Theor Appl Genet (1993) 86:875–879

Field performance of derived generations of transgenic tobacco

P. D. S. Caligari[1], Y. M. H. B. Yapabandara[1,], E. M. Paul[2,*], J. Perret[3], P. Roger[3], J. M. Dunwell[2]**

[1] Department of Agricultural Botany, School of Plant Sciences, University of Reading, Whiteknights, PO Box 221, Reading RG6 2AS, UK
[2] Zenaca Seeds, Jealott's Hill Research Station, Bracknell, Berkshire, RG12 6EY, UK
[3] BIOCEM Limagrain Group, Laboratory of cell and Molecular Biology, C.U. des Cézeaux, 24, Av des Landais, F-63170 Aubière, France

Received: 9 September 1992 / Accepted: 4 January 1993

Abstract. Two inbred cultivars of *Nicotiana tabacum* (tobacco), 'Samsun' and 'Xanthi', were transformed with the plasmid pBI 121 using Bin 19 in *Agrobacterium tumefaciens*. The plasmid carries the *nptII* gene conferring kanamycin resistance and the *uidA* gene encoding β-glucuronidase (GUS). Progeny carrying the genes in the homozygous condition were identified and selfed over several generations. One line homozygous for the introduced genes and one untransformed control from each cultivar were then selected and crossed reciprocally to give four families per cultivar. Seeds from each family were grown in a replicated field trial and all plants scored for a range of morphological and agronomic characters. In addition, leaf samples were taken and GUS activity measured. In the 'Samsun' material, which contained one copy of the introduced gene at a single locus and showed high levels of GUS expression, the transformed homozygote showed twice the level of GUS activity as the hemizygotes, wheareas in the 'Xanthi' line, which had a lower level of GUS, the hemizygotes showed the same level of GUS activity as the transformed homozygote. The agronomic data showed differences between the families, but the source of such differences could not be ascribed unambiguously. The results are discussed in the light of related information on gene expression and field performance from other transgenic material.

Key words: Transgenic plants – *Nicotiana tabacum* – Field performance

Communicated by J. W. Snape
* *Present address:* Department of Plant Sciences, University of Oxford, South Parks Road, Oxford, OX1 3RB, UK
** *Present address:* Res. St., Dept of Export Agriculture, Matale, Sri Lanka
Correspondence to: P. D. S. Caligari

Introduction

Within the last few years, the production of transgenic plants has become commonplace, and their value in fundamental (Lam 1990) and applied studies (Dunwell and Paul 1990; Lycett and Grierson 1990) has been described in numerous reports. However, before such plants are accepted as having commercial value (Knauf 1991) the stability and expression of the introduced gene(s) must be confirmed over several generations and preferably in a range of genetic backgrounds. Moreover, such studies need to be conducted under field conditions, and the agronomic performance of the material confirmed in relevant trials (Jefferson 1990; McHughen and Holm 1991; McHughen and Rowland 1991).

Because of its high level of responsiveness in tissue culture, *Nicotiana tabacum* (tobacco) has been the species of choice in many transformation experiments, and an extensive series of data has been produced on the genetic analysis of transformants produced either by the use of *Agrobacterium tumefaciens* (Otten et al. 1981; Budar et al. 1986; Heberle-Bors et al. 1988; Vyskot et al. 1989) or by direct gene transfer techniques (Potrykus et al. 1985; Bellini et al. 1989; Tomes et al. 1990). Several of these studies show that non-Mendelian as well as Mendelian segregation may occur amongst the progeny of the initial transformant and that, in addition, the level of expression of the selectable marker gene may vary considerably amongst different transformants. It has also been demonstrated with doubly transformed tobacco plants that the first introduced T-DNA may be inactivated by the introduction into the same genome of an unlinked second T-DNA, (Matzke and Matzke 1990, 1991). In a related study, notable for being the only one to include field data, Hobbs et al. (1990) described the relative expression of

the reporter gene *uidA* (encoding β-glucuronidase, GUS) in a range of transformants.

In the study reported here the selfed progeny of two independent transformants were selected and intercrossed with non-transgenic plants in order to study both the genetic aspects of the reporter gene expression as well as the relative agronomic performance of transgenic and non-transgenic material under field conditions.

Materials and methods

Two inbred cultivars of *Nicotiana tabacum* (tobacco), 'Samsun' (ICI) and 'Xanthi' (BIOCEM), were transformed by leaf disc methods in the respective laboratories with the plasmid pBI 121 using a Bin 19 vector in *Agrobacterium tumefaciens*. The plasmid carries the *nptII* gene conferring kanamycin resistance and the *uidA* gene encoding β-glucuronidase (GUS). The regenerated plants were selfed, and progenies that were homozygous for kanamycin resistance and GUS expression were selected and selfed over four (ICI) or three (BIOCEM) generations. The transformed plants proved to have inserts only at a single site and with one, or at most two, copies at such a site. Alongside these plants were lines which had been through similar in vitro procedures but which had not been subject to transformation procedures; these were selfed for a similar number of generations. This gave rise to a series of lines from which was taken one transformed 'Samsun' line (S^+) showing a high level of GUS expression, one untransformed 'Samsun' line (S), one transformed 'Xanthi' line (X^+) showing a lower level of GUS expression and one untransformed Xanthi line (X).

The two lines (i.e. transformed and untransformed) from each cultivar were crossed, by BIOCEM, to give, including reciprocals, four families per cultivar [i.e. 'Samsun' unmodified (S), 'Samsun' modified (S^+) and the reciprocal F_1 crosses between them; and similarly 'Xanthi' unmodified (X), 'Xanthi' modified (X^+) and the reciprocal F_1 crosses between them] (Table 1). Seeds from each of the eight families, along with the seeds from unmodified parents for use in guard rows, were sown three per pot, in Jiffy pots (35 mm × 35 mm) filled with J.I. No. 1 soil mixture and placed in a shaded glasshouse. These pots were laid out in the glasshouse in a randomized block design with 20 blocks. Three weeks after sowing, excess plantlets were randomly thinned out, leaving the one nearest to the label, in order to obtain a single plant per pot. Plants were maintained for a further 2 weeks in the glasshouse before being transferred to the field.

The experimental plots were laid out according to the randomized block design with 20 blocks (1 plant/family per block) with the plants in each block planted as two rows with a spacing of 1.0 m between the rows and 0.5 m within rows. These 20 blocks were surrounded by a guard row of plants (unmodified parents) established with the same spacing. An insecticide was sprayed to control insect damage, which started to appear 1 week after field planting, and the trial plots were regularly inspected and weeded.

In order to assess activity of the reporter gene, two half-circles of leaves, without the midrib, were taken from the tip of the eighth and the ninth leaf of each plant (6 weeks after planting). The samples were immediately brought to the laboratory. The protocol used for leaf extraction and GUS assay (fluorogenic assay) was as previously described by Jefferson (1987). Enzyme activity was expressed as nanomoles of methyl umbelliferone/milligram of protein per hour (nmol MU/mg protein per hour).

Plant height and length and width of the largest leaf were measured, and the number of leaves was counted 1 day before field planting and subsequently at 2 weeks, 4 weeks, 6 weeks and 8 weeks after field planting. The date of flowering was recorded as the day on which the first open flower appeared in each plant. An arbitrary scale of 1–9, (9 being maximum fruit set) was later used to estimate the fertility on each plant. Five mature seed capsules on the main branch were randomly chosen from each of the plants, collected as separate capsules into envelopes and allowed to dry at room temperature. Subsequently, the seeds were extracted, and the seed weight of each capsule was recorded. The weight of 100 seeds was measured after mixing the seeds from the five capsules taken from the same plant. Thereafter, the number of seeds in each capsule was calculated from the total seed weight of the capsule and the average weight of 100 seeds.

The characters recorded can therefore be summarized as:

Character	Abbreviation
β-glucuronidase activity	[GUS]
Height (cm) at 2, 4, 6 and 8 weeks	[2HT, 4HT, 6HT and 8HT]
Number of leaves at 2, 4 and 6 weeks	[2NLVS, 4NLVS, and 6NLVS]
Width (cm) of largest leaf at 2, 4 and 6 weeks	[2WLVS, 4WLVS and 6WLVS]
Length (cm) of largest leaf at 2, 4 and 6 weeks	[2LLVS, 4LLVS and 6LLVS]
Days to first flower (from August 1st	[F. DATE]
Estimate fertility	[FERT]
100 000 seed weight (g)	[10^5 SWT]
Seed weight per capsule (g × 10)	[CSWT]

Results

GUS activity

The results of assays for GUS activity, one of the specifically introduced characters, are presented in Table 1. As expected, no appreciable GUS activity was detected in the selfed, unmodified 'Xanthi' (X) and 'Samsun' (S) families. In the selfed, modified 'Xanthi' (X^+) and 'Samsun' (S^+) families GUS activity was clearly present. However, there was a considerable difference in activity between the 'Xanthi' (305) and 'Samsun' (2650) families, despite transformation with the same construct. GUS activities in the two F_1 families produced from crosses between modified and non-modified 'Xanthi' were similar to those of the selfed 'Xanthi' family in that while neither differed significantly from the selfed mean, they differed significantly from each other. The results confirm that the introduced character was inherited and that there was a tendency for dominance in the direction of high GUS expression. In contrast to this, activities in the two F_1 families produced from crosses between modified and non-modified Samsun were approximately half those of the selfed 'Samsun' family, suggesting an additive, gene dosage effect.

These data confirm that the introduced gene was expressed in the field-grown material and had been transmitted to the F_1 families. However, not only were

the activity levels different in the two cultivars, but the gene action appeared to be different. In addition, the progeny from crosses with the modified line as the male parent tended to have a higher level of expression (though not significantly so in the case of 'Samsun') than the reciprocal in both cases (391.2 versus 250.8 and 1450.8 versus 1398.7), suggesting the presence of factors modifying gene expression and/or enzyme activity.

Table 1. GUS expression in leaves 6 weeks after field transplanting. Results are given as means of the 20 plants per family in nmol methyl umbelliferone/mg protein per hour

	Parent	
	Male	Female
	X^+	X
X^+	305.0 ± 68.6	391.2 ± 55.7
X	250.8 ± 50.3	-2.0 ± 0.5
	S^+	S
S^+	2650.0 ± 94.5	1450.8 ± 66.8
S	1398.7 ± 69.3	-0.6 ± 0.6

Plant development

The data were analysed to determine whether the plants exhibited any differences in development when the introduced genes were present. No effect was expected as the introduced genes were markers and were not intended to affect plant development directly. The families obtained from crosses within each of the two cultivars will be considered separately. It is possible to apply orthogonal functions to the data in the analysis of variance to test whether, with respect to a particular character, there are significant differences between (a) modified and unmodified lines, (b) reciprocal F_1's and (c) mean of the F_1's and the mid-parental value.

The results from 'Xanthi' will be considered first. There were 4 significant effects detected among the 51 tests carried out. This is slightly more than would be expected by chance at the 5% probability level, but nevertheless the possibility of chance effects accounting for some of these apparent differences cannot be ignored. In Table 2 the means are presented for the characters that showed significant effects. The number of leaves at 2 weeks differed, but this was not maintained in later weeks, whereas length of the longest leaf showed more consistency with the difference and persisted into weeks 4 and 6. It is also interesting to note that for these characters the modified line tended to

Table 2. Means and levels of significance for the comparisons between the 'Xanthi' families for the characters where significant effects were detected

Character	Means				Probabilities		
	X^+	X	$F_1(X \times X^+)$	$F_1(X^+ \times X)$	P_1 *v* P_2	F_1 *v* F_1	P's *v* F_1's
2NLVS	8.6	7.6	7.9	7.9	*	ns	ns
4LLVS	32	30	31	31	*	ns	ns
6LLVS	36	32	34	36	**	ns	ns
CSWT	1.0	0.9	0.6	0.7	ns	ns	*

* $P < 0.05$; ** $P < 0.01$; ns, not significant ($P > 0.05$)

Table 3. Means and levels of significance for the comparisons between the 'Samsun' families for the characters where significant effects were detected

Character	Means				Probabilities		
	S^+	S	$F_1(S^+ \times S)$	$F_1(S \times S^+)$	P_1 *v* P_2	F_1 *v* F_1	P's *v* F_1's
6HT	116	132	124	126	***	ns	ns
8HT	140	153	146	144	***	ns	ns
6LLVS	34	37	36	33	**	ns	ns
F. DATE	20	18	19	19	**	ns	ns
FERT	6.2	7.6	6.4	6.3	***	ns	*
10^5 SWT	1.2	1.1	1.4	1.1	ns	ns	*
CSWT	1.3	1.5	0.9	0.7	ns	ns	*

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, not significant ($P > 0.05$)

show the higher values, i.e. more leaves (which is actually the case in later weeks for this character although not formally significant) and longer leaves. The difference between the parents and the F_1's was unexpected for seed weight per capsule and has no easy explanation, but is probably connected with our early harvesting of some of the capsules in order to terminate the whole experiment before any seed shedding occurred.

Results from the 'Samsun' families (Table 3) show that there were 8 significant comparisons out of 51, 5 of which were at a probability of less than 1%. It would seem that in this case the evidence for the existence of differences is more substantial. The first two significant comparisons reflect effects of height differences at 4 and 6 weeks, with the modified line showing the shorter stature. The modified line had shorter leaves and flowered later than its unmodified counterpart, and it also had a lower fertility score. It should be noted that this was a visual assessment of the flowers and capsules present at a point just before the earliest capsules began to open, which was the latest stage the plants could be left intact before the requirements of the licence that ripe capsules should be removed to avoid any seed shedding. The two characters related to seed output, namely weight of of 100 000 seeds and the weight of seed per capsule showed significant effects, but again relate to differences between parents and F_1's.

Discussion

There are two major conclusions to be drawn from this study. The first relates to the levels of GUS expression observed in the various F_1 combinations. Whereas the 'Samsun' transformants showed a level of GUS in the homozygous transformed parent that was almost twice that found in the hemizygous F_1's, the 'Xanthi' material showed levels in the F_1's that were approximately equal to those in the homozygous transformed parent. This finding is closely related to that made by Hobbs et al. (1990) who studied ten tobacco (cv 'Xanthi') transformants produced by *Agrobacterium* co-cultivation using the same vector as that used in present study, namely pBI 121. Seven of these Ro transformants were shown by progeny analysis to have inserts at single sites (as in the present study), and these seven could be divided into two groups on the basis of levels of GUS expression. Four of the group showed high GUS levels (384–516 nmol/mg protein per hour) in the homozygous R_1 plants and three showed low levels (66–144 nmol/mg protein per hour). The notable feature is that hemizygous R_1 plants of the high category showed GUS expression that was half the homozygote value, whereas hemizygous plants of the low category showed values equal to that of the homozygotes. Molecular characterization demonstrated that the highly expressing material had only a single gene inserted at the integration site, whereas the material with lower levels of expression had double insertions at the single site. To date, there is no obvious explanation of the apparent contradiction that two allelic copies of T-DNA gave double expression in the high group whereas two non-allelic copies gave a lower level of expression and that hemizygotes and homozygotes show this same low level.

There is a limited amount of relevant information from other studies. For example, Czernilofsky et al. (1986) studied relative NPT II activity in a small number of hemizygous and homozygous progeny from a single transformant and found the latter values were higher than the former. In a second, more recent, study, Dean et al. (1988) examined the levels of expression of petunia *rbcS* gene in tobacco and found that homozygous progeny had twice the value of the hemizygotes.

The second major conclusion relates to the agronomic data. It can be seen that there were several differences in whole plant characters between modified and unmodified lines and that the two cultivars were not affected in the same way or to the same extent. The changes in 'Xanthi' were less extensive and had only one character in common with those of 'Samsun'. In addition, while the changes in 'Xanthi' tended to favour the modified line (more and longer leaves), those in 'Samsun' tended to favour the unmodified line (the modified line being shorter in height and having horter leaves, later flowering and reduced fertility) although in some contexts these may be agronomically advantageous. These effects may be due to:

1) 'Carry-over' effects from the treatments applied;
2) Variation present in the original inbreds;
3) The direct effect of the inserted genes (pleiotropy);
4) Somaclonal variation;
5) Insertional mutagenesis.

'Carry-over' effects cannot completely be dismissed but after three to four rounds of selfing before crossing these seem unlikely. Such effects seem even less likely when it is remembered that the controls were subject to the same in vitro treatments. With respect to the second explanation, the original cultivars were inbred and appeared to be true-breeding, but we cannot ignore the possibility of variation in the original lines. As to the remaining three possibilities, we cannot distinguish these in our present experiment, and it will require further investigations to do so, but each of these may have contributed.

These results can be viewed alongside those of the only other detailed reports of field performance of transgenic material, namely those on flax (McHughen and Holm 1991; McHughen and Rowlands 1991), which both show no confirmed detrimental effect of the

introduced T-DNA. Clearly, examination of a larger number of examples is required before generalizations can be made, but it does appear that some variation, if somewhat minor, can be induced during transformation. Whether carefully observed field trials, as performed routinely in plant breeding programmes, will enable the selection of transformants with good agronomic performance depends on the source of such variation and the relative effects of introduced and induced variation. Since there have now been several hundred field trials of such material across the world (e.g. Jefferson 1990; Arnoldi et al. 1992) and transformation techniques have been devised for most major crops, it will soon be possible for plant breeders to consider transformation and its potential as a further adjunct in increasing the range of variation available to them – and the possible complications of induced variation will need to be borne in mind.

Acknowledgements. The authors are grateful to IPSR, Cambridge (now the Cambridge Laboratory, John Innes Centre, Norwich) for the gene construct (pBI 121). We also acknowledge that the work was carried out under Licence PHF 1239/115(61) issued by the Ministry of Agriculture and Fisheries and Food (MAFF).

References

Arnoldi M, Baszcynski CL, Bellemare G, Brown G, Carlson J, Gillespie B, Huang B, MacLean N, MacRae WD, Rayner G, Rozakis S, Wescott M, Kemble RJ (1992) Evaluation of transgenic canola under field conditions. Genome 35:58–63

Bellini C, Guerche P, Spielman A, Goujaud J, Lesaint C, Caboche M (1989) Genetic analysis of transgenic tobacco plants obtained by liposome-mediated transformation: absence of evidence for the mutagenic effect of inserted sequences in sixty characterised transformants. J Hered 80:361–367

Budar F, Thai-Toong L, Van Montagu M, Hernalsteens JP (1986) Agrobacterium-mediated gene transfer results mainly in transgenic plants transmitting T-DNA as a single Mendelian factor. Genetics 114:303–313

Czernilofsky AP, Hain R, Baker B, Wirtz V (1986) Studies of the structure and functional organisation of foreign DNA integrated into the genome of *Nicotiana tabacum*. DNA 5: 473–482

Dean C, Jones J, Favreau M, Dunsmuir P, Bedbrook J (1988) Influence of flanking sequences on variability in expression levels of an introduced gene in transgenic tobacco plants. Nucleic Acids Res 16:9267–9283

Dunwell JM, Paul EM (1990) Impact of genetically modified crops in agriculture. Outlook Agric 19:103–109

Heberle-Bors E, Charvat B, Thompson D, Schernthaner JP, Barta A, Matzke AJM, Matzke MA (1988) Genetic analysis of T-DNA insertions into the tobacco genome. Plant Cell Rep 7:571–574

Hobbs SLA, Kpodar P, DeLong CMO (1990) The effect of T-DNA copy member, position and methylation on reporter gene expression in tobacco transformants. Plant Mol Biol 15:851–864

Jefferson RA (1987) Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol Biol Rep 5:387–405

Jefferson RA (1990) New approaches for agricultural molecular biology: from single cells to field analysis. In: Gustafson JP (ed) Gene manipulation in plant improvement II. Plenum Press, New York, pp 365–400

Knauf VC (1991) Agricultural progress: engineered crop species, field trial results and commercialisation issues. Curr Opp Biotechnol 2:199–202

Lam E (1990) From footprint to function: an approach to study gene expression and regulatory factors in transgenic plants. In: Setlow JK (ed) Genetic engineering: Principles and methods 12. Plenum Press, New York, pp 73–86

Lycett GW, Grierson D (1990) Genetic engineering of crop plants. Butterworths, London

Matzke MA, Matzke AJM (1990) Gene interactions and epigenetic variation in transgenic plants. Dev Genet 11: 214–223

Matzke MA, Matzke AJM (1991) Differential inactivation and methylation of a transgene in plants by two suppressor loci containing homologous sequences. Plant Mol Biol 16: 821–830

McHughen A, Holm F (1991) Herbicide resistant transgenic flax field test: agronomic performance in normal and sulfonylurea-containing soils. Euphytica 55:49–56

McHughen A, Rowland GG (1991) The effect of T-DNA on the agronomic performance of transgenic flax plants. Euphytica 55:269–275

Otten L, De Greve H, Hernalsteens JP, Van Montagu M, Schieder O, Straub J, Schell J (1981) Mendelian transmission of genes introduced into plants by the Ti plasmids of *Agrobacterium tumefaciens*. Mol Gen Genet 183:209–213

Potrykus I, Paszkowski J, Saul MW, Petrusku J, Shillito RD (1985) Molecular and general genetics of a hybrid foreign gene introduced into tobacco by direct gene transfer. Mol Gen Genet 199:169–177

Tomas DT, Weissinger AK, Ross M, Higgins R, Drummond BJ, Schaaf S, Malone-Schoneberg J, Staebell M, Flynn P, Anderson J, Howard J (1990) Transgenic tobacco plants and their progeny derived by microprojectile bombardment of tobacco leaves. Plant Mol Biol 14:261–268

Vyskot B, Brzobohaty B, Karlovsa L, Bezdek M (1989) Structural and functional stability of foreign genes in transgenic tobacco plants. Fol Biol 35:360–372