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The influence of auxins on transformation of wheat and tritordeum and analysis of transgene integration patterns in transformants

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Abstract Although it is possible to transform wheat, broad application of the technology is limited because of the low overall efficiency and the lack of reliability of the technique. In addition there is little published data on transgene integration patterns and inheritance in wheat. We have generated a population of transgenic wheat and tritordeum lines under different auxin regimes and show that, under the conditions described, the presence of picloram results in higher transformation efficiencies than the presence of 2,4-D. Molecular analysis shows low-copy numbers and simple integration patterns to be prevalent in the transgenic lines. Mendelian inheritance of transgenes in T_1 progeny was observed for the majority of lines.

Key words Wheat · Auxins · Bombardment · Transformation efficiency · Transgene integration pattern

Introduction

The production of transgenic plants by direct gene transfer (DGT) relies on the following three requirements; (1) that DNA is delivered to the nucleus of cells of target tissues and integrated into the genome, (2) that those cells are in mitosis and proliferate and (3) that this tissue differentiates into a transgenic plant that is able to reach maturity. Transgenic cereals are most commonly generated using DGT technology, and particle bombardment is the most widely used method (Barcelo and Lazzeri 1995). In particle bombardment, DNA is delivered into the cells by being coated onto gold particles that are accelerated into target cells which then proliferate in response to auxin exposure and wounding (Dudits et al. 1991). Two auxins commonly used to induce somatic embryogenesis and allow regeneration from cereal tissues, are 2,4-dichlorophenoxyacetic acid (2,4-D) and picloram (Wernike and Milkovits 1987). Both are synthetic auxins with distinct effects on the induction of cell division, proliferation and further regeneration. They act by inducing auxin-sensitive non-dividing cells, arrested in G1, to re-enter S-phase and mitosis. The timing of this process depends on the auxin type and on the concentration applied (Wernicke and Milkovits 1987; Barcelo et al. 1992).

Wheat transformation by particle bombardment was first reported by Vasil et al. (1992), and reports have followed from several other laboratories. Transformation efficiencies between 0.15–0.5% (Weeks et al. 1993, Blechel and Andersen 1996 and Altpeter et al. 1996a, b) and between 0.5–1.5% (Vasil et al. 1993, Nehra et al. 1994, Becker et al. 1994 and Barro et al. 1997) have been reported. In tritordeum (a fertile hexaploid amphiploid between *Hordeum chilense* and *Triticum durum*, Martin and Sanchez-Monge 1982) 2% transformation efficiency was reported by Barcelo et al. (1994). Taking the best individual experiments from these reports, high transformation efficiencies for wheat have been reported by Nehra et al. (1994) (2.5%), Becker et al. (1994) (3.6%) and Altpeter et al. (1996b) (2%), but all studies report high levels of experiment-to-experiment variation in efficiency. This variation is currently hard to control due to our poor understanding of the initial factors influencing stable transformation. One important area of which we have little knowledge is the process of DNA integration and on

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how cells may be treated in order to improve the integration of foreign DNA.

In the study presented here, we analysed the effect of the auxin included in the culture medium for the induction of embryogenesis on the efficiency of wheat and tritordeum transformation. Our rationale was that if cultured cells are induced to re-enter S-phase and mitosis at different timings depending on the type of auxin and the concentration applied (Wernicke and Milkovits 1987), then this could also affect the recovery of transformed plants: potentially by synchronization of cells in a phase of the cell cycle receptive to foreign DNA integration. We also examined whether the selection system (PPT or G418 selection) interacts with the auxin used and with the target explant in affecting the recovery of transgenic plants. Previous studies on wheat transformation have used 2,4-D as the auxin source, and there is no report of the effect of other auxins on transformation efficiency.

Materials and methods

Plant material, explants and media

Immature scutella of wheat (*Triticum aestivum* L.) cv 'Florida' and immature inflorescences of tritordeum lines HT28 and HT31 were used as target tissues for transformation by particle bombardment. Plants were grown in growth cabinets illuminated with 400W HQI lamps with a 16-h photoperiod and a day/night temperature regime of 16°–18°/14°C. Caryopses and tillers containing immature inflorescences were harvested and explants isolated as described by Barcelo and Lazzeri (1995). For each bombardment 35 immature scutella or approximately 1-mm inflorescence segments were placed in the centre of a 90-mm petri dish containing induction medium. Explants were precultured in darkness at 24°C for 1 day prior to bombardment. The induction media tested were; MD1, MD2, MP2 and MP4 for immature scutella and L7D2, L7D4, L7P4 and L7P6 for immature inflorescences (see Table 1).

Plasmids and microprojectile bombardment

Three plasmids were used for the transformations: plasmid pAHC25 (Christensen and Quail 1996) which contains the *uid*A and *bar* genes, both under the control of the maize ubiquitin promoter and the ubiquitin intron; plasmid pAct1-DGus (McElroy et al. 1990) containing the *uid*A gene under the control of the actin-1D promoter from rice; plasmid pCaI-neo (constructed by S. Luetticke, University of Hamburg, modified from the plasmid pCaI-gus described in Callis et al. 1987) which contains the *neo* gene under the control of the CaMV 35S promoter and the Adh1 intron from maize. Plasmids pAct1-DGus and pCaI-neo were used together in a 1:1 equimolar mixture, while pAHC25 was delivered alone. Plasmid DNA was precipitated onto gold particles following the protocol of Barcelo and Lazzeri (1995). Bombardments were carried out at a distance of 5 cm from the stopping plate using a PDS 1000/He gun (BioRad) at a helium pressure of 1100 psi.

Experimental design

A total of 96 replicates (each replicate was 1 petri dish containing explants) was used for this experiment from which 48 were bombarded with plasmid DNA-coated particles and the rest were used as controls (bombarded with particles without plasmid DNA). Thirty-five explants *per* replicate were bombarded from which 5 were taken for transient gene expression assays and the remainder were cultured further.

Culture, selection and plant regeneration

Immediately after bombardment, explants were spread over the surface of the medium in the original dishes and cultured at 24°C in darkness for 3 weeks. Embryogenic capacity was then assessed by counting the number of explants bearing somatic embryos (data not shown). Explants from control dishes were then divided into two sets: one was regenerated without selection, whereas the other was selected under the same conditions as the explants bombarded with plasmid DNA. For selection and regeneration, calli were transferred to regeneration (Rz) medium (Table 1) supplemented with either 2 $mg l^{-1}$ L-phosphinothricin (L-PPT, the active ingredient of the herbicide BASTA) or 50 $mg l^{-1}$ G418 (geneticin disulphate) and cultured for a further 3 weeks. Regeneration capacity (number of

Table 1 Culture media for wheat scutella and tritordeum inflorescences

Medium	Component
M	MS macrosalts (Murashige and Skoog 1962), L microsalts (Lazzeri et al. 1991), MS vitamins (Murashige and Skoog 1962), FeNaEDTA (Sigma, 10 $mg l^{-1}$), L1 amino acids (Lazzeri et al. 1991) and 30 $g l^{-1}$ sucrose
MD1	M medium plus 1 $mg l^{-1}$ 2,4-D
MD2	M medium plus 2 $mg l^{-1}$ 2,4-D
MP2	M medium plus 2 $mg l^{-1}$ picloram
MP4	M medium plus 4 $mg l^{-1}$ picloram
L7	L7 macrosalts (250 $mg l^{-1}$ NH_4NO_3, 150 $mg l^{-1}$ KNO_3, 200 $mg l^{-1}$ KH_2PO_4, 350 $mg l^{-1}$ $MgSO_4 \cdot 7H_2O$ and 450 $mg l^{-1}$ $CaCl_2 \cdot 2H_2O$), L microsalts FeNaEDTA, L1 amino acids, L2 vitamins (Barcelo and Lazzeri 1995), inositol 400 $mg l^{-1}$ and maltose 30 $g l^{-1}$
L7D2	L7 medium plus 2 $mg l^{-1}$ 2,4-D
L7D4	L7 medium plus 4 $mg l^{-1}$ 2,4-D
L7P4	L7 medium plus 4 $mg l^{-1}$ picloram
L7P6	L7 medium plus 6 $mg l^{-1}$ picloram
R	L7 medium lacking L1 amino acids
Rz	R medium plus 0.1 $mg l^{-1}$ 2,4-D, 5 $mg l^{-1}$ zeatin

calli producing shoots/total explants) was then assessed in control plates under non-selection conditions. Surviving explants were transferred to a second regeneration medium (R) (Table 1) again containing 2 mg l^{-1} L-PPT or 50 mg l^{-1} G418, and successive 3-week selection passages were applied on this medium until all control cultures grown under selection were dead [even though selection pressure was not released until all the control plants were dead, the regeneration of untransformed plants (escapes) was observed in most of the experiments]. Surviving plantlets from transformation treatments were transferred to soil and grown to maturity in the greenhouse.

Extraction of plant DNA

Genomic DNA was extracted from leaf tissue harvested from primary transformants and their progeny using a modification of the method described by Stacey and Isaac (1994). Briefly, 100–200 mg of fresh leaf material was ground into small fragments (approx. 1–3 mm^2) under liquid nitrogen. Samples were then incubated in 1 ml extraction buffer (see below) for 30 min at 65°C and allowed to cool at room temperature for 15 min before a single protein extraction with approximately 1 ml chloroform/octanol (24 : 1). DNA was precipitated by incubation in ethanol at −20°C for 15 min. DNA threads were spooled onto a glass hook, washed in 75% ethanol, 0.2 M ammonium acetate, air-dried and resuspended in TE buffer (10 m*M* TRIS-HCl pH 8.0, 1 m*M* Na_2EDTA). The extraction buffer consisted of 2% CTAB (hexadecyltrimethylammonium bromide – Sigma H5882), 0.02 *M* EDTA, 0.1 *M* TRIS-HCl (pH 8.0), 1.4 *M* NaCl, 25 m*M* dithiothreitol (added immediately prior to use).

Polymerase chain reaction (PCR) screening

The presence or absence of transgenes in primary transformants and their progeny was determined using PCR. Generally, 100–200 ng template DNA was used in a 30-µl PCR reaction containing 1 × enzyme buffer (10 m*M* TRIS-HCl pH 8.8, 1.5 m*M* $MgCl_2$, 50 m*M* KCl, 0.1% Triton X-100), 200 m*M* dNTPs, 0.3 m*M* primers (see Table 1) and 0.66 units recombinant thermostable DNA polymerase (Dynazyme II – Flowgen). Thermocycling conditions were as follows for 30 cycles: denaturation at 94°C for 30 s, annealing for 30 s and extension at 72°C for 2 min. The primer sequences (F = forward; R = reverse) were: *bar* (F) 5′-GTCTGCACCATCGTCAACC-3′ (R) 5′-GAAGTCCAGCTGCCAGAAAC-3′; *uid*A (F) 5′-AGTGTACGTATCACCGTTTGTGTGAAC-3′ (R) 5′-ATCGCCGCTTTGGACATACCATCCGTA-3′; *neo* (F) 5′-GAGGCTATTCGGCTATGACTG-3′ (R) 5′-ATCGGGAGCGGCGATACCGTA-3′. Annealing temperatures and approximate product lengths were: *bar* = 57°C, 420 bp; *uid*A = 62°C, 1020 bp; *neo* = 57°C, 700 bp.

Southern blotting and hybridization

Digested DNA was separated by electrophoresis through 0.8% agarose and transferred to Hybond N + (Amersham) using standard procedures (Sambrook et al. 1989). Digoxigenin-labelled probes were prepared using a PCR DIG probe synthesis kit (Boehringer Mannheim) using primers to amplify fragments internal to the *bar*, *neo* and *uid*A coding regions according to manufacturers instructions. Hybridization and chemiluminescent detection of probes was carried out according to the 'Optimised Hybridization and Detection Protocol' described in Engler-Blum et al. (1993).

Transgene expression

Transient β-glucuronidase (GUS) expression was assayed 2 days after bombardment as described by Barcelo and Lazzeri (1995). The same procedure was used to assay GUS expression in the leaf or root samples of all primary regenerants and controls and also leaves and roots from selected T_1 progeny from transgenic lines containing the *uid*A gene, with the modification that chlorophyll was extracted after staining by incubation in 70% ethanol for 1 h and then 100% ethanol overnight. Determination of BASTA resistance was carried out by topical application of 0%, 0.1%, 0.5%, 1% and 5% BASTA solutions containing 0.1% Tween-20 to mature leaves of putative and control plants using cotton buds. Levels of NPTII enzyme expression were assayed in crude leaf protein extracts from selected T_1 progeny of transgenic lines using a NPTII ELISA kit (CP Laboratories) following the manufacturer's instructions.

Transgene inheritance

For each of 12 confirmed independent lines, a minimum of 22 and maximum of 47 T_1 progeny plants were analysed. Transmission of the transgene(s) to each progeny plant was confirmed using PCR. Segregation ratios were calculated from these data. In addition, transgene activity was monitored in selected progeny from each line using the expression assays described above.

Statistical analysis

Fisher's Least Significant Difference (LSD) pairwise comparison of means was used to determine the significance of differences in embryogenesis and regeneration capacities from immature scutellum and immature inflorescence cultures. For analysis, data were transformed using arcsin transformation. The Chi square test was used to confirm the probability that ratios conformed to Mendelian segregation patterns.

Results

Effect of auxin type and concentration on regeneration and transformation efficiency

To examine the influence of auxin on embryogenesis and shoot production in the absence of selection, we assessed scutellum and inflorescence explants from bombarded controls after 3 and 6 weeks of culture, respectively. It was observed that both explants reacted similarly to picloram and 2,4-D but that picloram-based induction media induced significantly more explants to proceed towards embryogenesis and regeneration than media containing 2,4-D. For both auxins, no difference between the two concentrations tested was found, irrespective of the type of explant used (see Table 2 for regeneration data).

For the assessment of the effect of auxin type on the recovery of transgenic plants, scutellum and inflorescence explants bombarded with constructs containing either the *bar* gene (pAHC25) or the *neo* gene (pCaI-neo) were selected on PPT or G418, respectively. Inflorescence cultures gave rise to transgenic plants when cultured on either picloram or 2,4-D, but in scutellum cultures only picloram-based media produced transgenic plants (Table 2). Picloram was more

Table 2 Effect of auxin type and concentration and selection system on the recovery of transgenic wheat and tritordeum plants from bombarded scutellum and inflorescence cultures (*TE* transmission efficiency)

Harmone	Regeneration (%)	PPT		G418		Total no. transgenic plants/no. explants	Total TE (%)
		Transgenic plants/no. explants	TE (%)	Transgenic plants/no. explants	TE (%)		
Scutella							
2,4-D							
1 mg l^{-1}	44[a] a	0/83	0	0/90	0	0/173	0%
2 mg l^{-1}	31 a	0/87	0	0/89	0	0/176	0%
Picloram							
2 mg l^{-1}	61 b	2/86	2.3	1/80	1.2	3/166	1.8%
4 mg l^{-1}	66 b	2/80	2.5	2/86	2.3	4/166	2.4%
Total		4/336	1.2	3/345	0.9	7/681	1.0%
Inflorescence							
2,4-D							
2 mg l^{-1}	44 a	0/115	0	1/85	1.2	1/200	0.5%
4 mg l^{-1}	45 a	2/101	2.0	0/89	0	2/190	1.0%
Picloram							
4 mg l^{-1}	76 b	3/94	3.2	2/88	2.3	5/182	2.7%
6 mg l^{-1}	74 b	0/97	0	1/86	1.2	1/183	0.5%
Total		5/407	1.2	4/348	1.1	9/755	1.2%

[a] Means within species and columns with the same letter are not significantly different at the 0.05 level according to Fisher's Least Significant Difference (LSD) Test.

efficient than 2,4-D, in producing transgenic plants from scutellum cultures; the overall transformation efficiency was 1.86% for picloram-based media (average of 1.8%, 2.4%, 2.7% and 0.5% efficiencies) and 0.4% for 2,4-D-based media (average of 0%, 0.5% and 1% efficiencies). This result may partly be explained by the fact that picloram induced higher regeneration frequencies than 2,4-D, although the difference of 1.7-fold in regeneration between the two auxins (average 40% versus 70% for 2,4-D and picloram, respectively) is not as great as the difference in transformation efficiency of 4.6-fold (0.4% versus 1.86%). The highest frequencies of production of transgenic plants were 3.2% for inflorescence cultures and 2.5% for scutellum cultures, in both cases from cultures induced on media containing 4 mg l^{-1} picloram.

Overall, PPT and G418 appeared to have a very similar efficiency for selecting transgenic plants; 1.2% transformation efficiency under PPT selection and 1% under G418 selection (averaged over the two explant types and the two auxins), although the stringency of selection with G418 was better than with PPT; there were approximately 30% escapes under G418 and approximately 80% under PPT selection. In total, 16 transgenic lines were produced in the experiment with an overall efficiency of 1.2% from tritordeum inflorescence and 1% from wheat scutellum cultures. All transgenic wheat plants developed normally and set seed (see below and Fig. 1d), however 3 out of the 9 tritordeum plants produced were sterile. This lack of fertility in some tritordeum lines was related to poor pollen fertility, which was also observed in untransformed control plants.

PCR screening of regenerants

All plants resistant to the selection agents were screened first by PCR. Figure 2 shows an example of results from PCR screening of selected plants recovered from selection, which had been bombarded with the construct pAHC25 (panel A) and the constructs pCaI-neo and pAct1-DGus (panel B). In panel A, tracks 3, 8, 9 and 12 show PCR bands corresponding to the expected sizes for the *bar* and *uid*A genes. The remaining tracks do not show any PCR product for these genes, and they correspond to plants which had escaped selection. This panel also shows that all 4 positive plants are co-transformed with both genes. Panel B shows similar results for primary regenerants from co-bombardment with pCaI-neo and pAct1-DGus. Tracks 1, 5, 7, 10 and 11 correspond to transformants containing the *neo* gene, while only the plant analysed in track 10 is co-transformed with both genes. Results of PCR screens were subsequently corroborated by Southern analysis (see Table 3).

Determination of transgene integration patterns in wheat and tritordeum transgenic plants

To examine transgene integration patterns and estimate numbers of transgene insertions, we digested

Fig. 1 a Histochemical GUS assay for expression of the *uid*A gene in primary wheat transformants, **b** NPTII ELISA for *neo* gene expression in T_1 progeny, **c** Basta resistance in primary wheat transformants, **d** fertile primary transformants

DNA from putatively transformed plants with an enzyme which cuts only once in the inserted plasmid and probed with either a *uid*A, *bar* or *neo* coding region fragment. The size of hybridizing bands is determined by the distance between the restriction site in the plasmid and the next occurrence of the

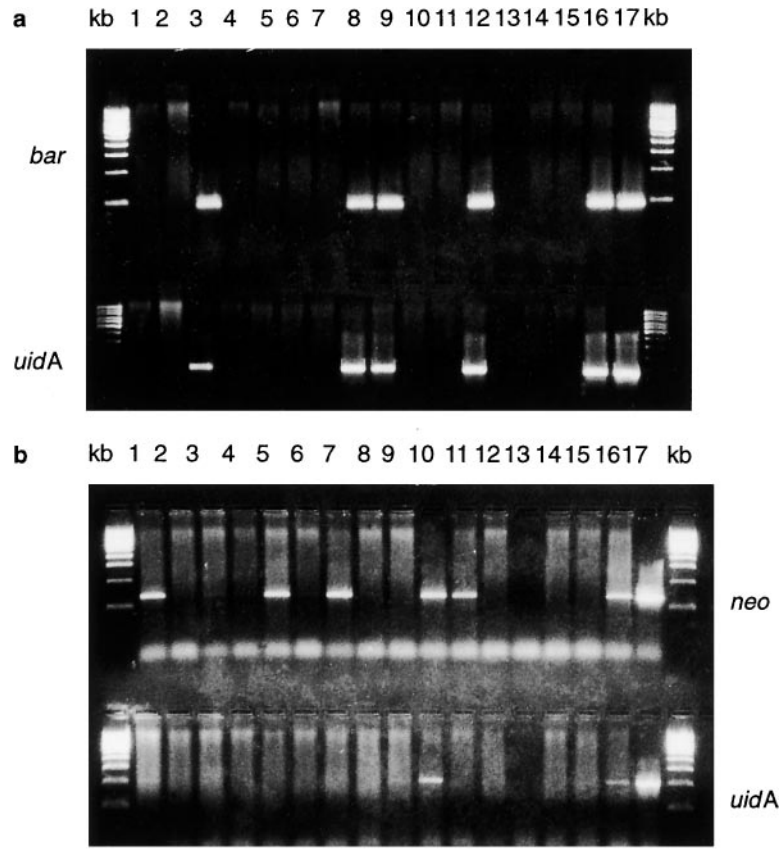

Fig. 2A, B Example of PCR screening of selected putative transformants. Agarose gels contain PCR products of the *bar* and *uid* A, *neo* or *neo* and *uid*A genes, amplified from DNA of selected primary wheat and tritordeum regenerants containing either pAHC25 (**A**), pCaI-neo alone or pCaI-neo and pAct1-DGus (**B**). *Lanes 13–15* Negative controls, *lanes 16, 17* Positive controls, kb 1-kb ladder

Table 3 PCR analysis, estimated coding region copy number, expression and segregation in T_1 of *bar*, *neo* and *uid* A transgenes in wheat and tritordeum transformants *WT* wheat transgenic line; *HTT* tritordeum transgenic line; *NA* not applicable; *n*d not determined

Line	Plasmid used	Medium	PCR			Estimated copy number			Segregation in T_1	Expression in T_1		
			neo	*bar*	*uid*A	*neo*	*bar*	*uid*A	χ^2	NPTII	BASTA	GUS
Wheat												
WT1	pAHC25	MP2	NA	+	+	NA	> 10[cd]	> 10[cd]	2.7[a]	NA	+	+
WT2	pAHC25	MP4	NA	+	+	NA	6	6	0.655[a]	NA	+	+
WT3	pAHC25	MP4	NA	+	+	NA	5[c]	5[c]	Skewed (2:34)	NA	+	+
WT4	pAHC25	MP2	NA	+	+	NA	5[c]	5[c]	0.04[a]	NA	+	+
WT5	pCaI-neo	MP2	+	NA	–	2[c]	NA	–	3.2[b]	+	NA	NA
WT6	pCaI-neo	MP4	+	NA	–	3	NA	–	nd	+	NA	NA
WT7	pCaI-neo	MP4	+	NA	–	2	NA	–	0.466[a]	+	NA	NA
Tritordeum												
HTT1	pCaI-neo	L7D2	+	NA	–	nd	NA	nd	Sterile		NA	NA
HTT2	pCaI-neo/ pAct1DGus	L7P4	+	NA	+	3	NA	3[c]	1.14[a]	+	NA	+
HTT3	pCaI-neo	L7P6	+	NA	–	2[c]	NA	–	0.67[a]	+	NA	NA
HTT4	pCaI-neo	L7P4	+	NA	–	2[c]	NA	–	0.07[a]	+	NA	NA
HTT5	pAHC25	L7D4	NA	+	+	NA	4–10[c]	4–10[c]	Skewed (5:23)	NA	–	–
HTT6	pAHC25	L7D4	NA	+	+	NA	nd	nd	Skewed (2:24)	NA	+	+
HTT7	pAHC25	L7P4	NA	+	+	NA	> 10[cd]	> 10[cd]	Sterile		NA	NA
HTT8	pAHC25	L7P4	NA	+	+	NA	3[c]	3[c]	0.47[a]	NA	–	–
HTT9	pAHC25	L7P4	NA	+	+	NA	> 10[c]	> 10[c]	Sterile	NA	NA	NA

[a] In good agreement with 3:1 ratio at $P = 0.05$.
[b] In good agreement with 15:1 ratio at $P = 0.05$.
[c] Coding region rearrangement.
[d] Concatamers.

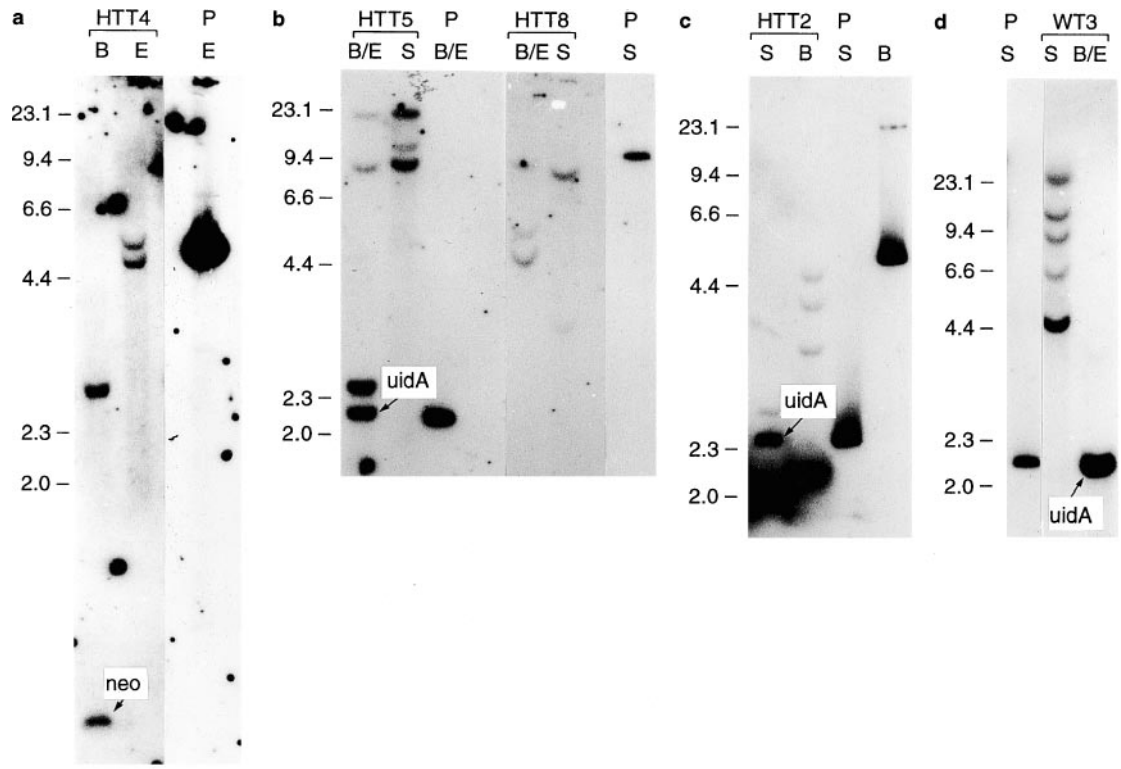

Fig. 3a–d Southern hybridization in five primary transformants. *WT* wheat transgenic line, *HTT* tritordeum transgenic line, *B Bam*HI, *E Eco*RI, *S Sac*I, *B*/*E Bam*HI/*Eco*RI, *P* plasmid control. *Panel a* Probed with *neo*, *panels b–d* probed with *uid*A

restriction site in the plant DNA flanking the inserted plasmid. The banding patterns of independent transformants will therefore almost always be different. Since coding region DNA is used as a probe, the number of hybridizing bands gives an indication of the number of transgene insertions, providing rearrangements or truncations within the coding region did not occur during integration. The occurrence of multiple plasmid insertions which are joined together (concatamers) is signified by the presence of a band the same size as the plasmid (intensity of this band is proportional to the number of plasmids inserted in this way, but accurate estimation of copy numbers is difficult). In addition to this analysis and in order to detect rearrangements within the coding sequence, an additional digest with an enzyme(s) which releases a fragment containing the intact coding sequence was also probed. The detection of a single hybridizing band of a size which corresponds to the distance between the enzyme(s) sites strongly suggests that only intact coding regions are present. Rearrangements within or between the enzyme sites will prevent them cutting, resulting in other, bigger or smaller hybridizing bands in this track. It should be noted that the type of analysis described above provides little information on the insertion of plasmid backbone sequences since the probe only detects coding region sequences. However, since the overall aim was to concentrate on the analysis of the inserted genes of interest, the lack of this type of information was not considered significant.

For plasmids pAHC25, pCaI-neo and pAct1-DGus the single-cutting enzymes were *Sac*I, *Eco*RI and *Bam*HI respectively; the coding region-releasing enzymes were *Bam*HI/*Eco*RI (this double digest releases an approximate 2.2 kb fragment containing the *uid*A coding region from pAHC25), *Bam*HI (releases an approximate 0.85 kb fragment containing the *neo* coding region from pCaI-neo) and *Sac*I (releases an approximate 2.4 kb fragment containing the *uid*A coding region from pAct1-DGus), respectively.

Figure 3 shows Southern analysis of 5 primary transformants obtained either from co-bombardments with pCaI-neo and pAct1-DGus (HTT4 and HTT2) or from bombardments with pAHC25 (HTT5, HTT8 and WT3). Panel a shows the detection in plant HTT4 of an approximate 0.85 kb *neo*-hybridizing band in DNA digested with *Bam*HI (see HTT4, lane B), suggesting the presence of at least 1 intact *neo* coding region. The second hybridizing band (approx. 3 kb) suggests the presence of a rearranged *neo* coding region fragment (joined to plant DNA). The detection in *Eco*RI-digested DNA of two hybridizing bands (see HTT4, lane E) provides good evidence for the insertion of 2 *neo* coding regions (one complete and one truncated). Panel b shows the detection in *Bam*HI/*Eco*RI-digested DNA from plant HTT5 of a single approximate 2.2-kb *uid*A-hybridizing band and three other bands, suggesting the presence of at least 1 intact *uid*A coding region and also

the insertion of 3 other rearranged *uid*A fragments. The detection in *Sac*I-digested DNA of three hybridizing bands (see HTT5, lane S), one of which appears to be the same size as linearized plasmid DNA (see plasmid control), suggests the presence of a small concatamer of pAHC25. The relative band intensities and patterns in both lanes are, however, unusual, making an accurate estimation of insertion number difficult. Panel b also shows the detection in *Bam*HI/*Eco*RI-digested DNA from plant HTT8 of three *uid*A-hybridizing bands, none of them of the expected approximate 2.2-kb size, representing 3 rearranged *uid*A coding region fragments (*uid*A gene expression was not detected in this plant). The detection in *Sac*I-digested DNA of three hybridizing bands (see HTT8, lane S) suggests the insertion of 3 *uid*A fragments. Panel c shows the detection in *Sac*I-digested DNA from plant HTT2 of an approximate 2.4-kb *uid*A-hybridizing band and an additional hybridizing band, suggesting the insertion of at least 1 complete *uid*A coding region and also the presence of a rearranged *uid*A coding region fragment. The detection in *Bam*HI-digested DNA (see HTT2, lane B) of three *uid*A-hybridizing fragments suggests the insertion of 3 *uid*A coding regions – 2 complete and 1 rearranged. Panel d shows the detection in *Bam*HI/*Eco*RI-digested DNA from plant WT3 of an approximate 2.2-kb *uid*A-hybridizing band, suggesting the insertion of at least 1 complete *uid*A coding region. The two weakly hybridizing bands in this lane may represent small fragments of rearranged coding regions. The detection in *Sac*I-digested DNA (see WT3, lane S) of five *uid*A-hybridizing fragments of similar intensity, suggests the insertion of around 5 *uid* A coding regions. The Southern analysis of each transformant is accompanied by control plasmid DNA digested either with the plasmid-linearizing enzyme or coding region-releasing enzyme(s) (or both) to give fragment size controls.

Figure 4 panels a and b show Southern analysis of 9 putative transgenic wheat plants (labelled as E or WT) obtained from co-bombardment with pCaI-neo and pAct1-DGus. Panel b shows *neo*-probed DNA digested with *Bam*HI which releases the *neo* coding region as an approximate 0.85-kb fragment. The presence of only a single approximate 0.85-kb hybridizing band in plants WT6 and WT7 suggests that only intact coding regions are present. The detection in *EcoRI*-digested DNA from WT6 and WT7 (see panel a) of three and two bands, respectively, suggests the insertion of 3 and 2 *neo* coding regions, respectively. In plant WT5 the detection of an approximate 0.85-kb band in *Bam*HI-digested DNA (see panel b) suggests the presence of at least 1 complete *neo* coding region (NB: this lane ran faster than lanes WT6 and WT7, probably due to uneven setting of the gel). The presence of an additional, larger, single band suggests the presence of a rearranged *neo* coding region fragment. The detection

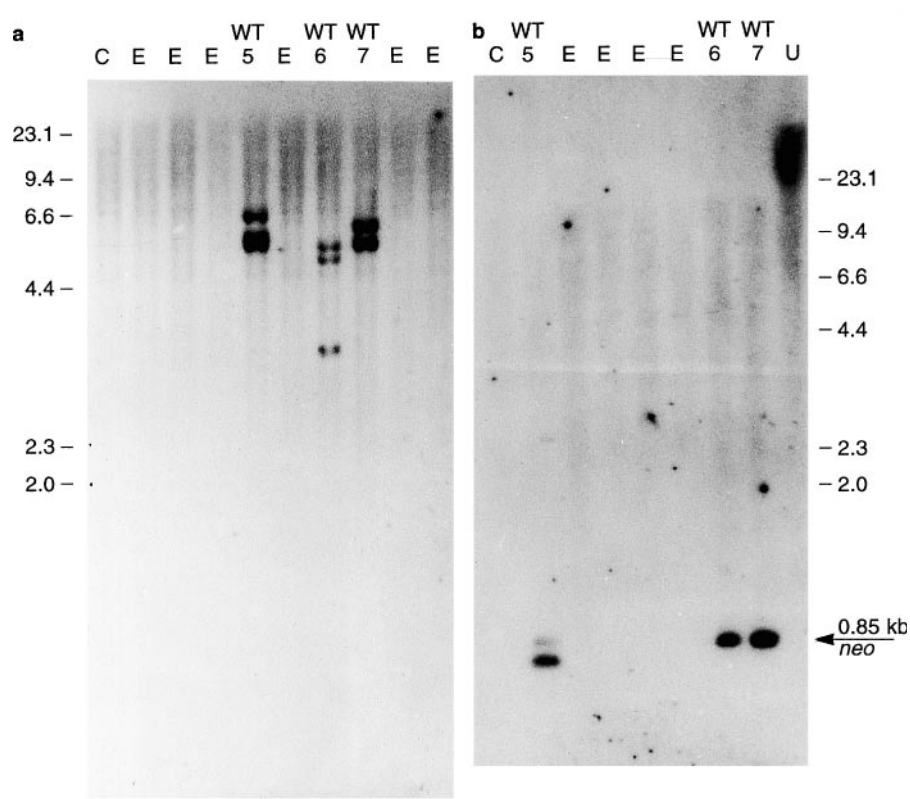

Fig. 4a, b Southern hybridization of *neo* probe to DNA from primary wheat regenerants transformed with pCaI-neo. DNA digested with either *Eco*RI (**a**) or *Bam*HI (**b**). *C* control DNA from untransformed plant, *E* untransformed primary regenerant (escape), *WT* wheat transgenic line, *U* undigested DNA from WT7

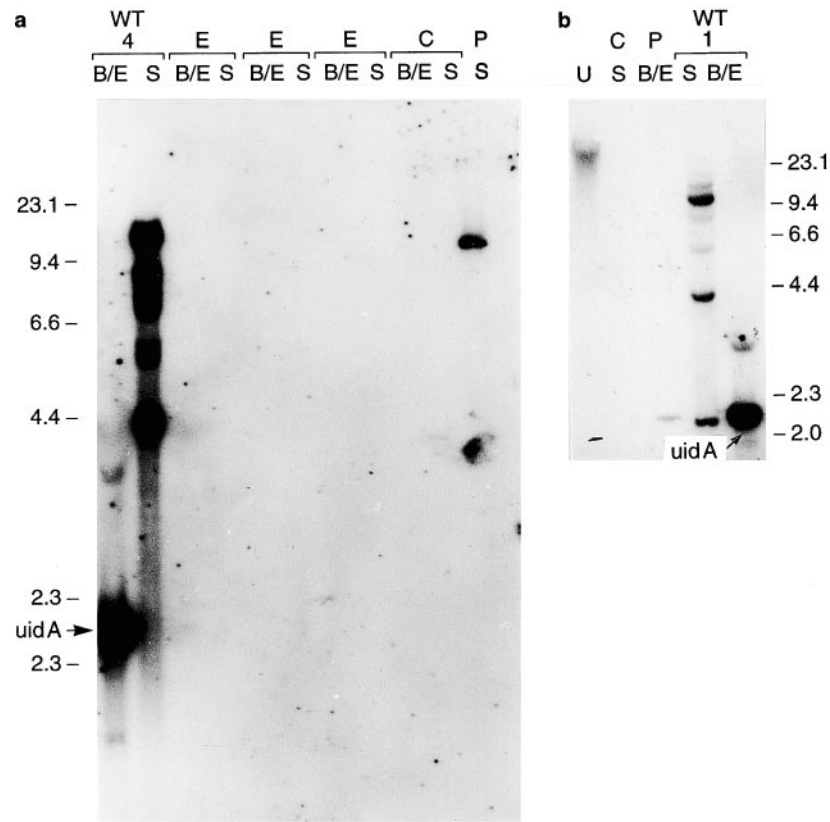

Fig. 5a, b Southern hybridization of *uid*A probe to DNA from primary wheat regenerants transformed with pAHC25. *P* plasmid control, *B/E* *Bam*HI/*Eco*RI, *S* *Sac*I, *C* control DNA from untransformed plant, *E* untransformed primary regenerant (escape), *WT* wheat transgenic line, *U* undigested DNA from WT2

in *Eco*RI-digested DNA (panel a, lane WT5) of 3 hybridizing bands suggests the insertion of 3 *neo* coding regions – 2 complete and 1 truncated. The absence of integrated *uid*A gene sequences in plants WT5, WT6, WT7, HTT4, HTT2 was confirmed upon hybridization of membranes with a *uid*A probe (data not shown).

Figure 5 shows Southern analysis of 5 putative transgenic wheat plants obtained from bombardment with pAHC25 (labelled as E, WT4 and WT1). The detection in plant WT4 of a single major *uid*A-hybridizing fragment in DNA digested with *Bam*HI/*Eco*RI (see WT4 in panel a, lane B/E) suggests the presence of mainly complete copies of the *uid*A coding region. The two weakly hybridizing bands suggest the presence of small, rearranged *uid*A fragments. The detection of five *uid*A-hybridizing fragments in DNA digested with *Sac*I (see WT4, lane S) suggests the insertion of around 5 copies of the *uid*A coding region. Interestingly, seven fragments were detected in *Sac*I-digested DNA from WT4 and nine bands in *Bam*HI/*Eco*RI digested DNA, when probed with *bar*, suggesting multiple rearrangements of the *bar* coding region (data not shown). The presence of a strong *uid*A-hybridizing fragment at approximate 2.2-kb molecular weight in *Bam*HI/*Eco*RI-digested DNA from plant WT1 (panel b, lane B/E) suggests the release of multiple *uid*A fragments from pAHC25. The two other weakly hybridizing bands detected in this lane suggest the presence of small, rearranged *uid*A fragments. The detection of a relatively strong hybridizing band of approximate 9.7-kb molecular weight in *Sac*I-digested DNA (panel b, lane S) suggests the presence of concatamers of construct pAHC25. The bands of other molecular weights in this lane correspond to plant restriction fragments joined to either end of the concatamer and other inserted *uid*A sequences. The approximate number of inserted coding regions is difficult to estimate in this case but based on comparisons between band intensities it is probably around 10–20. Plasmid controls are included in the analyses of transformants WT4 and WT1.

Southern analysis was performed in 14 out of the 16 lines produced in the experiment and the results are summarized in Table 3. Of the plants obtained 79% (11/14) had low estimated numbers of coding region insertions (50%; 7 plants contained around 2 or 3, and 29%; 4 plants around 4 to 7 copies), and only 21% (3 plants) contained an estimated 10 or more inserted transgenes out of 16 transgenic plants were obtained from cultures induced under 4 $mg\,l^{-1}$ picloram, and within those picloram-induced plants 7 (78%) contained low estimates of transgene copies.

Co-integration frequencies were very different for the two sets of genes when delivered in the same plasmid or in separate plasmids. All 9 plants transformed with

pAHC25 proved to contain both *bar* and *uid* A sequences, whereas only one plant of the 7 transformants recovered after co-bombardment with pAct1-DGus and pCaI-neo contained both the *neo* and *uid*A sequences. This co-transformation frequency is low compared with other published data and also compared with all previous and subsequent experiments conducted in our laboratory. We have no data to suggest why.

Transgene segregation and expression in the T_1 generation

Positive expression of the *bar* gene in the T_1 generation was scored only when plants showed resistance to 1% BASTA (some controls showed resistance to 0.1% Basta). Some lines showed levels of resistance of up to 5% BASTA (10 $g l^{-1}$ PPT), suggesting a range of levels of transgene expression (Fig. 1c). Of the 4 wheat lines containing pAHC25 (WT1, WT2, WT3, and WT4) all expressed both the *uid*A and *bar* genes in T_1 progeny (see Table 3). Of the 3 tritordeum lines containing pAHC25 (HTT5, HTT6 and HTT8), only 1 (HTT6) expressed both *uid*A and *bar* genes. Figure 1a shows an example of the expression of the *uid*A gene in leaf tissue of T_1 transgenic plants. The 3 wheat lines and 2 tritordeum lines containing pCaI-neo only (WT5, WT6, WT7, HTT3 and HTT4) were all shown to express the *neo* gene. The single tritordeum line containing both pCaI-neo and pAct1-DGus (HTT2) was shown to express both the *neo* and the *uid*A genes. Figure 1b shows an ELISA test on 10 T_1 progeny plants from line HTT2. Of these, 6 out of 10 showed expression of the *neo* gene (see data on expression in T_1 generation in Table 3).

Transgene segregation ratios were not significantly different (at the 5% probability level) to Mendelian segregation ratios consistent for the presence of a single locus in 67% of the lines (8 out of 12 – see Table 3). One wheat line (plant WT5) gave rise to 47 T_1 plants all of which contained and expressed the *neo* transgene. Chi square analysis of this ratio suggests the presence of two loci ($P = 0.1$–0.05 for 15 : 1 Mendelian ratio). Three lines showed segregation ratios deviating towards the loss of the transgene.

Discussion

In this paper we have shown that the type and concentration of auxin used for induction of embryogenesis influenced the efficiency of recovery of transgenic plants from wheat and tritordeum and that other factors such as the target explant and the selection system applied had less influence on transformation efficiency. Picloram gave improved regeneration and transformation efficiencies compared with 2,4-D (particularly in scutellum cultures), with the optimum concentration in these experiments being 4 $mg l^{-1}$. The effect of picloram on transformation was not simply due to its influence on regeneration efficiency as transformation efficiency showed proportionally greater increases. The mechanism of action of picloram cannot be determined from these data; however, it is known that auxins play a role in the activation of genes involved in cell dedifferentiation and division (Dudits et al. 1991) and that cells in the S-phase (DNA synthesis phase) of the cell cycle are more predisposed to the integration of foreign DNA (Villemont et al. 1997). To clarify whether, in our transformation system, cells at DNA replication and mitosis phases are indeed more likely to integrate foreign DNA, a more detailed study of the cell-cycle phases in the pre-cultured tissues used for DNA delivery in wheat transformation would be required. The practical conclusion from the results presented here, however, is that auxins can have a strong effect on wheat transformation efficiency and that they should be tested for a given variety and explant.

The two different selection systems we have studied in the present work appeared to have no influence on the number of transgenic plants obtained either from immature scutella or inflorescence cultures. It is difficult to compare these results to previously published data since no other study has directly compared these selection systems in wheat. However, an important observation was the difference in the percentage of escapes seen under the two selection agents. This varied from experiment to experiment but overall the percentage of escapes was much higher (approx. 80%) from cultures under PPT selection than from those selected on G418 (approx. 30%) as has been observed by other groups working on wheat transformation (Nehra et al. 1994). Our mean transformation efficiency of 1% for wheat scutellum cultures (ranging from 0 to 2.5%) and 1.2% for tritordeum inflorescence cultures (range: 0–3.2%) are comparable to previous reports published in wheat (Altpeter et al. 1996a, mean transformation efficiency 0.45%, range 0–2%).

Most transgenic plants produced in the present study exhibited simple integration patterns with few (generally fewer than 10) transgene coding region copies inserted and mainly at one locus. Evidence of coding region rearrangements in most lines was observed, but intact copies were present in 13 of 14 lines analysed. In addition, loss of expression was not observed in the following generation (2 tritordeum lines that had no detectable expression in the T_1 also did not show expression in the T_0). No new integration patterns were observed among the T_1 populations, although we cannot discount the possibility that instability could occur in later generations. Published information on the stability and inheritance of transgenes in wheat is very limited. The results from the single study known contrast surprisingly with our own. Srivastava et al. (1996) studied six transgenic wheat

plants from three different cultivars and analysed transgene integration and expression in the T_2 and T_3 generations. In agreement with our results, they observed relatively low copy numbers integrated (1–5), and five of the lines were reported to transmit the transgenes to the following generations. However, five of the six lines did not show expression of the *uid*A gene, and in most cases the lack of expression corresponded with re-arrangement of transgene sequences. In addition, in four out of five lines they detected a different integration pattern in some of the T_2 progeny. The Southern data presented by Srivastava et al. (1996) are, however, difficult to interpret due to a poor resolution of restriction fragments. In addition, there is some uncertainty concerning the distinction between independent and clonal transgenic lines as some lines appearing to have very similar integration patterns are scored as independent. Our results regarding structural re-arrangements during transgene inheritance are in agreement with those observed in transgenic maize and rice as described by Register et al. (1994) and Cooley et al. (1995), respectively. The first paper reports a large set of data on the analysis of patterns of integration of *uid*A and *bar* genes carried out in 112 transgenic maize callus lines and 22 independent transgenic plants, and the second, a similar analysis performed in 56 rice plants. These two reports, as well as others cited in Cooley et al. (1995), always reported integration patterns being inherited unchanged over generations.

In the present study, little difference in the complexity of integration pattern between non-selectable and selectable transgenes was observed. However, Register et al. (1994) reported that the integration patterns of non-selectable genes such as the *uid*A gene were more complex than those of selectable genes such the *bar* gene. Both genes in this case, however, were delivered on plasmid pBARGUS as opposed to plasmid pAHC25 used in the present study. It may be that inherent differences between the two plasmid constructs are the reasons for the contrasting results since Cooley et al. (1995) concluded that the instability of the *uid*A gene was inherent to that particular gene rather than its lack of exposure to selection pressure. This is, however, an example of the difficulty of drawing general conclusions from different transformation experiments.

Considering transgene segregation, 75% of our lines showed Mendelian segregation of transgenes. We identified 3 lines exhibiting skewed segregation ratios; these produced less than the expected proportion of transgenic progeny indicating reduced transmission of the transgene. This type of distorted segregation is known to occur naturally at surprisingly high frequencies in many species and is known to be associated with specific chromosomal regions in rice (Xu et al. 1997). The causes are related to a variety of physiological and genetic phenomena, the most common of which include male gametophyte selection by the gynoecium and differences in competitive ability shown by pollen, which is genetically variable. In transgenic plants there are additional factors which may potentially contribute to distorted segregation. These include (1) increased genetic variation resulting from tissue culture (2) physical/chromosomal disruption caused by transgene integration within or close to genes/chromosomal regions which are important for meiosis (3) chimerism in the primary transformant and (4) inherent instability of the recipient genotype (see review by Pawlowski and Somers 1996).

As it has been made clear in the discussion, there is presently very little information on the identity of major factors affecting transformation in wheat and how transgenes are inherited and expressed in the following generations. Such data are, however, needed to underpin the application of genetic manipulation in wheat crop improvement. The present paper is a contribution in this area, and the study of our transgenic lines in successive generations should add useful information on the long-term stability and heritability of transgenes in cereals.

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