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A population of wheat and tritordeum transformants showing a high degree of marker gene stability and heritability

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Abstract The stability and heritability of three marker genes was investigated in a population of twelve independent transgenic cereal lines (six wheat and six tritordeum). Integration patterns, inheritance of structural transgenes and inheritance of expression were analysed in the T_0 and T_1 generations for all 12 lines. Transmission and expression were analysed in the T_2 generation for 9 lines and in the T_3 generation for the six wheat lines. Inheritance of integration patterns was highly stable, and transmission of the transgenes and inheritance of their expression followed Mendelian ratios in the majority of lines. A gradual reduction in *uid*A expression was observed over three generations, which was not accompanied by a similar reduction in *bar* expression. Some unexpected phenomena associated with transgene inheritance were also observed and are discussed.

Key words Transgenic wheat · *Triticum aestivum* · Tritordeum · Transgene inheritance · Marker gene stability

Introduction

Compared with most major crop species, wheat transformation has only been achieved relatively recently (Vasil et al. 1992, 1993; Weeks et al. 1993) and, therefore, few reports describing the stability and heritability over several generations of transgenes in wheat have been published (see Srivastava et al. 1996). Although the *Agrobacterium*-mediated transformation of wheat (Cheng et al. 1997) and barley (Tingay et al. 1997) has recently been reported, most current wheat transformation systems use direct gene transfer (DGT) technology, principally particle bombardment. Copy number, stability and heritability of marker genes in plants produced in this way are often variable, and the factors influencing these parameters are poorly understood (for review see Pawlowski and Somers 1996), although they have previously been investigated in other cereal crops such as maize and rice (Register et al. 1994; Cooley et al. 1995).

Information regarding the long-term stability of transgenes underpins the application of genetic manipulation (GM) in wheat crop improvement and is central to any programme attempting to genetically modify an agronomic trait or phenotype. In addition there is increasing concern over the implications of transgene silencing for the application of GM technology [recently reviewed in Kumpatla et al. (1998) and highlighted by reported cases of silencing in rice Kumpatla and Hall (1998); and Kumpatla et al. (1997)]. Clearly, therefore, it is important to have an understanding of the expected frequency of such instability before GM programmes are initiated.

In order to address this, and as part of a programme to develop a robust wheat transformation system, a population of transgenic wheat (cv 'Florida') and tritordeum plants was generated under conditions described previously (Barro et al. 1998). Tritordeum is a fertile amphiploid which is genotypically similar to wheat, containing the homeologous group A and B chromosomes from *Triticum durum* and the H (barley) genome from *Hordeum chilense*. The aim of the study presented here was to survey the stability of integration patterns, inheritance and expression of the *uid*A, *bar* and *neo* marker genes in this population over three generations.

Materials and methods

Plant material

The production of the 12 transgenic wheat (cv 'Florida') and tritordeum (*Hordeum chilense*×*Triticum durum*: $H^{ch}H^{ch}AABB$) lines described in the present study is detailed in Barro et al. (1998). Lines WT1, WT2, WT3, WT4, HTT5, HTT6 and HTT8 had been transformed with the plasmid pAHC25 encoding both the *bar* and

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*uid*A genes under the control of the maize ubiquitin promoter and intron (Christensen and Quail 1996). Lines WT5, WT7, HTT3 and HTT4 had been transformed with the plasmid pCaI-neo (constructed by S. Luetticke, University of Hamburg, modified from the plasmid pCaI-gus described in Callis et al. 1987) encoding the *neo* gene under the control of the CaMV 35 S-maize Adh1 intron promoter. Line HTT2 had been transformed with both pCaI-neo and also pAct1-DGus (McElroy et al. 1990) encoding the *uid*A gene under the control of the rice actin promoter.

Polymerase chain reaction (PCR) screening of progeny plants

Extraction of plant DNA was as described in Barro et al (1998). Approximately 100–200 ng plant genomic DNA was used as templates in 30-µl PCR reactions containing 1× enzyme buffer [10 m*M* TRIS-HCl pH 8.8, 1.5 mM $MgCl_2$, 50 m*M* KCl, 0.1% Triton X-100), 200 µ*M* dNTPs, 0.3 µ*M* primers and 0.66 units recombinant thermostable DNA polymerase (Dynazyme II – Flowgen)]. Primer sequences: *bar* (5′-GTCTGCACCATCGTCAACC-3′, 5′-GAAGTCCAGCTGCCAGAAAC-3′, annealing temperature 57°C, approximate product length 420 bp), *uid*A (5′-AGTGTACGTATCACCGTTTGTGTGAAC-3′, 5′-ATCGCCGCTTTGGACATACCATCCGTA-3′, annealing temperature 62 C, approximate product length 1020 bp), *neo* (5′-GAGGCTATTCGGCTATGACTG-3′, 5′-ATCGGGAGCGGCGATACCGTA-3′, annealing temperature 57°C, approximate product length 700 bp). Approximately 30 T_1 progeny from each of the 12 lines were screened in the T_1 generation; 15–20 T_2 progeny from 5 out of 9 lines were screened in the T_2 generation; 45 progeny from each of 5 lines and 15 from 1 line were screened in the T_3 generation.

Expression assays

Inheritance of *uid*A expression in the progeny of lines transformed with pAHC25 or pAct-1DGus was confirmed using the GUS histochemical staining method described in Barcelo and Lazzeri (1995). Leaf material was assayed three times during plant growth. Flowers (developing anthers and ovaries) were also assayed by this method. Inheritance of *bar* gene expression was confirmed in selected T_1 progeny and all T_2 and T_3 progeny of plants transformed with pAHC25 using a herbicide resistance assay. The following dilutions of the herbicide BASTA (or equivalent concentrations of the herbicide Challenge) were prepared in 0.1% Tween 20: 0%, 0.5% and 1%. Herbicide was applied by 'painting' the distal, upper halves of leaf surfaces with cotton buds. Resistance was measured 7 and 14 days post-application as the percentage leaf area showing dessication and browning. Inheritance of *neo* gene expression in crude leaf protein extracts of progeny from lines transformed with pCaI-neo was confirmed using an NPTII ELISA kit (CP laboratories, UK). Protein extracts were made thus: approximately 50–100 mg leaf tissue was homogenised in a pestle and mortar containing 0.5 ml cold extraction buffer (0.25 *M* TRIS-HCl, pH 7.8, 1 m*M* PMSF) and a pinch of sand. Sand grains and cell debris were removed by microcentrifugation at 4°C twice for 30 min each, transferring the supernatant to a fresh tube after each spin. Protein concentrations were determined using the Bio-Rad protein assay dye-reagent, and the ELISA was carried out according to the manufacturer's instructions. On average, 12 progeny plants from each of the 12 lines were screened for marker gene expression in the T_1 generation, 15–20 plants out of 8 expressing lines were screened in the T_2 generation and approximately 40 plants per line were screened in the T_3 generation.

Southern analysis

Extraction of plant DNA was as described in Barro et al. (1998). Between 10 and 15 µg of DNA per sample was digested. DNA blotting, hybridisation and chemiluminescent detection of probes was carried out according to the DIG System User's Guide for Filter Hybridization (Boehringer Mannheim). Digoxigenin-labelled probes were prepared using a PCR DIG probe synthesis kit (Boehringer Mannheim) and primers (see above) to amplify fragments internal to the *bar, neo* and *uid*A coding regions. Approximately 7 T_1 progeny of each line were analysed. DNA from the 6 primary transformants containing pAHC25 (WT1, WT2, WT3, WT4, HTT5 and HTT8) and their T_1 progeny was digested with *Sac*I, which cuts once in pAHC25, and also with a *Bam*HI/*Eco*RI double digest, which releases an approximately 2.2-kb fragment containing the *uid*A coding region and an approximately 0.85-kb fragment containing the *bar* gene coding region. DNA from the 5 lines containing pCaI-neo (WT5, WT7, HTT2, HTT3, HTT4) and their T_1 progeny was digested with *Eco*RI, which cuts once in pCaI-neo, or with *Bam*HI, which releases an approximate 0.85-kb fragment containing the *neo* gene coding region. DNA from the single line containing pAct1-DGus, (HTT2) and its T_1 progeny was digested with *Bam*HI which cuts once in pAct-1DGus, or with *Sac*I, which releases an approximate 2.4-kb *uid*A coding region fragment.

Results

The present study provides data on three aspects of the inheritance of transgene loci. Firstly, their rate of transmission to progeny populations, secondly, the integration patterns of transgene loci and their stability through sexual transmission and thirdly, the inheritance of transgene expression. Transgene transmission rates were determined using PCR to identify segregants and null segregants of marker gene sequences in progeny populations. The inheritance of the molecular structures of transgene loci was examined by comparison between integration patterns of primary transformants and their T_1 progeny using Southern analysis. Inheritance of transgene expression was followed by the application of marker gene assays to populations of progeny plants. All three aspects were investigated in the T_1 generation of 12 transformed lines in the present study. Only the transmission rate and inheritance of expression were examined in a subset of 9 lines in the T_2 generation and 6 lines in the T_3 generation.

Transmission of transgenes from primary transformants (T_0) to the T_1 generation

Of the 12 primary transformants, 7 contained both the *bar* and *uid*A genes (WT1, WT2, WT3, WT4, HTT5, HTT6 and HTT8), 4 contained the *neo* gene alone (WT5, WT7, HTT3 and HTT4) and 1 contained both the *neo* and *uid*A genes (HTT2). For the analysis of the transmission rates of transgenes from the T_0 to the T_1 generation, approximately 30 T_1 progeny plants from each transformant were screened by PCR: genomic DNA was isolated from each progeny plant, and transmission of the relevant marker gene(s) was confirmed by PCR amplification – null segregants being identified by the absence of a PCR product. For all 7 pAHC25-transformed lines the *uid*A and *bar* genes were always inherited together and for the only line co-transformed with pCaI-neo and pAct1-DGus (HTT2), both genes were also always co-inherited (Table 1). An example of PCR screening of T_1 progeny of a pAHC25-containing line (WT1) is shown in Fig. 1.

Table 1 Transgene heritability from the T_0 to T_1 generation

	Transmission[a] to T_1 (PCR)	Expression[b] in T_1		χ^{2} [d] (3:1)	*P* value
	*uid*A/*bar* +ve: –ve	GUS +ve	BASTA +ve		
Lines[c] containing pAHC25					
WT1	22:13	22/22	22/22	2.75	0.2–0.1
WT2	29:12	22/22	7/7	0.655	0.5–0.3
WT3	2:34	2/2	2/2	nd	nd
WT4	22:8	16/16	16/16	0.04	0.9–0.8
HTT5	5:23	0/3	0/3	nd	nd
HTT6	2:24	2/2	2/2	nd	nd
HTT8	18:8	0/18	0/9	0.47	0.5–0.3
Lines[c] containing pCalneo	*neo* +ve: –ve	NPTII +ve			
WT5	47:0	7/7		nd	nd
WT7	28:7	14/14		0.466	0.5–0.3
HTT3	20:4	10/10		0.67	0.3–0.5
HTT4	24:7	7/7		0.07	0.8–0.7
Lines[c] containing pCal-neo & pAct1-DGus	*uid*A/*neo* +ve: –ve	NPTII +ve	GUS +ve		
HTT2	19:3	19/19	10/10	1.14	0.3–0.2

[a] For transmission data, numbers refer to the *ratio* of +ve plants to –ve plants
[b] For expression data, numbers refer to the number of +ve plants out of the total number of PCR +ve plants tested
[c] WT, Wheat transformant; HTT, tritordeum transformant
[d] Chi-square taken from PCR data
nd not determined

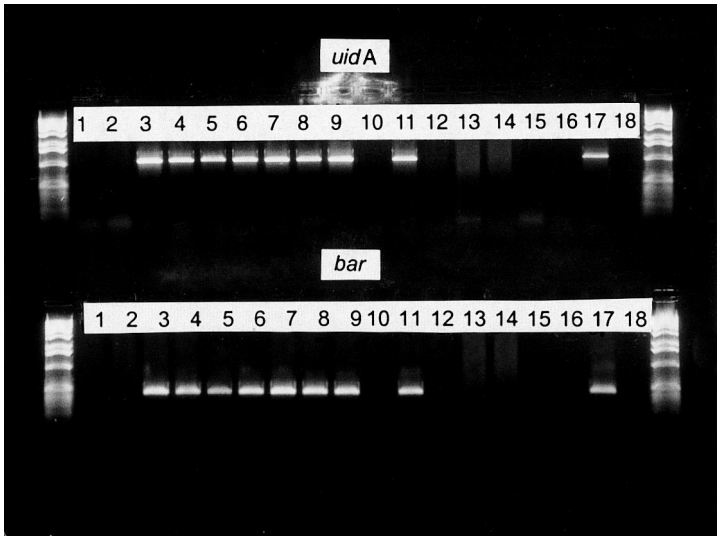

Fig. 1 Agarose gel containing *uid*A and *bar* PCR products from T_1 progeny of line WT1. *Marker lanes*=1 kb ladder, *lanes 1–14*=progeny nos. 1–14, *lane 15* and *16*=negative controls, *lane 17*=pAHC25 positive control, *lane 18*=water control

Transmission ratios were shown to be Mendelian in 8 out of 12 lines, whereas 4 lines (WT3, WT5, HTT5 and HTT6) gave highly distorted segregation ratios. In lines WT3, HTT5 and HTT6, very few T_1 progeny inherited a transgene. Tritordeum is known to be relatively genetically unstable because is it has a relatively novel genomic combination. The distorted segregation observed in lines HTT5 and HTT6 is therefore perhaps not so surprising. However, because the other line with reduced transmission (WT3) was a wheat transformant, more detailed analysis of inheritance in the only 2 T_1 transgene-positive progeny of WT3 was undertaken (see below). In line WT5, all 47 T_1 progeny screened were shown to have inherited the *neo* transgene. The classical explanation for such a ratio is that multiple loci are present. However, molecular analysis of the integration patterns in large numbers of T_1 progeny discounted this explantation and suggested that WT5 was homozygous for the *neo* gene insertion (see below).

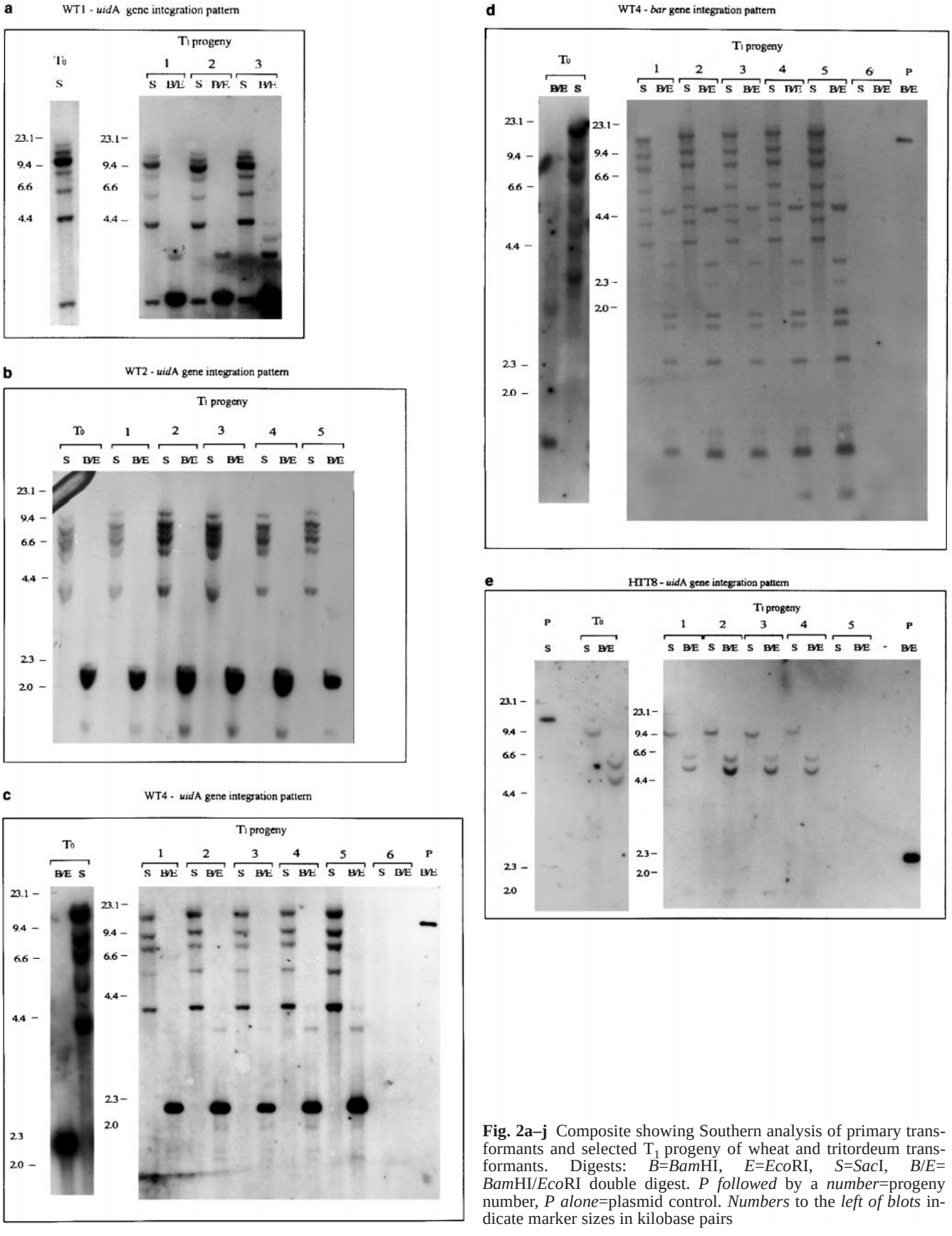

Fig. 2a–j Composite showing Southern analysis of primary transformants and selected T_1 progeny of wheat and tritordeum transformants. Digests: *B*=*Bam*HI, *E*=*Eco*RI, *S*=*Sac*I, *B*/*E*=*Bam*HI/*Eco*RI double digest. *P followed* by a *number*=progeny number, *P alone*=plasmid control. *Numbers* to the *left of blots* indicate marker sizes in kilobase pairs

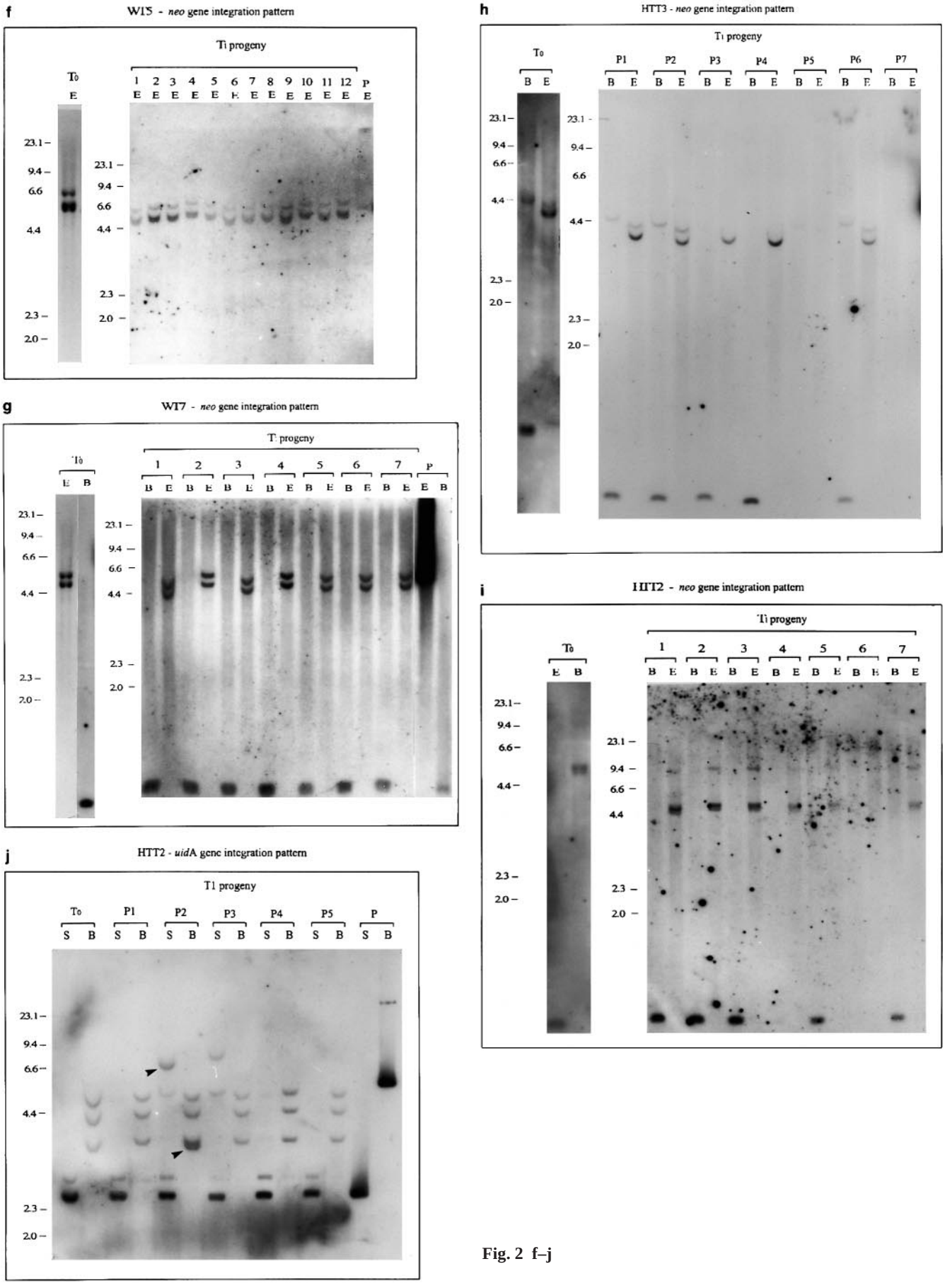
f
W15 - neo gene integration pattern
T1 progeny
T0
1 2 3 4 5 6 7 8 9 10 11 12 P
E E E E E E E E E E E E E
23.1
9.4
6.6
4.4
2.3
2.0
g
W17 - neo gene integration pattern
T1 progeny
T0
1 2 3 4 5 6 7 P
B E
h
HTT3 - neo gene integration pattern
T1 progeny
T0
P1 P2 P3 P4 P5 P6 P7
i
HTT2 - neo gene integration pattern
T1 progeny
T0
1 2 3 4 5 6 7
j
HTT2 - uidA gene integration pattern
T1 progeny
T0
P1 P2 P3 P4 P5 P
S B

Fig. 2 f–j

Stability of integration patterns between T_0 and T_1 progeny

Marker gene integration patterns in 12 primary (T_0) transformants and T_1 progeny (around 7 individuals from each line) were examined using Southern hybridisation. Results from 4 lines (WT1, WT2, WT4 and HTT8) containing pAHC25 (Fig. 2a–e), 3 lines (WT5, WT7 and HTT3) containing pCaI-neo alone (Fig. 2f–h) and 1 line (HTT2) co-transformed with both pCaI-neo and pAct-1Dgus (Fig. 2i, j) are shown. The estimated number of insertions was generally low and varied from approximately two to three in lines WT5, WT7, HTT2, HTT3 and HTT8 to five or more in the remaining lines analysed.

In lines WT2, WT4, WT5, WT7, HTT2 (*neo* gene only, Fig. 2i) and HTT8, the hybridisation patterns remained unchanged between T_0 and T_1 progeny. In line WT1, two additional bands are visible in progeny number 3, lane B/E (Fig. 2a), this is due to overloading of the track (the same bands are faintly visible in the other progeny of this line). Thus, all lines shown in Fig. 2 except for HTT2 (*uid*A gene only, Fig. 2j) exhibited stability of integration patterns following their transmission to the T_1 generation. The same stability was observed in all remaining lines in the population (data not shown), although in some cases fewer progeny were examined due to poor transgene transmission. In line HTT2, the integration pattern of the *neo* gene insertion (Fig. 2i) event in the T_0 and 5 T_1 progeny was identical (the signal in lane B of progeny no. 4 is very faint due to under-loading of DNA. Progeny no. 6 is a null segregant), indicating stability of the *neo* integration pattern following transmission to the T_1 generation. However, following the analysis of another set of T_1 progeny from line HTT2, some minor differences in banding patterns were observed when probed with the *uid*A gene. Lane S in T_0 DNA of HTT2 (Fig. 2j) shows the detection of the expected approximately 2.4-kb fragment corresponding to the insertion of at least one intact *uid*A coding region fragment and also the detection of a more slowly migrating fragment probably corresponding to the insertion of a truncated *uid*A coding region fragment. Both bands are present in all 5 T_1 progeny probed, but in progeny numbers 2 and 3, two additional, larger bands were detected. Lane B in T_0 DNA shows the detection of three bands corresponding to the insertion of three fragments containing the *uid*A coding region. However, an additional fragment was detected in progeny no. 2 (shown by arrow). The DNA of progeny number 2, lane B was digested with *Bam*HI which cuts once in the transforming plasmid. The number of bands detected in this digest gives an estimate of the number of plasmid molecules inserted, with band sizes determined by the distance between restriction sites originating either from plant DNA or adjacent inserted plasmids. It is possible therefore that the extra band observed in this plant represents a sequence duplication event.

Southern analysis of line HTT3 showed a phenomenon not previously evident from the PCR and expression data (Fig. 2h). The different banding patterns observed between the T_0 and T_1 progeny of HTT3 are explained by the insertion of *neo* genes at two loci (PCR data gave a segregation ratio consistent with the presence of a single locus; see Table 1). Lane B in T_0 DNA shows the detection of the expected approximately 0.85 kb *neo* fragment and a fragment of a different size, suggesting the presence of at least one intact copy of the *neo* coding region and a rearranged *neo* gene, respectively. Lane E in T_0 DNA shows the detection of two fragments, suggesting the insertion of two plasmid copies. The difference in relative band intensities of each support the suggestion that these correspond to one complete and one truncated *neo* gene insertion. The detection of the same fragment sizes in different combinations in the T_1 progeny confirms this – the banding patterns observed for each progeny plant resulted either from the inheritance of the complete *neo* fragment (P3 and P4), the truncated fragment (P5) or both fragments (P1, P2 and P6). Analysis of line HTT4 (not shown) also indicated the occurrence of a similar event, i.e. a rearranged *neo* fragment was inserted at one locus and a second, intact fragment at a second locus. This line also gave a 3:1 segregation ratio following PCR screening of T_1 progeny (see Table 1), presumably because the rearranged *neo* gene insertion was not amplifiable.

A further observation was made following the analysis of line WT4. Figure 2c shows the *uid*A integration pattern in *Sac*I-digested DNA (five fragments detected) and the extent of coding region rearrangement in *Bam*HI/*Eco*RI digested DNA (little rearrangement) from line WT4 and five T_1 progeny. Figure 2d shows the same membrane reprobed with the *bar* gene. There are two extra fragments detected in DNA digested with *Sac*I and the appearance of a number of bands in *Bam*HI/*Eco*RI-digested DNA gives evidence for more extensive rearrangement of the *bar* gene than the *uid*A gene in this line. Most pAHC25-containing lines were not probed with the *bar* gene, therefore it is not known if this was a general phenomenon.

No segregation of the *neo* transgene was apparent from the Southern analysis of all T_1 progeny of line WT5 (Fig. 2f). In this line, the parental hybridisation pattern was present in all 12 progeny on this blot and also in a further 8 on another gel (data not shown). The failure to detect a change in hybridisation pattern or a null segregant in these 20 progeny (and a further 27 screened by PCR; see Table 1) suggests that T_0 line WT5 was homozygous for the *neo* gene insertion. This is surprising since all primary transformants generated by particle bombardment of somatic tissue should be heterozygous (hemizygous) for transgenes due to the extreme improbability that insertion will occur at the same (allelic) position on the opposing homologous chromosome.

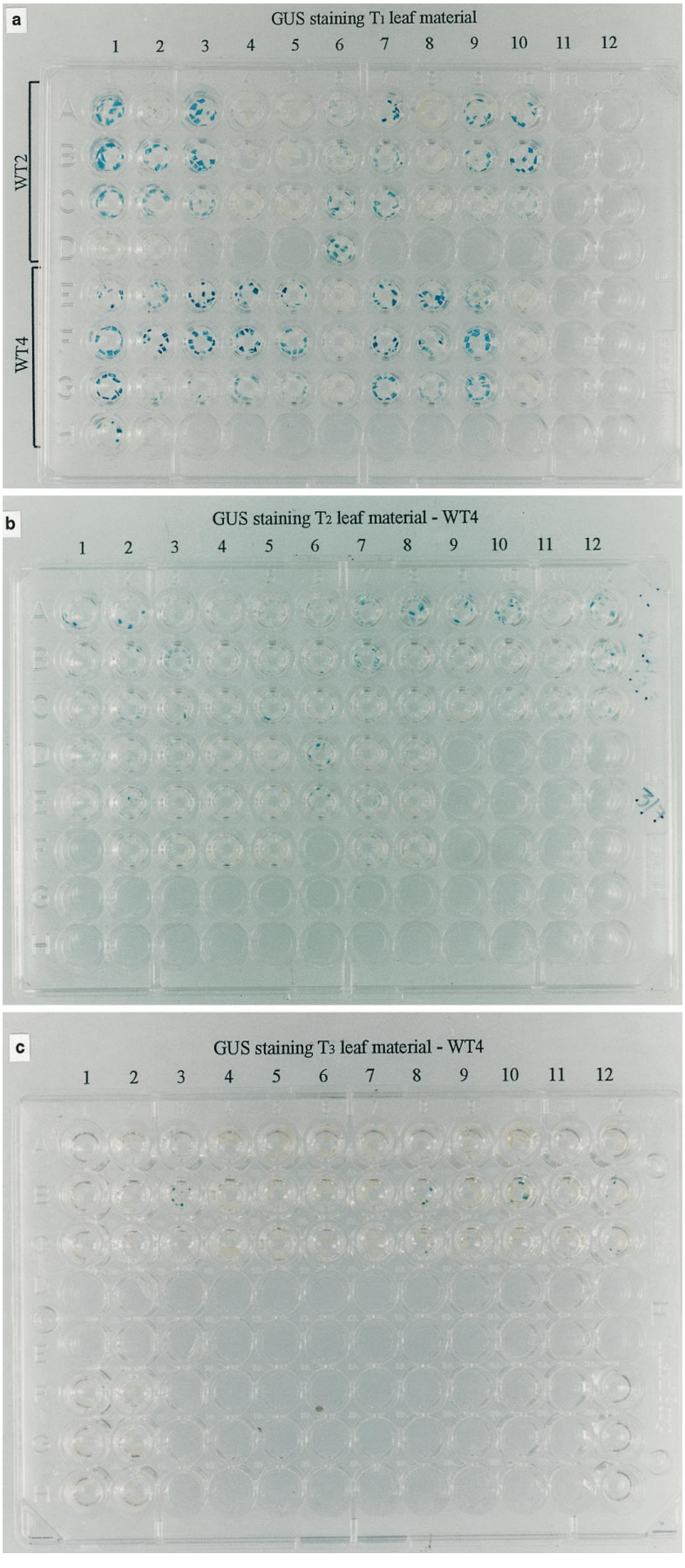

Fig. 3a–c Histochemical GUS assays on T_1, T_2 and T_3 progeny.**a** Leaf material from 10 individual T_1 plants of lines WT2 and WT4. *Numbers* indicate progeny number. Individual *wells A*, *B* and *C* contain material from three individual leaves of WT2 progeny (nos. *4*, *5* and *8* null segregants, *D6* additional material). Individual *wells E*, *F* and *G* contain material from three individual leaves of WT4 progeny (WT4 nos. *6* and *10* null segregants; *H1* additional material. **b** Material from 20 individual T_2 plants of line WT4. *Wells A-C/1–12* material from progeny numbers 1–12, *wells D-F/1–8* material from progeny numbers 13–20. **c** Material from 14 individual T_3 plants of line WT4. *Wells A-C/ 1–12* material from progeny numbers 1–12, *wells F-H/1–2* material from progeny numbers 13, 14

Fig. 4a–d BASTA resistance assay on T_1 progeny of line WT4. Four leaves from – **a** control plant, **b** null segregant, **c** and **d** segregants containing pAHC25 – showing BASTA resistance

Inheritance of transgene expression from the T_0 to the T_1 generation

Inherited *uid*A gene expression was screened for in randomly selected T_1 individuals including some null segregants. All assayed progeny that inherited the *uid*A gene from pAHC25-transformed lines expressed it (if the parent also expressed it). The parent lines HTT5 and HTT8 did not express *uid*A because neither line apparently contained a structurally complete *uid*A coding sequence (data shown for HTT8, see Fig. 2e). All progeny assayed from the pAct-1DGus-transformed line (HTT2) also expressed *uid*A. Figure 3a shows an example of histochemical GUS screening on T_1 progeny of lines WT2 and WT4. Table 1 shows the number of progeny screened for each line. In all cases, PCR screening confirmed GUS assay results. Most progeny screened for *uid*A expression were also assayed for *bar* expression by herbicide painting. All tested progeny that inherited the *bar* gene expressed it and were resistant to 1% BASTA. Figure 4 shows an example of the BASTA resistance assay on T_1 progeny of line WT4. Table 1 shows the number of progeny screened for each line. In all cases, PCR screening confirmed BASTA resistance assays. Inheritance of *neo* gene expression was screened for in randomly selected T_1 individuals containing pCaI-neo. All tested progeny expressed the inherited *neo* gene. Figure 5 shows an NPTII ELISA on 14 T_1 progeny of line WT7: 11 gave colorimetric levels higher than in the controls, and 3 gave levels comparable with controls (null segregants). Table 1 shows the number of progeny screened for each line. In all cases, PCR screening confirmed the ELISA results.

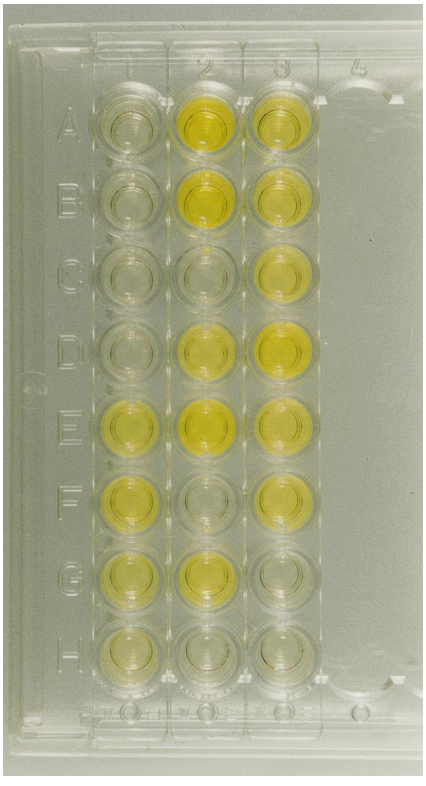

Fig. 5 NPTII ELISA of 14 selected T_1 progeny of line WT7. *Wells A1-D1*=Blanks; *E1-H1*=NPTII enzyme standard dilutions, *A2-F3* segregating T_1 progeny of WT7 (*C2*, *F2* and *H2*=null segregants), *G3*, *H3*=–ve controls

Transmission of transgenes and inheritance of expression from the T_1 to the T_2 generation

Analyses were made of the progeny of 9 transformants selected from the original population of 12 T_0 lines. Five lines (WT1, WT2, WT3, WT4, HTT8) contained pAHC25, and 4 lines (WT5,WT7, HTT3, HTT4) contained pCaI-neo. The lines HTT5 and HTT6 were discarded because they gave distorted transmission ratios in the T_1 generation as was the line HTT2 because T_2 seeds were heavily infected with mildew and gave only 1–5% germination frequencies.

In 7 out of the 9 lines, inheritance of marker gene expression was screened in the T_2 generation in progeny grown from a single seed from 15–20 randomly chosen T_1 parents of unknown zygosity. The 2 exceptions were HTT8 and WT3. In line HTT8 (which did not contain a functional copy of the *uid*A gene), transmission of marker genes only was assayed by PCR. The remaining line (WT3) gave rise to 2 T_1 progeny (WT3A and WT3B) which had inherited the *uid*A and *bar* genes. Analysis of 16 T_2 progeny from each was undertaken. The T_2 progeny of WT3A contained only 1 plant which had inherited and expressed the *bar* and *uid*A genes (confirmed by PCR screening), whereas the progeny of WT3B inherited the expression of both genes in a normal 3:1 ratio (Table 2). In all progeny, the *uid*A and *bar* genes were co-inherited.

The ratio which indicated Mendelian inheritance in the other 7 T_2 populations was predicted to be 5:1 due to the presence of hemizygotes and homozygotes (single locus) in the population of T_1 parent lines chosen at a predicted ratio of 2:1. Inheritance of marker gene expression was screened in these plants and the segregation ratios compared to the expected 5:1. In lines not following a 5:1 ratio, progeny plants were further screened by PCR for the presence/absence of the *bar*, *uid*A or *neo* genes.

In 5 out of the 7 lines, the results of *bar*, *uid*A and *neo* gene expression assays followed 5:1 ratios, suggesting Mendelian inheritance of expression to the T_2 generation (at the 5% level, see Table 2). The progeny of one line (WT2) deviated from 5:1 based on the expression data – 8 plants expressed both the *bar* and *uid*A genes, and 8 plants gave no detectable expression of either gene. However, the results of PCR analysis showed a 12:4 ratio, demonstrating that some progeny had inherited both the *bar* and *uid*A genes but were not expressing either gene at detectable levels, thus indicating the occurrence of gene silencing. Similarly, 3 progeny of WT4 were also shown to contain both genes but not to express them at detectable levels. Finally, the results of PCR screening progeny of HTT8 showed a 5:1 ratio.

Overall, 7 out of 9 lines gave ratios of transgene inheritance to the T_2 generation consistent with Mendelian genetics. Two lines (WT2 and WT4) gave rise to some progeny not expressing their inherited marker genes. In general, the GUS assay showed a reduction in the intensity and area of leaf tissue stained blue from the T_1 to the T_2 generation (Fig. 3a, b), although levels of BASTA resistance were the same between generations. Similarly, the levels of *neo* expression were comparable in the T_1 and T_2 progeny of all lines.

Transmission of transgenes and inheritance of expression from the T_2 to the T_3 generation

For four of the six wheat lines examined in the T_3 generation (WT1, WT2, WT4 and WT7), three populations of

Table 2 Transgene heritability from the T_1 to T_2 generation

	Transmission[a] to T_2 (PCR)	Expression[a] in T_2		$\chi^{2\,b}$ (5:1)	*P* value
	*uid*A/*bar* +ve: –ve	GUS +ve: –ve	BASTA +ve: –ve		
Lines containing pAHC25					
WT1[b]	nd	19:2	19:2	0.77	0.5–0.3
WT2[b]	12:4	8:8	8:8	0.76	0.5–0.3
WT4[b]	19:4	16:7	16:7	0.01	0.95–0.9
HTT8[b]	13:2	nd	nd	0.12	0.8–0.7
WT3a[c]	1:15	1:15	1:15	nd	nd
WT3b[c]	nd	12:4	12:4	0	1
Lines containing pCalneo	*neo* +ve: –ve	NPTII +ve: –ve			
WT5[b]	nd	10:0		1.9	0.2–0.1
WT7[b]	nd	13:2		0.12	0.8–0.7
HTT3[b]	nd	14:1		1.08	0.3–0.2
HTT4[b]	17:3	17:3		0.04	0.9–0.8

[a] For transmission and expression data, numbers refer to the *ratio* of +ve plants to –ve plants
[b] Single seeds taken from individual T_1 parent lines
[c] All seeds taken from a single T_1 parent line
[d] Chi-square taken from PCR data where present
nd not determined

Table 3 Transgene heritability from the T_2 to T_3 generation

	Transmission to T_3 (PCR)	Expression in T_3		$\chi^{2\,c}$	*P* value
	*uid*A/*bar*	GUS	BASTA		
Lines[b] containing pAHC25	+ve: –ve	+ve: –ve	+ve: –ve	(3:1)	
WT1a	10:5	10:5	10:5	0.56	0.7–0.5
WT1b	13:2	13:2	13:2	1.11	0.3–0.2
WT1c	15:0	5:0	5:0	nd	nd
WT2a	11:4	11(F)[d]:4	3(P)[e]:12	0.03	0.9–0.8
WT2b	11:4	10(F):5	10(P):5	0.03	0.9–0.8
WT2c	15:0	4(F):0	0:4	nd	nd
WT3Ba	11:4	11:4	11:4	0.03	0.9–0.8
WT3Bb	11:4	11:4	11:4	0.03	0.9–0.8
WT3c	15:0	5:0	5:0	nd	nd
WT4a	11:3	11:3	11:3	0.09	0.8–0.7
WT4b	12:3	12:3	12:3	0.2	0.7–0.5
WT4c	15:0	5:0	5:0	nd	nd
Lines[b] containing pCalneo	*neo* +ve: –ve	NPTII +ve: –ve			
WT5	15:0	15:0		nd	nd
WT7a	12:3	12:3		0.2	0.7–0.5
WT7b	9:6	9:6		1.7	0.2–0.1
WT7c	15:0	nd		nd	nd

[a] For transmission and expression data, numbers refer to the *ratio* of +ve plants to –ve plants
[b] a, b, Hemizygous T_2 parent lines; c, probable homozygous T_2 parent line (based on segregation ratio of 15:0)
[c] Chi-square taken from PCR data
[d] F, Data from GUS assays on flowers
[e] P, Partial resistance phenotype
nd not determined

15 T_3 progeny were grown and screened by PCR and the relevant expression assays. Two of the three populations were derived from hemizygous T_2 parents and one from a probable homozygous parent (based on a segregation ratio of 15:0). The latter parent type was chosen to investigate any potential differences in expression levels and patterns between hemizygotes and homozygotes. In line WT2, the three parents selected were ones which had tested positive for *uid*A and *bar* expression (as opposed to using silenced lines). In the case of line WT3, because of its distorted segregation in the T_1 generation, the progeny of two hemizygous T_2 parents and one probable homozygous T_2 parent (all originating from the same T_1 line – WT3B) were screened by *bar* and *uid*A expression assays. For the remaining line (WT5), the progeny of a single T_2 parent were screened by PCR and NPTII ELISA.

Mendelian segregation of marker genes was confirmed by PCR in all lines examined (Table 3). As observed in previous generations, the progeny of lines containing pAHC25 always co-inherited the *uid*A and *bar* gene. No non-expressing T_3 progeny of WT4 were identified, although some had been observed in the T_2 generation.

Expression of marker genes was detected in all 'segregant' progeny from every line, except in some individuals from line WT2. In the T_3 progeny of WT2a, no *uid*A expression was detected in the leaves, whereas it was clearly present in the flowers of 11/11 WT2a plants tested. *Uid*A expression was detected in the leaves of 9/11 T_3 progeny of WT2b (not shown) and in the flowers of 10/11 plants. Regarding BASTA resistance, although 8/11 progeny of WT2a were shown to have lost BASTA resistance, 3/11 were shown to be partially resistant i.e. the area of leaf dessication/browning was significantly less than 100%; usually 40–50%. This partially resistant phenotype was observed in 10/11 progeny of WT2b. All 4 progeny of WT2c were shown to have completely lost BASTA resistance.

Although inheritance of expression was Mendelian in all but one line (WT2), in general the GUS assay showed a further reduction in the intensity and area of leaf tissue stained blue from the T_2 to the T_3 generation (Fig. 3b, c). In some cases, this effect was so marked that expression could only be detected in some individual guard cells using a stereo-microscope at high magnification. However, levels of BASTA resistance were the same in T_2 and T_3 progeny. Levels of NPTII were similar in the T_2 and T_3 progeny of all lines. No significant differences in expression levels and patterns between hemizygotes and probable homozygotes were detected.

Discussion

The study presented here is a survey on different aspects of transgene stability (transmission rates, stability of integration patterns and inheritance of expression) in the T_1, T_2 and T_3 generations and gives an overall indication of the stability of three marker genes in a population of wheat and tritordeum transformants. The overall finding was that the integration, inheritance and expression of these marker genes in this population were, with a few exceptions, stable and predictable.Out of 12 lines 8 were found to be normal in every respect following examination of T_1 progeny (line WT5 is included, as it appears normal in all aspects except that the T_0 was probably homozygous for the transgene). At least 5 out of 9 lines were normal in every respect following examination of T_2 progeny [a few T_2 progeny of line WT4 showed evidence of silencing, which then disappeared in the T_3

generation, and progeny of another line (WT3) which had transmitted poorly to the T_1 had reverted to normal segregation and expression in the T_2]. Out of 6 lines 5 were normal in all aspects other than a reduction of GUS staining following examination of T_3 progeny.

Transmission rates

Segregation ratios from PCR and expression screening followed Mendelian laws in 9 out of 12 lines in the T_1 (assuming WT5 was homozygous at the *neo* locus), 8 out of 9 lines in the T_2 and 5 out of 6 lines in the T_3 generation (at the 5% level). This suggests that the transformation procedure, transgene integration and marker gene expression had little effect on the normal transmission of transgenes. The identification of 3 lines with reduced levels of transmission to the T_1 generation may be within the normal frequency of occurrence of loci with distorted segregation which are present in nature (Xu et al. 1997). However, the results from further analysis of the progeny of 1 of these lines (WT3) were notable. Following the screening of 36 T_1 progeny, only 2 were identified which contained and expressed the *uid*A and *bar* genes. Segregation continued to be distorted in the progeny of only 1 of these plants but reverted to a normal 3:1 ratio in the other. Further investigation of progeny lines are being undertaken to improve our understanding of the events which may have caused this phenomenon.

Inheritance of integration patterns

A high level of integration pattern stability was observed in the present study with 11/12 lines transmitting parental banding patterns to all T_1 progeny tested. This contrasts with results from a similar study on a population of six transgenic wheat lines. Srivastava et al. (1996) reported that rearrangements between the integration patterns of parents and T_2 progeny had occurred in 5/6 lines. However, this may reflect some inherent instability in the population examined. A direct comparison between the two populations is not possible because no Southern analysis of T_2 progeny was undertaken in the present study. Thus, we cannot discount the possibility that rearrangements may have occurred in our T_2 progeny, despite none being observed from the T_0 to the T_1 generation in 11 out of the 12 lines examined. However, if rearrangements did occur they had little effect on the normal inheritance of transgene expression.

In 1 line containing pAHC25 (WT4), although integration patterns were inherited faithfully between the T_0 and T_1 generation, digestion with the insert excision enzymes showed that multiple rearrangements in the coding region of the *bar* gene (Fig. 2d) but not the *uid*A gene (Fig. 2c) had occurred upon integration. A similar observation was recorded by Srivastava et al. (1996). This contrasts with observations made by Register et al. (1994) who reported a higher frequency of *uid*A gene than *bar* gene rearrangements in maize. Also, Cooley et al. (1995) reported higher frequencies of non-expression of the *uid*A gene than the *bar* gene in rice plants and suggested that this may be due to more frequent rearrangements of the *uid*A gene. A clear interpretation of these observations is difficult due to the smaller number of wheat plants analysed, the use of different constructs in each experiment and also the use of different species and different transformation conditions.

Experimental evidence for a duplication of the *uid*A sequence (or part of it) in line HTT2 is presented in the Results section (Fig. 2j). Register et al. (1994) reported the occurrence of a similar phenomenon where amplification of the *uid*A gene occurred in some T_3 progeny of a maize transgenic line. However, further progeny of line HTT2 must be analysed and in detail to support the sequence duplication hypothesis, which may have important implications for transgene stability.

Southern analysis showed that insertions had occurred at unlinked loci in 2 out of 12 lines in the present study (17%). Results from a different population of wheat lines from our laboratory have given even higher frequencies (up to 30% – L. Rooke, unpublished data) of multiple locus insertions of marker genes and genes of interest. This represents a relatively high frequency compared to that reported in transgenic rice and maize populations produced by particle bombardment (Kohli et al. 1998; Pawlowski and Somers 1998; Cooley et al. 1995; Register et al. 1994). However, in comparison with the other cereals, wheat and tritordeum have large, complex, hexaploid genomes, and it is possible that this has an influence on the frequency of such events. The identification of plants with marker genes and genes of interest at separate loci may be useful in applied wheat GM projects, as marker genes may eventually be removed by segregation.

The inserted *neo* genes in the T_0 line WT5 gave rise to segregation ratios and Southern banding patterns in T_1 progeny which were consistent for the presence of a locus that was homozygous for the insertion event. The most likely mechanism which would cause this is sister chromatid exchange (mitotic crossing-over) during the early stages of embryogenesis and regeneration of a bombarded embryo. Normally, mitotic division of a hemizygous cell should result in two hemizygous daughter cells because replicated chromatids from a homologous pair of chromosomes are shared between them. However, during or after DNA replication, homologues occasionally become aligned so that exchanges of chromatid sections may occur between them. These exchanges become more frequent in wheat under tissue culture conditions (Pijnacker and Ferwerda 1994). Thus, a copy of the transgene on one chromatid could be passed to the allelic position on the opposing homologue. The daughter cell which inherited a transgene-containing chromatid from each homologue would be homozygous for the transgene, the other daughter cell would be null for the transgene. The former genotype

would proliferate under selection, whereas the latter would die.

A further possible explanation is that the transgene was inserted into a chromosome whose homologue was subsequently lost, giving rise to a line which was monosomic for the transgenic chromosome. The transgene locus in this case would also be unable to segregate. However, monosomic wheat lines are sometimes sterile and grow with poor vigour, whereas line WT5 was fertile and appeared to be physiologically and morphologically normal.

Inheritance of expression

Inheritance of expression followed Mendelian laws in the majority of lines. However, some T_2 progeny of line WT2 were shown to have lost both *uid*A expression (in leaves and flowers) and BASTA resistance (expression of both genes was strongly detected in the T_1 progeny). PCR data and Southern analyses (not shown) of the T_2 plants of WT2 confirmed that transgene eliminations and rearrangements were not the cause. Thus, transgene silencing may have been responsible. In the T_3 generation of WT2, although loss of *uid*A expression in leaf material and loss of BASTA resistance was observed in some progeny, we were able to detect *uid*A expression in the flowers of nearly all plants tested, plus in some cases a partial BASTA resistance phenotype. The difference between the expression characteristics of the two genes between the two generations may result from environmental influences. However, the observation of transgene inactivation in only some T_2 and T_3 progeny of previously expressing WT2 parents suggests that epigenetic phenomena may also be involved. Similar instances of epigenetic silencing in rice have recently been reported by Kumpatla et al. (1998) who studied the inheritance of expression of a complex locus containing multiple copies of the maize ubiquitin promoter/intron driving the *bar* gene. In this case, loss of *bar* gene expression was shown to occur in 5–40% of progeny of a previously expressing parent. Methylation of promoter sequences was shown to be associated with this recurrence of silencing in progeny of later generations, suggesting that the inactivation occurred at the transcriptional level.

In the present study, a reduction in the overall amounts and intensity of GUS-stained tissue was observed between the T_1 and T_2 and the T_2 and T_3 progeny of three wheat lines containing pAHC25 at a single locus. A similar reduction in the resistance to BASTA was not observed. This suggests a form of uniform, 'progressive' transgene silencing specific to the *uid*A gene only. Since the *bar* and *uid*A genes on plasmid pAHC25 are both controlled by the same promoter and intron, this silencing of the *uid*A gene may not be of the promoter homology-mediated type described in Park et al. (1996), in which the methylation of promoter sequences was shown to cause silencing at the transcriptional level. Such observations suggest that the progressive *uid*A silencing in these three lines may be post-transcriptional. Interestingly, Srivastava et al. (1996) also reported *uid*A-specific inactivation in wheat lines containing both *uid*A and *bar* genes. However, in some cases the *bar* gene was expressed even though it was methylated.

Future work

Transgene loci in the tritordeum lines appeared less stable than in the wheat lines. This observation suggests that background genotype may have an effect on overall transgene stability, independent of other factors thought more commonly to have an effect (such as copy number and integration pattern complexity). In order to investigate this possibility in more detail we have recently crossed several of the transgenic lines described here with a number of different elite wheat varieties and are analysing transgene stability in the resulting progeny.

While the present work suggests relative stability in wheat transformants, it provides information up to the T_3 generation only. Clearly for the application of GM technology, information on longer term stability is required. With this aim, analysis of this marker gene population is being continued into the T_4 and T_5 generations and beyond. In addition, some of the lines for which unexpected phenomena were observed are being analysed in more detail since a better understanding of them may lead to possible strategies to avoid them.

The high frequency of insertion pattern stability and Mendelian inheritance of expression observed in this study overall has positive implications for the application of GM technology in wheat breeding programmes. However, the observation in some lines of *uid*A-specific silencing suggests that transgene instability may be specific to the sequence inserted and that further studies on a wider range of homologous and heterologous transgenes are needed for a broader understanding of transgene stability in wheat.

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