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Insertional tagging of regulatory sequences in tritordeum; a hexaploid cereal species

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Abstract As an approach to isolate novel cereal promoters, promoterless *uid*A constructs and particle bombardment were used to transform tritordeum. Five of eight transgenic lines containing *uid*A sequences showed evidence of promoter tagging. Expression of *uid*A was detected in four lines as: constitutive expression, expression in short cells of the epidermis of the spikelets, expression in pollen grains and in cells of the epidermis of the spikelet, and expression in anther primordia and pollen grains. In the fifth line, the *uid*A was shown by RT-PCR to be transcribed, but no GUS activity was detected. The different patterns of *uid*A expression indicate that different regulatory sequences were tagged in each of these lines. Analysis of the progeny resulting from self-fertilisation of the primary tagged plants, indicate that the transgenes integrated at one or two loci and the patterns of expression were stably inherited. To our knowledge, this is the first report of promoter tagging in cereals by direct gene transfer.

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Introduction

Transformation of cereals has progressed steadily in the past decade (see reviews by Lazzeri and Shewry 1993; Brettell and Murray 1996; Barcelo et al. 2001). This progress has led to improvements in transformation efficiency, to a widening of the range of varieties transformed (Christou et al. 1991; Iser et al. 1999; Rasco-Gaunt et al. 2001; Pastori et al. 2001), and to the development of *Agrobacterium tumefaciens*-mediated transformation systems for the major cereals (Hiei et al. 1994; Ishida et al. 1996; Cheng et al. 1997; Tingay et al. 1997).

Transformation technology has now broad application in both basic research and cereal crop improvement, but there is a lack of well-characterised, tissue-specific and developmentally regulated promoters. This need is most immediate in the temperate cereals, wheat and barley, which have lagged behind rice and maize in the development of genetic modification technology.

Methods to isolate promoter sequences fall generally into two broad categories: those based on known gene sequences and those designed to isolate promoters from unknown genes. The first type of approach has been in use for longer. It relies on screening genomic libraries with probes derived from a known gene, or, more recently, walking into the 5′ region from known gene sequences. The second type of method is a gene-tagging approach and relies on the random insertion of promoterless marker genes, or marker genes with a minimal promoter, into plant genes. A recent modification of this approach is the development of the GAL4-minimal promoter system (Brand and Perrimon 1993; Kiegle et al. 2000). Successful tagging of a regulatory sequence is assessed by detection of marker gene expression in the transformants (see reviews by Topping and Lindsey 1995; Springer 2000). This method has been used to tag regulatory sequences in *Arabidopsis*, potato, tobacco and *Nicotiana plumbagini-*

folia (Koncz et al. 1989; Kertbundit et al. 1991; Topping et al. 1991; Lindsey et al. 1993). A recent adaptation of this method included transposon donor and acceptor sites flanking the promoterless marker gene and was successful in tagging regulatory sequences in *Arabidopsis* (Klimyuk et al. 1995; Nussaume et al. 1995; Sundaresan et al. 1995). In all these applications, which utilised *A. tumefaciens* transformation, the average frequency of tagging reported in the species tested so far ranges between 20 and 25% of the total of transgenic lines recovered. To-date, only a few genes and promoters identified by tagging have been cloned and sequenced (reviewed by Springer 2000); however, the potential of these approaches for gene and/or promoter isolation is clear.

We were particularly interested in using a promoter-tagging approach to isolate novel promoter sequences for genetic manipulation of cereals because, as well as the promoter sequence, it provides information about its regulation and specificity. We were also interested in testing the use of direct gene transfer in promoter tagging, allowing application of the method in species where *Agrobacterium* transformation is not well established.

With these objectives, transgenic lines of tritordeum, a hexaploid cereal containing the A and B genomes of wheat and the H^{ch} genome of *Hordeum chilense*, were produced using different types of promoterless *uid*A constructs: a simple promoterless *uid*A gene construct, or two different constructs in which maize intron sequences were introduced upstream from the *uid*A gene. Introns are known to have a role in enhancing expression in maize (Luehrsen and Walbot 1991) and were introduced in these constructs to increase the chances of detecting expression where weak promoters were tagged.

Materials and methods

Plasmid construction

To construct the promoterless tagging plasmids, the bacterial *uid*A gene and the *nos* terminator were isolated as a *Bam*HI/*Xba*I fragment from pAct1-DGUS (McElroy et al. 1990) and introduced into a pBluescript II backbone (SK+, Stratagene, Cambridge, UK) producing plasmid pPLGUS, represented in Fig. 1. The *Bg*/II/*Bam*HI fragment from pAHCI7 (Christensen and Quail 1996), which includes 40 bp of exon sequence and the ubiquitin-1 intron from maize, was cloned in sense orientation into the *Bam*HI site of pPLGUS to make pPLubiGUS (Fig. 1). The *Pst*I/*Bam*HI fragment from the p35SC1 plasmid (provided by V. Chandler, University of Oregon), with 14 bp of the first exon and the intron 1 from maize alcohol dehydrogenase I, was cloned into the respective sites of the pPLGUS plasmid to make pPLadhGUS (Fig. 1). Plasmids were purified using the Qiagen plasmid purification system (Qiagen, Crawley, UK), following the manufacturer's instructions. All plasmid manipulations followed standard cloning techniques. The newly created junctions in all the plasmids were checked by restriction analysis and by sequence analysis, using the ABI PRISM dye terminator cycle sequencing ready reaction kit (ABI, Warrington, UK) and the ABI 373 DNA sequencing system and sequencing analysis software.

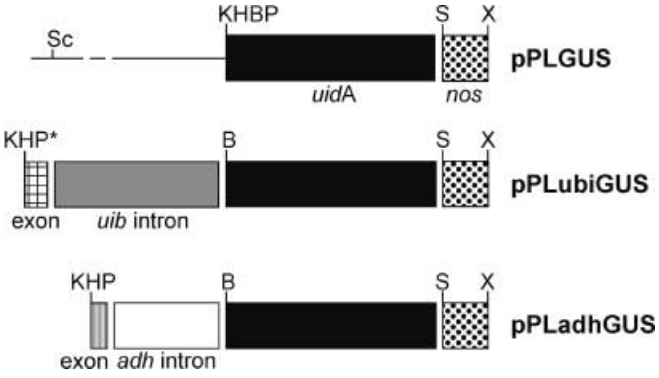

Fig. 1 Schematic representation of the promoterless tagging constructs. All plasmids have the *uid*A gene (*black box*) with the *nos* terminator (*stipled box*). Plasmid pPLubiGUS is based on pPLGUS and has part of the maize ubiquitin-1 exon 1 (*hatched box*) and intron 1 (*grey box*) preceding the *uid*A gene. Plasmid pPLadhGUS is also based on pPLGUS and has part of the maize alcohol dehydrogenase-I exon 1 (*striped box*) and intron 1 (*white box*). *Letters* indicate the position of restriction sites used during cloning and in the Southern-blot analysis: *X – Xba*I; *S – Sac*I; *B – Bam*HI; *P – Pst*I; *H – Hind*III; *K – Kpn*I; *Sc – Sca*I; * – ligation of *Bam*HI to *Bg*/II. *Line* in pPLGUS represents the plasmid backbone

Plant material and bombardment

Immature inflorescences of tritordeum were harvested and isolated as described by Barcelo et al. (1994). For each bombardment, immature inflorescences of 0.5 to 1.2 cm in length were cut transversally into sections of approximately 1 mm. Approximately 30 sections were placed in the center of each 90-mm Petri dish containing induction medium L7P4-V (Rasco-Gaunt and Barcelo 1999). Explants were pre-cultured in darkness at 26 °C for 1 day prior to bombardment.

The promoterless plasmid pPLGUS was delivered in three forms: supercoiled, digested with *Kpn*I or with *Sca*I. Both these enzymes linearise the plasmid; the *Kpn*I site is 67-bp upstream from the ATG start codon of the *uid*A gene and the *Sca*I site is approximately 1.1-kb upstream from the ATG start codon (Fig. 1). Digested plasmids were ethanol-precipitated and the pellets were resuspended in TE (Tris-HCl 10 mM, EDTA 1 mM, pH 8.0). Plasmids pPLubiGUS and pPLadhGUS were delivered in supercoiled form. In all experiments, promoterless plasmids were cobombarded with plasmids containing selectable marker genes. These were either pAHC20 (Christensen and Quail 1996), which contains the *bar* gene and *nos* terminator driven by the maize ubiquitin-1 promoter cassette, or the plasmid pCaI-neo, which has the cauliflower mosaic virus 35S promoter with the maize alcohol dehydrogenase-I intron 1 driving the *neo* gene, followed by the *nos* terminator (provided by S. Luetticke, University of Hamburg). Plasmid DNA, either in equimolar amounts or, in some experiments, as a 2:1 molar ratio of promoterless plasmid to selectable marker plasmid, was precipitated onto gold particles following the procedures described in Rasco-Gaunt and Barcelo (1999). Coated gold particles were delivered at acceleration pressures of 900 or 1,100 psi using a Bio-Rad PDS-1000/He device (Bio-Rad, Hemel Hempstead, UK).

Regeneration of transgenic plants

Immediately after bombardment, explants were distributed over the whole surface of the medium in the same Petri dishes and cultured in the dark at 26 °C for 5 to 6 weeks. Embryogenic calli were transferred to dishes containing Rz medium (Rasco-Gaunt and Barcelo 1999) supplemented with the selection agents L-phosphinothricin (PPT) at 2 mg/l or 50 mg/l of geneticin sulphate

(G418), as appropriate. Half of the calli in the control plates, i.e. containing explants bombarded with gold onto which no DNA was precipitated, were transferred to dishes with Rz medium without selecting agent in order to assess the full regeneration potential in each experiment, while remaining control calli were selected under the same conditions as the treated ones. Plates were cultured in the light for 3 further weeks. Surviving explants were transferred to R medium (Rasco-Gaunt and Barcelo 1999) supplemented with 3 mg/l of PPT or 50 mg/l of G418 and successive 3-week rounds of culture were applied until all controls on selection media were dead. Resistant plantlets were transferred to soil and grown to maturity in the glasshouse.

Analysis of the transgenic plants

Total genomic DNA was isolated from leaf tissue of putative transformed plants using a cetyltrimethylammonium bromide (CTAB) method (Stacey and Isaac 1994). PCR analysis was carried out using as template 50 to 150 ng of plant genomic DNA in a 30-µl reaction volume containing 50 mM of KCl, 10 mM of Tris-HCl (pH 8.8), 1.5 mM of $MgCl_2$, 0.1% Triton X-100, 200 µM of dNTPs, 300 nM of each primer and 0.4 U of Dynazyme DNA polymerase (Flowgen, Lichfield, UK). Primers for amplifying the *bar* gene were 5′ GTCTGCACCATCGTCAACC 3′ (forward) and 5′ GAAGTCCAGCTGCCAGAAAC 3′ (reverse), resulting in amplification of a 440-bp fragment and for the *neo* gene were: 5′-GAGGCTATTCGGCTATGACTG 3′ and 5′ ATCGGGAGCGGCGATACCGTA 3′ resulting in amplification of a 700-bp fragment. To amplify the *uid*A gene, primers were 5′ AGTGTACGTATCACCGTTTGTGT 3′ (starting at position 379 in the coding region of the *uid*A, forward), and 5′ ATCGCCGCTTTGGACATACCATCCGTA 3′ (starting at position 1,402 of the *uid*A coding region, reverse), resulting in amplification of a 1,050-bp fragment. Conditions for the PCR were as follows: 95 °C for 5 min, 57 °C (*bar* or *neo* amplification) or 62 °C (*uid*A amplification) for 45 s, 72 °C for 2 min, followed by 30 cycles of incubations at 95 °C for 1 min, 57 °C (*bar* and *neo*) or 62 °C (*uid*A) for 45 s and 72 °C for 2 min (with a 3-s incremental increase in each cycle) in a Hybaid Omnigene thermocycler (Hybaid, Teddington, UK). The products of the PCR amplification were analysed by agarose-gel electrophoresis.

The patterns of integration of the *uid*A gene were analysed by Southern blotting. DNA from plants transformed with either of the pPLGUS forms (supercoiled or linearised) was digested with *Bam*HI, which cuts once within the plasmid sequence, or double-digested with *Bam*HI and *Sac*I which releases a fragment with the *uid*A gene of 1.9 kb. DNA from lines transformed with pPLubiGUS or pPLadhGUS was digested with *Hind*III, which linearises the plasmid sequence, and double-digested with *Hind*III and *Sac*I, releasing a fragment of approximately 2.9 kb or 2.3 kb corresponding to the maize ubiquitin-1 intron or the maize alcohol dehydrogenase-I intron 1 and the *uid*A gene, respectively. Tracks with DNA extracted from non-transformed plants, digested in the same manner as described above, were included in all gels, as well as plasmids digested with each of the enzyme combinations described. Digested DNA was separated by electrophoresis in 0.7% (w/v) agarose, transferred by capillary blotting onto positively charged nylon membranes (Boehringer Manheim, Lewes, UK) and cross-linked to the membrane with UV light. Filters were probed with a fragment of the *uid*A gene labeled using a Boehringer PCR labeling kit (PCR DIG Probe Synthesis kit, Boehringer Manheim, Lewes, UK) following the instructions of the manufacturer, synthesised with the same *uid*A specific primers described in the PCR screening assays. Filters were hybridised with the probe at 42 °C and subsequently washed. The hybridised probe was detected using a chemiluminescent detection system (DIG Luminescent Detection kit, Boehringer Manheim, Lewes, UK).

Reverse transcriptase-PCR

RNA was extracted from plant tissues using the RNeasy RNA extraction kit (Qiagen, Crawley, UK). Poly(A) RNA was purified from approximately 1 µg of total RNA using the Oligotex system (Qiagen, Crawley, UK). One half of the poly(A) RNA was used as template for first-strand cDNA synthesis reactions, using the *uid*A-specific reverse primer 5′-ATCGCCGCTTTGGACATACCATCC-GTA-3′ (position 1,402 in the *uid*A gene), the Superscript II reverse transcriptase (RT, Life Technologies, Paisley, UK) and following the instructions of the manufacturer. Five microliters of this reaction were used as a template in a PCR reaction with the *uid*A-specific primers (forward) 5′-AGTGTACGTATCACCGTTTGTGT 3′ (position 379) and (reverse) 5′-CCAGTCGAGCATCTCTTCAGCGTA 3′ (position 997), under the same PCR conditions as described before for amplification of the *uid*A gene, resulting in amplification of a fragment of 640 bp. PCR products were analysed by agarose-gel electrophoresis.

GUS activity assay

For histochemical analysis of transformed plants, the following tissues were collected: (1) root and leaf (leaf sheath and basal, mid and top sections of the blade) from four-leaf stage plants; (2) root and leaf from plants with three to four tillers (leaf material was from the second youngest leaf of one tiller), leaf sheath and basal, mid and top sections of the blade were tested; (3) immature inflorescences (6 mm to 15 mm in length) and the surrounding immature flag leaf; (4) the node immediately preceding immature inflorescences and the older node below; (5) the internode between first and second nodes; (6) florets (glume, lemma, palea, awn, carpel, stamen and pollen) at anthesis; (7) the immature embryo and immature endosperm of caryopses older than 16 days post-anthesis.

Tissues were washed briefly in 100% ethanol and then in sterile distilled water, prior to incubating in X-gluc buffer containing 1 mM of X-gluc, 100 mM of sodium phosphate buffer pH 7.0, 0.5 mM of potassium ferricyanide, 0.5 mM of potassium ferrocyanide and 0.1% (v/v) Triton X-100, for 16 h at 37 °C, followed by incubation for 24 h at 26 °C. Chlorophyll was extracted from green tissues to allow visualisation of GUS activity by washing tissues twice in 70% (v/v) ethanol, followed by several changes in 100% ethanol. Tissues were photographed with a camera attached to a Olympus SZ-PT stereomicroscope or to an Olympus BH-2 microscope.

Results

Transformations

A total of 13 plants scoring positive in PCR tests for the *bar* or *neo* genes were recovered. Eight of these plants also scored positive for the presence of the *uid*A gene, representing a co-transformation efficiency of about 60%. All but one of the plants recovered (TPGUS1) had normal appearance and were fertile.

Southern-blot analysis was used to estimate the *uid*A gene copy number in the transformed lines, using a fragment of the *uid*A coding region as a probe. The minimum number of transgene copies was estimated by counting the bands detected in tracks with DNA digested with *Hind*III or with *Bam*HI. These enzymes cut once within the plasmid sequence, upstream from the start of the *uid*A (Fig. 1) and in other unknown sites in the plant genome. These digests result in a number of bands of various sizes detectable in the Southern blots, each considered to correspond to at least one insertion of the *uid*A gene in the genome. Bands of the same size as the linearised plasmid (approximately 5.2 kb for plasmid

Table 1 Molecular and expression analyses of plants containing the *uid*A gene

Item	TPGUS1[a]	TPGUS2	TPGUS3	TPGUS4	TPaGUS1	TPuGUS1	TPuGUS2	TPuGUS3
Plasmid	pPLGUS[b] supercoiled	pPLGUS[b] *Kpn*I-digested	pPLGUS[b] *Kpn*I-digested	pPLGUS[b] *Sca*I-digested	pPLadhGUS[c] supercoiled	pPLubiGUS[b] supercoiled	pPLubiGUS[b] supercoiled	pPLubiGUS[b] supercoiled
*uid*A Copy no.[d]	>12	>10	5	4	>10	8	4	2
Evidence of tagging	Yes[e], transcripts	No[f]	Yes, expression	No[f]	No[f]	Yes, expression	Yes, expression	Yes, expression

[a] Plant name: *T*agged *P*lant
[b] Selectable marker: *bar* gene
[c] Selectable marker: *neo* gene
[d] Numbers represent the minimum number of copies of the *uid*A gene
[e] Sterile plant
[f] No evidence of expression under the conditions tested

pPLGUS; 5.6 kb for pPLadhGUS or 6.2 kb for pPLubiGUS) were considered to correspond to plasmid concatemers (see following section for results of these analyses). The range of *uid*A gene insertions in the transgenic lines was wide, from a minimum of 2 to over 12 (Table 1). Linearisation of the construct prior to transformation did not seem to influence the range of copies integrated, as plants transformed with linearised constructs ranged from a low (TPGUS3, TPGUS4) to a high (TPGUS2) number of transgene copies. However, it is difficult to draw further conclusions due to the limited number of lines obtained.

Characterisation of tagged lines

Five of the eight plants transformed with promoterless plasmids displayed evidence of tagging of a regulatory sequence. Details of the molecular analysis of these plant lines are shown on Figs. 2 and 3. Lines TPaGUS1, TPuGUS1, TPuGUS2 and TPuGUS3 (Fig. 2) were all transformed with plasmids containing introns. Line TPGUS3 (Fig. 2) was transformed with *Kpn*I-digested pPLGUS and line TPGUS1 (Fig. 3) was transformed with supercoiled pPLGUS. The top panels in Fig. 2 show the patterns of *uid*A integration in four of these transgenic lines, in decreasing copy number order, and the middle panels have an illustration of the corresponding patterns of GUS expression. The bottom panel represents an illustration of a cereal plant and flower.

Line TPuGUS1 has a minimum of eight copies of the *uid*A gene as revealed by the eight hybridising bands on the track with DNA digested with *Hind*III. Possibly one of them represents a plasmid concatamer since there are bands migrating around the size or the linearised plasmid (6.2 kb) (Fig. 2a, top panel, track L). Track E contains DNA from this plant, double-digested with *Hind*III and *Sac*I, which flank the ubiquitin intron-*uid*A cassette (see Fig. 1). This type of digest allows confirmation, in principle, of the integrity of this region of the construct, through detection of a band of approximately 2.9 kb hybridising with the *uid*A probe. For this line, track E shows that, in addition to the expected 2.9-kb band, four other bands are visible (Fig. 2a, top panel). These indicate that some of the integrated plasmid molecules have undergone rearrangements/deletions which resulted either in the elimination of one or both of the recognition sites for the restriction enzymes used, or in changes in the expected fragment size. In this line, it appears that approximately four copies might contain a complete intron-*uid*A cassette and that the other copies appear to be incomplete. GUS activity was detected in the pollen grains (Fig. 2a, middle panel) and in cells of the epidermis of the spikelet tissues but not confined to a specific cell type (data not shown, see Materials and methods for a complete list of the plant tissues tested).

Southern-blot analysis of DNA from line TPGUS3, revealed a minimum of five copies of the *uid*A gene (Fig. 2b, top panel, track L). At least one copy contained the intact *uid*A gene sequence as seen by the presence of a band of approximately 1.9 kb in track E (Fig. 2b, top panel). In addition, the four other bands in this track, with sizes different from that expected, indicate probable rearrangements/deletions in this area of the transgene. This line had very specific *uid*A expression exclusively in the short cells of the epidermis of the glume and lemma (Fig. 2b, middle panel). These were not the same type of cells where GUS activity was detected in line TPuGUS1.

Digestion of DNA extracted from line TPuGUS2 revealed a minimum of four *uid*A gene copies (Fig. 2c, top panel, track L). Of these, some contained the expected 2.9-kb fragments, as determined from track E of Fig. 2c. In this track, there were two additional bands of different sizes, one smaller and one larger than 2.9 kb. In this line, strong GUS staining was detected in root and leaves from four-leaf-stage plants; nodes and internodes of tillers with three nodes, immature inflorescences; floret tissues, including carpels and pollen grains (but not in the tapetum or filament) and in immature embryos at different stages of development, between 14 days and 24 days post-anthesis. GUS expression detected in pollen grains, the immature embryo and the immature inflorescence is shown (Fig. 2c, middle panel).

In the line TPuGUS3, when DNA was digested with *Hind*III and analysed by Southern blotting, a minimum of two copies of the *uid*A gene were revealed (Fig. 2d, top panel, track L). There was one strong band of approximately 2.9 kb in track E (Fig. 2d, top panel) and another faint band of approximately 2.0 kb. GUS activity

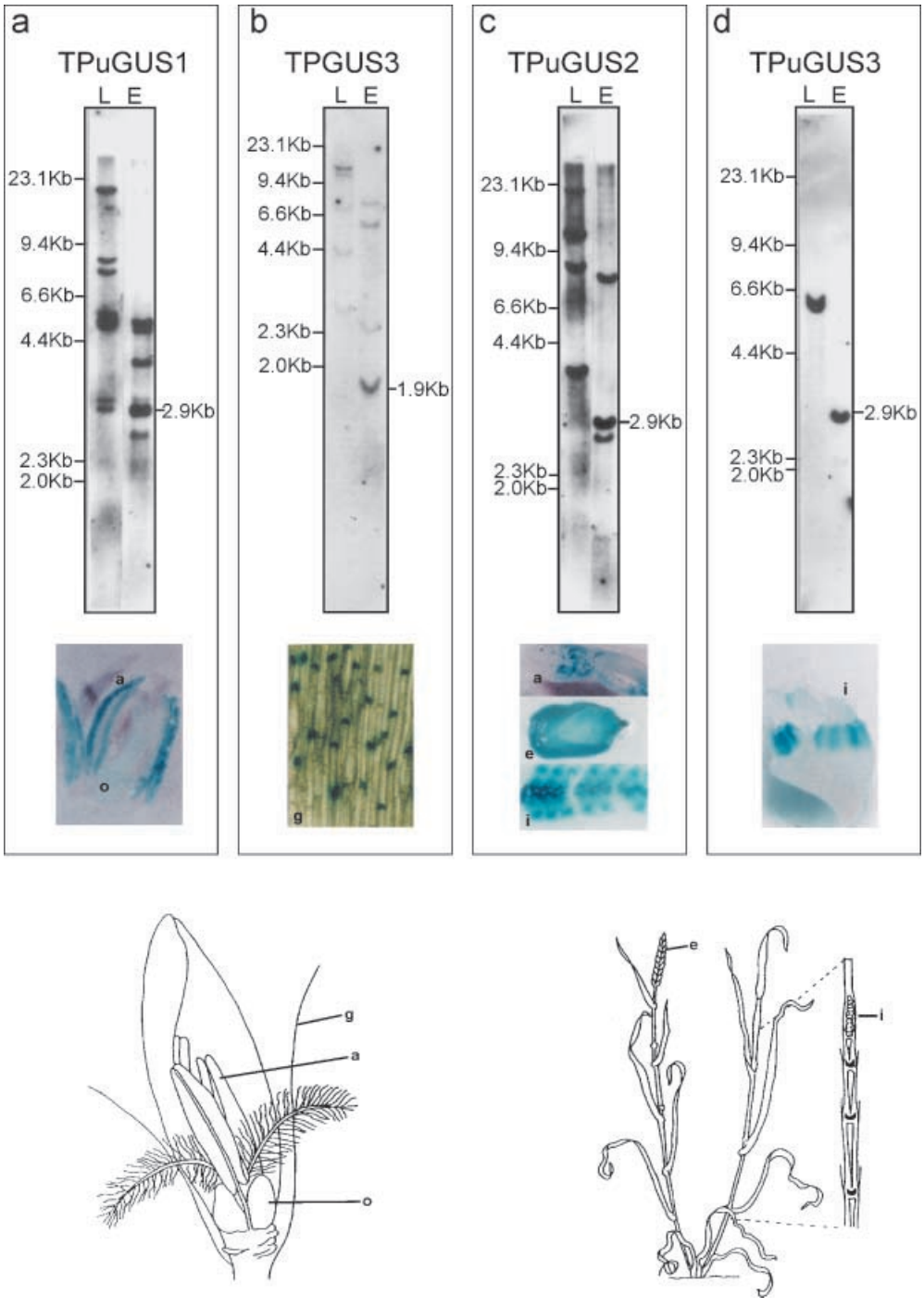

Fig. 2a–d Southern-blot analysis and patterns of *uid*A expression in tagged plants. Tracks marked *L*: genomic DNA was digested with an enzyme which cut once in the plasmid sequence. Tracks marked *E*: double-digested genomic DNA to release a restriction fragment of known size (indicated on the right-hand side) containing the *uid*A sequence. *Numbers* on the left-hand side of the Southern blots indicate the position and size in kb of the DNA molecular-weight markers. The bottom part of the figure includes schematic representations of a cereal flower (*left*) and a cross section of a tiller showing the position of the immature inflorescence (*right*). *a* anther, *o* carpel, *g* glume section, *e* immature embryo, approximately 2 mm, *i* immature inflorescence, approximately 1 cm in length

was detected specifically in the anther primordia of immature inflorescences (Fig. 2d, middle panel) and in pollen.

In summary, these four *uid*A-expressing lines include one with a low number of copies (TPuGUS3) and others with a relatively high copy number of the transgene (lines TPuGUS1 and TPGUS3), indicating that successful tagging is not copy number dependent.

The fifth tagged transgenic line (TPGUS1) had abnormal morphology: two ears emerged from each node and the florets also appeared doubled (Fig. 3a). The anthers in this plant seemed to be malformed, not developing to maturity, which could account for the fact that it was sterile. Southern analysis revealed the line to have over 12 *uid*A copies (data not shown). However, no GUS activity was detectable in any of the tissues analysed. RNA was isolated from various tissues (see legend for Fig. 3) and used in RT-PCR with *uid*A-specific primers. This analysis demonstrated that *uid*A transcripts could be detected in all the tissues analysed (Fig. 3b). This line represents a case of transcriptional fusion of the promoterless *uid*A to a regulatory sequence, where the coding sequence of the *uid*A gene is probably not in-frame and therefore transcription of the gene does not result in pro-

duction of the enzyme. The presence of transcripts in a wide range of tissues led us to suggest that in this case a constitutive regulatory sequence was tagged, as with line TPuGUS2.

Table 2 *uid*A inheritance in the T_1 generation of tagged lines

Tagged line	*uid*A$^+$:*uid*A$^-$ plants[a]	No. of loci[c]
TPGUS3	11:0	2 loci (1+4)[b]
TPuGUS1	7:2	1 locus
TPuGUS2	10:1	2 loci (1+3)[b]
TPuGUS3	1:10	1 locus

[a] PCR data
[b] Estimated minimum number of *uid*A copies (from Southern analysis) in each locus
[c] Estimated from Southern analysis

Inheritance of GUS expression

To test whether the T_0 patterns of GUS expression were inherited by progeny, and how that correlated with the presence of the transgene, we studied T_1 progeny from the four tagged lines showing GUS activity. A summary of the results is shown on Table 2.

All of the 11 T_1 individuals from line TPGUS3 analysed by PCR scored positive. Southern analysis of these plants indicated that insertions of the *uid*A had occurred in two loci, and that one locus appeared to have only one copy of the *uid*A gene with the remaining four copies inherited together at the second locus (data not shown). GUS histochemical assays on these 11 progeny plants revealed that all but the plant containing the locus with the single copy of *uid*A had retained the very specific pattern of GUS activity in the short cells of the lemma and glume, detected in the parental line. Thus, the single copy at the inactive locus was not responsible for the pattern of GUS expression characteristic of the tagged line.

From line TPuGUS1, nine T_1 progeny plants were analysed by PCR amplification of the *uid*A sequence from genomic DNA. Seven of these plants scored positive in this test, which corresponded to the plants that displayed GUS expression. Southern analysis of three of the *uid*A-containing T_1 progeny lines revealed the same number (a minimum of eight copies of the *uid*A gene) and the same pattern of integration of this gene (data not shown) as found in the parental line, indicating that all copies had integrated at a single locus. Analysis of the expression pattern in these T_1 lines also confirmed the pattern found in the parental line, of *uid*A expression in pollen grains and in cells in the epidermis of the spikelet.

Nine plants in a total of 11 T_1 plants analysed from the line TPuGUS2 scored positive when analysed by PCR for the presence of *uid*A sequences. Upon Southern-blot analysis, bands hybridising specifically to the *uid*A probe were detected in all of the nine PCR-positive T_1

Fig. 3a, b Line TPGUS1: plant morphology and RT-PCR analysis. **a** Plant with doubling of ears emerging from each node and abnormal ears. **b** 640-bp product resulting from RT-PCR in a variety of tissues from line TPGUS1: *fl* flower, *np* node and peduncle, *r* root, *l* leaf, *gf* spikelet, *i* immature inflorescence. Leaf material from a non-transformed plant (*p-*) and from a GUS-expressing plant (+) were also tested. *Lane m* molecular-weight markers. *Lane (-)* no-template PCR control

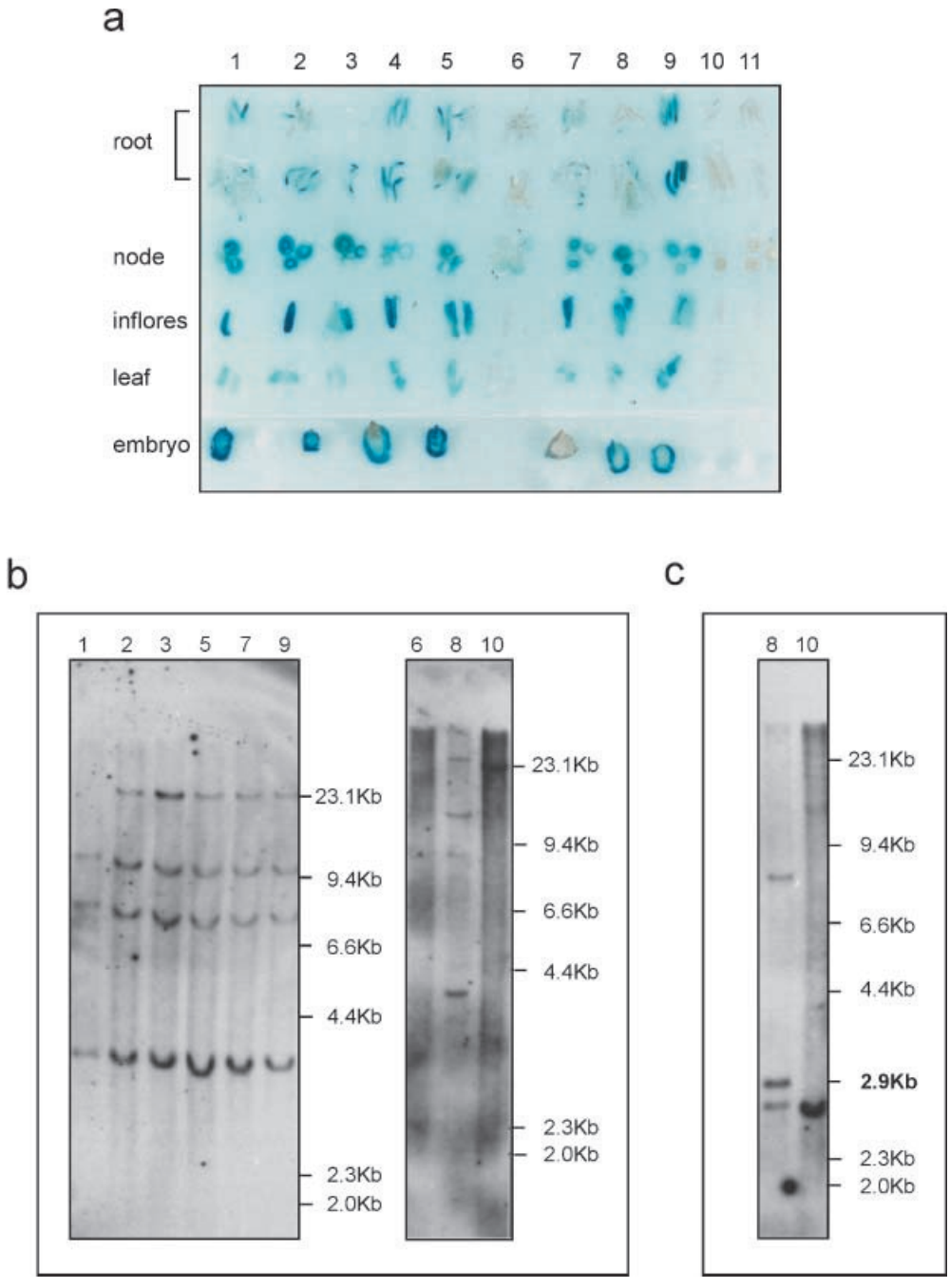

Fig. 4a–c Inheritance of the *uid*A gene in line TPuGUS2. Pattern of GUS expression in tissues of 11 T_1 plants. *Plants 6 and 11* were PCR negative for GUS (null segregants), the remainder were all positive with the exception of line 10 (*top panel*). The *bottom panel* (**b**) shows Southern analysis of nine lines showing segregation of the original integration pattern into one locus containing at least one transgene copy (23.1 kb, *plant 10*), which showed no GUS expression, and a second locus containing at least three copies (*plant 1*), which showed the same pattern of GUS expression as the parental T_0 line. The remaining plants contained all four copies. *Panel* **c** shows the results of a double digestion to excise the GUS gene. *Plant 8* has the same pattern as the parental T_0 line as shown in Fig. 2, *panel* **c**. *Plant 10* has no intact copies of the expected size (2.9 kb), which explains why no GUS expression was observed in this plant

plants (Fig. 4), confirming the PCR results. Two progeny plants (6 and 11), without integrated copies of the *uid*A had no GUS expression and represented null segregants. Southern analysis of the T_1 progeny confirmed the presence of *uid*A copies at two loci; one contained at least three insertions and correlated with gene expression, and the second locus contained at least one copy. The lines carrying this locus showed no evidence of GUS activity. The expression pattern observed in the T_1 lines was identical to that described for the original parental plant.

Eleven T_1 progeny plants from line TPuGUS3 were analysed by Southern blotting. Of these, only one had the same pattern of integration as the parental line, with at least two *uid*A gene copies, while no bands were detected in the remaining progeny (Table 2). The progeny plants containing the *uid*A gene were the only ones in which GUS activity was detected, restricted to anther primordia and pollen, exactly as in the parental line. The locus was inherited in subsequent generations at a lower frequency than would be expected from single-locus Mendelian ratios, (ten PCR and phenotype-positive plants out of 92 tested). One explanation for this is that the insertion may have disrupted the function of a gene involved in gametogenesis. Immature inflorescences at various stages of development were isolated from this

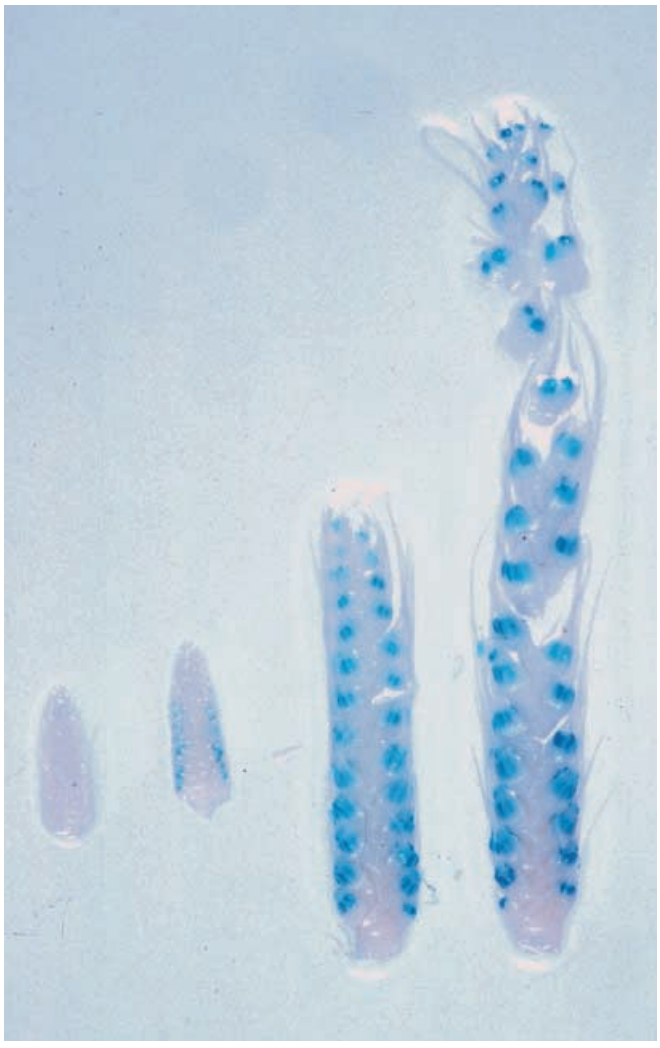

Fig. 5 Developmental regulation of de novo GUS activity in line TPuGUS3. Immature inflorescences of 4 mm (left), 5 mm, 12 mm and 18 mm

plant and tested for GUS activity. The results, shown in Fig. 5, indicated that the regulatory sequence tagged in this line was developmentally regulated as well as tissue-specific. GUS activity was not detected in very young inflorescences of 4 mm (Fig. 5), but de novo GUS activity was observed in more-developed anther primordia in the middle region of a 5 mm inflorescence (Fig. 5). In older inflorescences GUS activity was detected in developing anther primordia along the whole inflorescence and was also observed in pollen grains.

Discussion

A strategy for tagging novel tissue-specific regulatory sequences from cereals using the promoterless marker gene *uid*A is presented in this paper. Promoter tagging is an adaptation of gene-tagging techniques and has been successfully used to isolate very specific regulatory sequences in some plant species (e.g. *Arabidopsis*, Wei et al. 1997). The technique has been used in systems that support *Agrobacterium* transformation such as tobacco, *Arabidopsis* and potato (Lindsey et al. 1993). In this paper we show that it is possible to adapt the technique for use in transformation by direct gene transfer and for tagging regulatory sequences in cereals. Despite the use of direct gene-transfer techniques, the large hexaploid genome of tritordeum (similar to wheat, containing the genomes of durum wheat and *Hordeum*) and the very high percentage of non-coding sequences predicted in this genome (by analogy with the wheat genome), the insertion of the transgene into transcriptionally active regions seems to have occurred in a high proportion of the transformed lines. Koncz and collaborators (Koncz et al. 1989), have also suggested this preferential insertion to explain the similar percentages of T-DNA tagging in both *Arabidopsis* and tobacco (between 20 and 25%). This was a surprising finding, considering the differences in the structures of the two genomes; the *Arabidopsis* genome being small and with a relatively small proportion of repetitive sequences, and the tobacco genome much larger and with 60% repetitive DNA. Our results suggest that this preferential insertion of transgenic DNA in transcriptionally active areas of the genome is not a specific characteristic of *Agrobacterium*-mediated transformation but rather that it may be a general feature of transformation.

Analysis of the T_1 generation showed that the patterns of GUS activity detected in the T_0 tagged lines were inherited in the subsequent generations together with the *uid*A gene. In two of the tagged lines, TPGUS3 and TPuGUS2, in which *uid*A insertions were at two loci, segregation allowed us to recover progeny lines with the same pattern of expression as the parental line but having only one of the loci containing *uid*A insertions. In both cases, GUS activity correlated with a locus containing several copies of *uid*A. In tagged plants with more than one copy of the *uid*A gene, the patterns of expression observed might result from either a single copy or from a combination of tagged regulatory sequences.

In line TPuGUS3, the pattern of GUS activity detected indicated that a regulatory sequence involved in the development of the anthers, and possibly pollen, had been tagged. Analysis of several progeny plants, which inherited the transgene, showed that GUS expression was developmentally regulated in the anther primordia. Poor transmission of the transgene to the next generation has been frequently observed in transgenic populations. In this particular case it could be the result of the tag insertion causing a disruption in a gene involved in pollen development and/or the pollination process. This regulatory sequence may prove useful for engineering male-sterility wheat, for which a highly anther-specific promoter would be required.

Other regulatory sequences tagged in the lines described here are also potentially interesting; in line TPuGUS2 a constitutive promoter has been probably

tagged. Although constitutive promoters are available for monocot transformation, the isolation of the regulatory sequence responsible for the strong constitutive *uid*A expression observed in this line would represent the first constitutive promoter isolated from a temperate cereal. Line TPGUS3, with expression only in the short cells of the epidermis of glume and lemma, represents a good example of the type of tissue-specific sequences which are possible to tag using this type of strategy that would be very unlikely to be isolated in a conventional program for promoter or gene isolation.

The method described in this paper represents a significant alternative to more conventional methods for characterising genes. The process of studying the pattern of expression of the tagging marker gene, in this case *uid*A, supplies detailed information on the pattern of regulation of the gene and both its spatial and temporal specificity. This represents a progression from the more standard techniques of gene tagging which rely on loss of function of genes to produce a phenotype. Promoter tagging allows the regulation of the tagged gene to be studied through the expression of the marker gene, even when the insertion of the tag might have caused loss of gene function.

Work is now in progress to isolate and further characterise the regulatory sequences tagged in these lines which will be used in future cereal transformation experiments. To make full use of the new reports on the use of transposons in wheat (Takumi et al. 1998) and in barley (McElroy et al. 1997), work has now started at IACR-Rothamsted, UK, using the transposon-based promoter tagging technology in wheat.

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