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The relationship between homozygous and hemizygous transgene expression levels over generations in populations of transgenic rice plants

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Abstract Segregating T_1, T_2 and T_3 transgenic rice populations, derived from independent particle-bombardment-mediated transformation events were examined in order to assess the effect of gene dosage on transgene expression levels and stability. The expression level of the unselected β-glucuronidase (*gusA)* reporter gene was quantified in plants from these populations. The *gusA* gene dosage was determined by segregation analysis of progeny seedlings at the structural level (by PCR) and at the expression level. For some transformation events a gene dosage effect on transgene expression was observed, leading to higher transgene expression levels in homozygous progeny than in hemizygous progeny or primary transgenic plants. However, in many other transformation events, the homozygous state appears to be disadvantageous, being associated with lower transgene expression levels, gene silencing or counter-selection of homozygous plants across generations. Change of gene dosage is probably one of the key factors influencing transgene expression levels and stability in transgenic rice. This is particularly important when considering molecular genetic studies and crop improvement programmes. The possible influence of matrix attachment regions (MARs) in increasing the likelihood of an additive effect on transgene expression level is discussed.

Keywords Rice · Transformation · Transgene dosage · Homozygous · Matrix attachment regions (MARs)

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Introduction

The genetic transformation of cereals, either by direct transfer of DNA (Gordon-Kamm et al. 1990) or by *Agrobacterium*-mediated transformation (Hiei et al. 1994), is an important tool for both basic research and commercial plant breeding programmes. These applications require that transgenes be stably integrated and expressed, over several generations. However, expression levels and patterns of transgene inheritance generally show wide variation between independently transformed plants carrying the same construct (Peach and Velten 1991; Spencer et al. 1992; Walters et al. 1992). Many factors can contribute to variation in transgene expression. These include integration site, transgene copy number and transgenic locus configuration, as well as epigenetic silencing mechanisms (reviewed by Finnegan and McElroy 1994; Meyer 1998; Iyer et al. 2000; Matzke et al. 2000). The specific transformation system used (*Agrobacterium vs* direct transfer of DNA), the construct configuration (Breyne et al. 1992), the selection strategy (Bhattacharyya et al. 1994), the flanking matrix attachment regions (Allen et al. 2000) or the plant tissue analysed (Ülker et al. 1999) have all been reported to influence transgene structure or expression in plants.

For self-pollinated species, such as rice, wheat and barley, homozygous plant lines are often the basic material used for molecular genetic studies and crop improvement programmes. However, much of the early assessment of transgene expression and associated phenotype is generally made on hemizygous primary transformants, or segregating populations containing hemizygous progeny. It is therefore crucial to determine how transgenes behave in the homozygous state compared to the hemizygous state. To-date, most studies on the relationship between transgene dosage and expression level have focussed on tobacco plants transformed by *Agrobacterium*, often carrying a single transgene copy (For example Hobbs et al. 1990; Caligari et al. 1993; Bhattacharyya et al. 1994; Mlynárová et al. 1996). Hobbs et al. (1990)

and Caligari et al. (1993) both found that homozygous tobacco progeny from a highly expressing single-copy parent showed 2-fold higher *gusA* expression than hemizygous progeny. Interestingly, no difference was observed between homozygotes and hemizygotes from low expressing plant lines containing multiple transgene copies (Hobbs et al. 1990; Caligari et al. 1993). Work involving *Agrobacterium*-transformed *Torenia* (Aida and Shibata 1998) and lettuce (Pang et al. 1996) also showed 2-fold higher transgene expression in homozygous plants compared to hemizygous plants. Studies of gene dosage in cereal crops have been more limited and often contradictory, showing either no effect (Peng et al. 1995; Fearing et al.1997) or increased transgene expression levels with gene dosage (Duan et al. 1996; Baruah-Wolff et al. 1999). It is important, therefore, to determine how transgenes behave in the homozygous state compared to the hemizygous state of transformed cereal plants produced by direct transfer of DNA.

In transgenic studies, matrix attachment regions (MARs) have been reported to confer levels of position independent (Mlynárová et al. 1994) or copy number-dependent, transgene expression (Allen et al. 1996; Schöffl et al. 1993). To investigate any effect of MARs on the potential relationship between transgene dosage and expression levels, we evaluated transgene expression levels in homozygous and hemizygous plants from independently transformed rice clones, containing or not containing MARs. Segregating populations of transformed rice plants were propagated for three generations from single hemizygous primary transformed T_0 plants (Vain et al. 1999) for each transformation event. All T_0 plants carried the *gusA* reporter gene under control of the cauliflower mosaic virus 35S promoter (CaMV35 S) flanked or unflanked by MARs. Here we compared *gusA* expression levels to transgene dosage in T_2 rice plants.

Materials and methods

Rice transformation procedures

An Elite West African rice (*Oryza sativa* L.) variety, ITA212, was transformed by particle bombardment of immature embryos**,** as described previously (Vain et al. 1998). Two different plasmid combinations were introduced into rice: "D" clones contained plasmids pGHNC11 and pJIC201, whereas "A" clones contained plasmid pBAR-HYG-GUS. Plasmid pGHNC11 (Rb7MAR::CaMV35S::*gusA*::NOS-T::Rb7MAR) contains the *gusA* gene controlled by the Cauliflower Mosaic Virus (CaMV) 35S promoter and the *Agrobacterium tumefaciens* nopaline synthase *(nos)* polyadenylation site, flanked by Rb7 MARs from *Nicotiana tabacum* (Allen et al. 1996). Plasmid pJIC201 (UBI-5'region::*aphIV:*:NOS-T) contains the *aphIV* gene (conferring hygromycin resistance) controlled by the *Zea Mays* Ubiquitin-1 5′ region (promoter+exon1+intron1) and the *nos* polyadenylation site (Vain et al. 1999). Plasmid pBAR-HYG-GUS (CaMV35S::*gusA:*:NOS-T+CaMV35S::*aphIV*::NOS-T+CaMV35S::adh1int1::*bar*::NOS-T) contains the *gusA* gene controlled by the CaMV35S::AMV promoter/leader and the *nos* polyadenylation site, plus the *aphIV* gene controlled by the CaMV35 S promoter and the *nos* polyadenylation site, plus the *bar* gene (conferring resistance to phosphinothrycin, PPT) controlled by the CaMV35S promoter, the *Z. mays* alcohol dehydrogenase intron1 (*adh*1int1) and the *nos* polyadenylation site (Cooley et al. 1995).

Independently transformed rice callus clones were selected for hygromycin resistance (Vain et al. 1998). After 6 to 8 weeks, T_0 plants were regenerated from each clone, transferred to a controlled environment room for a further 8 weeks, and then to the greenhouse for seed setting (Vain et al. 1998). One T_0 plant per independent clone was randomly chosen for further inheritance studies.

Detection of transgenic plants by the polymerase chain reaction (PCR)

DNA was isolated 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 a 1.2-kb 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 1271-bp fragment containing the *aphIV* gene (forward: 5′-ACTCACCGCGACGTCTGTCG-3′; reverse: 5′-GATCTCCAATCTGCGGGATC-3′), and (3) the other to amplify a 2038-bp fragment of the CaMV35S::*gusA* expression cassette (forward: 5′-CCCACCCACGAGGAGCAT–3′; reverse: 5′-GCGCCAGGAGAGTTGTTGATT-3′).

Analysis of GUS activity

Fluorometric analysis for β-glucuronidase activity was carried out on leaf tissue from rice plants at the five-leaf stage, according to Jefferson et al. (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 pmoles of MU min^{-1} mg^{-1} of extracted protein. In order to reduce the error introduced by potential plant to plant variation (Elkind et al. 1995) transgene expression was measured in five different T_0 plants regenerated from each independent callus clone. The background GUS activity (35±4 pmol of 4-methylumbelliferone (MU) min^{-1} mg^{-1} of protein (P<0.05)) was subtracted from all fluorometric GUS activity values obtained from transformed plants (Vain et al. 1999). Due to the variation associated with background measurement, clones with less than twice the background value were considered as non/very low expressers. To minimise the influence of environmental conditions, all plants were grown in a fully controlled growth room and analysed at the same developmental stage (five-leaf stage) using standardised experimental procedures.

Segregation analysis of transgene expression

T_1, T_2 and T_3 seeds were obtained by self-pollination of T_0 (primary transformants), T_1 and T_2 rice plants respectively. Segregation analysis at the structural level was performed by PCR on seedlings germinated on MSR6 medium, without hygromycin selection (Vain et al. 1998). PCR analysis was conducted to test for the presence of the *aphIV* and *gusA* transgenes and the single-copy rice genomic probe C213 in up to 40 random seedlings. Segregation analysis of transgene expression was assessed qualitatively by histochemical GUS staining (Jefferson et al. 1987) of the seed endosperm and germination of the corresponding isolated embryo on hygromycin-containing medium (MSR6H50, Vain et al. 1998). Seedlings with abnormal growth on hygromycin (no root or browning) were scored as hygromycin sensitive. When GUS staining and hygromycin resistance did not correlate in a given rice progeny, or when the total number of seeds available was less than 40, segregation was assessed at the structural level.

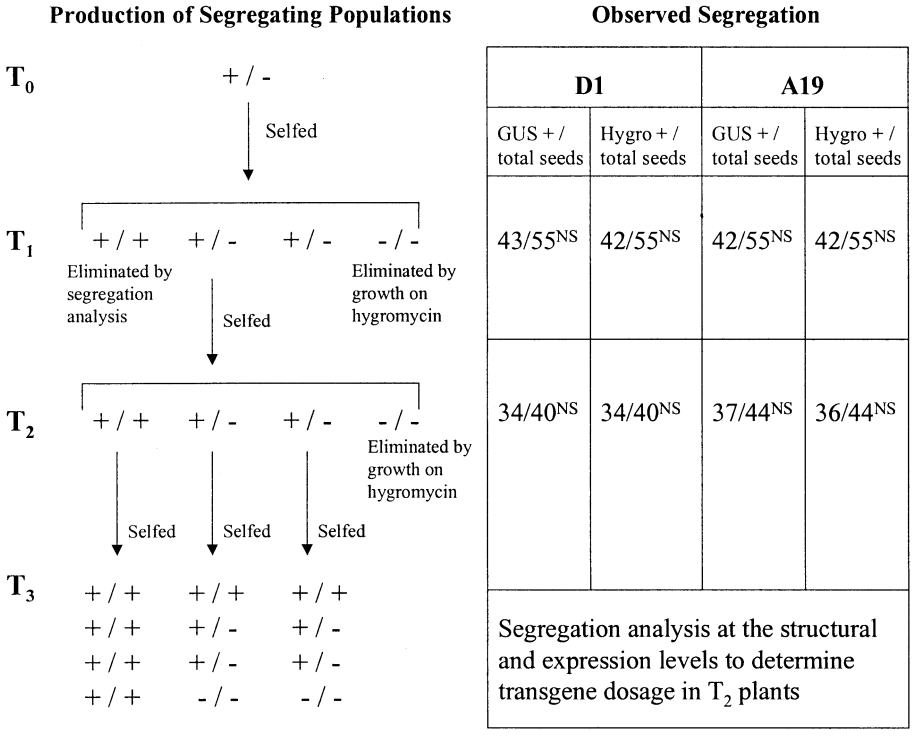

Fig. 1 Production of segregating T_1, T_2 and T_3 rice populations from clones D1 and A19. Clone D1: hemizygous T_1 plant D1–609 selected; 33 T_2 plants analysed. Clone A19: hemizygous T_1 plant A19–2 selected; 31 T_2 plants analysed. ^{NS} Segregation ratio not significantly different from 3:1 by the chi-square test (P>0.05)

Statistical analysis

Statistical analyses, following the requirements of each test (Snedecor and Cochran 1980; Stell and Torries 1980), were performed using Minitab 13.1 software. When variance was strongly dependent upon the mean, variability within each population was assessed using the coefficient of variation (CV=standard deviation/mean). CVs were compared using the Levene's test on data expressed as a percentage of the mean. Data sets were compared using the Kruskal-Wallis test, which does not require that the data follow a normal distribution. The likelihood of segregation data fitting expected models was assessed using the chi-square test.

Results and discussion

Production of segregating populations of rice plants over three generations

Three independently transformed rice clones expressing the *gusA* gene and showing Mendelian inheritance of a single transgenic locus (3:1) were used in this study. Clones D1 and D11 contained plasmids pGHNC11 (Rb7MAR::CaMV35S::*gusA*::NOS-T::Rb7MAR) and pJIC201 (UBI-5′region::*aphIV*::NOS-T); Clone A19 contained plasmid pBAR-HYG-GUS (ubi-5′region::*gusA*::NOS-T + CaMV35S::*aphIV*::NOS-T+CaMV35 S::adh1int1::*bar*::NOS-T). All plasmid copies were integrated at a single locus in clones D1, D11 and A19. One primary transformed rice plant (T_0) of each clone was selfed to produce a population of T_1 plants segregating for the transgenes. One T_1 hemizygous plant was selected from this population and selfed to give a segregating population of T_2 plants (Fig. 1). GUS activity was quantified at the 5-leaf stage of 33 random T_2 plants from D1–609 and 31 random T_2 plants from A19–2 by a fluorometric assay. T_3 seeds were collected from all 64 T_2 plants for segregation analysis to determine if the T_2 plants were homozygous or hemizygous. Transgene expression levels in the T_2 plants were then compared to transgene dosage. Clones D1 and A19 produced segregating T_1, T_2 and T_3 plant populations as expected, but clone D11 failed to produce any homozygous T_1 plants and therefore could not be used for further inheritance studies.

Transgene inheritance at the structural level

Transgene inheritance at the structural level was assessed by analysis of up to 40 T_3 plants germinated without hygromycin selection (Fig. 2). In T_2 plants homozygous for the transgene, such as A19–278, both the *gusA* and *aphIV* genes were present in all 40 progeny seedlings (Fig. 2A). In hemizygous T_2 plants, such as A19–279, the *gusA* and *aphIV* transgenes were present in 27/40 (67.5%) of the progeny and absent in 13/40 (32.5%) of the progeny (Fig. 2B). This was not significantly different from the 3:1 ratio expected for one locus (chi-square, P>0.05). Progeny showing no PCR amplification for the *gusA* or *aphIV* genes were analysed by the amplification of a rice genomic RFLP probe (C213) to ensure that each DNA sample was suitable for PCR amplification (Fig. 2B). The PCR data confirmed co-inheritance of the *gusA* and hygromycin resistance transgenes in all progeny tested from hemizygous and homozygous T_2 plants. Segregation analysis conducted at the expression level only is often insufficient to determine segregation ratios

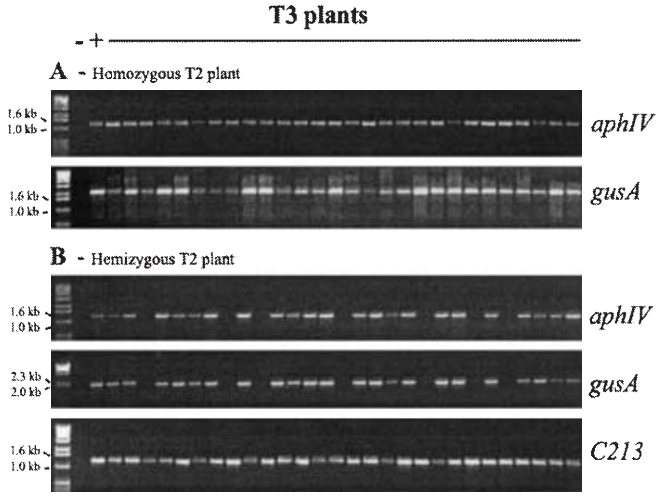

Fig. 2 Transgene inheritance at the structural level of the *gusA* and *aphIV* transgenes in the progeny of *A* homozygous (A19–278) and *B* hemizygous (A19–279) T_2 plants. Presence of the transgenes was determined by PCR analysis. Primers for the RFLP probe C213 were used to check the suitability of genomic DNA extracts for PCR amplification

and subsequent transgene dosage of the parent plant. Progeny without transgene expression can result either from transgene silencing (transgene present but not expressed) or from non-inheritance (transgene not present) of the transgene in those plants. Structural studies were therefore critical in determining whether a transgene segregates in a Mendelian fashion or not, and in the determination of factors underlying cases of skewed segregation.

Effect of gene dosage on transgene expression levels in clone D1

GUS activity was quantified by fluorometric assay in 33 transformed T_2 plants randomly chosen from a segregating population derived from clone D1, containing flanking MARs sequences (Table 1). Determination of transgene dosage in these T_2 plants was performed by segregation analysis at the structural level (PCR) and/or at the transgene expression level (GUS activity and hygromycin resistance), in at least 40 progenies from each T_2 plant. Transgene segregation data was collected from a

Table 1 Relationship between transgene expression level and gene dosage in T_2 rice plants from clone D1. */**/NS Chi-square test for a 3:1 segregation ratio. * and ** showed a significant difference at the 5% and 1% levels respectively (T_2 plant is homozygous). NS showed no significant difference at the 5% level (T_2 plant is hemizygous). PCR Segregation confirmed by PCR analysis. SEED Lower total seed number due to contamination or seed damage

T_2 plant number	GUS activity in T_2 plant leaf[a]	Segregation GUS	Segregation hygoR
		(Number of transgenic T_3/total T_3 progenies)	
Homozygous plants			
D1–100	2744	40/40**	40/40**
D1–120	2963	40/40**	40/40**
D1–122	5480	40/40**	40/40**
D1–126	2626	40/40**	40/40**
D1–127	1734	40/40**	39/40** SEED
D1–129	3432	40/40**	40/40**
D1–131	4610	40/40*	40/40**
D1–193	4437	80/80**	79/80** SEED
D1–194	2293	40/40**	40/40**
D1–199	1878	40/40**	40/40**
D1–200	1819	40/40**	40/40**
D1–201	2751	40/40**	40/40**
D1–204	3462	40/40**	40/40**
Hemizygous Plants			
D1–99	3280	27/40 NS	26/40 NS
D1–101	3031	31/40 NS	32/40 NS
D1–102	1646	26/40 NS	27/40 NS
D1–104	2609	30/40 NS	31/40 NS
D1–105	2153	31/40 NS	31/40 NS
D1–107	2100	27/40 NS	26/40 NS
D1–119	1699	28/40 NS	30/40 NS
D1–121	1874	60/80 NS	63/80 NS
D1–123	937	32/40 NS	33/40 NS
D1–124	1703	31/40 NS	32/40 NS
D1–125	2807	28/40 NS	28/40 NS
D1–128	2068	30/40 NS	34/40 NS
D1–132	1534	25/40 NS PCR	25/40 NS
D1–133	1181	28/40 NS	32/40 NS
D1–196	1572	33/40 NS	31/40 NS
D1–197	3078	33/40 NS	32/40 NS
D1–198	946	60/80 NS	59/80 NS
D1–202	1016	31/40 NS	31/40 NS
D1–203	669	35/40 NS	34/40 NS
D1–205	1100	31/40 NS	32/40 NS

[a] Data expressed as pmol of 4-methylumbelliferone (MU) min^{-1} mg^{-1} of extracted protein

Table 2 Average transgene expression levels in homozygous and hemizygous T_2 plants from clones D1 and A19. **A19: a** complete raw data set; **b** data set minus three outlier values. [p/q] column entries followed by different letters are significantly different at $P<0.05$ by the Kruskal-Wallis test; column entries followed by the same letter are not significantly different at $P>0.05$ by the Kruskal-Wallis test. [x] column 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

Clone number		Zygotic state	Number of T_2 plants	Mean GUS activity[a]	Interval of confidence ($P<0.05$)	Coefficient of variation
D1		Homozygous	13	3095[p]	±630	37[x]
		Hemizygous	20	1850[q]	±343	42[x]
A19	**a**	Homozygous	10	12241[p]	±3270	43[x]
		Hemizygous	21	19112[q]	±3311	41[x]
	b	Homozygous	9	13601[p]	±2491	24[x]
		Hemizygous	19	17085[p]	±2022	25[x]

[a] in pmol of MU min^{-1} mg^{-1} of extracted protein

total of 1440 T_3 plants. The progeny of homozygous T_2 plants were all expected to contain and express the *gusA* gene and to be hygromycin resistant. In contrast, the progeny of hemizygous T_2 plants were expected to segregate 3 (GUS positive and hygromycin resistant) : 1 (GUS negative and hygromycin sensitive). Deviation from this ratio was assessed using the chi-square test (Table 1). In the population of 33 T_2 plants belonging to clone D1, segregation analysis showed that 13 T_2 plants (39.4%) were homozygous and 20 T_2 plants (60.6%) were hemizygous. This ratio is in agreement with the Mendelian expectation.

The average *gusA* expression level in T_2 plants from the D1 population was 3095±630 pmol of MU min^{-1} mg^{-1} of protein for homozygous plants and 1850± 343 pmol of MU min^{-1} mg^{-1} protein for hemizygous plants (mean ± interval of confidence, $P<0.05$). The level of transgene expression observed in homozygous T_2 plants was significantly greater, 1.7-fold, than the expression level in hemizygous T_2 plants (Kruskal-Wallis test $P=0.002$; Table 2, D1). Thus, there appears to be an additive effect of transgene expression in homozygous offspring of the D1 clone, containing MARs. For this clone, transgene expression level is approximately doubled in plants homozygous for the transgene.

To-date there are only limited studies in cereals (Peng et al. 1995; Fearing et al. 1997; Baruah-Wolff et al. 1999) and little evidence to suggest a correlation between expression levels and transgene dosage. Whilst no difference in transgene expression was detected between homozygous and hemizygous rice plants carrying the *gusA* gene (Peng et al. 1995) or maize plants expressing the *cryIA(b)* gene (Fearing et al. 1997), Baruah-Wolff et al. (1999) observed that a subset of transgenic T_1 rice plants that expressed the firefly luciferase gene at high levels were scored as homozygous. High levels of transgenic protein have also been associated with the homozygous state in rice (Duan et al. 1996). In contrast to *Agrobacterium*-mediated transformation, which is generally associated with low transgene copy number (frequently one or two copies) and simple integration patterns, particle bombardment often gives rise to high copy numbers and more-complex transgene rearrangements. In this study the clone D1, generated by particle bombardment and containing approximately 14 *gusA* gene copies with flanking MARs (Vain et al. 1999), showed that an additive effect of transgene expression in homozygous plants was not adversely affected by high copy number or locus complexity. Further analysis of other transformation events should provide a better insight into how frequent this transgene behaviour is in populations of transformed plants generated by direct transfer of DNA.

Effect of gene dosage on transgene expression levels in clone A19

GUS activity in 31 segregating T_2 plants from clone A19 was determined by fluorometric assay (Table 3), and their gene dosage determined, as for clone D1; *gusA* expression levels were higher in clone A19 than in clone D1 due to differences in promoter and enhancer sequences. Segregation of transgenes was assessed in 1312 T_3 plants. In the segregating population of T_2 plants, 10 out of 31 T_2 plants (32.3%) were homozygous and 21 out of 31 T_2 plants (67.7%) were hemizygous. This ratio is in agreement with the Mendelian expectation.

In contrast to the D1 T_2 population, no transgene dosage effects were observed in the A19 population. The average *gusA* expression level amongst A19 plants was 12241±3270 pmol of MU min^{-1} mg^{-1} of protein for homozygous plants and 19112±3311 pmol of MU min^{-1} mg^{-1} protein for hemizygous plants (mean ± interval of confidence, $P<0.05$). The raw data showed that the average transgene expression level in homozygous plants was significantly inferior to those of hemizygous plants from A19 (Kruskal-Wallis test $P=0.013$) (Table 2, A19-a). Three outliers for *gusA* expression level were present in the population of 31 T_2 plants. One homozygous plant (A19–257) with very low/no *gusA* expression

Table 3 Relationship between transgene expression level and gene dosage in T_2 rice plants from clone A19. */**/NS Chi-square test for a 3:1 segregation ratio. * and ** showed a significant difference at the 5% and 1% levels respectively (T_2 plant is homozygous). NS showed no significant difference at the 5% level (T_2 plant is hemizygous). PCR Segregation confirmed by PCR analysis. SEED Lower total seed number due to contamination or seed damage

T_2 plant number	GUS activity in T_2 plant leaf[a]	Segregation GUS	Segregation hygoR
		(Number transgenic T_3/total T_3 progenies)	
Homozygous plants			
A19–248	15328	40/40**	40/40**
A19–249	9807	80/80**	36/38** SEED
A19–251	12595	40/40**	32/32** PCR
A19–252	14844	16/16**	16/16** PCR
A19–257	0.5	40/40**	36/36** SEED
A19–258	11625	16/16**	15/10 ** PCR/SEED
A19–259	19932	40/40**	40/40**
A19–261	13415	40/40**	40/40**
A19–262	9492	40/40**	40/40**
A19–278	15371	80/80**	40/40** PCR
Hemizygous plants			
A19–250	15252	35/40 NS	31/40 NS
A19–253	16725	31/40 NS	31/40 NS
A19–255	43932	29/40 NS	28/40 NS
A19–256	13060	30/40 NS	30/40 NS
A19–260	14101	33/40 NS	32/40 NS
A19–263	18638	30/40 NS	28/40 NS
A19–264	12389	29/40 NS	29/40 NS
A19–265	21013	31/40 NS	33/40 NS
A19–266	21258	29/40 NS	29/40 NS
A19–267	11781	26/40 NS	25/40 NS
A19–268	19571	32/40 NS	32/40 NS
A19–269	12724	29/40 NS	29/40 NS
A19–270	20125	28/40 NS	27/40 NS
A19–271	13229	27/40 NS	27/40 NS
A19–272	21549	25/40 NS	25/40 NS
A19–273	12041	35/40 NS	32/40 NS
A19–274	18033	32/40 NS	32/40 NS
A19–275	32801	27/40 NS	28/40 NS
A19–276	18844	30/40 NS	29/40 NS
A19–277	16996	30/40 NS	27/40 NS
A19–279	27289	66/80 NS	27/40 NS PCR

[a] Data expressed as pmol of 4-methylumbelliferone (MU) min^{-1} mg^{-1} of extracted protein

(0.5 pmol of MU min^{-1} mg^{-1} of protein) and two hemizygous plants (A19–255 and A19–275) with very high *gusA* expression (43932 and 32801 pmol of MU min^{-1} mg^{-1} of protein, respectively). When these three outlier values were omitted from the data set, the average transgene expression level in homozygous plants was not statistically different from that of hemizygous plants (Kruskal-Wallis test P=0.052) (Table 2, A19-b).

For clone A19, not containing MARs, there was no additive effect of transgene copy number in the population of T_2 plants. The homozygous state conveyed no advantage, or even imposed a disadvantage with relation to *gusA* expression. This penalty of being homozygous was also apparent in several other clones studied, which failed to produce homozygous progeny (see next section). The absence of an additive effect of transgene copy number on expression level is in accordance with previous studies of transgene expression in monocot species transformed by direct transfer protocols (Peng et al. 1995; Fearing et al. 1997).

The absence of additive transgene expression has also been reported in some dicotyledonous species transformed by *Agrobacterium*-mediated transfer (Hobbs et al. 1990; Caligari et al. 1993; Scott et al. 1998). Examination of transgene inheritance at the structural and expression levels in white clover showed no significant difference in *gusA* expression levels of homozygous and hemizygous plants (Scott et al. 1998). Interestingly, in tobacco, although a dosage effect was evident in plant lines showing high GUS activity, no difference was observed in the GUS activity of homozygous or hemizygous tobacco plants from low-expressing populations (Hobbs et al. 1990; Caligari et al. 1993). Our results indicate that transgene dosage effects can also be suppressed, or not present, in populations of rice plants expressing a transgene at high levels. High transgene copy numbers and complex transgenic loci produced by direct DNA transfer methods could promote the suppression of additive effects of transgene copy number by increasing transcriptional (Matzke and Matzke, 1995) and/or post-transcriptional (Jones et al. 1999) gene silencing.

In the present study, only one homozygous T_2 plant (A19–257) exhibited complete transgene silencing (no transgene expression) among the entire segregating population of plants. This could be the first indication of silencing progressively affecting progeny plants. Either transcriptional gene silencing of all transgene copies or post-transcriptional gene silencing could be responsible for this observation (Matzke and Matzke 1995; Iyer et al.

Table 4 Relationship between transgene expression level and zygotic state in T_1 rice plants from clones D11. * [NS] Chi-square test for a 3:1 or 2:1 segregation ratio. * Showed a significant difference at the 5% level. [NS] Showed no significant difference at the 5% level. [PCR] Results confirmed by PCR analysis

T_1 plant number	GUS activity[a]	Segregation GUS (Number transgenic/total seeds)			Segregation hygoR (Number transgenic/total seeds)		
			3:1 ratio	2:1 ratio		3:1 ratio	2:1 ratio
D11–267	7996	49/76[PCR]	*	NS	29/40	NS	NS
D11–991	3041	58/76[PCR]	NS	NS	32/40	NS	NS
D11–996	2956	54/82[PCR]	NS	NS	26/40	NS	NS
D11–414	4786	33/40	NS	NS	32/40	NS	NS
D11–266	N/A	28/40	NS	NS	28/40	NS	NS
D11–989	3075	31/40	NS	NS	31/40	NS	NS
D11–990	2573	29/40	NS	NS	30/40	NS	NS
D11–992	2575	29/40	NS	NS	29/40	NS	NS
D11–993	2815	32/40	NS	NS	32/40	NS	NS
D11–994	6319	26/40	NS	NS	26/40	NS	NS
D11–995	3755	28/40	NS	NS	28/40	NS	NS
D11–997	2108	23/39	*	NS	23/39	*	NS
D11–998	2465	31/40	NS	NS	30/40	NS	NS
D11–1000	4404	31/40	NS	NS	31/40	NS	NS
D11–1001	3223	28/40	NS	NS	28/40	NS	NS
D11–1011	4784	25/38	NS	NS	25/38	NS	NS

[a] Data expressed as pmol of 4-methylumbelliferone (MU) min^{-1} mg^{-1} of extracted protein

2000). Other examples of transgene silencing associated with the homozygous state have been reported in tobacco transformed by *Agrobacterium*-mediated transformation (Neuhaus et al. 1991; De Carvalho et al. 1992; Brandle et al. 1995). De Carvalho et al. (1992) detected complete suppression of β-1,3-glucanase transgene expression in homozygous tobacco plants, which could be restored upon backcrossing to produce hemizygous plants. Environmental conditions and early plant rearing conditions can also affect epigenetic silencing events (Hart et al. 1992; Brandle et al. 1995). Interestingly, nine out of ten homozygous T_2 plants from clone A19 exhibited transgene expression levels equivalent to hemizygous T_2 plants. To reduce homozygous expression levels to those of hemizygous plants, either half of all transgene copies were transcriptionally silenced (within one or both loci), or the expression level of each transgene was reduced by half as a result of transcriptional limitations.

Analysis of clones producing no homozygous progenies

Primary transgenic T_0 plants from clone D11 failed to produce any expressing homozygous T_1 progeny among the 16 plants studied (Table 4). Segregation analysis at the structural level of progenies from three random T_1 plants (D11–267, D11–991, and D11–996) showed that 73/114 (64%) of progeny plants contained the transgenes. This proportion was lower than the expected 75% from a hemizygous parent. Nevertheless, the progeny of D11–991 and D11–996 T_1 plants exhibited segregation ratios not significantly different from 3:1. The segregation of D11–267, however, was significantly different from a 3:1 ratio, but not from a 2:1 ratio (Table 4). A 2:1 ratio could be obtained if the transformed homozygous (+/+) plants were not present, so that the only progeny produced were either hemizygous (+/–) or homozygous non-transformed (–/–). In clone D11, the homozygous condition could be lethal. Segregation analysis performed at the expression level can add complexity to the interpretation of segregation analysis results. In this study, 10.9% (5/46) of the progenies without GUS activity did contain the *gusA* gene, but were silenced for transgene expression. In such a case, silenced homozygous progenies may be artificially counter-selected during germination on hygromycin, leading to a population enriched in hemizygous plants.

Segregation analysis of an additional 13 T_1 plants (Table 4) showed that 14 out of the 16 randomly tested T_1 plants exhibited a 3:1 segregation ratio. Two T_1 plants had ratios significantly different from 3:1 (chi-square, $P<0.05$). Interestingly, all 16 T_1 plants exhibited segregation ratios not significantly different from 2:1 (chi-square, $P>0.05$). Segregation analysis performed on 40 progeny seedlings, even at the structural level, was not always sufficient to distinguish between a 3:1 (expected Mendelian ratio for one locus) and a 2:1 (counter-selection of homozygous plants) segregation ratio. In practice, over 100 progenies should be analysed to statistically determine an exact 2:1 or exact 3:1 ratio using the chi-square test at the 5% level. In many transformation events it was therefore possible that levels of counter-selection of homozygous progenies went undetected as observed segregation results did not significantly differ from those of a stable (3:1) hemizygote. Instability associated with the homozygous state may therefore be more widespread than is currently appreciated because it is only detected in extreme cases such as clone D11.

In previous work (Vain et al. 1999), several transformation events among a population of 83 independent clones also produced lower than expected frequencies of homozygous plants within T_1 segregating plant populations (data not shown). Segregation analysis of 12 T_1 plants from clone H61 identified only one homozygous plant. The remaining 11 T_1 plants from clone H61 exhibited segregation ratios not significantly different from the 3:1 ratio expected for a hemizygous plant (chi-square, $P>0.05$). PCR analysis of seedling progeny from

two hemizygous T_1 plants confirmed that the plants were not homozygous with silencing. Unusually low frequencies of homozygous progeny were also detected from a genetic cross in white clover (Scott et al. 1998). Potentially, many clones could show some degree of discrimination against homozygous progeny. Clone D11 contained the same transgene construct as clone D1, with approximately the same copy number and expression levels. Despite similarities between the T_0 plants from these two clones, transgene locus position and/or configuration did appear to have a major influence when the transgene was in a homozygous state in progeny plants.

MARs effect

Transgene dosage effects observed in homozygous plants imply that transgene expression levels increased in proportion to copy number. Most transgenic studies involving plants created by particle bombardment showed no, or very little, correlation between transgene expression levels and transgene copy number in dicot (Allen et al. 1996) or monocot (Vain et al. 1999) species. It is therefore not surprising that gene dosage effects were not commonly observed in homozygous plants obtained by direct DNA transfer methods. Interestingly, in the present study, a gene dosage effect was observed in homozygous plants derived from clone D1, containing flanking MARs, whereas no gene dosage was seen in homozygous plants from clone A19, without MARs. Flanking MARs have been shown to provide copy number-dependent transgene expression up to 20 gene copies in rice plants transformed using particle bombardment (Vain et al. 1999). Clone D1 was known to contain approximately 14 copies of the *gusA* transgene and so even in the homozygous state might still exhibit copy number-dependent expression. Clones with significantly more transgene copies would not be expected to show any gene dosage effect since the homozygous state would have significantly more than 20 copies. MARs could therefore promote additive transgene expression only in clones with low copy numbers. The potential relationship between flanking MARs and gene dosage should be examined in a large population of independent and random transformation events, with and without MARs, in order to precisely quantify the impact of MARs on transgene expression levels and stability in homozygous versus hemizygous plants. Transgene dosage effects could also be affected by the complexity of transgenic loci created by particle bombardment. It would be interesting to determine if the relationship between gene dosage and expression levels is more frequent in rice carrying a single-copy locus generated through *Agrobacterium*-mediated transformation.

Conclusion

In this study we have shown that an additive effect of transgene expression can be observed in homozygous rice plants from some transformation events exhibiting stable transgene expression and segregation. However, in many other transformation events, the homozygous state appears to be disadvantageous, being associated with lower transgene expression levels, gene silencing or counter-selection of homozygous plants across generations. This finding suggests that transgene dosage is a key factor to consider when assessing transgene expression levels and stability in plants. It also indicates that phenotype and bioassays involving hemizygous T_0 primary transformants, or segregating populations rich in hemizygous plants (such as T_1 populations), may poorly reflect future transgene behaviour in homozygous plants. The absence of homozygous progeny for some transformation events is also a form of transgene instability, often overlooked because it can be difficult to detect. There is great benefit in conducting segregation analysis at the structural level and also in large numbers of progenies (100+) in order to distinguish 3:1 (expressing: non-expressing) Mendelian locus segregation from a 2:1 segregation ratio caused by lethality of homozygous progenies. Segregation ratios falling between 2:1 and 3:1 can be observed due to levels of counter-selection of homozygous plants in the progeny. As transgene instability is often a dynamic process during plant development and across generations, evidence of homozygote counter-selection or absence of transgene dosage in the progeny of a given transformation event should give cause for caution even if some of the homozygous plant progeny do exhibit gene dosage. This study shows that transgene dosage is of particular importance when assessing transgene expression levels and associated phenotype in molecular genetic studies and crop improvement programmes of species often stabilised as homozygous lines such as rice, wheat and barley.

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