 1995; Allen et al. 2000). This is in agreement with parallel studies using flanking Rb7 MARs in tobacco plants (Ülker et al. 1999). Overall Rb7 MARs exhibited some, but not all, of the characteristics predicted by the loop model (Mirkovitch et al. 1984). In contrast, the ARS1 MARs had a more limited "MAR effect" by exhibiting a less significant copy number-dependence of transgene expression (Vain et al. 1999).

Transgene inheritance at the structural level

T_1 seeds were obtained by self-pollination of primary transformed rice (T_0) plants. Only 61% of the initial co-transformed plant lines (51 out of 83 lines) were sufficiently fertile (i.e. more than 40 seeds) to allow further inheritance analysis (nf in Table 1).

Transgene inheritance at the structural level was analysed in up to 40 T_1 plants from each of 20 lines and in 5 to 8 T_1 plants from each of the remaining 31 fertile lines. Seeds were germinated without hygromycin selection and tested for the presence of the *gusA* and *aphIV* genes by PCR analysis (data not shown). Around 65% of the fertile lines analysed exhibited Mendelian inheritance of the *gusA* and *aphIV* transgenes at the structural level. Segregation frequencies indicated that transgenes were generally linked and integrated at only one locus (3:1 ratio after self-pollination). Two lines showed transgene integration in at least two unlinked loci (15:1 ratio after self-pollination) each containing both *gusA* and *aphIV* transgenes. When transmitted to the offspring, the *gusA* and *aphIV* transgenes always co-segregated in the T_1 progenies. Skewed segregation at the structural level was observed in around 35% (17/51) of the fertile lines tested. The progenies from these transformation events were analysed using PCR primers aimed at amplifying different regions of the *gusA* or *aphIV* transgenes. Detailed structural analysis showed that many plant progeny did not contain, or only contained fragments, of the *gusA* and/or *aphIV* transgenes. Among fertile lines, 18% (9/51) showed no transmission of *gusA* nor *aphIV* transgenes to any of their progeny, despite repeated confirmation that both intact transgenes were present in the parent T_0 transgenic plants by Southern analysis (Fig. 1) and by expression studies (Table 1). Six percent (3/51) of the lines (such as D28 described in the next section) exhibited transgene inheritance at a significantly lower frequency than 3:1 (as expected after self-pollination) but transgenes were co-inherited without detectable alteration of expression units. Ten percent (5/51) of the lines exhibited complex segregation patterns with some T_1 plants containing no transgene and others containing only fragments of the original *gusA* and/or *aphIV* transgenes. The occurrence of lines with this latter type of behaviour suggested that some transgenic loci generated by particle bombardment are likely to be altered from one generation to the next through recombination or deletion. Such processes could also have occurred without being detected in high-copy number-lines exhibiting Mendelian inheritance, as long as at least one intact expression unit of each transgene was inherited. Interestingly, the fertile lines showing non-transmission of intact transgenes to some or all progenies (17/51) exhibited significantly lower fertility (51 vs 124 seeds, $P = 0.007$, ANOVA) but the same *gusA* and *aphIV* gene copy number (23 vs 26 *gusA* gene copies and 10 vs 7 *aphIV* gene copies, $P > 0.05$, ANOVA) and the same *gusA* expression level (4,651 vs 3,703 pmol MU min^{-1} mg^{-1} of protein, $P = 0.176$, ANOVA) as lines transmitting intact transgenes. Similar loss of transgenes from one generation to the next has also been reported in transformed maize plants (Walters et al. 1992) and associated with low plant fertility (Register et al. 1994).

Non-transmission of intact transgenes to some or all progenies affected at least 14% of the lines in the population of plants without MARs. The presence of flanking MARs had no significant effect on this situation (21% and 28% were affected by poor transgene transmission in the presence of Rb7 and ARS1 MARs respectively). Non-transmission of intact transgenes to some or all progenies was a key factor in generational transgene instability. When it occurs at a low frequency it can easily been confused with levels of gene silencing if no structural study is conducted in parallel to the segregation study at the expression level. It can also be mistaken for out-segregation of co-transformed transgenes unless multi-primer PCR or Southern analysis is conducted on progenies. Study of transgene stability at the structural level was a prerequisite for characterising other forms of instability at the expression level, such as reduced expression levels or gene silencing.

Generational stability of transgene expression

Stability of transgene expression was first assessed by segregation analysis, then by quantitative measurement of transgene expression levels in progenies. Only 51 of the initial 83 co-transformed plant lines exhibited sufficient levels of fertility (i.e. more than 40 seeds) and only 34 of these fertile lines transmitted both transgenes to their progenies, allowing monitoring of *aphIV* and *gusA* gene expression across generations. Twenty one of the 34 lines expressed the *aphIV* and *gusA* genes in T_0 plants allowing inheritance studies of transgene expression to be conducted in parallel for both transgenes (Fig. 4A). The remaining 13 lines expressed the *aphIV* gene but not the *gusA* gene in T_0 plants, and therefore a transgene inheritance study at the expression level could only be conducted for the *aphIV* gene (Fig. 4B). More than 5,100 segregating T_1 seedlings were tested for expression of both transgenes in the T_1 generation. Segregation frequencies indicated that 28 (82%) and 17 (81%) lines showed Mendelian inheritance (1 or 2 loci, $P < 0.05$, chi-square analysis) at the expression level for the *aphIV* and the *gusA* genes, respectively (Fig. 4). Most of the remaining lines showed skewed inheritance at the expression level for only one of the transgenes. Interestingly, significantly more non-expressing GUS lines (4/13) exhibited skewed segregation for hygromycin resistance than did expressing lines (2/21) ($P < 0.05$, chi square analysis, Fig. 4A vs 4B). This suggests that inactivation of one transgene (*gusA*) could be associated with the destabilisation of expression of the other transgene (*aphIV*) co-integrated at the same locus (Fig. 4B). Segregation frequencies could not be correlated with the variability of GUS activity (CV) among T_0 plants regenerated from each line ($r = 0.16$, $P > 0.05$). However, lines with skewed segregation exhibited significantly higher intra-line *gusA* expression variability in parent T_0 plants than lines with Mendelian inheritance (Mann-Whitney skewed vs non-skewed segregation, $P = 0.014$). This suggests that variability of transgene expression among clonal T_0 plants may be a sign of further generational transgene instability.

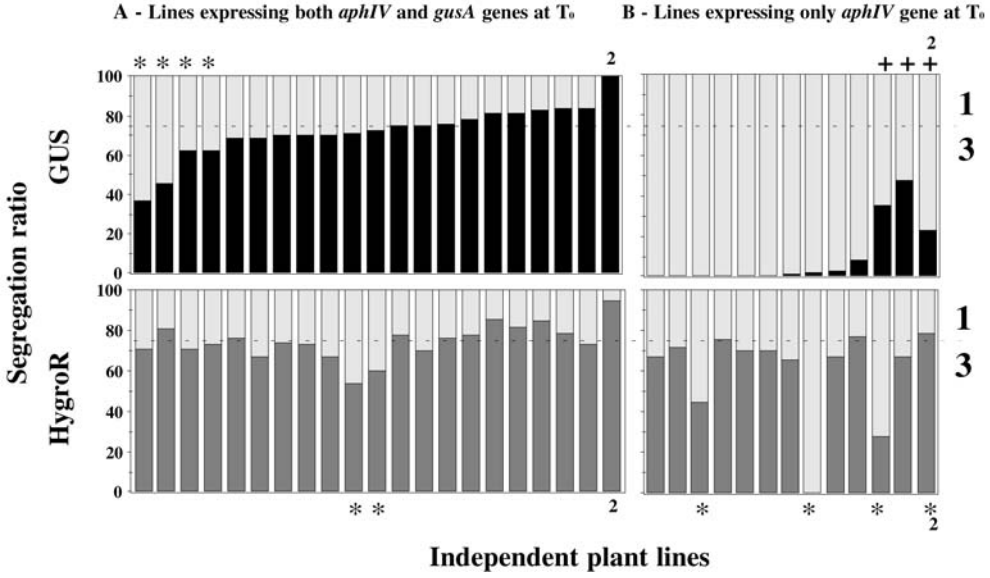

Fig. 4A, B Segregation analysis of transgene expression in T_1 transgenic rice seedlings. Each bar represents the segregation ratio of at least 40 seedlings expressing (in *black* or *dark grey*) or not expressing (in *light grey*) the transgenes. Among the 51 fertile plant lines with or without MARs studied, 17 lines did not transmit intact transgenes to their progeny (data not represented here), 21 lines expressed both the *gusA* and *aphIV* gene in the T_0 plants (**A**) and 13 expressed the *aphIV* but not the *gusA* gene in T_0 plants (**B**). *Star* (*) indicates the skewed segregation ratios significantly different from Mendelian 3:1 or 15:1 ratios (chi-square test, $P < 0.05$). *Cross* (+) indicates lines not expressing the *gusA* gene in T_0 and T_1 plants but expressing the *gusA* gene in vitro and in T_1 seeds. Two (*2*) indicates lines containing two independent transgenic loci

Among the 51 fertile lines with or without MARs, almost half of the lines showed skewed segregation at the expression level for the *aphIV* gene (45% of the lines) or the *gusA* gene (50% of the lines). This is comparable to other inheritance studies measured by the transgene phenotype in cereals (Register et al. 1994; Pawlowski and Somers 1996). In this study, deviation from Mendelian segregation of transgenes at the expression level was due to a combination of poor transmission of the transgene(s) (see previous section) and/or transgene silencing. In line D28, among 41 T_1 plants tested for the presence and expression of the *aphIV* and *gusA* genes, 44% (19/41) contained and expressed the transgenes, 10% (4/41) contained but did not express the transgenes (silencing) and 46% (18/41) did not contain the transgenes (25% expected to be non-transformed segregants + 21% non-transmission of transgenes). In lines such as D28 the non-transmission of transgenes (21% of T_1 plants) had a greater impact than transgene silencing (10% of T_1 plants) on skewing segregation ratios determined at the expression level. Skewed segregation of transgene expression was observed in the presence or in the absence of MARs suggesting that MARs do not eliminate skewed inheritance or transgene silencing.

Stability of transgene expression level was then assessed by comparison of GUS activity in T_0 and T_1 plants from the 21 lines containing and expressing the transgenes at both generations. Lines exhibiting Mendelian segregation at the structural level and skewed segregation at the expression level systematically showed reduced or silenced transgene expression levels in some T_1 plants. In the absence of MARs (pGHNC12), g*usA* expression levels were significantly heritable ($P < 0.001$); however, there was a significant 35% reduction of transgene expression levels in T_1 plants compared to the parental T_0 plants (Vain et al. 1999). Three lines exhibited an unstable transgene expression level in the progeny due to gene silencing. The overall impact of gene silencing in the entire population of plant lines without MARs (29 lines) was therefore around 10%.

As previously reported (Vain et al. 1999), the presence of flanking Rb7 MARs appeared to significantly improve the stability of transgene expression levels over two generations at the population level. These observations are similar to those seen in parallel studies in tobacco plants using the same experimental procedure (same gene construct introduced by particle bombardment, Ülker et al. 1999). Nevertheless in rice, generational instability of transgene expression was not eliminated by the presence of flanking MARs. In the population of plant lines containing MARs, 13% of the lines (7/54) exhibited an unstable (e.g. reduced or silenced) transgene expression level in the progeny. However, among these lines, only 7% (4/54) showed transgene silencing in some or all progenies. The remaining 6% (3/54) of lines showed only a decrease in transgene expression without any progeny being silenced. The overall benefit of flanking MARs in a population of transgenic rice plants was therefore mostly to increase the occurrence of stable lines (from 7% to 17%) rather than to reduce the production of unstable lines (from 10% silenced to 7% silenced plus 6% reduced expression). In other plant species, MARs have been shown not to protect

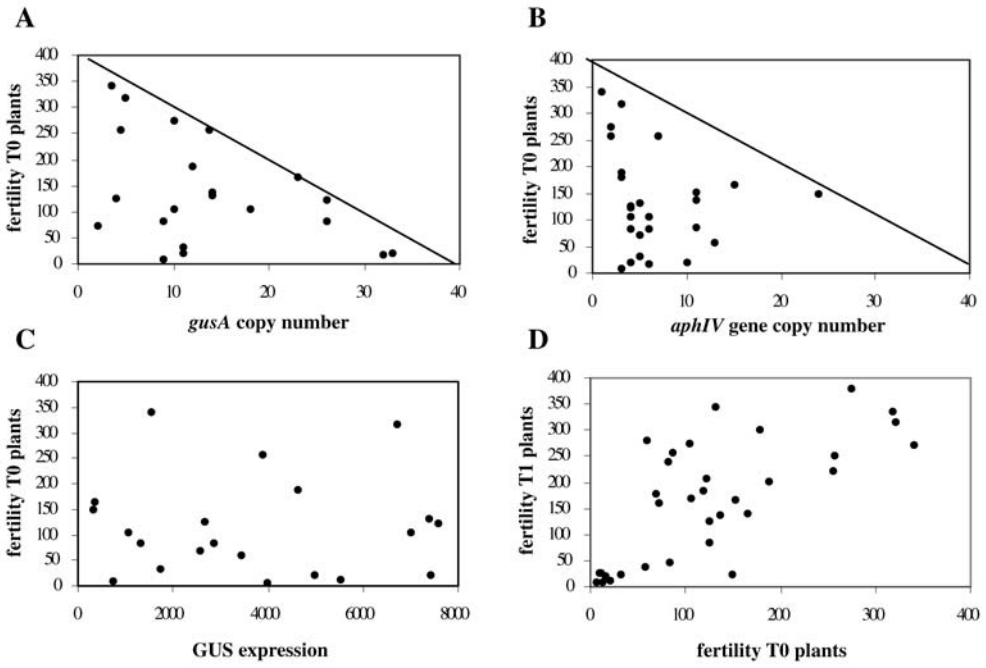

Fig. 5A–D Factors influencing T_0 and T_1 rice plant fertility. Fertility of each independently transformed plant line was determined by the average number of seeds produced by five T_0 and eight T_1 rice plants from each line. **A** and **B** Relationship between transgene copy number and T_0 rice plant fertility. **C** Relationship between transgene expression level and T_0 rice plant line fertility. **D** Fertility of T_0 vs T_1 rice plants. Each data point represents one independent plant line

against strong post-transcriptional gene silencing (Allen et al. 2000).

In the 21 lines, with or without MARs, expressing both the *aphIV* and *gusA* genes in T_0 and T_1 plants, a decrease/silencing of transgene expression across generations seemed independent of *gusA* expression level, *aphIV* copy number and fertility (Kruskal-Wallis and ANOVA tests on stable vs unstable lines, $P > 0.05$). However transgenic lines with unstable GUS expression contained significantly higher *gusA* copies than lines with stable GUS expression (average of 23 and 11 *gusA* copies respectively, Kruskal-Wallis and ANOVA, $P = 0.039$). This suggests that high copy number might interfere with transgene stability over generations. The importance of transgene copy number on transgene expression stability was confirmed when lines exhibiting other forms of instability such as those at the structural level (e.g. non-transmission of intact transgenes to some or all progenies) were also included in the analysis. Lines showing instability at the structural or expression levels contained significantly more *gusA* and *aphIV* gene copies (Kruskall-Wallis unstable vs stable lines $P = 0.032$ and $P = 0.017$ respectively).

Fertility of transformed rice plants

The fertility of transformed rice plants from each independent line was measured over two generations. In the first generation (T_0 plants), 55% (16/29) of independently transformed lines without MARs consistently produced fertile plants (84 seeds per T_0 plant, Table 1). The remaining lines (13/29) were sterile. In this population, 28% (8/29) of the lines were sterile expressers. Across the entire set of 83 independent lines with or without MARs, 61% (51/83) of the lines consistently produced fertile plants (nf in Table 1). There was no significant difference in fertility levels between the expressing (89 seeds per plant line on average) and non-expressing lines (120 seeds per plant line on average) ($P = 0.225$, ANOVA). Only 34 of the 51 fertile lines exhibited inheritance of both transgenes allowing monitoring of plant fertility in transformed plants across generations (nfh in Table 1). The presence of flanking MARs did not affect the fertility of transgenic plants ($P = 0.553$, ANOVA). Among lines, with or without MARs, there was no significant difference in GUS activity between sterile and fertile plants ($P = 0.132$, ANOVA). There was no correlation ($r = 0.009$, $P > 0.1$, Fig. 5C) between the fertility level of the transgenic plants and the expression level of the *gusA* gene. There was also no significant difference between fertile and sterile plants for *gusA* and *aphIV* copy number ($P > 0.05$, ANOVA). However, high *aphIV* or *gusA* gene copy numbers were never associated with high fertility (Fig. 5A and B). This suggests that high transgene copy number could interfere with gamete or seed development/viability. Since each primary transformant is hemizygous for the transgene(s), it is possible that long stretches of foreign DNA with no homology to the homoeologous chromosome might interfere with meiosis. In the second generation, the fertility of T_1 plants (161 seeds per plant line on average) was significantly improved (by 30%) when compared to the fertility of the T_0 plants ($P = 0.01$, *t*-test of means on fertility T_0 to fertility T_1 across independent lines). Improvement in fertility level in the progeny of primary transgenics has often been reported in studies on transgenic cereals, but this was, however, slight in the present study. Among the

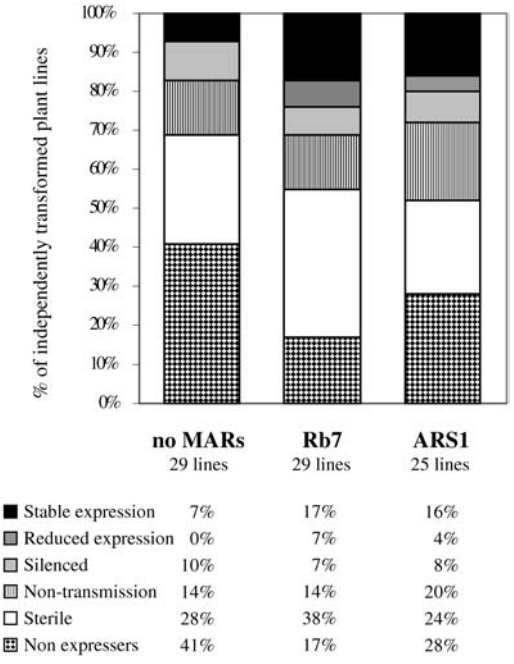

Fig. 6 Generational stability of transgene expression in rice plant lines, in the presence or in the absence of flanking MARs (from T_0 to the T_1 generation). T_0 plants were all hemizygous. T_1 plants were expected to be 2/3 hemizygous and 1/3 homozygous. Non expresser: line not expressing the *gusA* gene in all T_0 plants. Non-transmission: non-transmission of intact transgenes to some or all T_1 plants. Silenced: line expressing the *gusA* gene in all T_0 plants but with at least one T_1 plant silenced (containing but not expressing the transgenes). Reduced expression: no silencing observed in any T_1 plant

34 independent fertile lines transmitting the transgene(s) with or without MARs, no significant correlation could be shown between fertility levels at the T_0 and T_1 generations as the data set was not normally distributed even after data transformation. However, lines producing plants with low fertility levels consistently exhibited low fertility across generations (Fig. 5D).

Conclusion

Transgene behaviour in the population of transgenic rice plants generated in this study has been summarised in Fig. 6. Over two generations, only a small proportion of the plant lines without MARs (7%) exhibited Mendelian inheritance and stable expression of the unselected transgene (*gusA*). Transgene inactivation occurred in primary transgenic plants in 41% of the lines. In the next generation (T_1), loss or reduction of transgene expression in plants was mostly due to plant sterility (28% of lines), non-transmission of intact transgenes to some or all progenies (14% of lines) and transgene silencing (10% of lines). This profile is in accordance with many aspects of transgene behaviour described in other transgenic plant studies using particle gun bombardment (Pawlowski and Somers 1996; Ülker et al. 1999). The occurrence of transgene instability could also increase in subsequent generations (Kumpalta and Hall 1998) especially when plants are stabilised at the homozygous level (James et al. 2002). Alternative experimental conditions, transgene constructs, expression assays or plant material can negatively or positively influence transgene behaviour and consequently modulate this profile. Nevertheless, to-date most transgenic plants generated by direct transfer of DNA exhibit similar high levels of transgene instability, which probably originates from their common type of complex transgenic locus structure. Molecular studies have shown that direct DNA transfer often leads to integration at one locus (rarely two loci) of multiple fragmented and rearranged transgene copies as well as plasmid backbone sequences (Gordon-Kamm et al. 1990; Wan and Lemaux 1994). Massive rearrangements of genomic DNA including large scale duplication, deletion and translocation were also observed at the integration site (Takano et al. 1997). Extensive scrambling of transgene and intervening genomic DNA sequences has been identified by fiber-FISH experiments in oat plants transformed by particle bombardment (Svitashev and Somers 2001). The presence of plasmid backbone sequences in transgenic loci probably has a strong effect on transgene expression level and stability (Fu et al. 2000). However, it remains unclear whether the removal of plasmid backbone is enough to create simple transgenic loci through direct transfer of DNA (Breitler et al. 2002).

In this study, aspects of plant development and transgene stability across generations were significantly influenced by the copy number and to a further extent by the expression level of the unselected marker gene (*gusA*) itself. High *gusA* copy number significantly decreased transgene expression level and the stability of transgene expression across generations, and to a lesser extent plant fertility. The absence of *gusA* expression was associated with silencing of the co-transformed hygromycin resistance gene across generations. Sequential analysis of transgene inheritance at the structural then the expression levels, followed by quantification of transgene expression level in progeny, was crucial in identifying different types of transgene behaviour and characteristics. In this study, transgene instability at the structural level (i.e. non-transmission of intact transgenes to some or all progenies) was as important, if not more important, than instability at the expression level (reduced expression, silencing). Transgene inactivation, was also clearly a dynamic process occurring at any step of plant development or generation (data not shown). Transformation events exhibiting intra-line or developmental transgene instability in primary T_0 plants, were often affected by subsequent generational transgene instability. The structural and epigenetic mechanisms underlying such transgene instability in plants have been extensively described (references in Introduction) but to-date, the relative contribution of these mechanisms to transgene inactivation still remains unclear at the population level.

In this study, MARs exhibited some, but not all, of the characteristics predicted by the loop model. Flanking Rb7

MARs showed copy number-dependent transgene expression up to 20 gene copies and some reduction in position effects. In addition, and not directly predicted by the loop model, MARs increased the overall occurrence of stable lines over two generations. Studies over more generations, at different ploidy levels and using larger populations of transformed lines, will determine if this is a consistent feature of flanking MARs in transgenic studies.

To-date, the control of transgene integration, structure, and subsequent expression levels and stability remain key issues limiting transgenic studies. In cereals, the production of single-copy transgenic loci at high frequencies via *Agrobacterium*-mediated transformation (Hiei et al. 1994) and the prediction of transgene expression levels through copy number dependence using flanking MARs (Vain et al. 1999) have made a contribution towards this goal. They will also contribute to further improvements of cereal transgenic studies through unlinked transgene integration (Komari et al. 1996), the resolution of transgenic loci by secondary modifications (Srivastava et al. 1999) or by transposon-based strategies and gene targeting (Pazkowski et al. 1988).

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