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Transgenic rice as a system to study the stability of transgene expression: multiple heterologous transgenes show similar behaviour in diverse genetic backgrounds

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Abstract The success of contemporary breeding programmes involving genetic engineering depends on the stability of transgene expression over many generations. We studied the stability of transgene expression in 40 independent rice plant lines representing 11 diverse cultivated varieties. Each line contained three or four different transgenes delivered by particle bombardment, either by cotransformation or in the form of a cointegrate vector. Approximately 75% of the lines (29/40) demonstrated Mendelian inheritance of all transgenes, suggesting integration at a single locus. We found that levels of transgene expression varied among different lines, but primary transformants showing high-level expression of the *gna*, *gusA*, *hpt* and *bar* transgenes faithfully transmitted these traits to progeny. Furthermore, we found that *cry1Ac* and *cry2A* transgene expression was stably inherited when primary transformants showed moderate or low-level expression. Our results show that six transgenes (three markers and three insect-resistance genes) were stably expressed over four generations of transgenic rice plants. We showed that transgene expression was stable in lines of all the rice genotypes we analysed. Our data represent a step forward in the transfer of rice genetic engineering technology from model varieties to elite breeding lines grown in different parts of the world.

Keywords Transgenic rice · Insect resistance · Transgene expression · Genetic background

Introduction

The genetic manipulation of rice, either through direct DNA delivery by particle bombardment (Christou et al. 1991) or by *Agrobacterium*-mediated transformation (Hiei et al. 1994), is now routine in many laboratories world-wide. While the DNA transfer process may be relatively straightforward, there is still limited information concerning the fate and function of exogenous DNA in the progeny of primary transformants, particularly in lines carrying transgenes of agronomic value. Several studies have provided information concerning the inheritance and expression of marker genes in transgenic rice plants. Goto et al. (1993), Peng et al. (1995) and Zhang et al. (1996a, b) demonstrated the cointegration and coinheritance of markers carried on two separate plasmids, while Qu et al. (1996) analysed a large number of lines transformed with four different plasmids. Cooley et al. (1995) studied the integration, coinheritance and coexpression of multiple genes linked on a single plasmid, and transgene organisation has been investigated in plants transformed with three linked markers on one cointegrate vector (Kohli et al. 1998, 1999). Most recently, Chen et al. (1998) obtained a number of transgenic lines after bombarding rice tissue simultaneously with 14 different plasmids. They recovered plants cotransformed with up to 13 of the genes and investigated the expression of 4 marker genes in three individual lines.

While the information provided by such experiments is very useful, only certain marker genes (*gusA*, *bar*, *hpt* and *luc*) have been studied in detail. As genes of agronomic interest (e.g. those conferring disease or pest resistance) will ultimately be used in applied breeding programmes, it is important to confirm if the results obtained using marker genes can be extrapolated to agronomically important genes. Model japonica rice varieties (predominantly Nipponbare and Taipei-309) have been transformed with genes of agronomic interest. These encoded products such as phosphinothricin acetyltransferase (Cao et al. 1992; Toki et al. 1992) rice stripe virus coat protein (RSVCP; Hayakawa et al. 1992) Tungro virus coat protein (Qu et al. 1996) insecticidal endotoxins from *Bacillus thuringiensis* (Cheng et al. 1998; Fujimoto et al. 1993; Ghareyazhie et al. 1997; Wu et al. 1997) oryzacystatin (Hosoyama et al. 1994) corn-cystatin (Irie et

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al. 1996) potato proteinase inhibitor (Duan et al. 1996) soybean Kunitz trypsin inhibitor (Lee et al. 1999) Xa21 (Song et al. 1995; Wang et al. 1996, 1998) phytoene synthase (Burkhardt et al. 1997) fatty acid desaturase (Wakita et al. 1998) and pea legumin (Sindhu et al. 1997). However, only a limited number of elite breeding varieties have been transformed with agronomically useful genes, encoding products such as phosphinothricin acetyltransferase (Christou et al. 1991; Datta et al. 1990, 1992), *Bt* endotoxins (Alam et al. 1998; Datta et al. 1998; Maqbool et al. 1998; Nayak et al. 1997; Wunn et al. 1996; Tu et al. 1998b), Xa21 (Tu et al. 1998a; Zhang et al. 1998), chitinase (Lin et al. 1995), GNA (Rao et al. 1998; Sudhakar et al. 1998b), phytochrome (Clough et al. 1995) and oryzacystatin (Vain et al. 1998). The stability of useful transgenes in diverse and commercially significant breeding cultivars must be assessed over a number of generations to ensure the success of breeding programmes involving genetic engineering. The analysis of a large number of transgenic lines in diverse genetic backgrounds will provide the breeder with germplasm suitable for a broad spectrum of breeding programmes.

In our rice engineering programme, many different indica varieties have been successfully transformed with insect resistance genes. Bioassays have shown the efficacy of these insecticidal transgenes against common insect pests of rice, such as brown plant hopper, yellow stem borer and rice leaf folder (Maqbool et al. 1998; Rao et al. 1998; Sudhakar et al. 1998b). In the investigation reported here, we analysed 11 different indica and japonica varieties of rice (*Oryza sativa* L.) and studied the stability of transgene expression (including three insecticidal transgenes: *cry1Ac*, *cry2A* and *gna*). We showed that in most of the lines, marker and insecticidal transgene expression levels were stably transmitted to the R_1, R_2 and R_3 generations. Furthermore, despite the multicopy nature of the transgenic lines, we found that transgene silencing was an exceptional phenomenon and that the recovery of a high number of plants with desirable traits was possible.

Materials and methods

Transforming plasmids

We used a cointegrate vector containing three marker genes (*hpt*, *bar* and *gusA*), each driven by a separate copy of the CaMV 35S promoter (Cooley et al. 1995). Constructs pUbi1-*gna* and pRSs1-*gna* each carried the snowdrop lectin gene (*gna*), driven by the maize ubiquitin-1 promoter (constitutive) or the rice sucrose synthase-1 promoter (tissue-specific), respectively (Sudhakar et al. 1998b; van Damme et al. 1991). Two further plasmids carried *Bacillus thuringiensis* insecticidal genes: pIA2 carried a synthetic *cry1Ac* gene under the control of the maize ubiquitin-1 promoter (Sardana et al. 1996) and pROB6 carried a synthetic *cry2A* gene, driven by the CaMV 35S promoter (Maqbool et al. 1998). We used two different plasmids, both containing the selectable marker gene hygromycin phosphotransferase (*hpt*), in different cotransformation experiments. The *hpt* marker was driven by the CaMV 35S promoter in both constructs (Cooley et al. 1995; Maqbool and Christou 1999).

Plant material and transformation

We used the following rice varieties: Bengal, Cypress, Lido, IR 72, Koshihikari, Basmati 370, FX 97, M5, M7, M12, and ASD 16. The transformation process is described elsewhere (Maqbool and Christou 1999; Sudhakar et al. 1998a).

Polymerase chain reactions (PCR) analysis

DNA was extracted from rice leaves according to the method described by Edwards et al. (1991). The quantity of DNA was estimated against bacteriophage λDNA standards (Pharmacia) fractionated by 0.8% agarose gel electrophoresis. PCR analysis to detect the six transgene coding sequences was carried out using the following primers: for *hpt*, HPT1 (5′-GATCTCCAATCTGCGGGATC-3′) and HPT2 (5′-ACTCACCGCGACGTCTGTCG-3′); for *bar*, BAR1 (5′-GCGGTCTGCACCATCGTCAA-3′) and BAR2 (5′-GTCATGCC-AGTTCCCGTGCT-3′); for *gusA*, GUS1 (5′-ACGGCCTGTGGGC-ATTACGT-3′) and GUS2 (5′-GTTCGGCGTGGTGTAGAGCA-3′); for *gna*, GNA1 (5′-CGGATCCATGGCTAAGGCAAGT CTCCTC-3′) and GNA2 (5′-CGGTACCTCATTACTTTGCCGTCACAAG-3′); for *cry1Ac*, cry1Ac1 (5′-GCTCTCCGCGAGGAAATGCG-3′) and cry1Ac2 (5′-CACGTGGCTCAACCTGTGGG-3′); and for *cry2A*, cry2A1 (5′-GACATCCTCAGGGAGACC-3′) and cry2A2 (5′ ACCTGGAAGAGGGAGTAGAG-3′). PCR reactions were carried out as described by Maqbool and Christou (1999). PCR products were analysed by 0.8% agarose gel electrophoresis. The transforming plasmids were used as positive controls and DNA from wild-type plants as a negative control.

DNA isolation and Southern blot hybridisation

Genomic DNA was isolated from fresh leaf tissue using the Phytopure Plant DNA Extraction Kit (Amersham). Aliquots of DNA (5μg) were digested with *Sac*I or *Sph*I (there are unique sites for one or other of these enzymes in each of the transforming plasmids used) or *Sac*I and *Kpn*I (a combination that releases the *gna* expression cassette). The DNA was fractionated by 0.8% agarose gel electrophoresis, denatured, neutralised and blotted onto Hybond-N^+ nylon membranes (Amersham). For the insecticidal transgenes, specific probes were generated using the following enzymes: a *Pst*I digest of pIA2 to isolate a 1.4-kbp *cry1Ac* fragment, an *Xba*I-*Xho*I digest of pROB6 to isolate a 738-bp *cry2A* fragment; a *Hin*dIII digest of pRSs-*gna* to isolate a 450-bp *gna* fragment; and an *Xba*I-*Sac*I digest of the cotransformation plasmid to isolate a 988-bp *hpt* fragment. For the marker genes, PCR with gene-specific primers was used to generate the probes (a 423-bp *bar* product and a 1880-bp *gusA* product, using the plasmid as template). A gel-purified DNA fragment corresponding to each gene construct was used to generate a random-primed [^{32}P]-labelled probe (Feinberg et al. 1994). Southern blots were prepared and hybridised according to standard procedures (Sambrook et al. 1989).

Histochemical and fluorometric β-glucuronidase (GUS) assays

Crude leaf protein extracts were prepared according to the method of Jefferson et al. (1987). GUS activity was detected by incubating tissues with the substrate 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc, Sigma). GUS activity was quantified using the substrate 4-methylumbelliferone glucuronide (MUG, Sigma). The fluorescent product 4-methylumbelliferone (MU) was assayed using a Perkin Elmer LS-50 luminescence spectrophotometer, with an extinction wavelength of 365 nm and an emission wavelength of 455 nm. Protein concentrations were determined using the Bradford dye-binding method (Bradford 1976). Specific enzyme activity was expressed in pmol MU h^{-1} μg^{-1} protein.

Phosphinothricin acetyltransferase (PAT) assays

PAT assays for *bar* gene activity were carried out by spectrophotometry and thin-layer chromatography (TLC) as described by de Block et al. (1987).

Western blot analysis

Western blots were used to detect Cry1Ac, Cry2A and GNA proteins. Samples of leaf tissue were homogenised in 1× PBS buffer and centrifuged at 6000 *g* for 10 min at 4°C. The supernatants were collected and protein concentrations determined using the Bradford dye-binding assay (Bradford 1976). Aliquots (30 µg) of protein were combined with an equal volume of 2× SDS sample buffer and boiled for 10 min. The protein samples were fractionated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970) and transferred onto nitrocellulose membranes (Hybond-C, Amersham) according to standard methods (Sambrook et al. 1989). After transfer, filters were soaked in 2% periodic acid for 10 min and blocked by incubation in 10× PBS containing 10% non-fat dried milk and 0.1% Tween-20 for 2 h at room temperature. The filter was probed with the appropriate antibodies: polyclonal rabbit anti-Cry1Ac serum, rabbit anti-GNA serum or rabbit anti-Cry2A serum (1: 10000 dilution), and signals were detected using alkaline phosphatase (AP)- and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Bio-Rad) as the secondary antibody (1: 10000 dilution). Incubation, washing and detection procedures were carried out following standard protocols (Sambrook et al. 1989).

Results

Transformation and regeneration of transgenic plants

Independent transformation experiments were carried out using seven different transgene cassettes. A total of 40 independent transgenic plant lines were analysed for transgene stability over three and, in some cases, four generations. Of these plants, 12 carried *hpt*, *gusA* and *bar*, 8 carried Ubi-*gn*a, *gusA* and *hpt*, 6 carried RSs-*gna*, *gusA* and *hpt*, 8 carried *cry2A*, *gusA* and *hpt* and 6 carried *cry1A*c and *hpt.* Plants were regenerated in the presence of hygromycin, hence all surviving plants carried a functional *hpt* gene. Progeny from 40 independent plants were used to study the stability of transgene inheritance. Seeds were germinated in the presence of hygromycin and then transferred to the greenhouse where seedlings were grown to maturity.

Expression and inheritance of three marker genes delivered using a cointegrate construct

We analysed 12 transgenic plant lines representing five rice varieties (Bengal, Cypress, Lido, Koshihikari, and IR72) that had been bombarded with the cointegrate vector containing three markers, *gusA*, *hpt* and *bar*. Genetic analysis confirmed that all lines carried a single transgenic locus with one to seven copies of the three-gene construct (Kohli et al. 1998). Three to seven PCR-positive plants per line were used to determine PAT and GUS activities. Transgenic plants were grouped into three categories – high, moderate or low expressers – according to

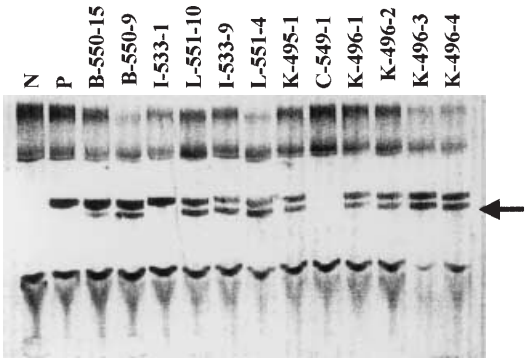

Fig. 1 Detection of PAT activity by thin-layer chromatography. Extracts were prepared from leaf tissue and reactions carried out with 20 µg total protein. [^{14}C]-labelled substrates and reaction products were visualised by autoradiography. *N* Negative control (wild-type plant), *P* positive control (PAT protein), *other lanes*: independent transgenic lines. The *arrow* shows the position of acetylated PPT bands

GUS levels in the R_0 plants (Table 1). GUS activity in progeny plants was determined by histochemical and/or fluorometric GUS assays. The activity of the *bar* gene was determined by TLC and spectrophotometric PAT assays. In some plants, PAT activity could be detected using the qualitative TLC assay (Fig. 1) but not the quantitative spectrophotometric assay, probably due to the low signal-to-noise ratio of the latter procedure at low expression levels. Figure 2 shows comparative results of quantitative expression analysis for *gusA* and *bar*. While it was not possible to make a direct comparison between the activities of the two enzymes, there was a strong correlation between the relative GUS and PAT activities in individual plants; i.e. the activities of both enzymes were either high or low. Quantitative analysis of specific activities revealed at least a threefold and ninefold variation in GUS and PAT activities, respectively, among independently derived transgenic lines. R_2 and R_3 plants of lines K496–4 and K496–1 consistently lacked detectable GUS activity, although the primary transformants and R_1 plants showed moderate GUS activity, indicating that the *gusA* transgene had undergone silencing. Plants from line C549–1 consistently lacked detectable PAT activity; however, in this case the primary transformants also lacked PAT activity. Southern blot hybridisation of K496–4, K496–1 and C549–1 genomic DNA revealed banding patterns identical to those of the primary transformants, showing that there had been no loss or rearrangement of the genes during transmission from R_0 to R_1. Stable inheritance of the *gusA*, *hpt* and *bar* transgenes was also found for the remaining (non-silenced) lines, from R_0 through to R_3 (Fig. 3).

Expression and inheritance of *gna* driven by two different promoters

To investigate the inheritance and expression of *gna* driven by the constitutive Ubi-1 promoter and the tissue-

Table 1 Rice lines used for transgene expression analysis, showing integrated transgenes and protein expression levels

Rice variety	Integrated transgenes[a]	Protein levels[a]	Transgenic lines
Bengal	*hpt-gusA-bar*	High	B-550–9
	hpt-gusA-bar	None	B-550–15
Cypress	*hpt-gusA-bar*	High	C-549–1
IR72	*hpt-gusA-bar*	High	I-533–1
	hpt-gusA-bar	Low	I-533–9
Lido	*hpt-gusA-bar*	High	L-551–10
	hpt-gusA-bar	Low	L-551–4
Koshihikari	*hpt-gusA-bar*	High	K495–1
	hpt-gusA-bar	Moderate	K-496–1
	hpt-gusA-bar	High	K-496–2
	hpt-gusA-bar	Moderate	K-496–3
	hpt-gusA-bar	Moderate	K-496–4
M5	*Ubi-gna*, *gusA-hpt*	High	M5–7
M12	*Ubi-gna*, *gusA-hpt*	High	M12–20
	RSsI-gna, *gusA-hpt*	High	M12–29
	RSsI-gna, *gusA-hpt*	Low	M12–48
	RSsI-gna, *gusA-hpt*	Low	M12–44
FX97	*Ubi-gna*, *gusA-hpt*	Moderate	FX97-15
	Ubi-gna, *gusA-hpt*	High	FX97–7
ASD16	*Ubi-gna*, *gusA-hpt*	Moderate	ASD16–32
	Ubi-gna, *gusA-hpt*	Low	ASD16–44
	Ubi-gna, *gusA-hpt*	Low	ASD16–45
	Ubi-gna, *gusA-hpt*	Low	ASD16–85
	RSsI-gna, *gusA-hpt*	High	ASD16–47
	RSsI-gna, *gusA-hpt*	High	ASD16–54
	RSsI-gna, *gusA-hpt*	Moderate	ASD16–78
M7	*cry2A*, *gusA-hpt*	High	M7–10
	cry2A, *gusA-hpt*	High	M7–12
	cry2A, *gusA-hpt*	High	M7–13
	cry2A, *gusA-hpt*	High	M7–14
	cry2A, *gusA-hpt*	High	M7–33
	cry2A, *gusA-hpt*	Moderate	M7–31
	cry2A, *gusA-hpt*	Low	M7–37
	cry1Ac, *hpt*	High	M7–42
	cry1Ac, *hpt*	High	M7–47
	cry1Ac, *hpt*	Moderate	M7–18
	cry1Ac, *hpt*	Moderate	M7–45
Basmati 370	*cry2A*, *gusA-hpt*	Low	Bas370–1
	cry1Ac, *hpt*	Low	Bas370-C1
	cry1Ac, *hpt*	Low	Bas370-C2

[a] Plants classified into high, moderate and low/non-expression categories according to the expression level of the underlined gene. Linkage between *gusA*, *hpt* and *bar* is shown where appropriate. Levels of protein expression (GNA, Cry1Ac, Cry2A) are shown as total soluble protein (high: >0.5%; moderate: 0.2–4.9%; low: <0.2%). *gusA* expression is determined by GUS activity in pmol MU μg^{-1} h^{-1} [high: >40; moderate: 21–40; low: 2–20; none <2 (this level is not detectable using the histochemical assay)]

specific RSs-1 promoter, we analysed 14 transgenic lines cotransformed with one of the two *gna* constructs and a second plasmid providing the *hpt* and *gusA* markers. The primary transformants were selected for hygromycin resistance and screened for GUS activity. Different lines were classified according to GNA expression levels, estimated visually on the basis of staining intensity on western blots (with equal amounts of protein loaded in each lane).

To investigate the stability of transgene expression, more than 30 seeds representing each expression category were germinated on hygromycin-supplemented medium. Plants thus derived were screened for the presence of the *gna* and *gusA* transgenes by PCR, and positive plants were used for Southern and western blot analysis. Additionally, GUS activity was determined by histochemical assay of leaf tissue. Southern blot analysis confirmed the structural integrity of the transgenes (data not shown). Western blot analysis showed that GNA expression levels were also stably transmitted, with high-expressing primary transformants giving rise to high-expressing R_1 and R_2 progeny. Moderate and low or non-expressers also gave rise to progeny with similar phenotypes. Figure 4A and 4B show western blot analysis of R_2 plants with *gna* driven by the Ubi-1 and RSs-1 promoters, respectively. All 14 lines analysed showed the presence of a 12-kDa protein corresponding to the expected size of GNA.

In most of the lines (11/14), the three transgenes segregated together as a single Mendelian trait (3:1 ratio), indicating cointegration of the two cotransforming plasmids at a single locus. Three lines showed an aberrant 1:1 ratio. In two lines (FX97–7 and M12–20) R_2 and R_3 progeny were homozygous. Table 2 shows transgene expression characteristics in a representative sample of the lines in the R_2 and R_3 generations.

Higher levels of GNA expression were observed in plants transformed with pUbi-*gna* (up to 2% total soluble protein) compared to those transformed with pRSs-*gna* (up to 1% total soluble protein).

Expression and inheritance of the Bt genes (*cry1Ac* and *cry2A*)

To study *cry1Ac* and *cry2A* transgene inheritance and the stability of Cry1Ac and Cry2A protein expression, we analysed 14 independent transgenic lines representing two elite indica rice varieties (Basmati 370 and M7). Transgenic plants were generated by cotransformation using either pIA2 (containing the *cry1Ac* gene) or pROB6 (containing the *cry2A* gene) together with a vector containing the selectable marker *hpt*. Transgenic lines were grouped into high, moderate or low and non-expression categories according to the level of Cry protein detected on western blots. More than 30 seeds were germinated from each line for molecular and expression analysis in the R_1 and R_2 generations.

We analysed 6 *cry1Ac*-transgenic lines. All lines showed 3:1 segregation for the transgenes in the R_1 and R_2 generations. One homozygous line (M7–18–5) was identified in the R_2 generation. Western blot analysis of R_2 plants showed the presence of a 65-kDa polypeptide corresponding to the purified Cry1Ac standard (Fig. 4 C).

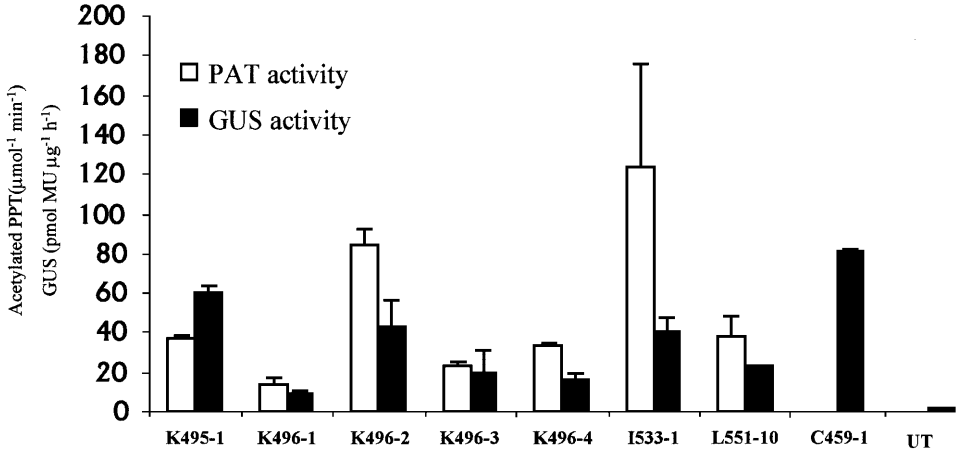

Fig. 2 GUS and PAT activities in a representative sample of R_1 transgenic rice plants, and in a wild-type plant (negative control). GUS activity expressed in pmol MU μg^{-1} h^{-1}; PAT activity expressed as the production of acetylated PPT (μmol^{-1} min^{-1}). Values represent an average over 3 R_1 plants, with standard errors as shown

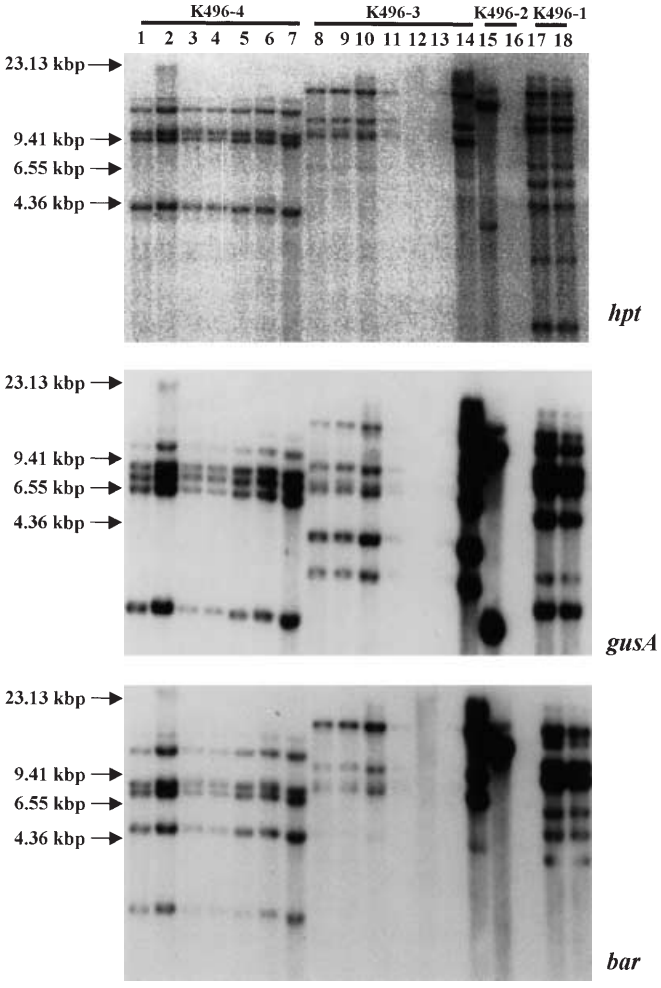

Fig. 3 Southern blot analysis of R_3 transgenic plants carrying the three-marker transformation plasmid. The filter was sequentially probed with the *hpt* (*top panel*), *gusA* (*middle panel*) and *bar* (*lower panel*) coding regions. *Lanes* represent individual plants grouped into sibling groups (*above bar*). The molecular-weight marker is *Hin*dIII-digested λDNA

Most seedlings from lines expressing Cry2a at high levels (M7–10, M7–12, M7–13 and M7–14) died within 2 weeks of emergence, probably due to the toxic effects of high-level endotoxin expression. However, we were able to grow a limited number of R_1 plants from lines M7–10 and M7–13, and recovered R_2 seeds. Western blot analysis of R_2 plants showed the presence of a 71-kDa polypeptide, corresponding to the Cry2 A standard, in only 4 of the 8 lines we studied (Fig. 4D). Two lines (M7–31 and M7–37) showed normal growth characteristics, comparable to those of the M7 wild-type plants, and displayed Mendelian inheritance and stable transgene expression in the R_1 and R_2 generations. These lines expressed the Cry2A protein at moderate and low levels (Table 1; Fig. 4D). However, plants from line M 7–13, which expressed Cry2A at high levels (2% total soluble protein) were stunted, set few seeds and matured earlier than wild-type plants. All 15 R_1 plants from this line analysed by western blot showed that high expression levels were faithfully transmitted to R_2 progeny (Fig. 4D and Table 3). Line Bas370–1 showed aberrant segregation (1:1) for *cry2A* and other genes in R_1 plants, but analysis of R_2 progeny showed that genetic stability was restored in one of the two lines (not shown). In some Bas370–1 progeny plants, protein expression was undetectable by western blot, although it was confirmed by insect feeding bioassays (Maqbool et al. 1998).

A number of additional bands were observed on the Cry protein western blots. These could be degradation products generated during the boiling process (Cheng et al. 1998) but might also represent proteins cross-reacting with the polyclonal antiserum we used.

Discussion

The success of national breeding programmes using transgenic germplasm will rely on the efficient generation of transgenic plants representing elite, locally-grown commercial rice varieties, and the stable inheritance and expression of multiple agronomically-important transgenes over many (all) subsequent generations.

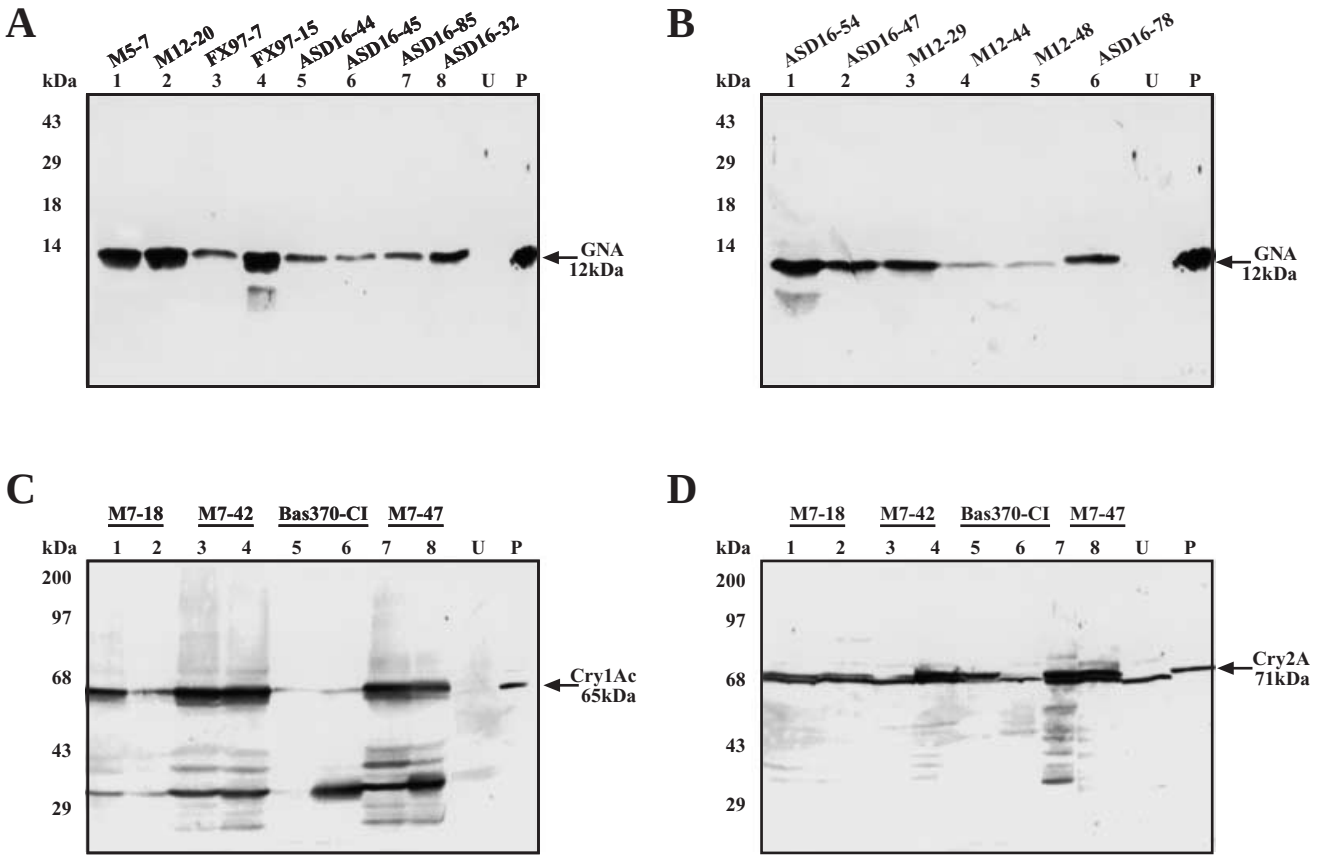

Fig. 4A–D Western blot analysis of R_2 transgenic rice lines expressing GNA protein (expression driven by the Ubi-1 promoter) (**A**), GNA protein (expression driven by the RSs-1 promoter) (**B**) Cry1Ac (**C**), Cry2A (**D**). *U* Untransformed control plant, *P* purified protein (size indicated by arrow). In **A** and **B** the individually labelled lanes represent independent transgenic lines; in **C** and **D** the grouped lanes represent siblings from the same transgenic line

Particle bombardment is the method of choice for generating useful transgenic germplasm because it allows the simultaneous introduction of multiple genes into rice in a genotype-independent manner (Sudhakar et al. 1998a; Vain et al. 1998; Valdez et al. 1998). For example, Chen et al. (1998) reported the integration of 13 out of 14 different plasmids they bombarded into rice. The multiple plasmids integrated at a single transgenic locus, which varied in size from 78 kbp to 200 kbp in different lines. The stable inheritance of such large transgenic loci confirms the suitability of particle bombardment for the stable transfer of multiple transgenes. However, the methodology has not been widely used to introduce multiple genes of agronomic importance into elite commercial rice varieties. Generally, agronomically important genes have been introduced only into model rice varieties, such as Nipponbare and Taipei 309, while very few commercial rice cultivars have been transformed with agronomically useful genes. A small number of indica breeding lines were used in most such studies, e.g. IR58, IR64 and IR72. Moreover, the stability of transgene expression was never studied in detail further than the R_1 generation. Datta et al. (1998) transformed six indica rice varieties (including two additional IR breeding lines and two locally important Indian varieties) with c*ry1Ab*. However, the analysis of transgenic plants was again limited to the R_1 generation.

In the present study we used particle bombardment to introduce marker genes and four genes of agronomic importance into a diverse range of elite rice cultivars. We then analysed transgene expression and stability up to the R_3 generation. The varieties we used represented 11 genotypes widely grown in the Americas, Europe, the Indian Peninsula, China, Japan, Southeast Asia and Africa. We used four transgenes conferring traits of agronomic interest: the *bar* gene for resistance to phosphinothricin (the active component of the herbicide Basta) and the insecticidal genes *gna*, *cry1Ac* and *cry2a* (see Table 1). We also evaluated three different promoters for transgene expression. We used two different gene delivery strategies: cotransformation of genes on different plasmids and transformation with a cointegrate vector carrying linked genes. Our results enabled us to determine the effects of these variables on transgene expression.

We used a cointegrate vector to introduce the *hpt*, *gusA*, and *bar* genes into rice varieties as shown in Table 1. One or more copies of this construct integrated at a single locus unique to each transgenic line (Kohli et al. 1998). Southern blots of genomic DNA from R_0, R_1 and R_2 plants of the same line, and from among R_2 siblings, showed identical band patterns. A single transgenic locus was evident from segregation analysis using PCR and

Table 2 Inheritance of *gna*, *gusA* and *hpt* transgenes in R_1, R_2 and R_3 generations. Plants were germinated on medium containing hygromycin, and resistance was scored 10 days post-emergence. The seedlings were then transferred to soil. Young leaves were analysed by PCR for the presence of *gna*, gusA and *hpt* transgenes

Generation	Transformant	Number of positive seedling assayed			Total number	Ratio analysed	χ^2	*P*
		gna	*gusA*	*hpt*				
R_1	ASD16–47	25	25	25	34	3:1 *gna*	0.04	0.83
						3:1 *gusA*	0.04	0.83
						3:1 *hpt*	0.04	0.83
	ASD16–54	28	28	28	40	3:1 *gna*	0.133	0.715
						3:1 *gusA*	0.133	0.715
						3:1 *hpt*	0.133	0.715
	FX97-7	20	20	20	28	3:1 *gna*	0.191	0.662
						3:1 *gusA*	0.191	0.662
						3:1 *hpt*	0.191	0.662
	M5–7	27	27	27	34	3:1 *gna*	0.353	0.55
						3:1 *gusA*	0.353	0.55
						3:1 *hpt*	0.353	0.55
	M12–20	26	26	26	34	3:1 *gna*	0.25	0.55
						3:1 *gusA*	0.25	0.55
						3:1 *hpt*	0.25	0.55
	M12–44	12	15	15	34	1:1 *gna*	2.454	0.11
						1:1 *gusA*		
						1:1 *hpt*		
R_2	ASD16–47–1	30	30	30	34	3:1 *gna*	3.18	0.08
						3:1 *gusA*	3.18	0.08
						3:1 *hpt*	3.18	0.08
	ASD16–47–2	46	46	46	57	3:1 *gna*	1.47	0.25
						3:1 *gusA*	1.47	0.25
						3:1 *hpt*	1.47	0.25
	ASD16–47–3	44	44	44	54	3:1 *gna*	1.22	0.25
						3:1 *gusA*	1.22	0.25
						3:1 *hpt*	1.22	0.25
	ASD 16–54–1	32	32	32	43	3:1 *gna*	0.002	0.9
						3:1 *gusA*	0.002	0.9
						3:1 *hpt*	0.002	0.9
	ASD 16–54–2	40	40	40	55	3:1 *gna*	0.055	0.7
						3:1 *gusA*	0.055	0.7
						3:1 *hpt*	0.055	0.7
	ASD 16–54–3	25	25	25	34	3:1 *gna*	0.039	0.83
						3:1 *gusA*	0.039	0.83
						3:1 *hpt*	0.039	0.83
	FX97–7–1	60	60	60	60	Homozygous		
	FX97–7–2	55	55	55	55	Homozygous		
	FX 97–7–3	69	69	69	93	3:1 *gna*	0.33	0.55
						3:1 *gusA*	0.33	0.55
						3:1 *hpt*	0.33	0.55
	M5–7–1	29	29	29	42	3:1 *gna*	0.79	0.46
						3:1 *gusA*	0.79	0.46
						3:1 *hpt*	0.79	0.46
	M5–7–2	40	40	40	54	3:1 *gna*	0.04	0.85
						3:1 *gusA*	0.04	0.85
						3:1 *hpt*	0.04	0.85
	M5–7–3	40	40	40	55	3:1 *gna*	0.055	0.8
						3:1 *gusA*	0.055	0.8
						3:1 *hpt*	0.055	0.8
	M12–20–1	60	60	60	83	3:1 *gna*	0.325	0.5525
						3:1 *gusA*	0.325	0.5525
						3:1 *hpt*	0.325	0.5525
	M12–20–2	25	25	25	34	3:1 *gna*	0.039	0.843
						3:1 *gusA*	0.039	0.843
						3:1 *hpt*	0.039	0.834
	M12–20–3	55	55	55	55	Homozygous		
R_3	ASD 16–54–1–1	53	53	53	66	3:1 *gna*	0.24	0.55
						3:1 *gusA*	0.24	0.55
						3:1 *hpt*	0.24	0.55
	ASD 16–54–1–2	40	40	40	55	3:1 *gna*	0.055	0.7
						3:1 *gusA*	0.055	0.7
						3:1 *hpt*	0.055	0.7

Table 2 (contiuned)

Generation	Transformant	Number of positive seedling assayed			Total number analysed	Ratio	χ^2	*P*
		gna	*gusA*	*hpt*				
R_3	ASD 16–54–1–3	44	44	44	61	3:1 *gna*	0.26	0.55
						3:1 *gusA*	0.26	0.55
						3:1 *hpt*	0.26	0.55
	FX97–1–1	48	48	48	48	Homozygous		
	FX 97–1–2	48	48	48	48	Homozygous		
	FX 97–1–3	48	48	48	48	Homozygous		
	M5–7–2–1	28	28	28	40	3:1 *gna*	0.133	0.71
						3:1 *gusA*	0.133	0.71
						3:1 *hpt*	0.133	0.71
	M5–7–2–2	30	30	30	30	Homozygous		
	M5–7–2–3	30	30	30	34	3:1 *gna*	3.18	0.08
						3:1 *gusA*	3.18	0.08
						3:1 *hpt*	3.18	0.08
	M12–20–3–1	48	48	48	48	Homozygous		
	M12–20–3–2	60	60	60	60	Homozygous		
	M12–20–3–3	60	60	60	60	Homozygous		

protein expression data. With the exception of line K495–1, PCR analysis confirmed a 3:1 Mendelian segregation in all lines at the DNA level. Many lines also showed Mendelian segregation for protein expression, but the ratio was distorted for *gusA* in lines K496–1 and K496–3 due to silencing (Kohli et al. 1999). Line C549–1 carried a single integrated copy of the construct, but *bar* gene expression was undetectable even in the R_0 plant. Further analysis showed that the CaMV 35S promoter driving *bar* in this line was rearranged (Fu et al. 2000). Using the cotransformation strategy, we delivered selectable markers on one plasmid and the insecticidal transgenes on another. In all transgenic lines thus transformed, Southern blots of genomic DNA from primary transformants and R_1 plants also showed identical banding patterns, indicating the presence of a single, stable transgenic locus. A number of models have been proposed to explain why unlinked transgenes integrate at a single locus and may become interspersed with genomic DNA (de Neve et al. 1997; Jorgensen et al. 1987; Kohli et al. 1998; Pawlowski and Somers 1998; Salomon and Puchta 1998).

To investigate the stability of transgene expression, we studied four types of variation in expression levels: variation between independent transformed lines, variation between parents and progeny, variation among siblings and covariation in the expression of different transgenes. As previously observed, there was significant variation in expression levels among independent transformants. This could reflect the influence of many factors, including position effects, copy number, transgene rearrangements, genetic background and the physiological and developmental states of the plant (Beaujean et al. 1998; Dean et al. 1988; Jones et al. 1987; Peach and Velten 1991; Scott et al. 1998; Shirsat et al. 1989;). However, there was much less variation between parents and progeny. Primary transformants with high or low PAT and GUS activities produced progeny plants with the same characteristics. Beaujean et al. (1998) observed a similar relationship between the GUS activities of parent and progeny tobacco plants. We found no significant differences in PAT or GUS activities among sibling plants (Fig. 2). Other studies have shown variable transgene expression levels among sibling plants (Hobbs et al. 1990; Peng et al. 1995). Scott et al. (1998) observed up to a fourfold variation in GUS activity among backcrossed (BC_1 and BC_2) and R_2 populations of white clover plants, but found no significant difference in median GUS activity values when the three populations were grouped together. We found a positive correlation between GUS and PAT activities in individual plants. Analysis of 18 transgenic wheat lines also showed a positive correlation between *gusA* and *bar* (E. Stoger, personal communication). However, there was no correlation in the levels of GUS and CAT activities in tobacco callus clones containing the *cat* and *gusA* genes both driven by the mannopine synthase (*mas*) promoter (Peach and Velten 1991). We found that GUS and PAT activities varied over the entire population of transformants, and similar variation was observed in all the rice varieties. This indicates that genetic background may not significantly affect the variability of transgene expression. Plants derived through the cotransformation strategy showed transgene transmission characteristics similar to plants obtained using the cointegrate vector.

We observed unusual segregation patterns in 15% of the transgenic lines. Such distorted ratios have been reported previously in rice (Goto et al. 1993; Peng et al. 1995) and in other plants (Christou et al. 1989; Deroles and Gardner 1988; Potrykus et al. 1985; Rhodes et al. 1988; Scott et al. 1998; Spencer et al. 1992; Walters et al. 1992). Segregation distortion may reflect sterility in one set of gametes (Christou et al. 1989) or homozygous lethality (Budar et al. 1986; Deroles and Gardner 1988; Scott et al. 1998). Table 2 shows expression and segregation data (R_1 to R_3) for transgenic lines generated by co-

Table 3 Inheritance of *Bt*, *gusA* and *hpt* transgenes in R1, R2 and R3 generations. Plants were germinated on medium containing hygromycin, and resistance was scored 10 days post-emergence. The seedlings were transferred to soil. Young leaves were then analysed by PCR for the presence of *Bt*, *gusA* and *hpt* transgenes

Generation	Transformants	Number of positive seedlings assayed			Total number analysed	Ratio	χ^2	*P*
		Bt	*gusA*	*hpt*				
R_1	M7–10–1	4	4	4	6	3:1 *cry2A* 3:1 *gusA* 3:1 *hpt*		
	M7–13–1	10	10	10	10	Homozygous		
	M7–31–1	26	26	26	35	3:1 *cry2A* 3:1 *gusA* 3:1 *hpt*	0.01 0.01 0.01	0.99 0.99 0.99
	M7–31–3	24	28	28	34	3:1 *cry2A* 3:1 *gusA* 3:1 *hpt*	0.353 0.353 0.353	0.55 0.55 0.55
	M7–37–3	12	21	23	30	1:1 *cry2A* 3:1 *gusA* 3:1 *hpt*	2.45 0.353 0.353	0.11 0.55 0.55
	Bas 370–1–1	15	0	26	30	1:1 *cry2A* 3:1 *hpt*	0.273 0.25	0.398 0.55
	Bas 370–1–7	9	0	9	20	1:1 *cry2A* 1:1 *hpt*		
	M7–18–5	22	22	22	30	3:1 *crylAc* 3:1 *gusA* 3:1 *hpt*	0.04 0.04 0.04	0.9 0.9 0.9
	M7–18–4	15	15	15	15	Homozygous		
	M7–42–4	28	28	28	40	3:1 *cry1Ac* 3:1 *gusA* 3:1 *hpt*	0.133 0.133 0.133	0.71 0.71 0.71
	M7–47–3	22	22	22	28	3:1 *cry1Ac* 3:1 *gusA* 3:1 *hpt*	0.19 0.19 0.19	0.66 0.66 0.66
	Bas 370–C1–2	12	na	15	34	1:1 *cry1Ac* 1:1 *hpt*	2.245 0.237	0.117 0.398
	Bas 370–C2–6	6	na	6	10	3:1 *cry1Ac* 3:1 *hpt*		
R_2	M7–18–5–3	30	30	30	30	Homozygous		
	M7–13–1–1	15	15	15	15	Homozygous		
	M7–31–3–1	40	40	40	54	3:1 *cry2A* 3:1 *gusA* 3:1 *hpt*	0.04 0.04 0.04	0.9 0.9 0.9
	M7–31–3–2	32	32	32	43	3:1 *cry2A* 3:1 *gusA* 3:1 *hpt*	0.02 0.02 0.02	0.96 0.96 0.96
	M7–31–3–3	32	32	32	39	3:1 *cry2A* 3:1 *gusA* 3:1 *hpt*	1.034 1.034 1.034	0.395 0.395 0.395
	M7–37–3–1	29	29	29	40	3:1 *cry2A* 3:1 *gusA* 3:1 *hpt*	0.133 0.133 0.133	0.7 0.7 0.7
R_3	M7–13–1–1–1	20	20	20	20	Homozygous		
	M7–13–1–1–2	20	20	20	20	Homozygous		
	M7–13–1–1–3	13	13	13	13	Homozygous		
	M7–31–3–1–1	44	44	44	55	3:1 *cry2A* 3:1 *gusA* 3:1 *hpt*	0.055 0.055 0.055	0.8 0.8 0.8
	M7–31–3–1–2	44	44	44	61	3:1 *cry2A* 3:1 *gusA* 3:1 *hpt*	0.268 0.268 0.268	0.55 0.55 0.55
	M7–31–3–1–3	32	32	32	43	3:1 *cry2A* 3:1 *gusA* 3:1 *hpt*	0.02 0.02 0.02	0.96 0.96 0.96

transformation. Since we generated homozygous lines, we discounted homozygous lethality as the reason for distortion. We have obtained preliminary evidence to support gamete lethality as the cause: in one line (C549–1) iodine staining of pollen revealed a number of intact but deformed pollen grains in some R_2 plants. R_3 progeny of one such plant showed a distorted segregation ratio of 1:1. (Fu et al. 2000). Scott et al. (1998) at-

tributed the distorted segregation ratio observed in later generations of transgenic white clover plants to the outbreeding nature of the species, which leads to changes in the genetic background. We observed similar distorted ratios even though rice, unlike white clover, is an inbreeding species. Aberrant phenotypic segregation ratios have also been observed in non-transgenic plants (Bradshaw and Stettler 1994).

Gene silencing also contributes to non-Mendelian transmission where segregation ratios are determined through the analysis of protein expression (Finnegan and McElroy 1994; McElroy and Brettel 1995). We observed *gusA* silencing in some R_1 plants, and this phenomenon occurred with equal frequency using the cotransformation and cointegrate vector strategies. The mechanisms of transgene silencing have been extensively reviewed (Meyer 1998). The presence of multiple transgene copies has been implicated in transgene silencing, but we found no correlation between transgene copy number and silencing in our plants. Other studies have also shown that the presence of multiple transgene copies does not necessarily lead to silencing (Beaujean et al. 1998; Kohli et al. 1999; Hobbs et al. 1993; Peach and Velten 1991; Shirsat et al. 1989).

In most of the lines we analysed, we found that high-level transgene expression did not affect the overall morphological phenotype of the plant. However, high level expression of the insecticidal Bt genes *cry1Ac* and *cry2A* (>1% total soluble protein) caused developmental and morphological defects such as sterility and stunted growth. It has been suggested that high levels of these toxins might be required for effective pest-management programmes in order to limit the build-up of insect resistance to transgenic plants (Boulter 1993). However, considerably lower levels of Cry1Ab (0.01% total soluble protein) were reported to confer 100% resistance to yellow stem borer and striped stem borer in rice (Wunn et al. 1996). The *Bt* gene expression levels observed in this study (0.01–2% total soluble protein) are within the range suitable for effective control of insect pests.

We used three promoters to drive transgene expression: the maize ubiquitin-1 promoter (Ubi-1), the rice sucrose synthase-1 promoter (RSs-1) and the cauliflower mosaic virus 35S RNA promoter (CaMV 35S). The Ubi-1 promoter has been reported to be more active than the CaMV 35S promoter in rice (Li et al. 1997), while the RSs1 promoter is tissue-specific (Shi et al. 1994). In the case of the *gna* transgene, we were able to perform direct comparisons between the Ubi-1 and RSs-1 promoters, and we showed that the Ubi-1 promoter conferred the highest transgene expression levels. However, this may be due to the low abundance of phloem cells (where the RSs-1 promoter is normally active) rather than any inherent difference in promoter strengths (Shi et al. 1994). Rao et al. (1998) found no difference in insect mortality levels using either the Ubi-1 or RSs-1 promoters to drive *gna* transgene expression.

In summary, we have demonstrated the stable expression of multiple transgenes (including markers and genes of agronomic importance) in diverse rice cultivars transformed by particle bombardment. Our analysis shows that transgenes are typically inherited and expressed in a stable form, regardless of the actual transgene construct, the transformation strategy (cointegrate vector or co-transformation) or the rice genotype. This is the first report of transgene stability and expression analysis up to the R_3 generation using agronomically important genes in a range of rice genotypes representative of world-wide commercially important cultivars. The ability to stably transform diverse rice cultivars with similar consequences in terms of transgene expression should allow transgenic material to be generated for a broad spectrum of breeding programmes and avoid the predominance of monoculture in crop improvement systems.

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