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(54) **PROMOTEUR D'ALEURONE DU GRAIN**

(54) **WHEAT ALEURONE PROMOTER**

(57) This invention is directed to a promoter obtained from a wheat aleurone gene LtpW1. This promoter, or truncated derivatives of this promoter, can be used to express heterologous genes of interest within the aleurone cells of a plant. This invention is also directed to vectors comprising these promoters operatively linked with a heterologous gene of interest, as well as plant cell cultures and transgenic plants comprising these vectors. A method for the preparation of a plant using the promoters of this invention are also disclosed. Furthermore, this invention is directed to a truncated LtpW1 promoter that exhibits constitutive activity within plants.

ABSTRACT OF THE DISCLOSURE

This invention is directed to a promoter obtained from a wheat aleurone gene LtpW1. This promoter, or truncated derivatives of this promoter, can be used
5 to express heterologous genes of interest within the aleurone cells of a plant. This invention is also directed to vectors comprising these promoters operatively linked with a heterologous gene of interest, as well as plant cell cultures and transgenic plants comprising these vectors. A method for the preparation of a plant using the promoters of this invention are also disclosed. Furthermore, this invention is directed to a
10 truncated LtpW1 promoter that exhibits constitutive activity within plants.

WHEAT ALEURONE PROMOTER**BACKGROUND OF THE INVENTION**

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The present invention relates to plant gene promoters and their use in the expression of genes of interest. More specifically, the present invention relates to the use of an aleurone promoter for organ and tissue specific expression of a gene of interest within aleurone tissues of plants.

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The endosperm of a seed is the site of deposition of storage products such as starch and proteins used by the developing embryo during germination. The endosperm surrounds the embryo of developing and mature cereal seeds. The endosperm comprises a peripheral layer of aleurone cells, which are specialized secretory cells.

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During germination, the aleurone layer is involved in the transfer of metabolites from the transport system to the endosperm. Furthermore, several antimicrobial compounds required to protect the seed during dormancy, imbibition and germination are synthesized within this tissue. The aleurone cells differentiate from primary endosperm cells 10-21 days after fertilization.

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Several aspects of hormonal regulation of gene transcription within aleurone tissue, in germinating barley seeds have been well characterized (Fincher 1989, Annu. Rev. Plant Physiol. Mol. Biol. 40:305-346). For example, genes encoding α -amylase, responsible for the digestion of the starch stored within the starchy endosperm, and β -glucanase, which digests the cell walls, have been isolated and characterized (WO 90/01551 Rogers; US 5,677,474 Rogers, issued October 14, 1997; Karrer et al 1991, Plant Mol. Biol.16:797-805; Slakeski and Fincher 1992). Furthermore, several structural and regulatory genes involved in anthocyanin biosynthesis within the aleurone have been isolated and characterized (Paz-Ares et al 1987, EMBO J. 5:829-833; Dellaporta et al 1988, pp263-282 18th Stadler Genet. Symp. ed. J.P. Gustafsan and R.Appels). Other genes representing differentially expressed transcripts

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- 2 -

within aleurone layers have also been reported including CHI26 (Lea et al 1991, J. Biol. Chem. 266:1564-73); pZE40 (Smith et al 1992, Plant Mol. Biol. 20:255-66); pHvGS-1, and pcHth3 (Heck and Ho 1996, Plant Mol. Biol. 30:611-23). Several genes encoding lipid transfer proteins (Ltp) have also been obtained from barley
5 aleurone tissues, including B11E- barley *Ltp1*, and B12A- barley *Ltp2* (Jakobsen et al 1989, Plant Mol. Biol. 12: 285-93). Only one of these genes, B12A, has been expressed ectopically in transgenic plants. In this case the promoter is active only during seed development (Kalla et al 1994 Plant J. 6:849-860)

10 Lipid transfer proteins are responsible for the transfer of phospholipids between membranes *in vitro*, and likely play a role during membrane biogenesis. This may be especially important in aleurone cells which are known to develop extensive membrane systems. Skriver et al (1992, Plant Mol.Biol. 18: 585-589) disclose the sequence of a genomic Ltp (*Ltp1*), including the promoter region, from barley. Northern analysis
15 demonstrated that this gene was specifically expressed in developing and germinating seeds, as well as in whole seeds and aleurone layers obtained from seeds 30 days post anthesis (dpa). No expression of *Ltp1* mRNA was observed in root, leaf, or shoot tissues, or coleoptiles of germinating seeds. Linnestad et al (1991, Plant Physiol 97: 841-843) also discloses the promoter sequence of the *Ltp1* promoter from barley which
20 was obtained using barley cDNA B12A as a probe. The *Ltp1* promoter, as well as a modified form of this promoter is disclosed in WO 95/23230 (February 23, 1995; Olsen et al). The modified form of the *Ltp1* promoter was not specific to directing expression within aleurone cells, and was active in a range of plant organs and tissues including aleurone cells, scutellar epithelial tissue and vascular tissue during
25 germination or in the plant, including root, leaves and stem.

The promoter of B12A (also termed *Ltp2*) directs expression specifically within the aleurone layer of developing grain as determined using transgenic cereal plants (Kalla et al 1994, Plant J. 6: 849-860). The sequence of the *Ltp2* promoter is disclosed
30 in CA 2,110,772 (filed December 6, 1993, Olsen and Kalla) and US 5,525,716 (Kalla et al). Dieryck et al disclose the incomplete cDNA sequence of a wheat (*Triticum*

- 3 -

durum) Ltp (pTd4.90). Ltp genes comprise a multigenic family and are ubiquitous in plants. Unfortunately as these genes or corresponding proteins have been isolated from various species there is no uniformity in the terminology used to identify the genes. Hence Ltp1 from tobacco, barley and Arabidopsis are not the same. As well, two
 5 barley Ltp2 genes are described in the literature; barley Ltp2 as described in Molina and Garcia-Olmedo (Plant J. 4:983-991) is a leaf Ltp, while barley Ltp2 as described in Kalla et al (1994 Plant J. 6:849-860) is aleurone specific.

It is desirable to provide promoters capable of controlling aleurone specific
 10 expression that is not detrimental to the developing embryo and seedling. Aleurone-specific promoters may be used for the regulation of the expression of heterologous or native genes within aleurone tissue of cereal seeds in order to modify grain development and germination. For example, placing genes of interest under the control of aleurone-specific promoters may be used to:

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- 1) mediate the unloading of metabolites from the transport system into the endosperm, since this metabolite unloading is processed through aleurone cells. By expressing genes of interest involved in this process specifically within the aleurone, the grain yield may be affected. For example, which is not to be
 20 considered limiting in any manner, these genes of interest may include Na⁺ and K⁺ ATPases functioning in active transport, modifiers of membrane pore exclusion parameters such as TMV movement proteins, invertase for sucrose transport etc.;
- 25 2) affect the quality of the grain, through the production of specific proteins or enzymes, lipids, secondary metabolites etc. and their secretion into the endosperm during endosperm development or endosperm digestion. For example, which is not to be considered limiting in any manner, such proteins may include starch synthase, ADP glucose pyrophosphorylase, monoclonal
 30 antibodies, glutenins, anticoagulants (eg hirudin), anti-pathogenic phenolics etc.. Furthermore, expression of a gene of interest within the aleurone may also be

- 4 -

used in order to express proteins for nutritional or medicinal purposes for feeding to animals or humans;

5 3) regulate pre-harvest sprouting by affecting dormancy, for example which is not to be considered limiting, by over-expression of ACC synthase to induce inhibitory levels of ethylene;

10 4) enhance alcohol production- introduction of novel high temperature resistant enzymes for industrial application, including, but not limited to, thermostable amylases, pectinases and invertase;

15 5) modify disease resistance of developing and germinating grains by expressing proteins, for example but not limited to, oxalate oxidase, glucose oxidase, chitinase, or lipid transfer proteins, in combination with a suitable signal peptide for targeting to the extracellular matrix and cell wall localization. This approach can be used to modify the matrix to provide a stronger physical barrier against invading pathogens or to direct specific anti-pathogen agents to the aleurone/pericarp interface.

20 This invention characterizes a novel wheat aleurone specific promoter active during embryo development and germination and which controls expression of heterologous genes of interest within transgenic plants.

- 5 -

SUMMARY OF THE INVENTION

The present invention relates to plant gene promoters and their use in the expression of genes of interest. More specifically, the present invention relates to the use of an aleurone promoter for stage and tissue specific expression of a gene of interest within aleurone tissues of plants.

According to the present invention there is provided an isolated DNA molecule comprising at least 40 contiguous nucleotides of the nucleotide sequence of SEQ ID NO:1. Also considered as part of this invention is an isolated DNA molecule that comprises the nucleotide sequence of SEQ ID NO:1.

Furthermore, this invention provides for an isolated DNA molecule comprising at least 21 contiguous nucleotides of the nucleotide sequence comprising nucleotides 1-481 of SEQ. ID NO:1, an isolated DNA molecule comprising at least 19 contiguous nucleotides of the nucleotide sequence comprising nucleotides 1-214 of SEQ ID NO:1, an isolated DNA molecule comprising nucleotides 1-481 of the nucleotide sequence of SEQ ID NO:1, an isolated DNA molecule comprising nucleotides 1-214 of the nucleotide sequence of SEQ ID NO:1, or an isolated DNA molecule comprising nucleotides 215-481 of the nucleotide sequence of SEQ ID NO:1.

This invention is also directed to an isolated DNA molecule comprising the nucleotide sequence of SEQ ID NO:2.

This invention also encompasses an isolated DNA molecule comprising the nucleotide sequence of SEQ ID NO:3.

Also included within this invention is a vector comprising an isolated DNA molecule, comprising at least 40 contiguous nucleotides of the nucleotide sequence of SEQ ID NO:1, and a gene of interest operatively linked thereto, wherein the isolated DNA molecule controls the expression of a gene of interest. This invention is also

- 6 -

directed to a vector, comprising an isolated DNA molecule comprising at least 21 contiguous nucleotides of the nucleotide sequence comprising nucleotides 1-481 of SEQ ID NO:1, or 19 contiguous nucleotides of the nucleotide sequence comprising nucleotides 1-214 of SEQ ID NO:1, and a gene of interest operatively linked thereto.

5 This invention also embraces a vector comprising nucleotides 1-481 of the nucleotide sequence of SEQ ID NO:1, nucleotides 1-214 of the nucleotide sequence of SEQ ID NO:1, or nucleotides 215-481 of the nucleotide sequence of SEQ ID NO:1. Furthermore, this invention provides for a vector comprising the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4, controlling the
10 expression of a gene of interest operatively linked thereto.

This invention also includes a transformed plant cell culture comprising a vector as defined above.

15 This invention is also directed to a transgenic plant transformed with a vector as defined above.

Also considered an aspect of this invention is a method of expressing a gene of interest within aleurone of a plant comprising;

- 20 i) operatively linking a gene of interest for which expression is desired with a promoter obtained from wheat aleurone to produce a chimeric gene construct; and
- ii) introducing the chimeric gene construct into the plant;
- wherein the promoter comprises the nucleotide sequence of at least 40
25 contiguous nucleotides of SEQ ID NO:1.

This invention also includes the method as described above, wherein the promoter comprises the nucleotide sequence containing at least 21 contiguous nucleotides of the nucleotide sequence comprising nucleotides 1-481 of SEQ ID NO:1,
30 19 contiguous nucleotides of the nucleotide sequence comprising nucleotides 1-214 of SEQ ID NO: 1, or comprising nucleotides 1-481 of the nucleotide sequence of SEQ

- 7 -

ID NO:1, nucleotides 1-214 of the nucleotide sequence of SEQ ID NO: 1, or nucleotides 215-481, or comprising the nucleotide sequence of SEQ ID NO: 1, SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO:3, or SEQ ID NO:4.

5 This invention also embraces a constitutive promoter obtained from wheat aleurone, characterized in that the constitutive promoter:

- i) is a truncated form of a native promoter, LtpW1,
- ii) lacks tissue and stage dependant regulation associated with the native promoter LtpW1; and that
- 10 iii) the promoter is active in at least aleurone scutellum and leaf tissues.

 The nucleotide sequence of the LtpW1 promoter as disclosed herein is different from the barley Ltp1 or 2 promoters, as is the range of activity of the LtpW1 promoter. Furthermore, the activity of the truncated LtpW1 promoter within the
15 aleurone, relative to barley Ltp2)Kalla et al 1994, Plant. J. 6: 849-860), is stronger.

- 8 -

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features of the invention will become more apparent from the following description in which reference is made to the appended drawings wherein:

5 **FIGURE 1** shows Ltp expression in aleurone tissues of *Hordeum* and *Triticum* species using Northern analysis hybridized with a barley Ltp1 DIG-labelled cDNA. Figure 1(a) shows *Hordeum vulgare* at 20 dpa; Figure 1(b) shows *Triticum aestivum* at 10 dpa; Figure 1 (c) shows *T. aestivum* at 20 dpa; Figure 1 (d) shows *T. tungidum* at 10 dpa.

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FIGURE 2 shows RNA *in situ* hybridization of ³⁵S-labelled barley Ltp ribo-probe in 73h germinating, and 18 dpa developing wheat grain. Figure 2 (a) and Figure 2 (b) show 73h germinating wheat grain, and Figure 2 (c) and Figure 2 (d) show 18 dpa developing wheat grain. Figure 2 (a) and Figure 2 (c) show hybridization results using anti-sense probe; Figure 2 (b) and Figure 2 (d) show hybridization with sense probe.

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FIGURE 3 shows the DNA sequence of the genomic LtpW1 gene. The coding region is underlined (the intron is not underlined). The ATG start and TGG stop codon are in bold type. The cap site, TATA, CAT boxes are italicized and double-underlined at positions -83, -117 and -222, respectively. SEQ ID NO 1 runs from -687 to -1, SEQ ID NO 2 runs from -473 to -1, and SEQ ID NO 3 runs from -206 to -1.

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25 **FIGURE 4** shows the DNA sequence alignment of LtpW1 and barley Ltp genes. Figure 4 (a) shows alignment of LtpW1 (top row) and barley Ltp1 (bottom row); Figure 4 (b) shows alignment of LtpW1 (top row) and barley Ltp2 (Kalla et al 1994 Plant J. 6:849-860). The ATG of the Ltp genes is overlined.

30 **FIGURE 5** shows the LtpW1 promoter constructs, in all three constructs the ADH1S6 intron lies between the LtpW1 promoter and the coding region

- 9 -

of the marker gene, GUS. Figure 5(a) p687LtpW1-GUS; Figure 5(b) p473LtpW1-GUS; Figure 5(c) p206LtpW1-GUS; Figure 5(d) pLC-GUS, the promoterless control used in transient assays.

5 **FIGURE 6** shows two constructs used for comparative studies containing prior art promoters. Figure 6(a) P35s-GUS, Figure 6(b) pACT-GUS.

10 **FIGURE 7** shows transient expression of LtpW1 promoter - GUS (p687LtpW1-GUS) fusion in aleurone of cereal grains delivered by microprojectile bombardment. Figure 7(a) shows *T. aestivum* at 15 dpa; Figure 5(b) shows *Zea mays* at 13 dpa, and Figure 7(c) shows *H. vulgare* at 12 dpa.

15 **FIGURE 8** shows GUS expression in aleurone layer (arrowed) of 3 days germinated kernel of *Z. mays*, T1 self progeny, transformed with p473LtpW1-GUS fusion.

DESCRIPTION OF PREFERRED EMBODIMENT

5 The present invention relates to plant gene promoters and their use in the expression of genes of interest. More specifically, the present invention relates to the use of an aleurone promoter for stage and tissue specific expression of a gene of interest within aleurone tissues of plants.

10 In the context of this disclosure, the term promoter or promoter fragment or promoter region refers to a sequence of DNA, usually upstream (5') to the coding sequence of a structural gene, which controls the expression of the coding region by providing the recognition for RNA polymerase and/or other factors required for transcription to start at the correct site.

15 Described below is a genomic Ltp sequence obtained from wheat termed LtpW1 (SEQ ID NO:4). The coding region of the LtpW1 gene sequence exhibits about an 85% homology with barley Ltp1, and includes a 26 amino acid transit peptide. The promoter of a wheat lipid transfer protein (Ltp) gene, LtpW1, has been isolated and characterized. This promoter comprises a novel oligonucleotide sequence (SEQ ID
20 NO:1), which is active in aleurone of wheat, maize and barley. The full length promoter is not active in leaf, root, or coleoptile tissues. The promoter region of LtpW1 compared to the barley Ltp1 promoter has 43% sequence similarity with the majority of sequence similarity (82%) occurring within 140 nucleotides upstream of the transcriptional start site (see Figure 4(a)). A minor sequence similarity was noted
25 between LtpW1 and a barley amylase protease inhibitor, however, no sequence similarity of any significance was observed between LtpW1 and Ltp2, or other known Ltp promoter sequences.

The full length LtpW1 promoter (687 nucleotides; p687LtpW1; SEQ ID NO:1),
30 or a truncated LtpW1 promoter, p473LtpW1 (SEQ ID NO:2; comprising a 473 nucleotide fragment of the full length promoter), can be used to drive the expression

- 11 -

of a gene of interest within the aleurone layer of a developing and germinating seed of a monocotyledonous plant, for example, but not limited to, wheat, rice, barley and maize.

5 LtpW1 exhibits 8.8% of 35S activity and 12.2 % activity of the strong rice action monocot constitutive promoter (Table 2, Example 3). Comparison of histological evidence of expression of Ltp2 (Kalla et al 1994, Plant J. 6:849-860)), with Figure 7 of the present invention (histological evidence of LtpW1 activity) indicates that LtpW1 is more than two times stronger than Ltp2.

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Experiments with deletions of the LtpW1 promoter indicate that the 473 nucleotide fragment (SEQ ID NO: 2; p473LtpW1) of the full length promoter is more active in aleurone tissue than the 687 base pair promoter, (SEQ ID NO: 1),
15 p687LtpW1 (Table 3). However, neither the full length promoter, nor the 473 bp truncated promoter were active in leaf tissue (see Table 4). A truncated promoter comprising a 206 bp nucleotide fragment (SEQ ID NO:3, p206LtpW1) of the full length promoter was active in aleurone, leaf, and scutellum tissue, functioning as a minimal promoter element. This 206 bp region therefore represents a novel,
20 potentially constitutive, promoter for monocotyledonous plants.

Together, these data indicate that nucleotides 1-214 and 215-481 of SEQ ID NO: 1 are responsible for imparting tissue specificity within this promoter, since once the nucleotides 1-481 are removed from the full length promoter, tissue specificity is
25 lost (Table 4). It is contemplated that either of these regions may be combined with any suitable promoter of interest, for example, which is not to be considered limiting, a minimal, constitutive, or viral promoter etc. in order to obtain aleurone-specific expression of a gene linked thereto. Both of these regions were found to comprise very low sequence similarity with other sequences present within gene sequence databases
30 such as GenBank.

- 12 -

Furthermore, the data presented in Table 3 indicates that the region comprising nucleotides 1-481 of SEQ ID NO: 1 is responsible for regulating the strength of promoter activity, and includes both silencer- and enhancer-type activities. For example, the fragment comprising nucleotides 215-481 of SEQ ID NO:1 may be used
5 as an enhancer like element as constructs comprising this region (e.g. p473LtpW1) resulted in increased expression when compared with either the full length promoter (p687LtpW1) or the truncated promoter p206LtpW1 (see Table 3). Similarly, nucleotides 482-687 also exhibit enhancer-type activity, since constructs comprising this region (p206LtpW1) exhibited higher levels of expression than the full length
10 promoter. Therefore, it is contemplated that nucleotides 214-481, or 482-687 may be combined with any suitable promoter of interest, for example, which is not to be considered limiting, a minimal, constitutive, or viral promoter etc., in order to obtain both aleurone-specific expression of a gene linked thereto, as well as increased gene expression.

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Similarly, the fragment comprising nucleotides 1-214 comprises silencer-type elements as constructs comprising this region (e.g. p687LtpW1) result in lower levels of expression compared with the levels of expression obtained with either of the truncated promoter constructs, p206LtpW1, or p473LtpW1 (see Table 3). It is
20 contemplated that nucleotides 1-214 may be combined with any suitable promoter of interest, for example, which is not to be considered limiting, a minimal, constitutive, or viral promoter etc., in order to obtain both aleurone-specific expression of a gene linked thereto, along with reduced gene expression.

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The truncated promoter, p473LtpW1, was used to transform maize, where it was noted that this promoter was active only in aleurone of developing and germinating cereal grain.

By "constitutive promoter" it is meant a promoter that directs the expression
30 of a gene throughout the various parts of a plant and continuously throughout plant

- 13 -

development. Examples of known constitutive promoters include those associated with the CaM35S transcript and *Agrobacterium* Ti plasmid nopaline synthase gene.

5 The gene constructs of the present invention can also include other optional regulatory motifs such as enhancers, either translation or transcription enhancers, as may be required. These enhancer regions are well known to persons skilled in the art, and can include, for example, the enhancer region of the 35S regulatory region, as well as other enhancers obtained from other regulatory regions, and/or the ATG initiation codon and adjacent sequences. The initiation codon must be in phase with the reading
10 frame of the coding sequence to ensure translation of the entire sequence. The translation control signals and initiation codons can be from a variety of origins, both natural and synthetic. Translational initiation regions may be provided from the source of the transcriptional initiation region, or from the structural gene. The sequence can also be derived from the promoter selected to express the gene, and can be specifically
15 modified so as to increase translation of the mRNA.

To aid in identification of transformed plant cells, the constructs of this invention may be further manipulated to include plant selectable markers. Useful selectable markers include, but are not limited to, enzymes which provide for
20 resistance to an antibiotic such as gentamycin, hygromycin, kanamycin, and the like. Similarly, enzymes providing for production of a compound identifiable by colour change such as *GUS* (β -glucuronidase), or luminescence, such as luciferase are useful.

Also considered part of this invention are transgenic plants containing the
25 chimeric gene construct of the present invention. Methods of regenerating whole plants from plant cells are known in the art, and the method of obtaining transformed and regenerated plants is not critical to this invention. In general, transformed plant cells are cultured in an appropriate medium, which may contain selective agents such as antibiotics, where selectable markers are used to facilitate identification of
30 transformed plant cells. Once callus forms, embryo or shoot formation can be encouraged by employing the appropriate plant hormones in accordance with known

- 14 -

methods and the shoots transferred to rooting medium for regeneration of plants. The plants may then be used to establish repetitive generations, either from seeds or using vegetative propagation techniques.

5 The constructs of the present invention can be introduced into plant cells using Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, particle bombardment, micro-injection, electroporation, etc, the techniques of which are known to one of skill in the art. For reviews of such techniques see for example Weissbach and Weissbach (1988) and Geierson and Corey (1988).

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While not to be considered limiting in any manner, the following examples are provided in order to exemplify embodiments of the present invention.

Example 1: Localization of Ltp1 Expression

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In order to isolate genes which are functional in aleurone of developing and germinating wheat grain, a barley cDNA probe of an aleurone specific lipid transfer protein gene (Ltp1,) was used to indicate activity of similar genes in wheat aleurones during seed development. Northern blot analyses using a DIG -labelled barley cDNA probe showed that Ltp transcripts were present in aleurone tissue 20 dpa (Figures 1 (a) and 1(c)). No activity was detected in early wheat grain development, 10 dpa (Figure 1(b) but could be detected in *T turgidum* (Fig. 1(d)).

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In situ hybridization (based on a modification of the procedure outlined in Cox and Goldberg, 1998, Analysis of Plant Gene Expression, In Plant Molecular Biology. A Practical Approach, pp. 1-34) performed on cross sections of developing and germinating grain showed that Ltp expression was limited to aleurone cells. A ³⁵S Ltp antisense ribo-probe hybridized strongly to aleurone cells (Figure.2 (a)), whereas no differential hybridization was observed with the sense RNA probe (Figure 2 (b)). Ltp expression was observed throughout grain development after 18 dpa and during germination up to 73 h post-imbibition at which time the endosperm was depleted. No

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- 15 -

hybridization was observed in developing endosperm, embryo or pericarp tissues (data not shown).

Example 2: Genomic DNA of LtpW1

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Genomic DNA was isolated from young leaf tissue of hexaploid wheat, (*T.aestivum*) and digested with XbaI. When this DNA was analysed by Southern blot using standard procedures and a DIG-labelled barley Ltp cDNA probe, three loci for the Ltp1 gene (at 1.5, 6.0, and 7.0 kb) were detected. The copy corresponding to the 10 1.5 kb XbaI band was cloned by screening a λ long C phage library of size-restricted XbaI fragments with a barley Ltp1 cDNA probe.

LtpW1 refers to the Ltp gene contained within the 1.5 kb XbaI digested *T. aestivum* genomic clone, the sequence of which is shown in SEQ ID NO: 4 (also see 15 Figure 3).

The coding sequence of LtpW1 shares 85% DNA identity with the barley Ltp1 (Figure 4 (a)), includes a 26 amino acid transit peptide for cell wall localization of the protein, and has one predicted 88bp intron which is 44 bp shorter than the equivalent 20 barley intron. The nucleotide sequences LtpW1 and barley Ltp 1 promoter (Linnestad 1991) are well conserved for approximately 140 bp upstream of the ATG start codon whereupon they diverge considerably (Figure 4 (a)). The conserved promoter region includes the putative cap and TATA sites but not the proposed CAT site or other regulatory elements (see Figures 3, and 4 (a)).

25

The nucleotide sequence of the LtpW1 promoter exhibits little or no identity with the barley Ltp2 promoter (Figure 4 (b)). The LtpW1 promoter was shown to be active in aleurone of developing and germinating cereal grain which is uniquely different from the barley Ltp2 promoter which is only active during grain development 30 but not during germination (Kalla et al 1994).

Example 3: Expression of GUS under the control of LtpW1 aleurone promoters.**p687LtpW1-GUS**

5 A 687 bp XbaI/BclII promoter fragment (SEQ ID NO: 1; Figure3) was subcloned from pLtpW1 and fused to a GUS promoterless reporter cassette (pLC-GUS). pLC-GUS was obtained by removing the 35S promoter as a SacI fragment from pZO1016 (designated p35S-GUS herein), which was a gift from R. Sinibaldi, Sandoz, Ca. A 687 bp XbaI/BclII fragment was isolated from pTALP1 (containing the
10 1.5 kb XbaI-digested *T. aestivum* genomic clone) and the sticky ends were filled-in with Klenow fragment of DNA polymerase. This fragment was blunt-end ligated into the SmaI site of pLCGUS (see Figure 5(d)), and the orientation of the insert was checked by digesting with BamHI. The activity of this promoter was compared with that of the promoterless construct (pLC-GUS) as well as to constructs comprising
15 constitutive CaMV35S and rice action promoters (see Figures 6 (a) and (b), respectively). These constructs were used for comparison of promoter activities. The 35S promoter is described in: Odel, J.T., Nagy, F. and Chua N-H (1985) Nature 313:810-812. The rice action promoter is described in: McElroy D., Zhang, W. Cao, J. and Wu, R. (1990) Plant Cell 2:163-171.

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 These constructs were introduced into the aleurone of cereal grains by microparticle bombardment using standard methods. LUC and GUS constructs were co-bombarded in equimolar amounts and GUS is expressed relative to LUC to minimize variability between reps (shots). LUC activity serves as an internal control
25 for the shot to shot variability.

 Tissues, 48 h post-bombardment, were incubated in reaction buffer containing 50mM NaH₂PO₄ (pH 7.0), 10 mM EDTA and 1 mM 5-bromo-4-chloro-3-indolyl-B-glucoronide (X-Gluc), 0.5mMK₃[Fe(CN)₆], 0.5 mM K₄[Fe(CN)₆] at 37°C for 4-20 h.
30 A blue precipitate in the bombarded cells indicates activity of B-glucoronidase. The promoter gave high expression of GUS in histological transient assays with wheat

- 17 -

aleurones (Figure 7(a)). Activity was also demonstrated in maize and barley aleurones (Figure 7(b) and (c)) The 687 bp promoter fragment showed no activity in leaf, root or coleoptile tissues of wheat (data not shown).

5 In quantitative expression assays in wheat aleurone the 687 bp promoter had 3.4% of the activity of the constitutive 35S promoter (Table 1). This underestimates the relative aleurone-directed activity of the LtpW1 promoter because of additional endosperm-derived activity of the constitutive 35S promoter.

10 **Table 1: Activity of p687LtpW1 in 12 dpa wheat aleurone**

Construct	Luciferase (mv/sec/mg/ protein)	GUS (pmol MU/min/mg protein)	GUS/LUC	%35S Activity
Au	200 ¹	0	0	-
pLC-GUS ² /p35S-LUC	2100	0	0	-
15 p35S-GUS/p35S-LUC	3400	30072	8.84	-
p687LtpW1-GUS/p35S-LUC	4200	1247	0.30	3.4

1: mean of three sets of bombardments

2: promoterless construct

20

P473LtpW1-GUS

A truncated version of the LtpW1 promoter (see SEQ ID NO:2; Figure 3) was
 25 prepared by digesting pTALTP1 with HincII and BelI, and the resulting 0.47kb
 fragment (after treatment with Klenow) was ligated into the SmaI site of pLC-GUS.
 Orientation of the insert was checked by digesting the resulting recombinant plasmid
 with BamHI. The construct (p473LtpW1-GUS) thus obtained showed 8.8% and
 12.2% activity of the constitutive 35S and rice action promoters, respectively (pAct-
 30 GUS was a gift from Ray Wu at Cornell). See Table 2 for results.

Table 2: Activity of p473LtpW1 in 12 dpa wheat aleurone

	Construct	Luciferase	GUS	GUS/LUC	%35S
		(mv/sec/mg/protein)	(pmol MU/min/mg protein)		Action Activity
5	Au	200 ¹	0	0	-
	pLC-GUS ² /p35S-LUC	1300	0	0	-
	p35S-GUS/p35S-LUC	3500	8077	2.31	-
	pAct-GUS/p35S-LUC	3300	5524	1.67	
	p473LtpW1-GUS/p35S-LUC	3200	651	0.20	8.8 12.2

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1: mean of three sets of bombardments

2: promoterless construct

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When compared within a single experiment, the 473 bp fragment was 170% as active as the 687 bp fragment (Table 3).

Table 3: Activity of p687LtpW1, p473LtpW1, and p206LtpW1 in 7 dpa wheat aleurone

	Construct	Luciferase	GUS	GUS/LUC	%35S
		(mv/sec/ mg/protein)	(pmol MU/min/mg protein)		Activity
5	pLC-GUS ² /p35S-LUC	9700 ¹	120	0.01	0.1
	p35S-GUS/p35S-LUC	2300	18305	7.96	-
	p206LtpW1-GUS/p35S-LUC	7900	1781	0.23	2.9
	p473LtpW1-GUS/p35S-LUC	6800	2399	0.35	4.4
	p687LtpW1-GUS/p35S-LUC	5100	1090	0.21	2.6

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1: mean of three sets of bombardments

2: promoterless construct

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P206LtpW1-GUS

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To generate P206LtpW1-GUS, pTALTP1 was digested with Bcl1, then with HindIII, and the 0.2 kb fragment was isolated from a gel and purified. The sticky ends were filled in with Klenow and the resulting fragment was ligated into the Sma1 site of pLC-GUS. Neither the 687 bp or 473 bp promoters was active in leaf tissue but the 206 bp HinII/BclII truncated promoter (SEQ ID NO:3; Figure 3) had 7.5% the activity of the 35S promoter in leaf (Table 4).

- 20 -

Table 4: Activity of p687LtpW1, p473LtpW1, and p206LtpW1 in wheat leaf tissue

5	Construct	Luciferase	GUS	GUS/LUC	%35S Activity
		(v/sec/mg/ protein)	(pmol MU/min/mg protein)		
	pLC-GUS ² /p35S-LUC	200 ¹	1.6	0.007	0.7
	p35S-GUS/p35S-LUC	200	204.3	1.020	-
	p206LtpW1-GUS/p35S-LUC	200	15.3	0.077	7.5
	p473LtpW1-GUS/p35S-LUC	700	1.3	0.002	0.2
10	p687LtpW1-GUS/p35S-LUC	700	1.7	0.002	0.2

1: mean of three sets of bombardments

2: promoterless construct

15

Similarly, in wheat scutellum tissue, only the 206 bp promoter fragment was active (Table 5) with activities of 11.4% of 35S and 8.5% of rice actin promoters.

Table 5: Activity of p687LtpW1, p473LtpW1, and p206LtpW1 in 20 dpa wheat scutellum tissue

5	Construct	Luciferase (v/sec/mg/ protein)	GUS (pmol MU/min/mg protein)	GUS/LUC	%35S Action Activity
	pLC-GUS ² /p35S-LUC	1300 ¹	37	0.028	0.23,0.17
	p35S-GUS/p35S-LUC	400	4873	12.182	-
	pAct-GUS/p35S-LUC	400	6530	16.325	-
	p206LtpW1-GUS/p35S-LUC	100	139	1.39	11.41, 8.51
10	p473LtpW1-GUS/p35S-LUC	200	2	0.01	0.08, 0.06
	p687LtpW1-GUS/p35S-LUC	200	6	0.03	0.24, 0.18

1: mean of three sets of bombardments

2: promoterless construct

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Thus the nucleotide sequence between 206 bp and 473 bp determines the tissue and stage dependent regulation of the LtpW1 promoter.

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Collectively, these data indicate that:

- nucleotides 1-214 and 215-487 of SEQ ID NO:1 are responsible for imparting tissue specificity within this promoter. Removal of either of these regions from the full length promoter results in greatly reduced tissue specificity (Table 4).

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- the region comprising nucleotides 1-487 of SEQ ID NO: 1 is responsible for regulating the strength of promoter activity, and includes both silencer- and enhancer-type activities:

- the fragment comprising nucleotides 214-481 of SEQ ID NO:1

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exhibits enhancer-like activity as constructs comprising this

- 22 -

5 region (e.g. p473LtpW1) resulted in increased expression when compared with either the full length promoter (p687LtpW1), or the truncated promoter p206LtpW1 (see Table 3). Similarly, nucleotides 482-687 also exhibit enhancer-type activity, since constructs comprising this region (p206LtpW1) exhibited higher levels of expression than the full length promoter;

- 10 • the fragment comprising nucleotides 1-214 comprises silencer-type elements as constructs comprising this region (e.g. p687LtpW1) result in lower levels of expression compared with the levels of expression obtained with either of the truncated promoter constructs, p206LtpW1, or p473LtpW1 (see Table 3);
- 15 • the 206 bp version of the LtpW1 promoter represents a novel, potentially constitutive promoter, for monocotyledonous plants.

Because of the relatively superior activity of the 473 bp fragment in aleurone tissue (Tables 1,2 and 3), this version was used for transformation of maize.

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Example 4: Preparation of transgenic plants of *Zea mays*

To verify that the 5' flanking sequence from the genomic clone LtpW1 contained the regulatory sequences required to confer expression in aleurone cells, the 473 bp LtpW1 /GUS fusion was co-bombarded with a bialaphos selectable plasmid (pAHC25) into embryogenic cultures of maize. Transgenic calli were selected on bialaphos media and transgenic plants regenerated. The transgenic plants were screened for GUS activity. The 473 bp LtpW1 promoter directed the expression of GUS only in the aleurone layer of developing and germinating transgenic maize kernels (Figure 6).

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- 23 -

The present invention has been described with regard to preferred embodiments. However, it will be obvious to persons skilled in the art that a number of variations and modifications can be made without departing from the scope of the invention as described herein.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(C) CITY: Nepean
(D) STATE: Ontario
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(F) POSTAL CODE (ZIP): K2E 6K9

(ii) TITLE OF INVENTION: Wheat Aleurone Promoter

25

(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(v) CURRENT APPLICATION DATA:

APPLICATION NUMBER: CA 2,230,975

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 687 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

(B) CLONE: LtpW1

(ix) FEATURE:

- (A) NAME/KEY: promoter
- (B) LOCATION:1..687

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CTAGAGAAAG AGTTT TAGAC CGGAGGTATT TGTTAGGAAG TACTTCTTGC CATACTAGTT

60

TCAATAAAGT AGCTTGAAAA GACATTTGTT AAGCAACCAT GTGTTTTTAA TATGAAGATC	120
CTCAATACCG AGAGCCTTTG GTCCCATGGA TGACACAAAA CTTCCCCTT GTTTTTTTTT	180
TTTGTGTGTG TGTGGGTAAA CTTCCCCTT GGTTAACCTA TACTTCGCT TATGTTTCATC	240
ACTTTGCCAG AAAATTGCAT ATGTGAAGGA AGTGCCAATA TTTAATACCG TCTGGTGTTA	300
TAAATTCATC TCCCAAATT ATTGGAGTTG AAGATTCCTT TGAAAAATA ATTTGACATA	360
TTAAAGATGT TGCCCTTGCG CGGGGTATCT GCAAATTGAG GATCCAAGGG ACGATTGCAT	420
CCAGTTCTAA ACACACCATT ATGATTTTCTG TGATAATGCA TGCTTCCAAA GCCCAGCTGC	480
AAGCTTGGGC CATCCTTCGG AAGGGAAAAA GAAAAGGGG TCCTGCTGCA CCAGCGACTA	540
AACCATCCAC GCATCTCTCG CTCGAACCCC TATTTAAGCC CCTCCATTCT TCCCTACATT	600
CTCCACACAA CCACGAGTTG CTCATCTCTC CACCCAATCA TCACTAGCTA ATACGGTGCA	660
CTGTTAGCTA CAGACCAAGA AGTGATC	687

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 473 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

(B) CLONE: LtpW1

(ix) FEATURE:

(A) NAME/KEY: promoter

(B) LOCATION:1..473

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

AACCTATACT TCCGCTTATG TTCATCACTT TGCCAGAAAA TTGCATATGT GAAGGAAGTG	60
CCAATATTTA ATACCGTCTG GTGTTATAAA TTCATCTCCC AAAATTATTG GAGTTGAAGA	120
TTCACCTGAA AAAATAATTT GACATATTAA AGATGTTGCC CTTGCGCGGG GTATCTGCAA	180
ATTGAGGATC CAAGGGACGA TTGCATCCAG TTCTAAACAC ACCATTATGA TTTCAGTGAT	240
AATGCATGCT TCCAAAGCCC AGCTGCAAGC TTGGGCCATC CTTCGGAAGG GAAAAAGAAA	300
AAGGGGTCCT GCTGCACCAG CGACTAAACC ATCCACGCAT CTCTCGCTCG AACCCCTATT	360
TAAGCCCCTC CATTCTTCCC TACATTCTCC ACACAACCAC GAGTTGCTCA TCTCTCCACC	420
CAATCATCAC TAGCTAATAC GGTGCACTGT TAGCTACAGA CCAAGAAGTG ATC	473

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 206 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

(B) CLONE: LtpW1

(ix) FEATURE:

(A) NAME/KEY: promoter

(B) LOCATION:1..206

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

AGCTTGGGCC ATCCTTCGGA AGGGAAAAAG AAAAAGGGGT CCTGCTGCAC CAGCGACTAA	60
ACCATCCACG CATCTCTCGC TCGAACCCTT ATTTAAGCCC CTCCATTCTT CCCTACATTC	120
TCCACACAAC CACGAGTTGC TCATCTCTCC ACCCAATCAT CACTAGCTAA TACGGTGCAC	180
TGTTAGCTAC AGACCAAGAA GTGATC	206

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1469 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

(B) CLONE: LtpW1

(ix) FEATURE:

(A) NAME/KEY: promoter

(B) LOCATION:1..687

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TCTAGAGAAA GAGTTT TAGA CCGGAGGTAT TTGTTAGGAA GTACTTCTTG CCATACTAGT	60
TTCAATAAAG TAGCTTGAAA AGACATTTGT TAAGCAACCA TGTGTTTTTA ATATGAAGAT	120
CCTCAATACC GAGAGCCTTT GGTCCCATGG ATGACACAAA ACTTCCCCTT TGTTTTTTTT	180
TTTTGTGTGT GTGTGGGTAA ACTTCCCCTT TGGTTAACCT ATACTTCCGC TTATGTTTCAT	240
CACTTTGCCA GAAAATTGCA TATGTGAAGG AAGTGCCAAT ATTTAATACC GTCTGGTGTT	300
ATAAATTCAT CTCCCAAAT TATTGGAGTT GAAGATTCAC TTGAAAAAAT AATTGACAT	360
ATTAAAGATG TTGCCCTTGC GCGGGGTATC TGCAAATTGA GGATCCAAGG GACGATTGCA	420
TCCAGTTCTA AACACACCAT TATGATTTC A GTGATAATGC ATGCTTCCAA AGCCCAGCTG	480
CAAGCTTGGG CCATCCTTCG GAAGGGAAAA AGAAAAAGGG GTCCTGCTGC ACCAGCGACT	540
AAACCATCCA CGCATCTCTC GCTCGAACCC CTATTTAAGC CCCTCCATTC TTCCCTACAT	600
TCTCCACACA ACCACGAGTT GTCATCTCT CCACCCAATC ATCACTAGCT AATACGGTGC	660
ACTGTTAGCT ACAGACCAAG AAGTGATCAT GGCCCGCGCT CAGGTAATGC TCATGGCCGT	720
CGCCTTGGTG CTCATGCTCG CGGCGGTCCC GCGCGCTGCC GTGGCCATCG ACTGCGGCCA	780
CGTTGACAGC TTGGTGAGAC CCTGCCTGAG CTACGTTTCAG GGCGGCCCCG GCCCGTCTGG	840

GCAGTGCTGC GACGGCGTCA AGAACCTCCA TAACCAGGCG CGATCCCAGA GCGATCGCCA	900
AAGCGCTTGC AACTGCCTCA AGGGGATCGC TCGTGGCATC CACAATCTCA ACGAGGACAA	960
CGCCCGCAGC ATCCCCCACA AGTGCGGTGT CAACCTCCCA TACACCATCA GTCTCAACAT	1020
CGACTGCAGC AGGTGATTAA TTCACATGCA AGCATATATA TATGAACACT CATCCACGTA	1080
AAATTTATTG ATATTAACAT TAATCAAATC TTTGCACTGC AGGGTGTAAT GGGCGACGAT	1140
CCGTCAAGCT GGTGCTCAGC TCATCCATCC ACGTGGAGTT GAAGCGCGCA GCCTCTATCC	1200
CTATGTAGTA TGGTCACTAG TTATGCGAGT TTATACTGAA TATGAATAAG AACTCTCTCC	1260
AGCTGGCTTG CTGGTACTCC TCTGGAGGAG ATCAGTATCT GTGTACCTGA GAGTTGAGAG	1320
TTTGTACCAT GGGCACTCCC AGTGTTTATG GACTTTAATA CATACAACTC GTTCTGTTCA	1380
GCGTGTGACT TATCTTTGTT TCCTCACGTT CGCCTGTCAT ATACTCCTTC CATCCGGTAT	1440
TAGTTGGCGT TCAAACGGAT ATATCTAGA	1469

- 31 -

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OF PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. An isolated DNA molecule comprising at least 40 contiguous nucleotides of the nucleotide sequence of SEQ ID NO:1
2. An isolated DNA molecule comprising at least 21 contiguous nucleotides of the nucleotide sequence comprising nucleotides 1-481 of SEQ ID NO:1.
3. An isolated DNA molecule comprising at least 19 contiguous nucleotides of the nucleotide sequence comprising nucleotides 1-214 of SEQ ID NO:1.
4. The isolated DNA molecule of claim 1 comprising the nucleotide sequence of SEQ ID NO:1.
5. The isolated DNA molecule of claim 1 comprising the nucleotide sequence of SEQ ID NO:2.
6. The isolated DNA molecule of claim 1 comprising the nucleotide sequence of SEQ ID NO:3.
7. The isolated DNA molecule of claim 2 comprising nucleotides 1-481 of the nucleotide sequence of SEQ ID NO:1.
8. The isolated DNA molecule of claim 3 comprising nucleotides 1-214 of the nucleotide sequence of SEQ ID NO:1.
9. The isolated DNA molecule of claim 1 comprising nucleotides 215-481 of the nucleotide sequence of SEQ ID NO:1.

- 32 -

10. A vector comprising the isolated DNA molecule of any one of claims 1 to 9 and a gene of interest operatively linked thereto, wherein said isolated DNA molecule controls the expression of said gene of interest.
11. A transformed plant cell culture comprising the vector of claim 10.
12. A transgenic plant transformed with the vector of claim 10.
13. A method of expressing a gene of interest within aleurone of a plant comprising:
 - i) operatively linking a gene of interest for which expression is desired with a promoter obtained from wheat aleurone, to produce a chimeric gene construct; and
 - ii) introducing the chimeric gene construct into said plant and allowing for expression of said gene of interest;wherein said promoter comprises the nucleotide sequence selected from any one of claims 1 to 9.
14. A constitutive promoter obtained from wheat aleurone, characterized in that said constitutive promoter:
 - i) is a truncated form of a native promoter, LtpW1,
 - ii) lacks tissue and stage dependent regulation associated with the native promoter LtpW1; and
 - iii) said promoter is active in at least aleurone scutellum and leaf tissues.

Figure 1

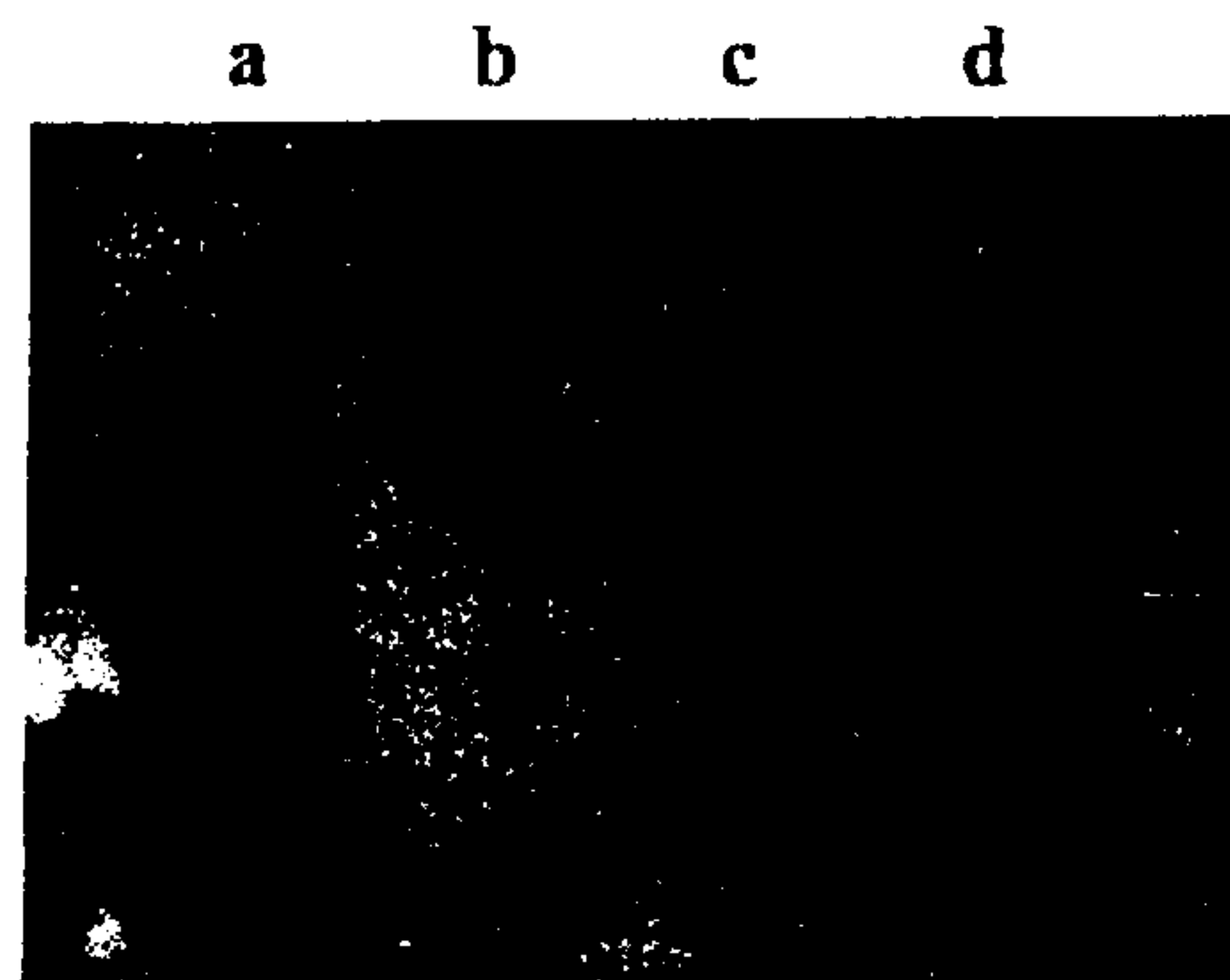


Figure 2

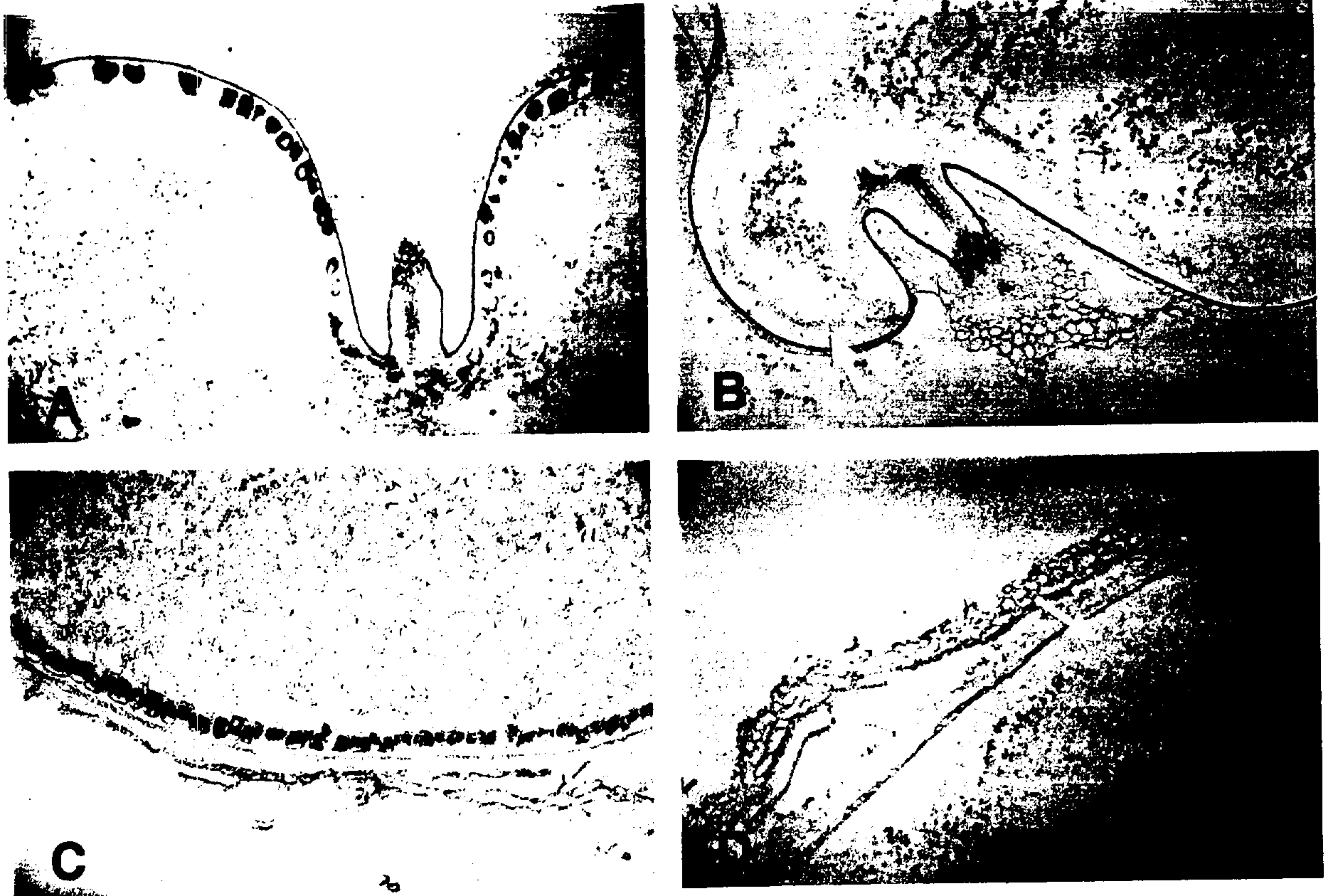


Figure 3.

*SEQ ID NO:1 (687 bp) >
-688 TCTAGAGAAAGAGTTTTAGACCGGAGGTATTTGTTAGGAAGTACTTCTTGCCATACTAGT
-628 TTCAATAAAGTAGCTTGAAAAGACATTTGTTAAGCAACCATGTGTTTTTAATATGAAGAT
-568 CCTCAATACCGAGAGCCTTTGGTCCCATGGATGACACAAAACCTCCCAGTTGTTTTTTTT
*SEQ ID NO:2 (473 bp) >
-508 TTTTGTGTGTGTGTGGGTAAACTTCCCAGTTGGTTAACCTATACTTCCGCTTATGTTTCA
-448 CACTTTGCCAGAAAATTGCATATGTGAAGGAAGTGCCAATATTTAATACCGTCTGGTGTT
-388 ATAAATTCATCTCCCAAATTATTGGAGTTGAAGATTCAGTTGAAAAATAATTTGACAT
-328 ATTAAAGATGTTGCCCTTGCGCGGGGTATCTGCAAATTGAGGATCCAAGGGACGATTGCA
-268 TCCAGTTCTAAACACACCATTATGATTTTCAAGTGATAATGCATGCTTCCAAAGCCCAGCTG
*SEQ ID NO:3 (206 bp) >
-208 CAAGCTTGGGCCATCCTTCGGAAGGGAAAAAGAAAAAGGGGTCCTGCTGCACCAGCGACT
-148 AAACCATCCACGCATCTCTCGCTCGAACCCCTATTTAAAGCCCCTCCATTCTTCCCTACAT
-88 TCTCCACACAAACCACGAGTTGCTCATCTCTCCACCCAATCATCACTAGCTAATACGGTGC
*+1
-28 ACTGTTAGCTACAGACCAAGAAGTGATCATGCCCCGCGCTCAGGTAATGCTCATGGCCGT
33 CGCCTTGGTGCTCATGCTCGCGGCGGTCCCGCGCGCTGCCGTGGCCATCGACTGCGGCCA
93 CGTTGACAGCTTGGTGAGACCCTGCCTGAGCTACGTTCAAGGGCGGCCCCGGCCCGTCTGG
153 GCAGTGCTGCGACGGCGTCAAGAACCTCCATAACCAGGCGCGATCCCAGAGCGATCGCCA
213 AAGCGCTTGCAACTGCCTCAAGGGGATCGCTCGTGGCATCCACAATCTCAACGAGGACAA
273 CGCCCGCAGCATCCCCCCTAAGTGCGGTGTCAACCTCCCATAACCATCAGTCTCAACAT
333 CGACTGCAGCAGGTGATTAATTCATATGCAAGCATATATATATGAACACTCATCCACGTA
393 AAATTTATTGATATTAACATTAATCAAATCTTTGCACTGCAGGGTGTAATGGGCGACGAT
453 CCGTCAAGCTGGTGCTCAGCTCATCCATCCACGTGGAGTTGAAGCGCGCAGCCTCTATCC
513 CTATGTAGTATGGTCACTAGTTATGCGAGTTTATACTGAATATGAATAAGAACTCTCTCC
573 AGCTGGCTTGCTGGTACTCCTCTGGAGGAGATCAGTATCTGTGTACCTGAGAGTTGAGAG
633 TTTGTACCATGGGCACTCCCAGTGTTTATGGACTTTAATACATACAACTCGTTCTGTTCA
693 GCGTGTGACTTATCTTTGTTTCCTCACGTTTCGCTGTCATATACTCCTTCCATCCGGTAT
753 TAGTTGGCGTTCAAACGGATATATCTAGA

Figure 4a

```

-679  GTTTTAGACCGGAGGTATTTGTTAGGAAGTACTTCTTGCCATACTAGTTTCAATAAAGTA
      |||| | | | | | | | | | | | | | | | | | |
-700  GTTTGATAACAAAGTAGTAAAAAACTAAAGTATTAAAAACTGCAGTAATTTTACGTGTA

-619  GCTTGAAAAGACATTTGTTAAGCAACCATGTGTTTTTAATATGAAGATCCTCAATACCGA
      | | |||| | | | | | | | | | | | | | | | |
-630  GATAGAAAATACCATGGTTTTAATATAATAATATTTTTTGCAGTATTCACAATGTAGAGA

-559  GAGCCTTTGGTCCCATGGATGACACAAAAC.....
      | | |||| | | | | |
-570  AACTGTTTGATTACGCCACATATTACTGCAGTTTAGATCGAGCAAGTACACGGGAAGAAG

-528  .....TCCCCTTGTTTTTTTTTTTTTGTGTGTGTGTGGGTAACTTCCCCTTGGT
      |||| | | |||| | | | | | | | | | |
-510  ATAACGACGTCCCACCCCTTCTTTTCGCCTTCTCTGTTTTTTAAAAAGAGGTCTGGGGT

-477  TAACCTATACTTCCGCTTATGTTCACTACTTGGCCAGAAAATTGCATATGTGAAGGAAGT
      | | | | | | | | | | | | | | | | | |
-450  AGTTTTTTCAATACTGCAGTTTTAAATCACAATTCTTAGAGGCAACCAAACACCTCATT

-417  GCCAATATTTAATACCGTCTGGTGTATAAATTCATCTCCCAAATTTATTGGAGTTGAAG
      | |||| | | | | | | | | | | | |
-390  GTAAATAAACTATGATAATCTCCAAAACGCAGTATTCTAAAAATACTAC.....

-357  ATTCCTTGAAAAAATAATTTGACATATTAAAGATGTTGCCCTTGCGCGGGGTATCTGCA
      |||| | | | | | | | | | | | | | |
-339  .....AAAATTCTTTGTTATCAACAGGGCCTAAGGAGTTAAAAAATTTAGCC

-297  AATTGAGGATCCAAGGGACGATTGCATC.....CAGTTCTAAACACACCATTATGATTTC
      | | | | | | | | | | | | | | | | | |
-289  GTAAGTGAAGTCTGGCGAGGCACCAGCAGCTAGCAGTCATCAACACT.....TGATGGT
      *SEQ ID NO:3 >

-242  AGTGATAATGCATGCTTCCAAAGCCCAGCTGCAAGCTTGGGCCATCCTTCGGAAGGGAAA
      | | | | | | | | | | | | | | | | | |
-235  TGGCAAAGGCGAGTCGACGTGTCGCGGGGCTCGGCCTGAGCGGGAGATACAATCTGTTCT

-182  AAGAAAAAGGGGTCCTGCTGCACCAGCGACTAAACCATCCACGCATCTCTCGCTCGAACC
      || | | | | | | | | | | | | | | | | |
-175  CCAGTAACCCCGTCGATTTGGCCCGCCGACTAAAGCATCCAGGCATCTCTCGCTCGAACC

-122  CCTATTTAAGCCCCTCCATTCTTCCCTACATTCTCCACACAACCACGAGTTGCTCATCTC
      |||| | | | | | | | | | | | | | | | |
-115  CCTATTTAAGCCCCTCCATTCTTCCCAACATTCTCCACACCTCCACGAGTTGC.....
      *

-62  TCCACCCAATCATCACTAGCTAATACGGTGCCTGTTAGCTACAGACCAAGAAGTGATCA
      |||| | | | | | | | | | | | | | | | |
-53  .....TCATCACTAGCTAGTACGTTGTACTGTTAGCTACAGATTAAGAAGTGATCA

2  TGGCCCGCGCTCAGGTAATGCTCATGGCCGTCGCCTTGGTGCTCATGCTCGCGGCGGTCC
   |||| | | | | | | | | | | | | | | | | | |
2  TGGCCCGCGCTCAGGTAATGCTCATGGCCGTCGCCTTGGTGCTGATGCTCACGGCGGCC

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Figure 4a cont'd

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62  CGCGCGCTGCCGTGGCCATCGACTGCGGCCACGTTGACAGCTTGGTGAGACCCTGCCTGA
    |||||
62  CGCGCGCTGCCGTGGCCCTCAACTGCGGCCAGGTTGACAGCAAGATGAAACCTTGCCTGA

122 GCTACGTTTCAGGGCGGCCCCGGCCCGTCTGGGCAGTGCTGCGACGGCGTCAAGAACCTCC
    |||||
122 CCTACGTTTCAGGGCGGCCCCGGCCCGTCCGGCGAATGCTGCAACGGCGTCAGGGATCTCC

182 ATAACCAGGCGCGATCCCAGAGCGATCGCCAAAGCGCTTGCAACTGCCTCAAGGGGATCG
    |||||
182 ATAACCAGGCGCAATCCTCGGGCGACCGCCAAACCGTTTGCAACTGCCTGAAGGGGATCG

242 CTCGTGGCATCCACAATCTCAACGAGGACAACGCCCGCAGCATCCCCCCAAGTGCGGGTG
    |||||
242 CTCGCGGCATCCACAATCTCAACCTCAACAACGCCGCCAGCATCCCCTCCAAGTGCAATG

302 TCAACCTCCCATACACCATCAGTCTCAACATCGACTGCAGCAGGTGATTAATTCACATGC
    |||||
302 TCAACGTCCCATACACCATCAGCCCCGACATCGACTGCTCCAGGTGATTAAATTTACACT

362 AAGCATA.....TATATATGAAC
    ||
363 CATCCAGAGTGAAATCTTTAAAAGAACTATATTTACGAACGGAGTGAGTATATAGGAAC

380 ACTCATCCACGTAAAATTTATTGATATTAACATTAATCAAATCTTTGCA.CTGCAGGGTG
    |
423 ATTCATCCACGTAAAATTTGTTGATATTAACATTAACACGCATGATTGACCTGCAGGATT

440 TAATGGGCGACGATCCGTCAAGCTGGTGCTCAGCTCATCCATCCACGTGGAGTTGAAGCG
    ||
483 TACTGAGCGACGATCCGTCAAGCTGGTGCTCAGCTCATCGATCCACGTGGAGCTGAAGCG

500 CGCAGCCTCTATCCCTATGTAGTATGGTCACTAGTTATG.CGAGTTTATACTGAATATGA
    |||||
543 CGCAGCCTCTGTCCCTATGTAGTATGGCTACCAGTTATGCCGAGTTTATGCTGA.....

559 ATAAGAACTCTCTCCAGCTGGCTTGCTGGTACTCCTCTGGAGGAGATCAGTATCTGTGTA
    |||||
597 ATAAGAACTCTCTCCT.....GTACTCCTTTGGAGGAGATCAGTATCTATGTA

619 CCTGAGAGTTGAGAGTTTGTACCATGGGCACTCCCAGTGTTTATGGACTT
    |
645 CGTGAGAGTTGAGAGTTTGTACCATCGGCACTCCCAGTGTTTATGGACTA

```

Figure 4b

```

-681 AAAGAGTTTTAGACCGGAGGTATTTGTTAGGAAGTACTTCTTGCCATACTAGT..TTCAA
    ||      |      | |      ||      || ||      ||      ||| ||||
-822 AACCGTGGCCTAAAAATAAGCCGATGAGGATAAATAAAATGTGGTGGTACAGTACTTCAA
-623 TAAAGTAGCTTGAAAAGACATTTGTTAAGCAACCATGTGTTTTTAATATGAAGATCCTCA
    |  |  ||      |||  |  ||  |  |  |  |  |  |  |  |  |||
-762 GAGGTTTACTCATCAAGAGGATGCTTTTCCGATGAGCTCTAGTAGTACATCGGACCTCAC
-563 ATACCGAGAGCCTTTGGTCCCATGGATGACACAAAACCTCCCACTTGTTTTTTTTTTTG
    |||||  |  |  |  ||      ||      |  ||      |  |  ||| ||| ||
-702 ATACCTCCATTGTGGTGAAATATTTTGTGCTCATTTAGTGATGGGTAAATTTTGTTTATG
-503 TGTGTGT..GTGGGTAAACTTCCCACTTGGTTAACCTATACTTCCGCTTATGTTTCATCAC
    |  |  |  ||      |  |||      |||      |  ||      ||      |  ||  |  ||
-642 TCACTCTAGGTTTTGACATTTGAGTTTTGCCACTCTTAGGTTTTGACAAATAATTTCCAT
-445 TTTGCC.....AGAAAATTGCATATGTGAAGGAAGTGCCAATATTTAATACCGTCTGGTG
    |  ||      || |||      |  |  |  |  |||  |  ||      |  |  ||
-582 TCCGCGGCAAAAGCAAAACAATTTTATTTTACTTTTACCCTCTTAGCTTTCACAATGTA
-390 TTATAAATTCATCTCCCAAATTATTGGAGTTGAAGATTCAGTTGAAAAAATAATTTGAC
    |  |  |||  |  |||      ||||  ||      ||      |  ||||| |||  |  ||
-522 TCACAAATGCCACTCTAGAAATTC.TGTTTATGCCACAGAATGTGAAAAAAACACTCAC
-330 ATATTA.....AAGATGTTGCCCTTGCGCGGGGTATCTGCAAATTGAGGATCCAAGGGA
    ||||      ||| |||      |  |  |  |  |||  |  |  |  |  |
-463 TTATTTGAAGCCAAGGTGTTTCATGGCATGGAAATGTGACATAAAGTAACGTTTCGTGTATA
-276 CGATTGCATCCAGT...TCTAAACACACCATTATGATTTGAGTGATAATGCATGCTTCCA
    ||      ||      || |||      |  |  |  |  |||      |  |  |  |||
-403 AGAAAAAATTGTACTCCTCGTAACAAGAGACGGAAACATCATGAGACAATCGCGTTTGA
    *SEQ ID NO:3 >
-219 AAGCCCAGCTGCAAGCTTGGGCCATCCTTCGGAAGGGAAAAAGAAAAAGGGGTCCTGCTG
    |  ||      ||  ||      ||||  ||  |  |||  ||      ||  |||      ||
-343 AGGCTTTGCATCACCTTTGGATGATGCGCATGAATGG.....AGTCGTCTGCTTG
-159 CACCAGCGACTAAACCATCCACGCATCTCTCGCTCGAACCCCTATTTAAGCCCCTCCATT
    |  |  |  |||  ||||  |  |  |  |||  |  ||      |  |  ||
-293 CTAGCCTTCGCCTACCGCCCACTGAGTCCGGGCGGCAACTACCATCGGCGAACGACCCAG
-99  CTTCCCTACATTCTCCACACAACCACGAGTTGCTCATCTCTCCACCCAATCATCACTAG.
    ||  |||  |  ||  ||      |  |  |  |  ||  |  |  |  ||
-233 CTGACCTCTACCGACCGGACTTGAATGCGCTACCTTCGTCAGCGACGATGGCCGCGTACG
    .....
-173 CTGGCGACGTGCCCCCGCATGCATGGCGGCACATGGCGAGCTCAGACCGTGCGTGGCTGG
-40  .....CTAATACGGTGCACTGTTAGCTA
    ||  |||      ||||  ||
-113 CTACAAATACGTACCCCGTGAGTGCCCTAGCTAGAACTTACACCTGCAACTGCGAGAGC

```

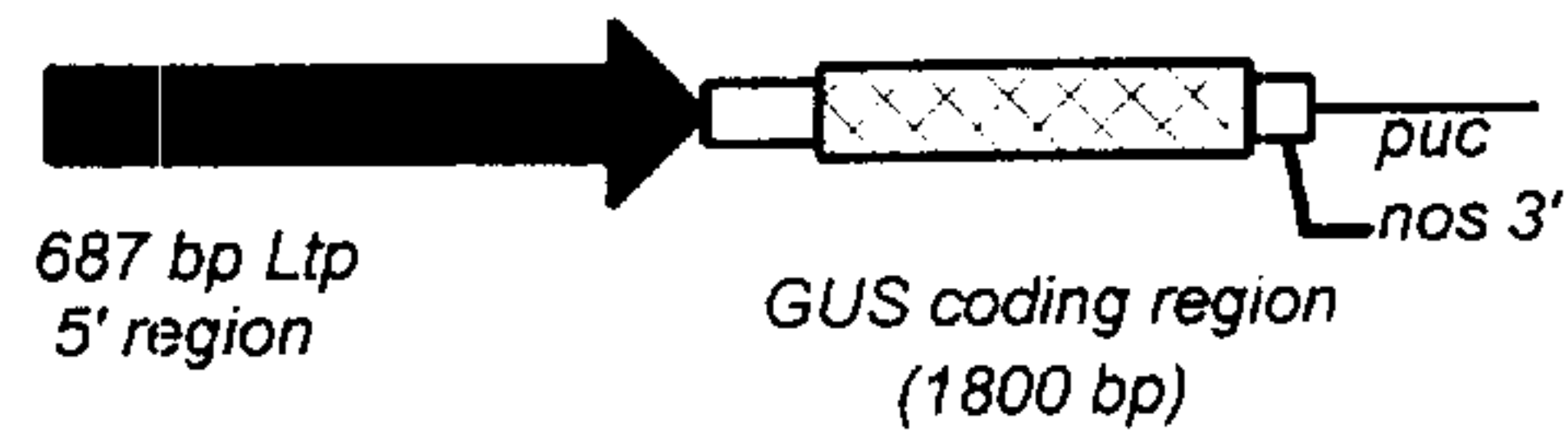
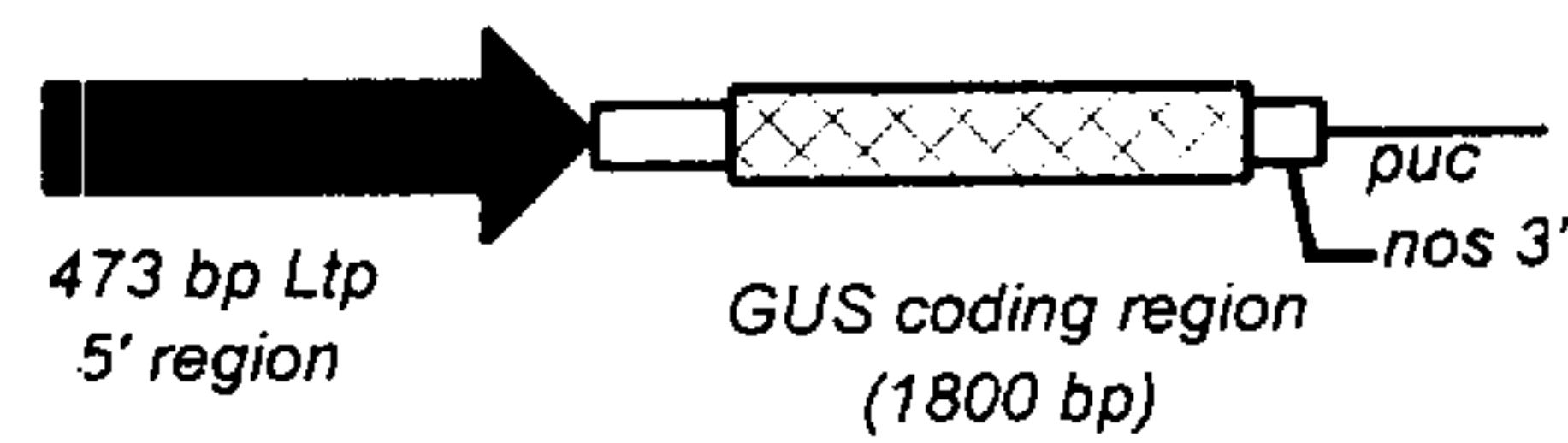
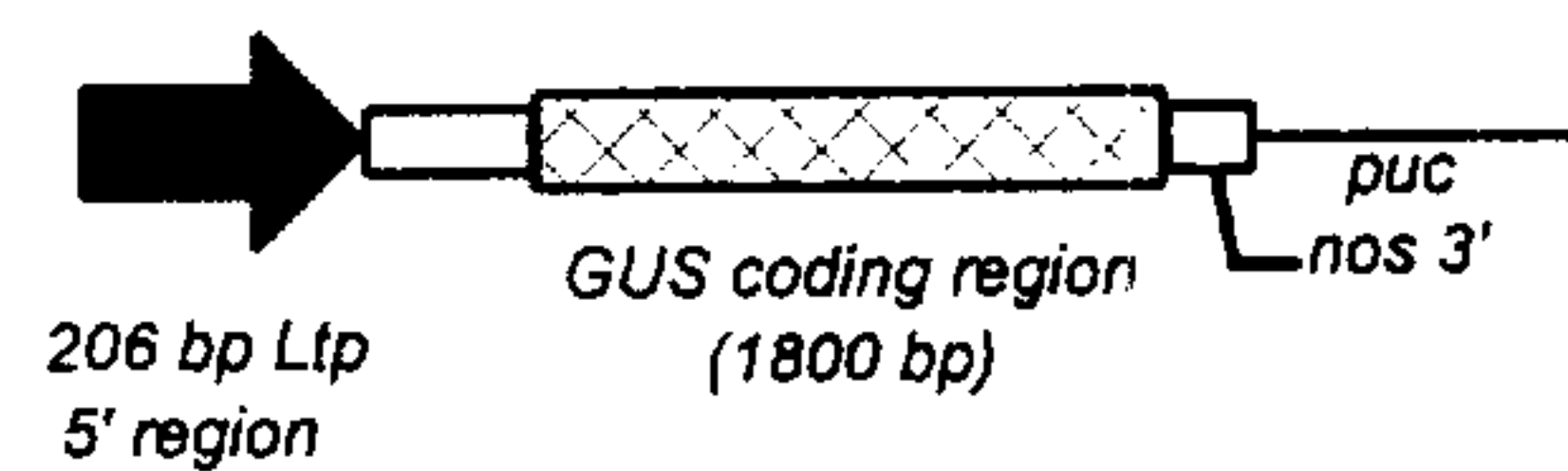
Figure 4b cont'd

*

-17 CAGACCAAGAAGTGATCATG

|| || |||

-53 GAGCGTGTGA.GTGTAGCCGAGTAGATCACCGTACGACGACGACGAGGGGCATG

Figure 5**a) p687LtpW1-GUS****b) p473LtpW1-GUS****c) p206LtpW1-GUS**

In all three constructs, the ADH1S6 intron lies between
The LtpW1 promoter and the GUS coding region.

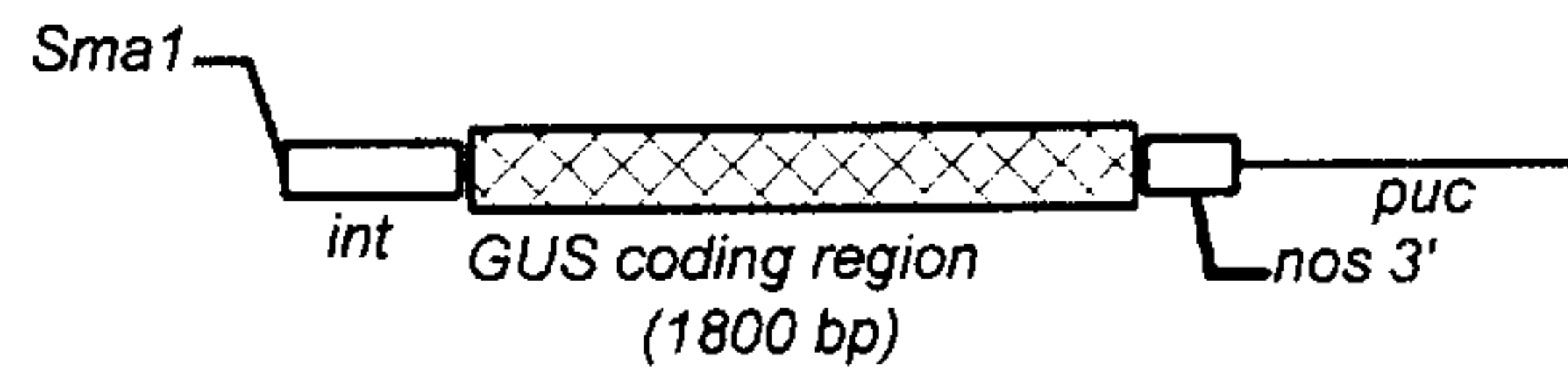
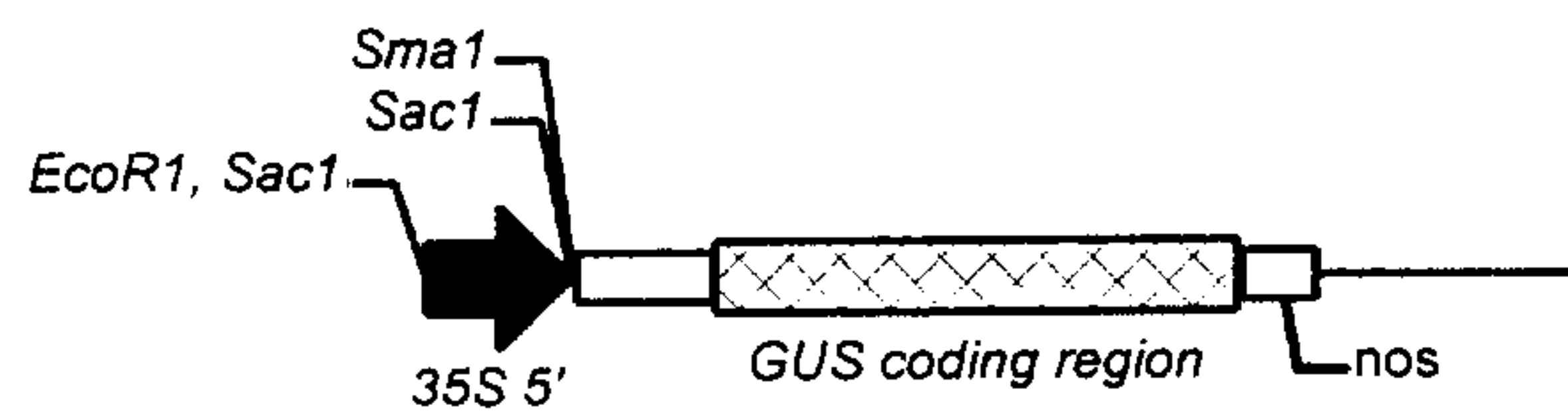
d) pLC-GUS

Figure 6**a) p35S-GUS**

The ADH1S intron lies between the promoter and the GUS gene

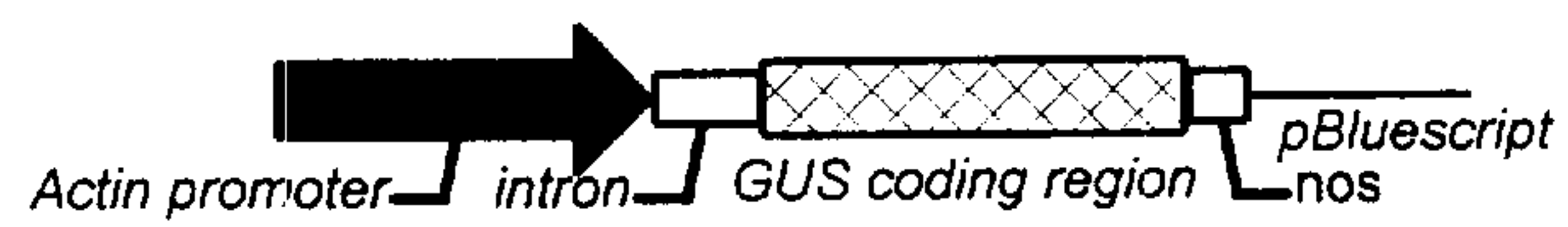
b) pACT-GUS

Figure 7

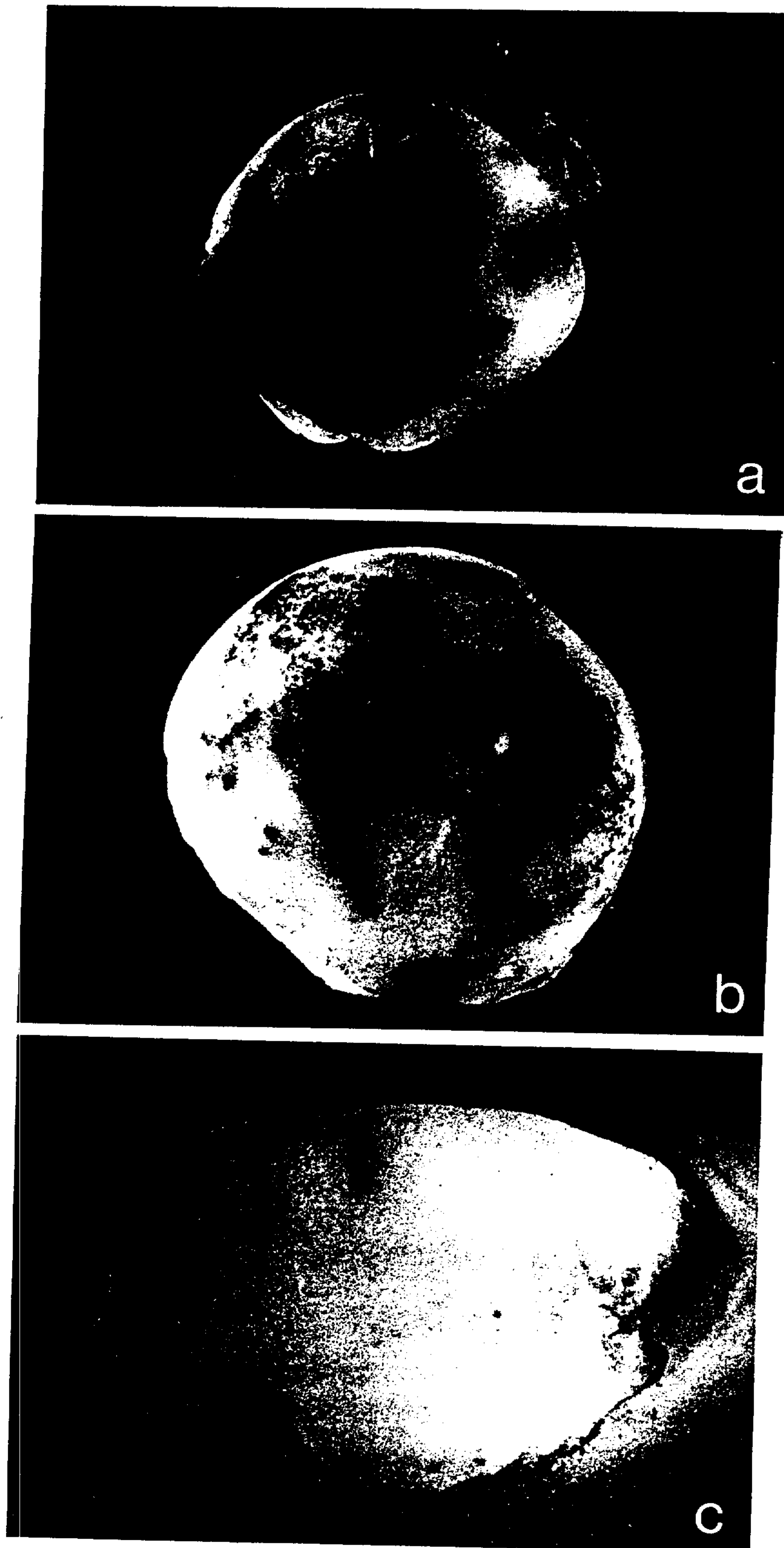


Figure 8

