

# The promoter of the barley aleurone-specific gene encoding a putative 7 kDa lipid transfer protein confers aleurone cell-specific expression in transgenic rice

Roger Kalla<sup>1</sup>, Ko Shimamoto<sup>2</sup>, Robert Potter<sup>3</sup>, Peter Stein Nielsen<sup>1</sup>, Casper Linnestad<sup>1</sup> and Odd-Arne Olsen<sup>1,\*</sup>

<sup>1</sup>Plant Molecular Biology Laboratory, Department of Biotechnological Sciences, Agricultural University of Norway, P.O. Box 5051, N-1432 Ås, Norway,

<sup>2</sup>Plantech Research Institute, 1000 Kamoshida, Midori-ku, Yokohama 227, Japan, and

<sup>3</sup>Cell and Tissue Culture Group NLVF, Department of Biology and Nature Conservation, Agricultural University of Norway, P.O. Box 5014, N-1432 Ås, Norway

## Summary

This paper describes the aleurone-specific gene *Ltp2* from barley, which encodes a putative 7 kDa non-specific lipid transfer protein. As shown by Northern and *in situ* hybridization analyses, the *Ltp2* transcript is present in barley aleurone cells shortly after the initiation of aleurone cell differentiation. The expression of *Ltp2* increases until grain mid-maturity, but the mRNA is absent from mature grains. The *Ltp2* transcript is undetectable in the embryo and vegetative tissues, confirming the aleurone specificity of the *Ltp2* gene. The ability of the isolated 801 bp *Ltp2* promoter to direct aleurone-specific expression in immature barley grains is demonstrated by particle bombardment experiments. In these experiments, the activity of the *Ltp2* promoter is 5% of the activity of the strong constitutive *Actin1* promoter from rice, as quantified by GUS activity measurements. In stably transformed rice plants containing the *Ltp2* promoter–*Gus* construct, the specificity of the *Ltp2* promoter is confirmed *in vivo* by the presence of GUS activity exclusively in the aleurone layer. This study demonstrates the conserved nature of the regulatory signals involved in aleurone-specific gene transcription in cereal grains.

## Introduction

The aleurone layer of the cereal grain is composed of specialized cells surrounding the starchy endosperm, the site for starch and storage protein accumulation in the developing grain (Bosnes *et al.*, 1992; Olsen *et al.*, 1992). After completion of the initial cellularization process of the barley endosperm during the cellularization stage (for endosperm staging, see Bosnes *et al.*, 1992), differentiation of aleurone cells is initiated in the periphery of the endosperm at 8 days after pollination (d.a.p.). Two components of microtubular arrays, which are absent during initial cellularization, are introduced in the endosperm life cycle at the onset of aleurone cell differentiation (Brown *et al.*, 1994): (i) cortical microtubular arrays with their main axis oriented perpendicular to the wall of the central cell and (ii) preprophase bands, predicting the future division plane of the cells. In conventional light microscopy, aleurone cell initials are discernible by the heavy vacuolation of the cytoplasm of the cells at the ventral side of the endosperm near the crease. Unlike the starchy endosperm, where the cells cease to divide after 14 days, aleurone cell mitosis continues until day 21, leading to the formation of three layers of cells. In most other cereal species, including rice, the aleurone layer is only one cell layer thick. The contents of the mature cereal aleurone cells, mainly lipid bodies and aleurone grains, differ dramatically from those of the inner starchy endosperm cells, most likely reflecting the expression of different gene programmes in these two endosperm tissues. Owing to its simple organization in only two main cell types, which can be separated mechanically, the endosperm is a good model system for plant development. In particular, aleurone cell differentiation is attractive due to the strict temporal and spatial control of the developmental process.

Two aspects of aleurone cell biology that have been intensively studied are the genetics of anthocyanin pigmentation in maize (McClintock, 1978) and the hormonal regulation of gene transcription in germinating barley grains (Fincher, 1989). Using transposon tagging, several structural and regulatory genes involved in the biosynthesis of anthocyanins have been isolated and characterized (Dellaporta *et al.*, 1988; Paz-Ares *et al.*, 1987). For example, Roth *et al.* (1991) have provided data suggesting that the anthocyanin structural gene *Bz1* is under the control of MYB- and MYC-like transcriptional activators encoded by the *C1* and *R* genes, respectively. In barley, two cDNAs representing transcripts that are differentially expressed in

Received 12 May 1994; revised 24 August 1994; accepted 30 August 1994.

\*For correspondence (fax + 47 64 94 14 65).

the aleurone layers of developing grains have been isolated, namely CHI26 (Leah *et al.*, 1991) and pZE40 (Smith *et al.*, 1992). To our knowledge, none of the promoters of these genes have been shown to be truly aleurone specific in transgenic cereal plants.

In our laboratory, we are interested in elucidating the molecular basis for the control of transcription in the aleurone cells of immature cereal grains. Besides providing increased understanding of the molecular biology of grain development, characterization of aleurone-specific promoters will be important in future efforts aiming at the modification of cereal grains through biotechnology (see, for example, McElroy and Brettell, 1994). To isolate such promoters, we first identified by differential screening nine homology groups of cDNAs representing transcripts expressed in the aleurone layer, but not in the starchy endosperm of 21 d.a.p. grains (Jakobsen *et al.*, 1989). Of these, only two groups represent aleurone-specific transcripts (Aalen *et al.*, 1994). One of them is represented by zB11E (Jakobsen *et al.*, 1989), the other group contains cDNAs corresponding to *Ltp1*, a 9 kDa non-specific lipid transfer protein (nsLTP) gene (Breu *et al.*, 1989; Linnestad *et al.*, 1991). For the remaining clones, including one clone encoding a protein putatively involved in the regulation of embryo dormancy (B15C), and one clone encoding an oleosin (B23D), transcripts are present also in the embryo, and, like B22E (Klemsdal *et al.*, 1991; Smith *et al.*, 1992), also in a limited number of maternal cell types (Aalen *et al.*, 1994).

Here we present a study of the gene and the transcript corresponding to the zB11E cDNA. In Northern and *in situ* hybridization analyses the transcript is detectable exclusively in barley aleurone cells from the beginning of the differentiation stage to half-way into the maturation stage. Considering the high identity to a previously isolated wheat protein (Monnet, 1990), we identify the protein derived from zB11E as a putative 7 kDa nsLTP, which we denominate LTP2. In particle bombardment experiments, the *Ltp2* promoter drives GUS expression in the aleurone layers of developing barley grains. A combination of histochemical and ultrastructural analyses show that the GUS activity in transgenic rice plants containing the *Ltp2-Gus* construct corresponds to the spatial and temporal pattern of *Ltp2* mRNA accumulation in barley.

## Results

### *The barley Ltp2 gene encodes a protein homologous to the 7 kDa wheat lipid transfer protein*

Using the zB11E cDNA (Jakobsen *et al.*, 1989) as a probe, the corresponding barley cv. Bomi genomic clone was isolated. Out of a total of  $2 \times 10^6$  plaques screened, four positive clones were identified. The restriction maps

of these four clones showed extensive overlap (data not shown). The sequences of the genomic clone BL53Ps17 and that of the zB11E cDNA are identical in overlapping regions and no intervening sequence was detected (Figure 1). This gene will in the following be referred to as *Ltp2*.

Computerized scanning of the genomic sequence for putative promoter elements using the method of Bucher (1990) identified the transcription start site as nucleotide 802 (+1 in Figure 1). This agrees well with the 600 nt estimated size of the *Ltp2* transcript (Jakobsen *et al.*, 1989). The *Ltp2* promoter contains MYB and MYC consensus binding sites (see Figure 1) previously identified in the promoter of maize *Bz1* (Roth *et al.*, 1991) and in the promoter of the barley *Ltp1* gene (Linnestad *et al.*, 1991). The 53 bp region of the *Ltp2* promoter containing these consensus binding site sequences is 49% identical to the corresponding region in the *Ltp1* promoter (data not shown; Linnestad *et al.*, 1991). While the two binding sites in the *Bz1* promoter are separated by only 7 nt, the corresponding putative *cis*-active elements in the *Ltp1* and *Ltp2* promoters are 36 and 16 nt apart, respectively. A similar arrangement with putative MYB- and MYC-binding sites is also present in the promoter of the barley chalcone synthase gene, *Chs* (Rohde *et al.*, 1991). In the *Chs* promoter, the spacing between the putative MYB- and MYC-binding sites is 34 nt.

In the zB11E cDNA, the poly(A) tail extends after the G at position 496 (Figure 1). The ATG codon initiating the

```

-801          AGATCTCGATGTGTAGTCTAGC
-780 AGAAGGGTTAACCGTCTCTTCGTGAGAATAACCGTGGCCATAAATAAGCCGATGAGGAT
-720 AAATAAAATGTGGTGGTACAGTACTTCAAGAGGTTTACTCATCAAGAGGATGCTTTTCCG
-660 ATGAGCTCTAGTAGTACATCGGACCTCACATACCTCCATTGTGGTGAAATATTTTGCTGCT
-600 CATTAGTGATGGGTAAATTTTGTATGTCACTTAGTGTGACATTTCAGTTTTCGTTGCC
-540 ACTTTAGGTTTGTACAAATAATTTCCATTCGCCGCGCAAGCAAAACAATTTTATTTTA
-480 CTTTACCACTCTTAGCTTTTCAATATGTATCAAAATGCCACTAGAAATTCGTTTAT
-420 GCCACAGATGTGAAAAAAACACTCACTTATTTCAAGCAAGGTGTTTCATGGCATGGAA
-360 ATGTGACATAAAGTAACTTTCGTGTATAGAAAAAATTTGACTCCTCGTACAGAGACG
-300 GAACATCATGACAAATCCGCTTTGGAAGGCTTGGCATCACCTTTGGATGATGCGCATG
-240 CATCGAGTCTGCTGCTTGTAGCTTCCGCTACCGCCCACTGAGTCCGGGCGGCAACTAG
-180 CACGCGGAACGACCCAGCTGACCTCTACCGACCGGACTTGAATGCGCTACCTTCTGTCAG
-120 CGACATGGCCGCTACGCTGCGGACGTGCCCCGATGATGCGGCGACATGGCGAGCT
-60 CAGACCGTGGCTGGCTACAAATACGTACCCCGTGAGTGCCTAGCTAGAACTTAC
      ↓
1  ACCTGCAACTGCGAGAGCGCGCTGTGAGTGTAGCCGAGTATGATACCGTACGACGAGC
   M A M A M G M A M R K E A A V A V
60  ACGAGGGCATGGCGATGGCGATGGGATGGCGATGAGGAAGGAGGAGCGGCTGGCCGTG
   M M V M V V T L A A G A D A G A G A A C
120 ATGATGGTATGGTGGTGACGCTGGCGCGGCTGGCGACCGGGAGCGGAGCGCGCTGC
   E P A Q L A V C A S A I L G G T K P S G
180 GAGCGGCGCAGCTGGCGGTGTGCGCTGCGCGATCTGGGCGGAGCAAGCCGAGCGGC
   E C C G N L R A Q Q G C L C Q Y V K D P
240 GAGTGTGCGGGAACCTGCGGGCGCAGCGGCGTGTGTGCGAGTACGTCAAGGACCCC
   N Y G H Y V S S P H A R D T L N L C G I
300 AACTACGGGCACTACGTGAGCAGCCACACGCGCGGACACCTCAACTTGTGCGGCATA
   P V P H C
360 CCCGTACCGCACTGTAGCGCGCTAGCCGATCGAGGGCTCCAGGACCGCATGCTGTTCC
420 TGTATGTGTATGTTGGAATAAAATGCTGGTATGATGATGCGGCTAGCTGCTTCTGCTG
480 TAGCAGCTGCTAATGAAATTTGTGTGCACTTTTATTTAGTCC
      ↑

```

**Figure 1.** Nucleotide sequence of *Ltp2*.

The putative transcription start site has been denoted +1. The putative TATA-box (TACAA) is shown in bold. Consensus MYB (CAACTA) and MYC (CAGCTG) binding sites are in bold italics. The predicted amino acid sequence of the longest ORF is shown in single letter code above the DNA sequence. The positions of the 5' end (+11) and the polyadenylation site (+497) of the zB11E cDNA are indicated by arrows.

longest open reading frame (ORF) in the *Ltp2* sequence is located 69 bp downstream of the putative transcriptional start site (Figure 1). This ORF contains five potential translation start codons within the first 30 nt. As the ATG flanking sequences do not allow for the determination of the most likely start codon (Cavener and Ray, 1991) we have used the first ATG codon for the deduction of the amino acid sequence of LTP2.

During our work with the zB11E cDNA and *Ltp2*, we became aware of the high identity between the protein derived from the ORF of *Ltp2* and the sequence of a 7 kDa nsLTP isolated from wheat grains by the standard *in vitro* lipid transfer assay (Monnet, 1990). By definition, non-specific lipid transfer proteins can mediate *in vitro* transfer of radiolabelled phospholipids from liposomal donor membranes to mitochondrial acceptor membranes (Kader *et al.*, 1984; Watanabe and Yamada, 1986). As shown in Figure 2, the identity in the 67 amino acid long overlapping region between LTP2 and the 7 kDa wheat protein is 78%. Based on this comparison, we conclude that LTP2 is an nsLTP and suggest that *Ltp2* and the gene encoding the wheat 7 kDa protein represent homologous genes.

The N-terminal residue of the mature 7 kDa wheat protein corresponds to the alanine in position 36 of the predicted barley LTP2 protein in the alignment presented in Figure 2. The N-terminal extension of the LTP2 protein shows all the features of a signal peptide. Owing to the high degree of sequence conservation between the barley

and wheat proteins, we postulate that the putative LTP2 preprotein is cleaved after the alanine residue at position 35 (arrow, Figure 2). A cleavage of the polypeptide at this position complies with the (-3,-1) rule of von Heijne (1988). N-terminal extensions representing signal peptide domains are also found in 9 kDa grain nsLTP sequences (see Figure 2) derived from wheat, barley, and maize cDNAs (Dieryck *et al.*, 1992; Mundy and Rogers, 1986; Tchang *et al.*, 1988).

Comparing the amino acid sequence of the 7 kDa LTP2 with the sequences of the preproteins of the 9 kDa nsLTPs from barley, wheat, and maize, the overall identities in the alignment shown in Figure 2 are only 21, 22, and 22%, respectively. However, in the 20 amino acid residue stretch from the cysteine at position 45 to the arginine in position 64 (overlined in Figure 2), LTP2 has 60% similarity to all three 9 kDa nsLTPs. Moreover, LTP2 contains the eight cysteine residues conserved in most plant LTPs. In contrast to the low overall identity between the sequences of the 7 kDa and the 9 kDa LTPs, the sequences of the 9 kDa LTPs from barley and wheat share 73% identity. The maize 9 kDa protein sequence has a somewhat lower identity to the corresponding 9 kDa barley and wheat sequences, 50% and 51%, respectively.

*The barley Ltp2 transcript accumulates specifically in the peripheral aleurone cells shortly after the onset of aleurone cell differentiation*

Aleurone cell differentiation is initiated over the ventral crease area in the developing grain at 8 d.a.p. (Bosnes *et al.*, 1992). These cells later develop into the modified aleurone cells. Shortly after, at 9 d.a.p., the second type of aleurone cell, characterized by extensive vacuolation (Figure 3a and b), appears in the peripheral endosperm close to the crease area, spreading first laterally and then to the dorsal side of the grain (see Figure 3a). At this stage, when whole de-embryonated grains are squeezed between the fingers, the extruded endosperm consists of the starchy endosperm, and the peripheral and modified aleurone cells (Figure 3a and b). The remaining maternal tissues consist of the pericarp, testa and the nucellar epidermis (Figure 3c). This is in contrast to later developmental stages, where the extruded endosperm consists only of the starchy endosperm and the modified aleurone cells (Figure 3d). The reason for this is that the cells of the aleurone layer at later stages adhere to the maternal tissues (Figure 3e). Aleurone cell formation is completed at 21 d.a.p., when cell divisions cease (Kvaale and Olsen, 1986). Using the *Ltp2* probe on Northern blots with total RNA, the obtained signal gradually becomes stronger in the pericarp fraction compared with the extruded endosperm, confirming the relocation of the aleurone cells from the endosperm to the pericarp fraction in the interval

|                  |                                                |     |
|------------------|------------------------------------------------|-----|
| LTP2             | MAMAMGMAMRKEAAVAVMMVVTLAGADAGAAACEP--AQLA      | 43  |
| 7kD <sub>w</sub> | ***QA--S***                                    | 8   |
| LTP1             | *ARAQVLIMAAAL*LM*T*APR*AVALN*GQVDSKMK          | 37  |
| 9kD <sub>w</sub> | AQVLMMAVAL*LM***VPR*AVAIID*GHVDSLVR            | 34  |
| 9kD <sub>m</sub> | **RTQQL*VVATA*VALV*L*A*TSE*AIS*GQVASAI*        | 39  |
| LTP2             | VCASAILG-GTKPSGEGCGNLR-----AQQGCLCQYVKD        | 76  |
| 7kD <sub>w</sub> | *****S-A*****F*****                            | 41  |
| LTP1             | P*LTYYQ--*PG*****NGV*DLNNQAQSSGDR*TV*N*L--*G   | 79  |
| 9kD <sub>w</sub> | P*L*YVQ--*PG***Q**DGVKNLHNQARSQSDR*SA*N*L--*G  | 76  |
| 9kD <sub>m</sub> | P*I*YAR*Q*S*G**AG**SGV*SLNNAARTTADRRAA*N*L--*N | 82  |
| LTP2             | PNYG-HYVSSPHARDTLNLCGIPVP-----HC               | 102 |
| 7kD <sub>w</sub> | *T*-Q*IR*****TS*LA*****                        | 67  |
| LTP1             | IAR*I*NLNLNN*ASIPSK*NVN*YTISPDIID*SRIY         | 117 |
| 9kD <sub>w</sub> | IAR*I*NLNEDN*SIPPK*VNL*YTISLNID*SRV            | 113 |
| 9kD <sub>m</sub> | AAA*VSGLNAGN*ASIPSK*GVSI*YTISTSTD*SRVN         | 120 |

**Figure 2.** Comparison of the amino acid sequence of the *Ltp2* ORF with the sequence of the 7 kDa wheat nsLTP and three cereal grain 9 kDa nsLTPs.

The sequence of the deduced LTP2 protein was aligned with that of the 7 kDa wheat protein (Monnet, 1990), the deduced amino acid sequences encoded by barley *Ltp1* (Linnestad *et al.*, 1991), and the 9 kDa proteins derived from the wheat cDNA pTd4.90 (9kD<sub>w</sub>) (Dieryck *et al.*, 1992) and the maize cDNA p9C2 (9kD<sub>m</sub>) (Tchang *et al.*, 1988). Gaps were introduced to optimize the alignment of the 7 and 9 kDa proteins. Identical amino acids are indicated with asterisks. The most likely cleavage point for the signal peptide in LTP2 is indicated by an arrow. Overlined sequence in LTP2 marks a block of amino acid residues with 60% similarity among the 7 kDa and the 9 kDa proteins. The eight conserved cysteines are shown in bold.

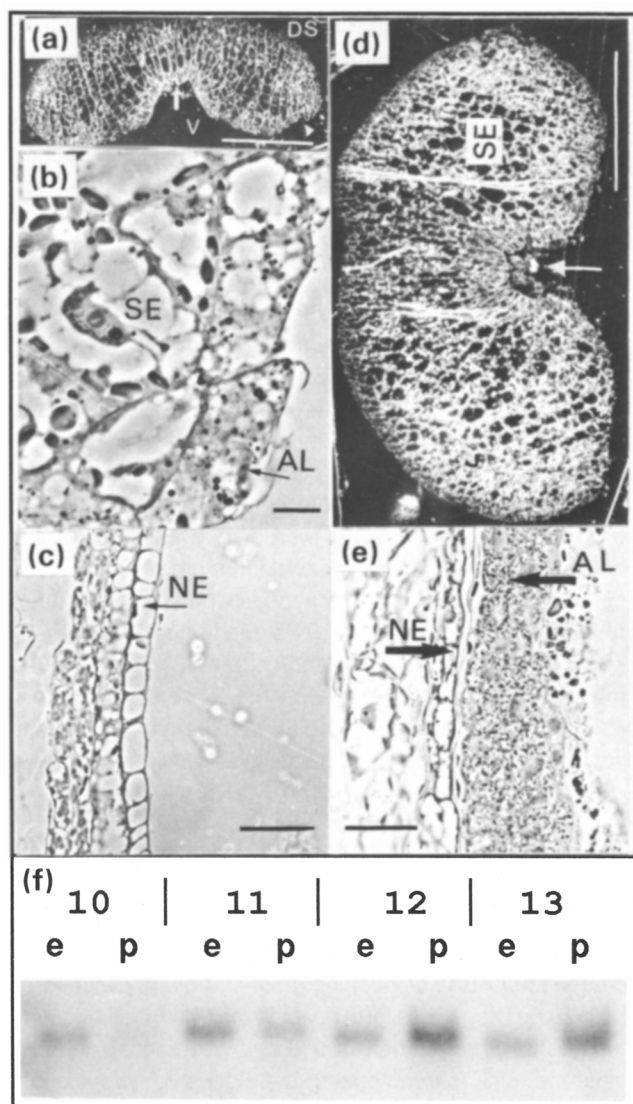


**Figure 3.** Transverse sections from the mid-region of barley grains (a–e) and steady-state levels of the *Ltp2* mRNA in different tissue fractions of developing barley endosperm (f).

(a) Endosperm (10 d.a.p.) isolated from the surrounding maternal tissues by manual extrusion. For maternal tissues, see (c). The extruded endosperm consists of the central starchy endosperm cells, a group of modified aleurone cells near the crease area on the ventral side (V) (arrow), and one layer of highly vacuolated peripheral aleurone cells (arrowhead). For modified aleurone cells, see Figure 4(c). Dorsal side (DS).

(b) Enlargement showing vacuolated peripheral aleurone cells (AL) and starchy endosperm cells (SE) in the area of (a) marked with an arrowhead. (c) Pericarp of 10 d.a.p. grain, after extrusion of the endosperm, showing the nucellus epidermal layer (NE) facing the endosperm cavity, which before extrusion contained the endosperm shown in (a).

(d) Extruded 15 d.a.p. endosperm with central starchy endosperm cells and modified aleurone cells (arrow), but without peripheral aleurone cells.

(e) (15 d.a.p.) Pericarp with adhering aleurone layer (AL) after extrusion of the endosperm (in d).

(f) Northern blot showing the steady state-level of *Ltp2* mRNA in the extruded endosperm fraction (e) and the pericarp fraction (p) in the interval from 10 to 13 d.a.p. Ten micrograms of total RNA were loaded in each lane.

between 10 and 13 d.a.p. (Figure 3f). Previously published data showed that the steady-state level of the *Ltp2* transcript is low at 10 d.a.p. compared with 25 d.a.p., when a 200-fold higher plateau level is reached (Olsen *et al.*, 1990). This level persists until around 35 d.a.p., after which the steady-state transcript level gradually is reduced, being undetectable at 50 d.a.p. (mature grains). From the experiments presented in Figure 3 we infer that the *Ltp2* transcript starts to accumulate in the aleurone layer shortly after the onset of aleurone cell differentiation.

To confirm the aleurone specificity of the *Ltp2* transcript, *in situ* hybridization analysis was carried out on transverse sections of 13 d.a.p. grains. Using  $^3\text{H}$ -labelled antisense transcript as probe, a signal is present in the peripheral aleurone cells after 10 weeks autoradiography (Figure 4a). No signal is present in the starchy endosperm, the maternal tissues, the modified aleurone cells (Figure 4a and b) or in the embryo (not shown). In the control experiment, using the *Ltp2* sense transcript as probe, no signal above background level was detected in any tissue (data not shown).

#### *The Ltp2 promoter is transiently expressed only in developing barley aleurone cells after particle bombardment*

The *Ltp2* promoter contained on a 842 bp *Bgl*II restriction fragment (from nucleotide –801 to +41 in Figure 1) was fused to the GUS reporter gene and delivered to exposed aleurone layers of 25 d.a.p. barley grains by microprojectile bombardment. In the first set of experiments, *Ltp2* promoter activity was assayed visually after histochemical staining with X-Gluc. Owing to a large variation between individual experiments, plasmid DNA containing the *Lc* (Ludwig *et al.*, 1989) and *C1* (Paz-Ares *et al.*, 1987) cDNAs from maize under the control of the 35S CaMV promoter were co-bombarded with the *Ltp2*–Gus construct to monitor shooting efficiency. In combination, but not individually, the latter two cDNAs gave high numbers of red anthocyanin spots in the barley aleurone cells after 1–2 days of incubation of the grains on solid nutrient medium (Figure 4c). In comparison, the *Ltp2*–Gus construct consistently gave only a small number of spots after histochemical staining in co-bombardment experiments (data not shown). A previous report showing that the insertion of a maize *Shrunken-1* intron into a promoter construct enhances the level of expression without interfering with the tissue specificity (Maas *et al.*, 1991), prompted us to insert this intron into the *Ltp2*–Gus construct giving the construct *Ltp2*–*Sh1int*–Gus. Using this construct, the number of spots in immature aleurone layers increased substantially (Figure 4d). Still, compared with aleurone layers bombarded with the *pAct1*–F construct (McElroy *et al.*, 1990), which contains the promoter of the constitu-

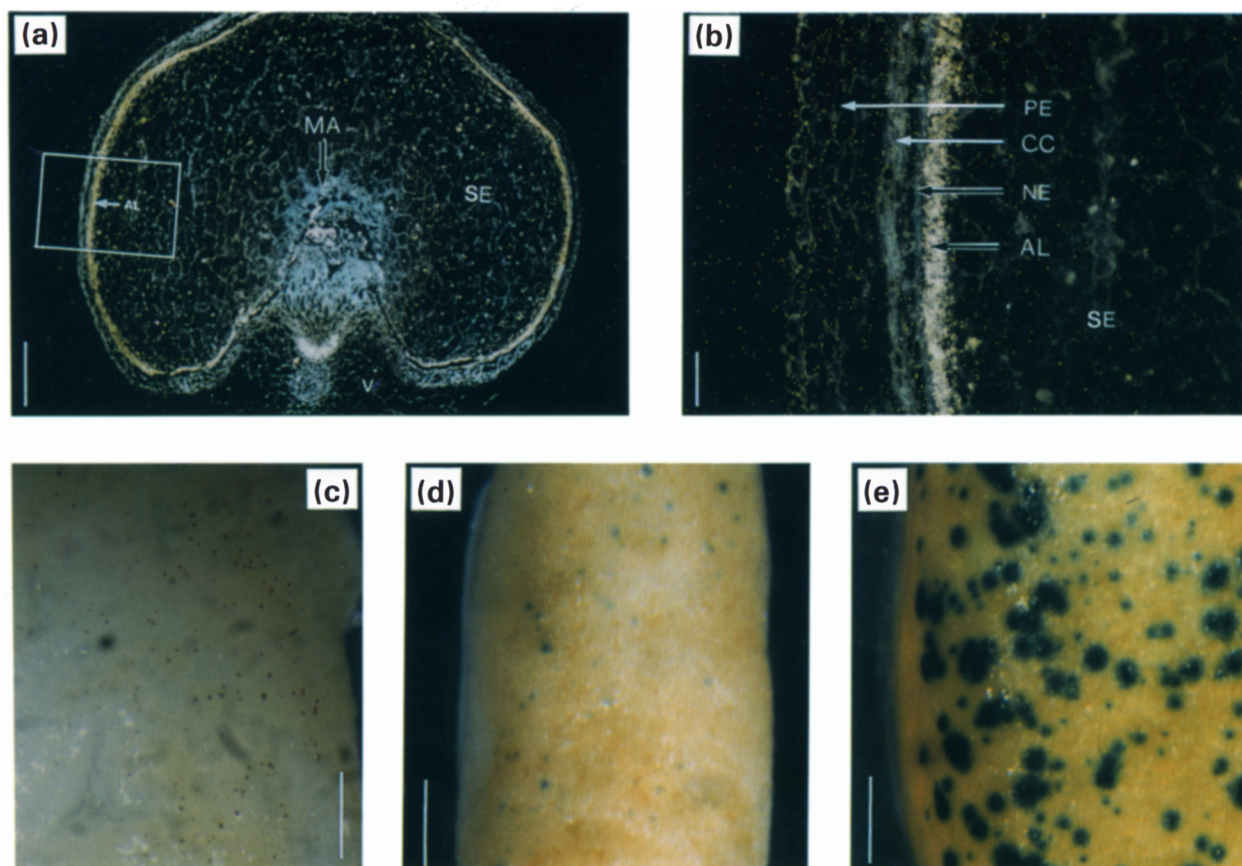


**Figure 4.** *In situ* hybridization of  $^3\text{H}$ -labelled *Ltp2* antisense probe to transverse sections of barley endosperm (a–b) and transient gene expression analysis of different promoter–reporter gene constructs in developing barley aleurone layers after particle bombardment (c–e).

(a) Dark-field micrograph of 13 d.a.p. grain showing hybridization with the *Ltp2* probe in the peripheral aleurone cells (AL), but not in the starchy endosperm (SE) or in the modified aleurone cells (MA) over the ventral crease (V).

(b) Magnification of peripheral endosperm (see frame in (a)) showing a signal in the aleurone layer (AL) but not in the starchy endosperm (SE), the nucellar epidermis (NE), the cross-cell layer (CC), or in the testa and pericarp (PE).

(c) Colourless barley aleurone layer co-bombarded with the *35S-C1* and *35S-Lc* constructs. Single aleurone cells synthesizing anthocyanin pigment appear as red spots.

(d) Exposed aleurone layer of 25 d.a.p. barley grains bombarded with the *Ltp2-Sh1int-Gus* construct. The transfected grain was incubated with X-Gluc for detection of GUS activity.

(e) Exposed aleurone layer of barley grain of the same stage bombarded with *pAct1-F* construct and histochemically stained as in (d).

Bars represent 0.1 mm in (a); 0.25 mm in (b); 9 mm in (c) and (e); 8 mm in (d).

tively expressed *Actin1* gene from rice, both the number and the size of the spots obtained with the *Ltp2-Sh1int-Gus* construct are significantly smaller (Figure 4e).

To quantify *Ltp2* promoter activity in particle bombardment experiments, a second control plasmid (Maas *et al.*, 1991) containing the LUC gene under the control of the *35S* promoter was co-bombarded with the *Ltp2-Gus* construct. Following incubation on tissue culture medium, protein was extracted from the grains in a buffer that allowed measurement of both LUC and GUS activity (for details, see Experimental procedures). In such experiments, when calculating GUS expression normalized to the LUC activity, the enzyme activity from *Ltp2-Gus* was not higher than the background level, corresponding approximately

to 1.5% of the *Actin1* promoter activity in parallel experiments. For the *Ltp2-Sh1int-Gus* construct, however, the activity was significantly higher than the background level, approximately 5% of the *Actin1* promoter. Blue spots from the *Ltp2-Gus* constructs were never observed in tissues other than the aleurone layer in developing grains (data not shown).

#### *The Ltp2 promoter directs aleurone-specific expression of the GUS reporter gene in transgenic rice grains*

The *Ltp2-Gus* construct used in the particle bombardment experiments was transformed into rice by electroporation of embryogenic protoplasts according to Shimamoto *et al.*

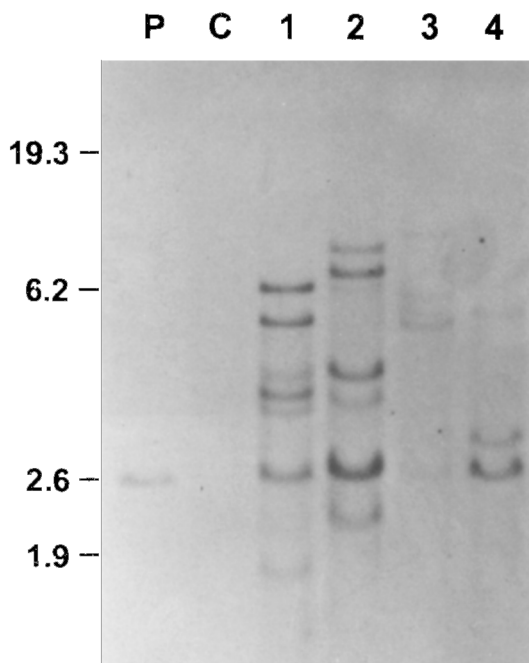


**Figure 5.** Southern blot analysis of the *Ltp2-Gus* construct in transgenic rice plants.

Lane P, *Ltp2-Gus* plasmid DNA digested with *Xba*I and *Hind*III; lane C, non-transformed control plant; lane 1, transgenic line 3-15; lane 2, transgenic line 4-13; lane 3, transgenic line 2-6; lane 4, transgenic line 4-6. Lane C and lanes 1-4 contain 4 µg rice DNA digested with *Xba*I and *Hind*III.

(1989). Four fertile transgenic rice plants were obtained and the integration of *Ltp2-Gus* was examined by Southern blot analysis (Figure 5). These results demonstrated that all four transgenic lines contained the 2.6 kb fragment expected from the integration of the complete *Ltp2-Gus* construct into the rice genome. In addition to the 2.6 kb band, hybridizing fragments of variable sizes were detected in all the lines. These bands most likely represent rearranged copies of the transgene as often observed in transgenic plants obtained after direct transfer of plasmid DNA (for example, see Terada *et al.*, 1993). A preliminary histochemical analysis demonstrated that all four plants carried grains expressing the GUS reporter gene in the aleurone layer.

As a basis for the analysis of *Ltp2-Gus* activity in transgenic rice, wild-type rice grains were fixed at daily intervals from 0 to 15 d.a.p. to determine the pattern of aleurone cell differentiation under the growth conditions used in our laboratory. As in barley, aleurone cell differentiation in rice starts in the periphery of the cellularized endosperm over the ventral vascular strand and spreads laterally to the dorsal region (Figure 6a-d). In rice, this process starts at 6 d.a.p. resulting in a complete aleurone layer at 7-8 d.a.p. (Figure 6c and d). To monitor the activity of the *Ltp2* promoter in rice aleurone cells, grains from the transgenic line 3-15 were harvested and stained for GUS activity (Figure 6e-h). This analysis shows that the *Ltp2* promoter is acti-

**Figure 6.** The *Ltp2* promoter drives aleurone layer-specific GUS expression in developing transgenic rice grains.

(a-d) Phase-contrast micrographs of transverse sections (2 µm) from nontransformed grains, (e-j, m and o) grains from the homozygous *Ltp2-Gus* progeny of transgenic line 3-15.

(a) Initial aleurone cell (AL) differentiation in the periphery of the endosperm (EN) above the ventral vascular strand (VS) at 5 d.a.p. (for orientation of the section, see lower frame in e) (NU = nucellus). Typically, the aleurone cells have dense cytoplasm and are smaller than the cells in the inner layers of the endosperm.

(b) Dorsal region of the same grain as in (a) lacking aleurone cells in the peripheral endosperm adjacent to the nucellus (NU) (for orientation see the upper frame in e).

(c) Ventral region of a 7 d.a.p. grain with aleurone cells over the ventral vascular strand (orientation of the section corresponds to the lower frame in e).

(d) Dorsal region of the same grain as in (c) showing differentiated aleurone cells.

(e) Six days after pollination grain showing no GUS activity.

(f) Seven days after pollination grain showing GUS activity in the aleurone cells in the dorsal region of the grain (arrow). Owing to diffusion of the CIBr-indigo stain, blue colour is also seen outside of the aleurone layer.

(g) Nine days after pollination grain with GUS activity in most of the aleurone layer.

(h) Thirteen days after pollination grain with strong GUS activity in the entire aleurone layer, but not in the embryo (EM).

(i) Section (10 µm) of the ventral part of the grain in (h) showing the location of CIBr-indigo in the aleurone layer (white arrow), the nucellar epidermis (arrow-head) and the starchy endosperm (black arrow).

(j) Separation of the maternal tissue from the endosperm prior to the addition of X-Gluc leads to blue staining only in the aleurone cells (arrow) and not in maternal tissues.

(k) GUS activity in a low number of aleurone cells (sectors) in one of the *Ltp2-Gus* 3-15 progeny lines. The grain has been cut in slices to facilitate the penetration of X-Gluc.

(l) Transverse section (10 µm) of grain in (k). The presence of GUS activity in some cells (white arrow), but not in others (black arrow) allows unambiguous identification of the aleurone cells as the source of the GUS activity.

(m) Stem section from the homozygous 3-15 *Ltp2-Gus* transgenic plant with no detectable GUS activity.

(n) GUS-positive stem section (control) from a *Ltp1* promoter-*Gus* transgenic plant (Linnestad *et al.*, unpublished results).

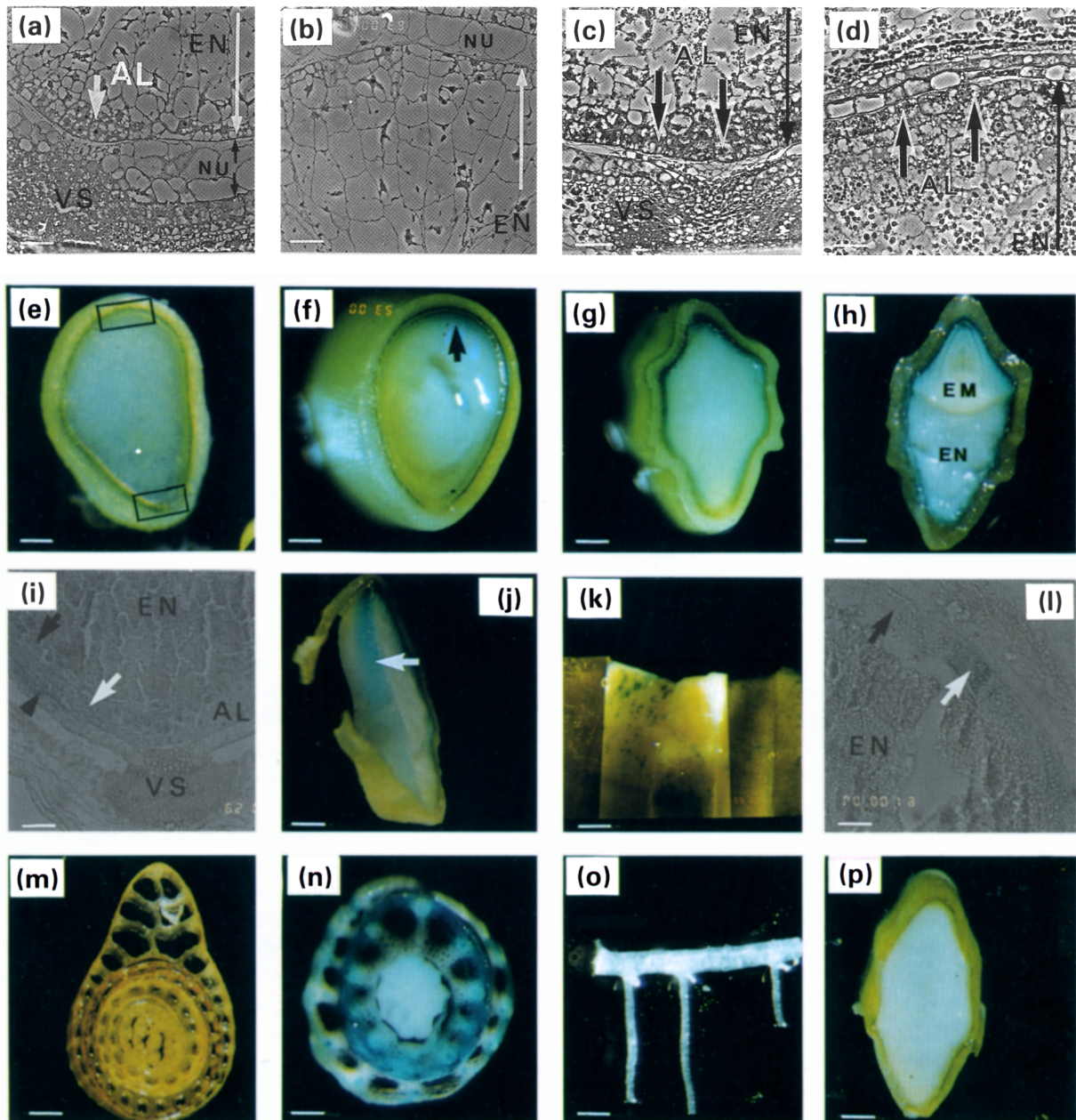
(o) Root segment from the homozygous 3-15 *Ltp2-Gus* transgenic plant with no detectable GUS activity.

(p) Wild-type 13 d.a.p. grain with no detectable GUS activity following incubation in X-Gluc as in (h).

Bars represent 0.1 mm in (a-d), (i) and (l); 0.3 mm in (e) and (f); 0.5 mm in (g), (h), (k), (o) and (p); 0.4 mm in j; 0.8 mm in (m); 0.6 mm in (N).

vated at 7 d.a.p. in a region corresponding to the location of the dorsal aleurone layer (Figure 6e and f). During the following 2 days, the *Ltp2* promoter becomes active in the whole perimeter of the endosperm, corresponding to the entire aleurone layer (Figure 6g and h). Notably, the GUS staining is equally strong in the aleurone cells over the embryo as in those surrounding the starchy endosperm (Figure 6h). The aleurone cells over the embryo, which to our knowledge have not been described in rice, are termed germ aleurone in barley (Cochrane, 1994 and references therein). As is well known from numerous previ-

ous investigations, the diffusion of the ClBr-indigo dye from the site of synthesis makes it difficult to unambiguously identify the cells producing the GUS activity. Diffusion of the stain also represented a problem in our study, making it hard to exclude GUS expression in tissues adjacent to the aleurone cells (Figure 6f–h). To overcome this problem, three different approaches were taken. First, fixation and sectioning of histochemically stained transgenic grains consistently showed staining first in the aleurone layer and only subsequently in the nucellus and in the starchy endosperm (Figure 6). Secondly, when maternal



tissues were separated from the aleurone layer before incubation in the staining buffer, GUS staining was present in the aleurone layer and not in the maternal tissues (Figure 6j). Thirdly, in some grains from a progeny plant of the transgenic line 3-15, in which GUS activity is seen only in a low percentage of the aleurone cells (Figure 6k), the blue staining of the nucellus and the starchy endosperm only occurs adjacent to a GUS-positive aleurone cell (Figure 6l). Thus, the most likely explanation for these observations is that the aleurone cells are the site for GUS activity, and that the CIBr-indigo diffuses to the neighbouring tissues from these cells.

In addition to developing grains, the distribution of GUS staining was studied in mature, germinating rice grains. In these grains, the intensity of the blue stain decreases from the level found in dry grains to that of grains germinated for 5 days (data not shown). Careful analysis failed to detect GUS activity in any other tissues of the homozygous transgenic rice plant, including stems and roots (Figure 6m and o). In contrast, rice plants transgenic for a *Ltp1* promoter-*Gus* construct (Linnestad *et al.*, unpublished) showed strong activity in the vascular tissue of the stem (Figure 6n). No GUS activity was detectable in the endosperm of non-transformed rice grains (Figure 6p).

## Discussion

This paper describes the barley aleurone-specific gene, *Ltp2*, and identifies its gene product as a 7 kDa nsLTP, which we designate LTP2. The identification of the predicted LTP2 amino acid sequence as an nsLTP is based on high identity to a 7 kDa protein that was isolated from wheat grains using the *in vitro* lipid transfer assay (Monnet, 1990). From the high degree of sequence identity between the 7 kDa LTPs, and between the members of the class of 9 kDa grain nsLTP proteins in barley and wheat, we infer that the aleurone layers of these cereals contain two classes of evolutionarily related nsLTPs. While the sequence identity is more than 70% within each of the two weight classes, it is only around 20% between the 7 kDa and 9 kDa grain LTPs. Thus, whether the aleurone 7 kDa and 9 kDa nsLTPs are encoded by members of one gene family remains an open question. It is noteworthy, however, that in the alignment of the amino acid sequences of barley LTP2 and LTP1, the best score is obtained by inserting three gaps in the LTP2 sequence (Figure 2). In addition, the LTP2 protein lacks the four C-terminal residues present in LTP1 (Figure 2). One possibility, therefore, is that the *Ltp1* and *Ltp2* genes belong to the same gene family, and that deletions of sequences corresponding to the introduced gaps in Figure 2, plus the four C-terminal amino acids from an aleurone-specific *Ltp1*-like progenitor gene, may have led to *Ltp2*.

Hybridization experiments using *Ltp1* and *Ltp2* probes

on barley genomic Southern blots suggest that the barley haploid genome contains only one copy of each gene (Jakobsen *et al.*, 1989; Skriver *et al.*, 1992). However, using the *Ltp1* probe under low-stringency conditions, three bands are detectable (Skriver *et al.*, 1992), demonstrating that the barley 9 kDa nsLTP family consists of at least three members. Recently, this conclusion was supported by the cloning of five new barley nsLTP cDNAs representing transcripts expressed in the vegetative plant tissues, but not in the endosperm (Gausing, 1994; Molina and García-Olmedo, 1993).

To date, all plant nsLTPs derived from cDNAs, including LTP2, contain N-terminal extensions with signal peptide-like features (Figure 2). In keeping with this, the barley preLTP1 protein was shown to be processed correctly and to enter the lumen of the microsomal membrane fraction in an *in vitro* study (Madrid, 1991). Two recent studies of the spatial accumulation pattern of transcripts encoding 9 kDa nsLTPs in maize and carrot embryos showed that the transcripts were restricted to cell layers facing the endosperm (Sossountzov *et al.*, 1991; Sterk *et al.*, 1991). Furthermore, nsLTPs were found to be located in the cell wall, mainly in epidermal cells of *Arabidopsis* (Thoma *et al.*, 1993), and in the surface wax of broccoli leaves (Pyee *et al.*, 1994). Our *in situ* hybridization data show that the *Ltp2* transcript is present exclusively in the aleurone cells. Interestingly, these cells are located at the interface between the endosperm and the maternal tissues of the seed coat. According to the suggestion by Sterk *et al.* (1991), plant nsLTPs may be involved in the extracellular transport of cutin or other lipid monomers from the endoplasmic reticulum to the site of synthesis of extracellular matrix components, such as the cuticle. Therefore, one possible role for the aleurone-specific nsLTPs in barley and wheat could be in the formation of the earlier described amorphous layer on the outside of the aleurone cells in wheat grains (Evers and Reed, 1988). Another possibility, as advocated by Molina and García-Olmedo (1993) for the nsLTPs expressed in vegetative tissues, is that the nsLTPs are involved in the protection of plant tissues against pathogen attacks. In our view, because of the low efficiency and specificity of the *in vitro* LTP assay, the possibility that the nsLTPs may have different functions in the various tissues should also be kept open.

One of the main goals of our molecular studies of endosperm is to isolate promoters specifically directing transcription in aleurone cells of developing cereal grains. The data presented strongly suggest that the barley *Ltp2* promoter directs transcription exclusively in the aleurone cells from shortly after the onset of aleurone cell differentiation to mid-maturity of the grain. This conclusion is based on three types of evidence, i.e. the aleurone-specific accumulation of the *Ltp2* transcript in barley, as revealed by Northern and *in situ* hybridization experiments, the ability

of the *Ltp2* promoter to direct transient expression of the GUS reporter gene in immature barley aleurone layers, and aleurone-specific expression of the GUS reporter gene in transgenic rice grains.

The *Ltp2* transcript is detectable in the aleurone layer of barley grains from shortly after the onset of aleurone cell differentiation (Figure 4a and b). In rice, as demonstrated by the high GUS activity around the embryo (Figure 6h), the *Ltp2* promoter is fully active also in the germ aleurone. Following this observation, we performed additional *in situ* hybridizations of transverse sections of developing barley grains using the *Ltp2* probe labelled with the high-energy isotope  $^{32}\text{P}$ . In long time exposures we interpret that there is some *Ltp2* promoter activity also in barley germ aleurone. In germinating barley grains,  $\alpha$ -amylase accumulates in the germ aleurone (Pogson *et al.*, 1989) suggesting that this tissue utilizes a gene programme resembling that of the 'normal' aleurone layer.

Owing to the lack of a routine protocol for stable barley transformation, demonstration of the activity of the isolated *Ltp2* promoter in the aleurone cells of developing barley grains must presently rely on transient assays using particle bombardment. Using this method, we show that the -801 bp *Ltp2* promoter upstream region is capable of driving the expression of the GUS reporter gene in immature barley aleurone layers. The *Ltp2* promoter does, however, appear weak compared with the constitutive cereal promoter from the *Actin1* gene, even after the introduction of the *Sh1*-intron into the *Ltp2-Gus* construct. Interestingly, the increase in the expression level was only around threefold in our assay, and not 100-fold as reported by Maas *et al.* (1991) for a reporter gene expressed in rice and maize protoplasts. In cultured barley cells, inclusion of the *Sh1*-intron in a *35S-Gus* construct increased the expression level about sevenfold (Chibbar *et al.*, 1993). In our hands, the expression from the *Ltp2* promoter in transient assays in barley aleurone cells is not sufficiently strong to allow quantitative studies of a promoter deletion series to map accurately the location of sequences responsible for directing aleurone-specific expression. One possible explanation for the low activity of the *Ltp2* promoter in transient assays using isolated barley grains is that the maintenance of a maximal steady state-level of the transcript depends on the grain being attached to the plant (Olsen *et al.*, 1990). Thus, when 24 d.a.p. isolated aleurone layers are incubated on MS medium, the *Ltp2* transcript disappears completely within 2 h (See Figure 2 in Olsen *et al.*, 1990). It may therefore be that detachment of the grain and the removal of the maternal tissues to expose the aleurone layer prior to particle bombardment reduces the level of *Ltp2* expression. This could explain the apparent discrepancy between the level of *Ltp2* expression obtained in transgenic rice grains and in transient assays in barley.

As described in this paper, the differentiation pattern of the aleurone layer is very similar in rice and barley. We may therefore expect that genes, such as *Ltp2*, are expressed in the same manner in the aleurone layers of these two species. This expectation is borne out using the GUS reporter gene to monitor the activity of the *Ltp2* promoter in rice. As observed for barley, the activity of the *Ltp2* promoter is detectable immediately after the onset of aleurone cell differentiation, appearing first on the dorsal side of the endosperm, later spreading to the whole aleurone layer. Owing to the high stability of the GUS enzyme, the decline in the *Ltp2* expression in barley towards grain maturity cannot easily be measured in transgenic rice. However, the *Ltp2* promoter is not active in the aleurone layer of mature germinating rice grains, as demonstrated by the reduction of GUS activity during germination. Taken together, therefore, the data presented from barley and rice show that the *Ltp2* promoter is under strict temporal and spatial control, directing transcription exclusively in the aleurone layer, from shortly after the onset of aleurone cell differentiation to mid-maturity of the grain. Because of the relatively large evolutionary distance between barley and rice, the described pattern of expression from the *Ltp2* promoter is likely to be found in other cereals as well, including maize and wheat. This conclusion is supported by previous investigations in which maize (Kyoizuka *et al.*, 1991) and wheat (Terada *et al.*, 1993) promoters direct highly regulated expression of the GUS reporter gene in transgenic rice. Not unexpectedly, therefore, our data support the view that the molecular mechanisms underlying aleurone cell-specific transcription in developing grains are conserved among the cereal species. Whether the MYB and MYC consensus binding sites found in the *Ltp2* promoter are involved in the regulation of *Ltp2* transcription in a manner similar to that suggested for the maize *Bz1* (Roth *et al.*, 1991) is currently under investigation in our laboratories.

## Experimental procedures

### Barley plants

Grains of barley (*Hordeum vulgare* cv. Bomi) were collected from plants grown in a phytotron chamber as described in Kvaale and Olsen (1986). The plants used in Figure 3 and Figure 4(a and b) were emasculated and pollinated by hand to ensure accurate determination of grain age. For all other grains, age determination is based on the day of anthesis.

### cDNA and genomic clones

The isolation and sequencing of the aleurone-specific cDNA clone zB11E has been described by Jakobsen *et al.* (1989). A custom-made barley cv. Bomi genomic library, constructed by partial *Mbol* digestion of total genomic DNA and subsequent ligation of the 8–22 kb size fraction with *Bam*HI-digested lambda

EMBL3 DNA, was purchased from Clontech. A total of  $2 \times 10^6$  plaques were screened using radioactive random primed (Boehringer-Mannheim) zB11E cDNA insert as a probe. Four positive clones (gHv29-101, gHv38-201, gHv53-201 and gHv59-101) were identified after repeated rounds of plaque hybridization. DNA purified from these clones was restricted with several enzymes and characterized by Southern blot analysis. One 12 kb clone, gHv53-201, was chosen for further analysis and a 6 kb *Pst*I subfragment was subcloned into pBluescript (Stratagene) giving the subclone BL53Ps17. A *Nhe*I restriction fragment of 0.7 kb covering the coding region of the *Ltp2* gene was cloned into the *Xba*I site of M13mp18 and sequenced as described below. In order to characterize the 5' and 3' sequences of the *Ltp2* gene, the following DNA fragments were generated by PCR amplification: (i) a 1.2 kb fragment covering the 5' end of the gene using a vector primer (KS) and the PLT11 primer located within the 5' end of the cDNA; (ii) A 0.3 kb PCR product generated by amplification directed by the internal primers LTP13 and PLT15, of which the latter is based upon sequence information from the cDNA clone zB14A, which is overlapping and identical to zB11E but contains an additional 30 bp downstream of the polyadenylation site as indicated at position 496 in Figure 1. The primer sequences are:

KS: 5'-CGAGGTCGACGGTATCG-3',  
 PLT11: 5'-TACGGTAGATCTACTCGGCTA-3',  
 LTP13: 5'-ACGAAGCCGAGCGGCGAGT-3',  
 PLT15: 5'-GGACTAAAAAAGATTGCAACACAAATTC-3'

The PLT11 sequence contains an extra nucleotide (shown in bold) introducing a *Bgl*II restriction site. The 1.2 kb PCR product containing the 5' end was restricted with *Bgl*II to give a 0.84 kb fragment that was subsequently cloned into the *Bam*HI site of pBluescript. The 0.3 kb PCR product of the 3' end was treated with T4 DNA polymerase (Sambrook *et al.*, 1989) and subsequently cloned into the *Sma*I site of M13mp18. Sequencing were performed using the Sequenase protocol (USB) after isolation of single-stranded DNA template using PCR amplification and magnetic beads (Dynabeads M280-Streptavidin, Dynal).

### Northern analyses

Total RNA was extracted from barley grain tissues essentially as described by Logemann *et al.* (1987), except that LiCl precipitation was used in place of ethanol precipitation. The RNA was denatured using formaldehyde and separated on 1.2% agarose gels as described by Selden (1987) and blotted on to Gene-Screen (NEN) membranes using a Stratagene posiblitter apparatus according to the supplier's instructions. Hybridization was done according to the GeneScreen instruction manual using radioactively labelled zB11E cDNA insert as probe.

### In situ hybridization

For *in vitro* transcription of antisense (and sense) RNA, zB11E (Jakobsen *et al.*, 1989) was linearized with *Pst*I (*Hind*III) and transcribed with T7 (T3) RNA polymerase using MAXIsript (Ambion) and [5,6-<sup>3</sup>H]Uridine 5'-triphosphate (40–60 Ci mmol<sup>-1</sup>, Amersham) according to the supplier's specifications. The probes were hydrolysed to fragments of about 100 bp as described by Somssich *et al.* (1988). Grain tissues were fixed in 1% glutaraldehyde, 100 mM sodium phosphate (pH 7.0) for 2 h and embedded in Histowax (Histolab, Sweden). Sections of 10 µm were pre-treated with pronase (Calbiochem) as described by Schmelzer *et al.* (1988) and hybridized with 25 µl of hybridization mix (200 ng probe ml<sup>-1</sup>, 50% formamide, 10% (w/v) dextran sulphate, 0.3 M NaCl, 10 mM Tris-HCl, 1 mM EDTA (pH 7), 0.02% poly-

vinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin) for 15 h at 50°C. Post-hybridization was carried out according to Somssich *et al.* (1988) and autoradiography was done as described by Schmelzer *et al.* (1988), except that sections were exposed for 10 weeks.

### Plasmid constructs

For the microprojectile bombardment experiments, the following constructs were used: pAct1-F (McElroy *et al.*, 1990) containing the rice *Actin1* promoter fused to the *uidA* reporter gene encoding the GUS enzyme and the 3' non-coding region of the nopaline synthase gene; pRT-ex/s-int/s-LUC (Maas *et al.*, 1991) containing the 35S CaMV promoter plus the maize *Sh1* first exon/intron fused to the firefly luciferase gene and the polyadenylation signal of CaMV 35S; pRT101C1 (Wienand, personal communication) containing the 35S CaMV promoter, the maize *C1* cDNA (Paz-Ares *et al.*, 1987) and the polyadenylation signal of CaMV 35S; pMF6Lc (Blowers, personal communication) containing the 35S CaMV promoter-*Adh1* intron, the *Lc* cDNA (Ludwig *et al.*, 1989) and the 3' non-coding region of the nopaline synthase gene. The *Ltp2* promoter contained on the 0.84 kb *Bgl*II fragment (sequence presented in Figure 1) was inserted into the *Bam*HI site of pBluescript in front of the GUS reporter gene and the 3' non-coding region of the nopaline synthase gene to give the *Ltp2*-Gus construct. For the transient expression studies in barley aleurone layers, the first intron of the maize *Sh1*, carried on a 1.1 kb *Hinc*II fragment (Maas *et al.*, 1991), was inserted into the *Sma*I site of the *Ltp2*-Gus construct between the promoter and the reporter gene to give the *Ltp2*-*Sh1*int-Gus construct.

### Transient expression in barley aleurone layers by particle bombardments

Barley grains were harvested at 25 d.a.p., surface sterilized in 1% sodium hypochlorite for 5 min and then washed four times in sterile distilled water. The maternal tissues were removed to expose the aleurone layer and the grain was then divided in two, longitudinally along the crease. The pieces of tissue were then placed, endosperm down, on to MS (Murashige and Skoog, 1962) medium with 10 g l<sup>-1</sup> sucrose solidified with 10 g l<sup>-1</sup> agar in plastic petri dishes (in two rows of four endosperm halves per dish). Single bombardments were performed in a DuPont PDS 1000 device, with M-17 tungsten pellets (approximately 1 µm in diameter) coated with DNA as described by Gordon-Kamm *et al.* (1990) and using a 100 µm mesh 2 cm below the stopping plate. Equal amounts (25 µg per preparation) of the GUS (promoter-reporter gene) and LUC (internal standard) plasmids were mixed before adding the microprojectiles. One tenth of this amount, 2.5 µg, was used for the *Lc* and *C1* cDNA constructs. Bombarded tissue was incubated at 24°C for 3–4 days before extraction and measurement of GUS and LUC activities. Anthocyanin pigmentation could be observed in the bombarded aleurone layers without further treatment. Histochemical staining for GUS expression was performed with X-Gluc as described by Jefferson (1987) at 37°C for 2 days. Extraction of proteins for quantitative analysis was performed by grinding four to eight half grains in a mortar and pestle with 0.5 ml of extraction buffer (50 mM Na-phosphate pH 7.0, 1 mM DTT, pH 7.0). After grinding, a further 0.5 ml of extraction buffer was added and two 400 µl aliquots were taken. To one of these, 100 µl of 5x Luciferase cell lysis buffer (Promega) were added and the sample vortexed before clearing by centrifugation at 9000 g. A 20 µl aliquot was then measured for LUC activity in a scintillation counter (Tri-Carb 4000), using the luciferase assay

system of Promega (E1500). To the other 400 µl aliquot, 100 µl of 5× lysis buffer (500 mM Na-phosphate pH 7.0, 50 mM EDTA, 10 mM DTT, 0.5% Sarcosyl, 0.5% Triton X-100) were added, the mixture vortexed and cleared as above and assayed for GUS activity using 4-methylumbelliferone, β-D-glucuronide as described by Jefferson (1987) modified to include 5% methanol in the reaction mixture (Kosugi *et al.*, 1990). Production of 4-methylumbelliferone (MU) was measured after 1 and 4 h using a TKO 1000 Mini-Fluorimeter (Hoefer Scientific Instruments). In the analysis of promoter activities, the GUS readings (MU produced per h) were normalized relative to the LUC values (photons produced over 30 sec, beginning 60 sec after mixing) from the same extract.

### Rice transformation

The *Ltp2-Gus* construct described above was co-transformed into rice (*Oryza sativa* L., cv. Kinuhikari) protoplasts with a hygromycin-resistance selection marker by electroporation as described previously (Shimamoto *et al.*, 1989). Hygromycin-resistant calli were screened for GUS activity by incubating a part of each calli in X-Gluc solution (Jefferson, 1987). The GUS-positive calli were further cultured and plants were regenerated from these cultures. For the histochemical GUS analysis of developing transgenic and wild-type rice grains, plants were grown at Ås under a regime of 16 h light (25 000 lux) at 29°C/8 h dark at 22°C. After sectioning with razor blades, longitudinal and transverse sections were incubated in 5 mM X-Gluc solution containing 20% methanol for 1–12 h at 37°C. For detailed localization of the GUS stain in light microscopy, grains were prepared as described for the *in situ* hybridization analysis. Sections for phase contrast light microscopy were prepared as described in Bosnes *et al.* (1992).

### Southern blot analysis of transgenic rice plants

Total genomic DNA was isolated from mature leaves, digested with *Xba*I and *Hind*III, separated by gel electrophoresis and transferred to a nylon membrane (Amersham). The coding region of the GUS gene was labelled and amplified with digoxigenin 11-dUTP by polymerase chain reaction and used for probing the *Ltp2-Gus* construct. Hybridization and chemiluminescence signal detection were performed according to the manufacturer's specifications (Boehringer-Mannheim).

### Acknowledgements

We thank K. Itho and J. Kyojuka of Plantech Research Institute for help in rice transformation and Southern blot analysis. The skilful technical assistance of Hege Munck is acknowledged. We are grateful to Drs U. Wienand, Cologne for the generous gift of the maize *C1* construct and to C. Maas, Cologne for the LUC and the maize *Sh1* intron constructs. We thank Dr S. Wessler, Athens GA and Dr A. Blowers, Ithaca, NY for their gifts of the maize *Lc* DNA constructs. Drs R. Wu and D. McElroy, Ithaca, NY, are acknowledged for providing the rice *pAct1-F* construct. This work was funded by the Norwegian Agricultural Research Council (NLVF). P.S.N. is a recipient of a postdoctoral fellowship from the Danish Agricultural and Veterinary Research Council (SJVF).

### References

- Aalen, R.B., Opsahl-Ferstad, H.-G., Linnestad, C. and Olsen, O.-A. (1994) Transcripts encoding an oleosin and a dormancy-related protein are present in both the aleurone layer and the embryo of developing barley (*Hordeum vulgare* L.) seeds. *Plant J.* **5**, 385–396.
- Bosnes, M., Weldeman, F. and Olsen, O.-A. (1992) Endosperm differentiation in barley wild-type and sex mutants. *Plant J.* **2**, 661–674.
- Breu, V., Guerbette, F., Kader, J.C., Kannangara, C.G., Svensson, B. and Von Wettstein-Knowles, P. (1989) A 10 kD barley basic protein transfers phosphatidylcholine from liposomes to mitochondria. *Carlsberg Res. Commun.* **54**, 81–84.
- Brown, R.C., Lemmon, B.E. and Olsen, O.-A. (1994) Endosperm development in barley: Microtubule involvement in the morphogenetic pathway. *Plant Cell*, **6**, 1241–1252.
- Bucher, P. (1990) Weight matrix descriptions of four eukaryotic RNA polymerase II promoter elements derived from 502 unrelated promoter sequences. *J. Mol. Biol.* **212**, 563–578.
- Cavener, D.R. and Ray, S.C. (1991) Eukaryotic start and stop translation sites. *Nucl. Acids Res.* **19**, 3185–3192.
- Chibbar, R.N., Kartha, K.K., Datla, R.S.S., Leung, N., Caswell, K., Mallard, C.S. and Steinhauer, L. (1993) The effect of different promoter-sequences on transient expression of gus reporter gene in cultured barley (*Hordeum vulgare* L.) cells. *Plant Cell Rep.* **12**, 506–509.
- Cochrane, M.P. (1994) Observations on the germ aleurone of barley. Morphology and histochemistry. *Ann. Bot.* **73**, 113–119.
- Dellaporta, S.L., Greenblatt, I., Kermicle, J.L., Hick, J.B. and Wessler, S. (1988) Molecular cloning of the R-nj allele by transposon tagging with Ac. In *Chromosome Structure and Function: Impact of New Concepts*, 18th Stadler Genetics Symp. (Gustafson, J.P. and Appels, R., eds). New York: Plenum Press, pp. 263–282.
- Dieryck, W., Gautier, M.-F., Lullien, V. and Joudrier, P. (1992) Nucleotide sequence of a cDNA encoding a lipid transfer protein from wheat (*Triticum durum* Desf.). *Plant Mol. Biol.* **19**, 707–709.
- Evers, A.D. and Reed, M. (1988) Some novel observations by scanning electron microscopy on the seed coat and nucellus of the mature wheat grain. *Cereal Chem.* **65**, 81–85.
- Fincher, G.B. (1989) Molecular and cellular biology associated with endosperm mobilization in germinating cereal grains. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **40**, 305–346.
- Gausling, K. (1994) Lipid transfer protein genes specifically expressed in barley leaves and coleoptiles. *Planta*, **192**, 574–580.
- Gordon-Kamm, W.J., Spencer, T.M., Mangano, M.L., *et al.* (1990) Transformation of maize cells and regeneration of fertile transgenic plants. *Plant Cell*, **2**, 603–618.
- von Heijne, G. (1988) Transcending the impenetrable: How proteins come to terms with the membranes. *Biochim. Biophys. Acta*, **947**, 307–333.
- Jakobsen, K., Klemsdal, S., Aalen, R., Bosnes, M., Alexander, D. and Olsen, O.-A. (1989) Barley aleurone cell development: molecular cloning of aleurone-specific cDNAs from immature grains. *Plant Mol. Biol.* **12**, 285–293.
- Jefferson, R.A. (1987) Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Mol. Biol. Rep.* **5**, 387–405.
- Kader, J.-C., Jullienne, M. and Vergnolle, C. (1984) Purification and characterisation of a spinach-leaf protein capable of transferring phospholipids from liposomes to mitochondria or chloroplasts. *Eur. J. Biochem.* **139**, 411–416.
- Klemsdal, S.S., Hughes, W., Lönneborg, A., Aalen, R.B. and Olsen, O.-A. (1991) Primary structure of a novel barley gene differentially expressed in immature aleurone layers. *Mol. Gen. Genet.* **228**, 9–16.
- Kosugi, S., Ohashi, Y., Nakajima, K. and Arai, Y. (1990) An improved assay for β-glucuronidase in transformed cells:

- methanol almost completely suppresses a putative endogenous-glucuronidase activity. *Plant Sci.* **70**, 133–140.
- Kvaale, A. and Olsen, O.-A.** (1986) Rates of cell division in developing barley endosperms. *Ann. Bot.* **57**, 829–833.
- Kyozuka, J., Fujimoto, H., Izawa, T. and Shimamoto, K.** (1991) Anaerobic induction and tissue-specific expression of maize *Adh1* promoter in transgenic rice plants and their progeny. *Mol. Gen. Genet.* **228**, 40–48.
- Leah, R., Tommerup, H., Svendsen, I. and Mundy, J.** (1991) Biochemical and molecular characterization of three barley seed proteins with antifungal properties. *J. Biol. Chem.* **266**, 1564–1573.
- Linnestad, C., Lönneborg, A., Kalla, R. and Olsen, O.-A.** (1991) The promoter of a lipid transfer protein gene expressed in barley aleurone cells contains similar Myb and Myc recognition sites as the maize *Bz-McC* allele. *Plant Physiol.* **97**, 841–843.
- Logemann, J., Schell, J. and Willmitzer, L.** (1987) Improved method for isolation of RNA from plant tissues. *Anal. Biochem.* **163**, 16–20.
- Ludwig, S.R., Habera, L.F., Dellaporta, S.L. and Wessler, S.R.** (1989) *Lc*, a member of the maize *R* gene family responsible for tissue-specific anthocyanin production, encodes a protein similar to transcriptional activators and contains the *myc*-homology region. *Proc. Natl Acad. Sci. USA*, **86**, 7092–7096.
- Maas, C., Laufs, J., Grant, S., Korfhage, C. and Werr, W.** (1991) The combination of a novel stimulatory element in the first exon of the maize *shrunk-1* gene with the following intron1 enhances reporter gene expression up to 1000 fold. *Plant. Mol. Biol.* **16**, 199–207.
- Madrid, S.M.** (1991) The barley lipid transfer protein is targeted into the lumen of the endoplasmic reticulum. *Plant Physiol. Biochem.* **29**, 695–703.
- McClintock, B.** (1978) Development of the maize endosperm as revealed by clones. In *The Clonal Basis of Development* (Subtelny, S. and Sussex, I.M., eds). New York: Academic Press, pp. 217–237.
- McElroy, D. and Brettell, R.I.S.** (1994) Foreign gene expression in transgenic cereals. *Trends Biotechnol.* **12**, 62–68.
- McElroy, D., Zhang, W., Cao, J. and Wu, R.** (1990) Isolation of an efficient actin promoter for use in rice transformation. *Plant Cell*, **2**, 163–171.
- Molina, A. and García-Olmedo, F.** (1993) Developmental and pathogen-induced expression of three barley genes encoding lipid transfer proteins. *Plant J.* **4**, 983–991.
- Monnet, F.-P.** (1990) Ph.D. Thesis. Montpellier: Université des Sciences et Techniques du Languedoc.
- Mundy, J. and Rogers, J.** (1986) Selective expression of a probable amylase/protease inhibitor in barley aleurone cells: comparison to the barley amylase/subtilisin inhibitor. *Planta*, **169**, 51–62.
- Murashige, T. and Skoog, F.** (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**, 473–497.
- Olsen, O.-A., Jakobsen, K.S. and Schmelzer, E.** (1990) Development of barley aleurone cells: temporal and spatial patterns of accumulation of cell-specific mRNAs. *Planta*, **181**, 462–466.
- Olsen, O.-A., Potter, R.H. and Kalla, R.** (1992) Histo-differentiation and molecular biology of developing cereal endosperm. *Seed Sci. Res.* **2**, 117–131.
- Paz-Ares, J., Ghosal, D., Wienand, U., Peterson, P.A. and Saedler, H.** (1987) The regulatory *c1* locus of *Zea mays* encodes a protein with homology to myb proto-oncogene products and with structural similarities to transcriptional activators. *EMBO J.* **6**, 3553–3558.
- Pogson, B.J., Ashford, A.E. and Gubler, F.** (1989) Immunofluorescence localization of  $\alpha$ -amylase in the scutellum, germ aleurone and “normal” aleurone of germinated barley grains. *Protoplasma*, **151**, 128–136.
- Pyee, J., Yu, H. and Kolattukudy, P.E.** (1994) Identification of a lipid transfer protein as the major protein in the surface wax of broccoli (*Brassica oleracea*) leaves. *Arch. Biochem. Biophys.* **311**, 460–468.
- Rohde, W., Dörr, S., Salamini, F. and Becker, B.** (1991) Structure of a chalcone synthase gene from *Hordeum vulgare*. *Plant Mol. Biol.* **16**, 1103–1106.
- Roth, B.A., Goff, S.A., Klein, T.M. and Fromm, M.E.** (1991) *C1*- and *R*-dependent expression of the maize *Bz1* gene requires sequences with homologies to mammalian myb and myc binding sites. *Plant Cell*, **3**, 317–325.
- Sambrook, L., Fritsch, E.F. and Maniatis, T.** (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Schmelzer, E., Jähnen, W. and Hahlbrock, K.** (1988) *In situ* localization of light-induced chalcone synthetase mRNA, chalcone synthetase, and flavonoid end products in epidermal cells of parsley leaves. *Proc. Natl Acad. Sci. USA*, **85**, 2989–2993.
- Selden, R.F.** (1987) Analysis of RNA by Northern hybridization. In *Current Protocols in Molecular Biology* (Ausubel, F.M. et al., eds). New York: Green Publishing Associates and Wiley-Interscience, pp. 4.9.1–4.9.8.
- Shimamoto, K., Terada, R., Izawa, T. and Fujimoto, H.** (1989) Fertile transgenic rice plants regenerated from transformed protoplasts. *Nature*, **338**, 274–276.
- Skriver, K., Leah, R., Müller-Urli, F., Olsen, F.-L. and Mundy, J.** (1992) Structure and expression of the barley lipid transfer protein gene *Ltp1*. *Plant Mol. Biol.* **18**, 585–589.
- Smith, L.M., Handley, J., Li, Y., Martin, H., Donovan, L. and Bowles, D.J.** (1992) Temporal and spatial regulation of a novel gene in barley embryos. *Plant Mol. Biol.* **20**, 255–266.
- Somssich, I.E., Schmelzer, E., Kawalleck, P. and Hahlbrock, K.** (1988) Gene structure and *in situ* transcript localization of the pathogenesis-related protein 1 in parsley. *Mol. Gen. Genet.* **213**, 93–98.
- Sossountzov, L., Ruiz-Avila, L., Vignols, F., et al.** (1991) Spatial and temporal expression pattern of a maize lipid transfer protein gene. *Plant Cell*, **3**, 923–933.
- Sterk, P., Boel, H., Schellekens, G.A., Van Kammen, A. and De Vries, S.C.** (1991) Cell-specific expression of the carrot EP2 lipid transfer protein gene. *Plant Cell*, **3**, 907–921.
- Tchang, F., This, P., Stiefel, V., et al.** (1988) Phospholipid transfer protein: Full-length cDNA and amino acid sequence in maize. *J. Biol. Chem.* **263**, 16 849–16 855.
- Terada, R., Nakayama, T., Iwabuchi, M. and Shimamoto, K.** (1993) A wheat histone H3 promoter confers cell division-dependent and -independent expression of the *gusA* gene in transgenic rice plants. *Plant J.* **3**, 241–252.
- Thoma, S., Kaneko, Y. and Somerville, C.** (1993) A non-specific lipid transfer protein from *Arabidopsis* is a cell wall protein. *Plant J.* **3**, 427–436.
- Watanabe, S. and Yamada, M.** (1986) Purification and characterization of a non-specific lipid transfer protein from germinated castor bean endosperms which transfers phospholipids and galactolipids. *Biochim. Biophys. Acta*, **876**, 116–123.

The EMBL Data Library accession number X69793 (*Ltp2*). Please note that since the entry of this EMBL submission (23 December 1992), the designation *Ltp2* has been used for another nsLTP cDNA clone from barley (Molina and García-Olmedo, 1993).