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Cloning of the promoter for a novel barley gene, *Lem1*, and its organ-specific promotion of *Gfp* expression in lemma and palea

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Abstract

The differential display method was used to identify a novel barley gene, *Lem1*, expressed primarily in the outer organs (lemma and palea) that enclose developing florets and seeds. The promoter was isolated from a BAC genomic clone and used in a translational fusion with a green fluorescent protein gene (*Gfp*) to produce a transient expression vector. After particle bombardment, *Gfp* was expressed only in lemmas, paleas and awns of developing spikelets. *Lem1* did not promote *Gfp* expression in vegetative leaves or in mature spikes, although expression of co-bombarded *uidA* (*GUS)* occurred under the regulation of a ubiquitin promoter. This reproduced the developmentally regulated pattern of mRNA accumulation. Deletion studies showed that the promoter activity is confined to a *cis* element within 80 bp of the transcription start site. Upstream from this, the promoter contains putative auxin-, ethylene- and gibberellin-responsive elements or homologues. *Lem1* was found to be a single intronless gene encoding an acidic 102 amino acid protein, possibly associated with membranes. In a two-rowed barley, *Lem1* mRNA was absent in the lateral spikelets, which fail to develop, and present only in the developing median spikelets. This suggests that *Lem1* may play a role in organ development.

Introduction

Numerous genes specific to various plant developmental, metabolic and response systems have been cloned. Among those cloned from cereals, few corresponding tissue-specific promoters have been characterized, particularly among the genes preferentially expressed in the organs of the developing floral meristem. These are particularly important, as they could provide insights into improving grain yields and resistance to pathogens, such as *Fusarium graminearum*, that attack the tissues surrounding the developing seed. In barley and wheat, the lemma is the main external organ enclosing the developing floret and seed. It would therefore be advantageous to utilize specific promoters for targeting antifungal gene expression to this organ.

The lemma of barley and wheat encloses the palea, which in turn encloses the other developing floral organs and, later, the seed (Figure 1). After the apical meristem ceases production of leaf initials, it produces spike primordia, which lead to the development of the typical barley spike consisting of numerous seed-bearing spikelets (Briggs, 1978). In the differentiation of each spikelet, the glume initials form first, followed inwardly by the lemma, palea, three papillae that grow into stamens (later, bearing anthers) and, lastly, the pistil. In six-rowed barleys, such as Morex, three fertile spikelets develop at each node of the spike. In two-rowed barleys, such as Golden Promise, only the

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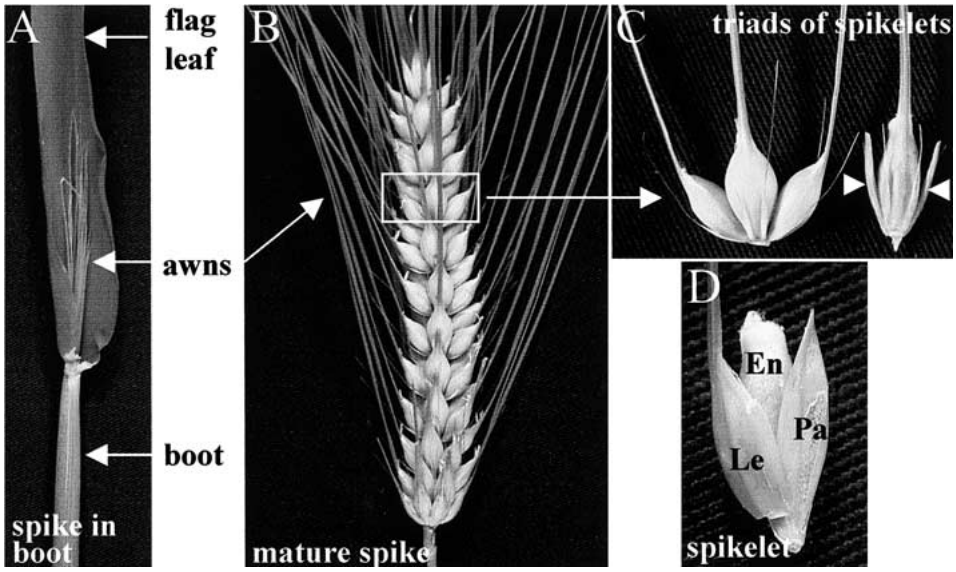

Figure 1. Lemma and palea organs in barley spikes. A. Barley spike in an early (awn extension) stage of development. The spike is enclosed within the boot (flag leaf sheath). B. Mature seed spike during the dough stage of seed development. C. Triads of spikelets in a 6-rowed barley (Morex, left) vs. a 2-rowed barley (Golden Promise, right). In the latter, only the median spikelet develops, while the lateral spikelets (marked by wedges) fail to develop. D. Spikelet organs. The palea (Pa) envelopes the endosperm (En). These are partially enclosed by the lemma (Le), which ends with an extended awn.

median spikelet of each triad is fertile; the two lateral spikelets grow slowly after the appearance of the floral primordia and develop into reduced sterile spikelets that still contain the glumes, lemma and palea. A thin awn elongates from the tip of the lemma and protrudes from the boot (flag leaf sheath) shortly before pollination. As the fertilized seed develops, the palea, and then the lemma, adhere to the pericarp and senesce.

The development of tissue-specific promoters for floral organs of cereal grains has lagged behind developments in dicots. Ogihara *et al.* (1998) used the differential display technique to clone genes that are preferentially expressed in wheat spikelet tissues at the time of heading. These were divided into genes preferentially transcribed in specific floral organs or genes continuously transcribed in multiple floral organs. Many of these were homologous to known floral-specific genes in *Arabidopsis*. The maize *KNOTTED1* gene is involved in meristem formation and has a homologue in barley (Müller *et al.*, 1995). Expression of *KNOTTED1* in barley induces meristem formation on the lemma/awn (Williams-Carrier *et al.*, 1997). In rice, a gene belonging to the AP1/AGL9 family of MADS domain proteins plays an essential role in spikelet development (Jeona *et al.*, 2000), and in maize *INDETERMINATE SPIKELET1* affects spikelet formation (Chuck *et al.*, 1998). However, more research is needed to identify genes expressed in more developed floral organs. These may include genes for specialized metabolism, cell signaling or cell structure.

This study describes the cloning and expression analysis of a novel gene, *Lem1*, expressed in the lemma/palea of developing barley spikelets. The promoter's organ-specificity and active region were determined through transient expression assays. For ease of discussion, references to the lemma will also apply to the palea, as both were combined for analyses.

Materials and methods

Materials

Seeds of barley (*Hordeum vulgare* L.) cultivars Morex and Golden Promise were obtained from the USDA-ARS Small Grains Germplasm Research Facility, Aberdeen, ID. Restriction enzymes were purchased from Promega, *Taq* DNA polymerase from Applied Biosystems, culture media from Difco, and RAPD primers from Biotechnology Laboratory, University of British Columbia. Oligonucleotide PCR primers were synthesized by Amitof Biotech. All chemical reagents were purchased from Sigma, unless otherwise stated.

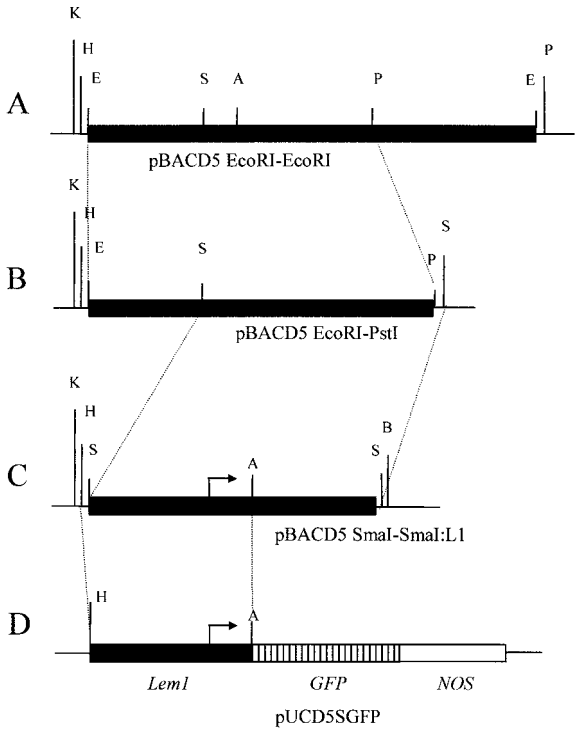

Figure 2. Cloning steps used to produce the *Lem1::Gfp* expression vector. An *Eco*RI fragment from an initial 100 kb genomic BAC clone was sequentially subcloned in pBluescript to produce a fragment containing the putative *Lem1* promoter in the region upstream from the initiation codon (panels A–C). This *Hin*dIII-*Apa*I fragment was inserted into a previously constructed ubi-GUS expression vector (Kaeppler *et al*., 2000) by replacing the *Ubi* promoter and first intron (panel D). *Lem1* sequences are denoted by a black box, the *Gfp* coding sequence by a striped box and the NOS termination sequence by an open box. A, *Apa*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I. The horizontal lines in panels A–C represent pBluescript vector and in panel D, pAHC25 vector.

Barley plants were grown in pots in a greenhouse and in growth chambers. The greenhouse was maintained at 21–29 °C and received supplemental lighting with sodium arc vapor lights to produce a 16 h photoperiod. Growth chambers were maintained at 16–18 °C under the same photoperiod. *Lem1* mRNA levels were analyzed in developing spikes, staged as follows. 'Pre-lemma' spikes had not formed lemmas or paleas (visible to the naked eye) or awns and ranged in length from 0.7 to 4.3 cm. In 'elongating' stage spikes, lemmas had elongated to 2–7 mm and had elongated awns. In 'awn extension' stage spikes, awns extended up to 2 cm beyond the auricle of the boot and had green anthers (Figure 1A). 'Emerging' spikes had finally emerged through the side of the boot and had awns protruding 5–7 cm beyond the auricle of the boot; pollination had commenced or was completed, but no ovule elongation had yet occurred. Other tissues analyzed included separate lemmas and paleas from the late awn extension stage, combined lemmas and paleas from the awn extension and dough stages, epicotyls and roots of 8-day old etiolated seedlings, internodal stems and flag leaves. Ovaries and stamens were collected at the elongating stage. Seeds in the milky through dough stages were dissected into starchy endosperm, pericarp/aleurone and embryos. RNA for differential display was extracted from Morex spikes pooled from several stages of development, ranging from the elongating stage of spike development to the dough stage of seed development. Seeds and about 80% of awns were removed prior to freezing in liquid nitrogen. As a control, flag leaves were harvested from the same plants. Young spikes for transient expression assays of promoter activity were collected at the elongating stage.

RNA extraction and differential display

Total RNA from all tissues, except ovaries, anthers and developing seeds, was extracted with guanidinium thiocyanate (Chirgwin *et al*., 1979). Ovary and anther RNAs were extracted by using an RNeasy kit (Qiagen). Developing seed RNAs were extracted as described (Skadsen, 1993). Pooled spike (see above) and flag leaf mRNAs were purified on an oligo-d(T) cellulose column (type III, Collaborative Biomedical Products). Syntheses of first-strand spike and leaf cDNAs were carried out according to Krug and Berger (1987). Routine molecular procedures were performed as described (Sambrook *et al*., 1989).

Differential display of cDNAs was performed as described (Miele *et al*., 1998), with modifications. A primary polymerase chain reaction (PCR) was performed in 25 μL with 1.5 μg of spike or leaf cDNA, 40 μM of dNTPs, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 400 ρM ETVN 24-mer downstream primer, 1.29 μM 10-mer RAPD primer, and 1.5 units of *Taq* DNA polymerase. The core ETV sequence was 5′-CGGAATTCGG$(T)_{12}$V, which includes an *Eco*RI recognition sequence and where V represents an equal mixture of the four dNTPs; four specific ETVN primers were synthesized by terminating the sequence with dATP, dCTP, dGTP or dTTP. The primary PCR program consisted of a 94 °C hot start for 105 s, followed by 25 cycles of annealing (42 °C, 30 s), extension (72 °C, 1 min) and denaturation (94 °C, 30 s). This was followed by a 72 °C extension for 7 min. A secondary 35 cycle PCR was

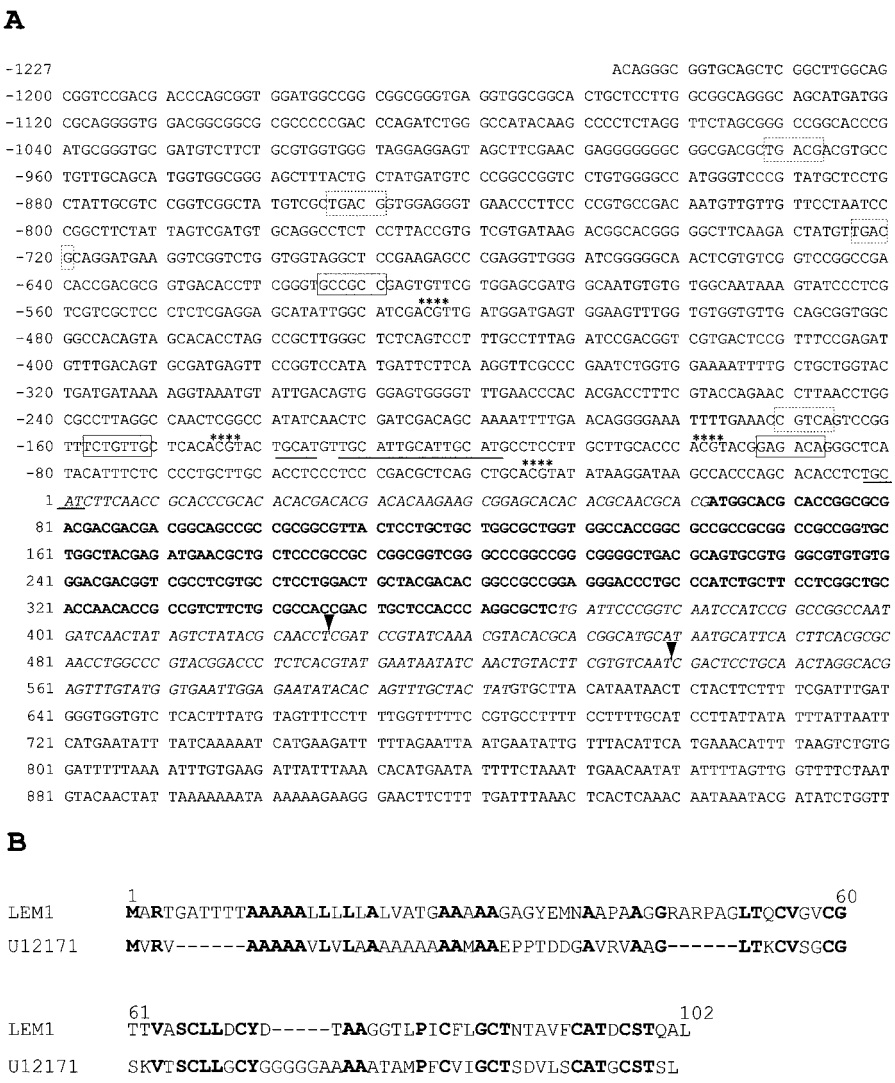

Figure 3. Sequences of *Lem1* genomic clone and the protein it encodes, LEM1. A. Selected portion of the sequence of genomic clone pBACD5 *Sma*I-*Sma*I:L1 (see Figure 2C). Coding sequence is given in bold font. Sequences of the mRNA 5′- and 3′-UTRs are given in italics, starting at +1. Sequences of putative ethylene-responsive element (−615), auxin-responsive element (−93), and gibberellin-responsive element (−158) are in solid boxes. Methyl jasmonate-responsive elements and homologues are in dashed boxes. Core G-box sequences (ACGT) are marked with asterisks. Repeated TGCAT sequences are underlined. The restriction cutting sites that produced the 113 bp 3′ *Lem1* probe are marked with wedges. B. Deduced amino acid sequence of LEM1 and an anther-specific rice protein (U12171). Identical amino acids are marked in bold. Dashes were introduced to give maximal alignment.

conducted as above, with 5 μCi of [^{35}S]-dATP (Amersham) and 6 μl of the primary PCR as the template. The differential display products were separated electrophoretically in a 6% denaturing polyacrylamide gel, and autoradiographed using Kodak X-OMAT AR film (Eastman Kodak).

Cloning and sequencing of cDNA

A radiolabeled DNA band (designated *Lem1*) that was prominent among spike products but absent in flag leaf products was excised from the gel and used (in gel) as a template in unlabeled tertiary PCRs, performed as above. As a control, the corresponding area from the leaf product lane was also used as a PCR template. Because each differential display product could represent more than one gene, the tertiary

PCR products were digested with *Hpa*II and *Taq*I. The digested DNAs were electrophoretically separated in a NuSieve GTG Agarose (FMC BioProducts). A unique 113 bp fragment found in spike products, but not in leaf products, was extracted from the gel with AgarACE (Promega), made blunt-ended with T4 DNA polymerase, further purified in a Sea Plaque agarose gel (FMC BioProducts), ligated into the *Sma*I site of pBluescript SK (Stratagene), and used to transform *Escherichia coli* DH5α cells. The cloned 113 bp insert was sequenced using Big Dye fluorescent terminators (Applied Biosystems); the insert was also used as a hybridization probe. Sequences were determined by the University of Wisconsin Biotechnology Center. Amino acid sequence, pI determination and DNA sequence analyses were done with the GCG software package (GCG, 1996), unless stated otherwise.

Determination of the 5′ and 3′ sequences of *Lem1* mRNA was performed by rapid amplification of cDNA ends (RACE) with the GeneRacer kit (Invitrogen), as described by the supplier. A GeneRacer RNA oligonucleotide (5′-CGACUGGAGCACGAGGACACUGACAUGGACUGGAAGGAGUAGAAA) was ligated to the 5′ end of developing spike mRNA. A GeneRacer oligo(dT) primer (5′-GCTGTCAACGATACGCTACGTAACGGCATGACAGTG(T)$_{18}$) was annealed and used to prime transcription with AMV reverse transcriptase. Two gene-specific primers were designed by using the available sequence information from the 113 bp *Lem1* fragment: GSP1 (5′-CCGTACGGGCCAGGCCAGGTTGCGCGTGAACTGAA) and GSP2 (5′-CAAACGTACACGCACGGCATGCATAATGCA). GSP1 and GSP2 were used to amplify the 5′ and 3′ cDNA ends of *Lem1* from the developing spike RACE-ready cDNA using GeneRacer 5′ primer (5′-CGACTGGAGCACGAGGACACACTGA) and 3′ primer (5′-GCTGTCAACGATACGCTACGTAACG), respectively, in a PCR reaction. The 3′ and 5′ RACEs began with a 94 °C denaturation (hot start) for 90 s, followed by 30 cycles of denaturation (94 °C, 30 s), annealing (65 and 67 °C, respectively, 30 s) and extension (72 °C, 1 min). This was followed by a final 30 min extension at 72 °C. Products were separated by electrophoresis on a 1% agarose gel and cloned into the pCR4-TOPO vector using the TOPO TA Cloning Kit (Invitrogen) as described by the supplier. A clone resulting from the 5′ RACE contained the 5′-UTR (5′-untranslated region), the coding sequence and half of the 3′-UTR; this was named *Lem1* cDNA and was used as a northern blot probe.

Northern and Southern blot analyses

For Southern blot hybridization, the 3′ 113 bp *Hpa*II/*Taq*I *Lem1* fragment was used as a probe (50 °C). For northern blot hybridizations, this or a longer probe, '*Lem1* cDNA' (representing bases 1–495 in Figure 3), was used. Preparation of Southern and northern gel blots, preparation of ^{32}P-dCTP-radiolabeled probe (Feinberg and Vogelstein, 1983), hybridization and washing conditions, and autoradiography were conducted as previously described (Skadsen *et al*., 1995). Northern blots were hybridized and rinsed at 42 °C (3′ 113 bp probe) or 50 °C (*Lem1* cDNA probe). All northern blots contained 10 μg RNA per lane. Southern blots contained 10 μg of Morex genomic DNA per lane; DNA was digested to completion with *Eco*RV, *Bam*HI, and *Hin*dIII.

Cloning of Lem1 *promoter and transient expression analysis*

The 113 bp *Lem1* clone was used to probe a BAC library of Morex barley genomic DNA (Yu *et al*., 2000) (provided by Dr Andris Kleinhofs, Washington State University, as part of the North American Barley Genome Mapping Project). We digested the BAC clone with *Eco*RI, Southern-blotted the fragments and hybridized blots with the above sequence. A fragment containing the *Lem1* sequence was subcloned into the *Eco*RI site of pBluescript SK (pBACD5 *Eco*RI-*Eco*RI, Figure 2A). A *Pst*I fragment was removed, producing pBACD5 *Eco*RI-*Pst*I (Figure 2B). This was digested with *Sma*I to release a fragment containing upstream, coding, and downstream regions. This fragment was subcloned into the *Sma*I site of pBluescript SK (pBACD5 *Sma*I-*Sma*I:L1, Figure 2C) and sequenced. A 1400 bp *Hin*dIII-*Apa*I fragment containing the *Lem1* promoter and partial N-terminal coding region was subcloned, replacing the *Ubi1* promoter contained in the *Hin*dIII-*Bam*HI fragment in pAHCS-GFP (Figure 2D) vector. The pAHCSGFP vector was derived from expression vector pAHC17 (Christensen and Quail, 1996) by inserting the coding sequence for the *Aequorea victorea* green fluorescent protein (GFP) between the *Ubi1* first intron and the NOS termination sequence (Kaeppler *et al.*, 2000). A translational fusion between *Lem1* and *Gfp* was created by removing four bases at the *Lem1*/*SGFP* junction. This vector, pUCD5SGFP (Figure 2D) was used to analyze *Lem1* promoter organ specificity through transient expression studies.

Particle bombardment and tissue sterilization were carried out as described by Wan and Lemaux (1994) with minor modifications. Barley spikes and flag leaf samples were harvested from greenhouse-grown barley plants on the day of bombardment. After sterilization, samples were mildly plasmolysed in 1.5% maltose, 6.4% mannitol and 6.4% sorbitol for 3 h (Vain *et al.*, 1993) before bombardment in a Biolistic PDS-1000 He gun (BioRad) with 0.4 mg of 1 μm gold particles. Spike tissues were bombarded twice at 9.3 MPa. Leaves were bombarded once at 7.6 MPa. As an internal control, the Ubi-GUS expression vector, pAHC25 (Christensen and Quail, 1996), was co-bombarded into leaf tissue along with pUCD5SGFP. The bombarded tissues were incubated at 24 °C in darkness for up to 72 h. Expression of *Gfp* was monitored using a Stemi-2000-C binocular microscope with a short-wave blue light (Carl Zeiss) and recorded as described (Kaeppler *et al.*, 2000). After examination of *Gfp* expression, leaves were stained for GUS activity (Jefferson *et al.*, 1987).

Lem1 *promoter deletion analysis*

The pUCD5SGFP vector (Figure 2), containing the 1227 bp *Lem1* upstream region, was used as a template for creating promoter deletions through PCR reactions employing *Pfu* polymerase (Promega). For creating 5′ deletions, 16–18 bp upstream primers were synthesized to correspond to sequences beginning at −1227 (control), −534, −396, −216 and −80 (Figure 3). The 5′ sequence of each primer was extended with TTTAAGCTT to provide a *Hin*dIII site. The downstream primer in each PCR was GFP106L (TCGCCGGACACGCTGAACTT), the reverse complement of a sequence beginning 106 bp from the *Gfp* start of translation. For creating 3′ deletions, the −1227 primer (above) was used upstream. For downstream primers, 20–23 bp reverse complement sequences were synthesized corresponding to positions beginning at −3, −438, and −785. The 5′ sequence of each was extended with GGAATTC to provide an *Eco*RI site. An expression vector was produced by removing the *Lem1* promoter or a *Lem1-Gfp* fragment from pUCD5SGFP and ligating the PCR products (after appropriate restriction digestion) into the vacated site. Particle bombardments of spike and leaf tissues were conducted as above; unaltered pAHC25 vector was again co-bombarded into leaves a control. Each deletion construct was bombarded two or more times on separate days.

Results

The differential display method was used to produce 100–500 bp 3′ cDNAs corresponding to mRNAs found in the spikes but not in flag leaves. RAPD primers often produced 3 to 8 spike-specific PCR products with each set of ETVN downstream primers. One of the cDNAs, named *Lem1* (for lemma/palea), was selected for further analysis due to its signal strength and specificity. Screening of the Morex barley genomic DNA BAC library with the 113 bp 3′ *Lem1* fragment probe detected one clone (*Lem1*) with a 100 kb insert. A 2.8 kb *Sma*I-*Sma*I fragment, containing upstream, coding and downstream regions, was subcloned and sequenced (Figure 3A). The endogenous *Lem1* mRNA sequence (Figure 3A) was derived from sequencing of the differential display product and 5′ and 3′ RACE products. These sequences matched that of the genomic clone. Thus, *Lem1* contains no introns. A putative TATA box occurs at −33, and putative CAAT boxes may occur at −821, −589 and −577. The best match to known polyadenylation sequences is AATATA, at position 581 (Joshi, 1987). Using the PLACE database (www.dna.affrc.go.jp/htdocs/ PLACE/), matches to several known promoter motifs were found in the upstream region, including auxin, ethylene, abscisic acid, and methyl jasmonate-responsive elements (see Discussion).

A BLAST (Altschul *et al.*, 1997) analysis found only a limited DNA sequence homology (33 identical bases in a 74 bp span with 6 introduced gaps) to a rice gene, *RTS2* (GenBank U12171), which encodes an anther-specific protein (Figure 3B). The deduced sequence of LEM1 reveals a 102 amino acid peptide that is hydrophobic through most of its length (Kyte and Doolittle, 1982) and has a pI of 4.54. A likely signal peptide cleavage site was predicted to occur between amino acids 29 and 30 (Nielsen *et al.*, 1995).

A Southern blot of Morex genomic DNA, hybridized with the 3′ 113 bp *Lem1* fragment, revealed that the *Lem1* gene exists as a single copy (Figure 4). The expression of *Lem1* was analyzed by northern blots. Significant mRNA levels occurred only in developing spikes (Figure 5A and B). When blots were probed at moderate stringency with the *Lem1* cDNA clone, there was no hybridization to other vegetative or seed organs. *Lem1* mRNA was abundant in lemma/palea tissues from spikes in the awning stage of development but near zero levels in lemma/paleas from spike in the dough stage of seed development

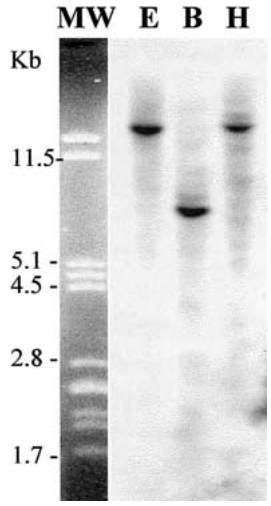

Figure 4. Southern blot analysis of *Lem1* in Morex barley genomic DNA. Each lane represents 10 μg completely digested by *Eco*RI (E), *Bam*HI (B) or *Hin*dIII (H). Fragment size markers (MW) are Lambda phage DNA hydrolyzed with *Pst*I. The band above the 11.5 kb band is uncut Lambda.

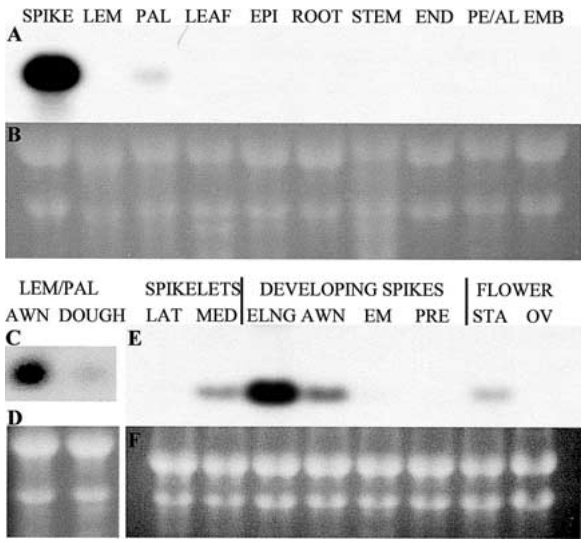

Figure 5. Northern blot analysis of *Lem1* mRNA levels in barley organs. A. Blot of RNA from panel B, hybridized with *Lem1* cDNA probe. SPIKE, spikes from the elongating stage of development; LEM and PAL, lemmas and paleas from the late awning stage; LEAF, flag leaves; EPI and ROOT, epicotyls and roots from 8-day old etiolated seedlings; STEM, internodal stems from mature plants; END, PE/AL and EMB, endosperm, combined pericarps and aleurones, and embryos of seeds from the milky through dough stages. B. Total RNA used for panel A, stained with ethidium bromide. C. RNA from combined lemmas and paleas from the awn extension stage and the dough stage. D. Total RNA used for panel C, stained with ethidium bromide. E. RNA from spikes at various stages of development, spikelets and flowers. LAT and MED, lateral and median spikelets from Golden Promise, taken at the elongating stage of spike development; ELNG, AWN, EM and PRE, whole Morex spikes taken at the elongating, awn extension, emerging, and pre-lemma stages of development, respectively; STA and OV, stamens and ovaries from Golden Promise spikes taken at the elongating stage. F. (bottom right) Total RNA for panel E stained with ethidium bromide. Blots in panels A and E were probed with *Lem1* cDNA probe, while the blot in panel C was probed with the *Lem1* 113 bp 3′-end fragment.

(Figure 5C and D). To define the period of expression in spikes, four stages of development were analyzed (Figure 5E and F). *Lem1* mRNA first appeared and was strongest in the period of spike development in which recognizable lemmas were elongating (elongating stage, see Materials and methods). As the awns of the elongating spikes began to emerge from the boot (Figure 1A), *Lem1* expression sharply declined and disappeared by the time when the spikes began to emerge from the side of the boot (at or near pollination; Figure 5D). Traces of *Lem1* mRNA can remain from the late awning stage to the dough stage.

Expression was also analyzed in isolated stamens and ovaries in elongating stage spikes of Golden Promise barley. *Lem1* mRNA occurred at low levels in stamens and was absent in ovaries (Figure 5E). Because these organs constitute only a small fraction of the spike, the signal seen in spike lanes resulted almost totally from mRNA in the lemma and palea. In Golden Promise, a two-rowed barley, the median spikelet of each triad develops fully, while the two lateral spikelets remain in a vestigial stage of development (Figure 1C). To further analyze the association between *Lem1* and lemma/palea development, the developing median spikelets and lateral spikelets were analyzed. *Lem1* mRNA was found only in the median spikelets, suggesting an association with successful spikelet development (Figure 5E).

The ability of the *Lem1* upstream region to serve as a promoter and its organ specificity were demonstrated by particle bombardment. The *Lem1* promoter drove *Gfp* expression in lemmas, paleas and awns of barley and wheat spikes (Figure 6B and F). The developmental specificity of the *Lem1* promoter was also retained; in spikes older than the elongating stage, *Lem1* failed to drive *Gfp* expression. *Lem1* did not promote *Gfp* expression in even a single cell of barley or wheat leaves (Figure 6I and M). The expression of *GUS* from the co-bombarded Ubi-GUS pAHC25 vector demonstrated that the leaves were competent to express transgenes (Figure 6J and N). Under the control of *Lem1*, *Gfp* was slow to achieve maximal expression in spike tissues. GFP fluorescence appeared brightest at 48 h

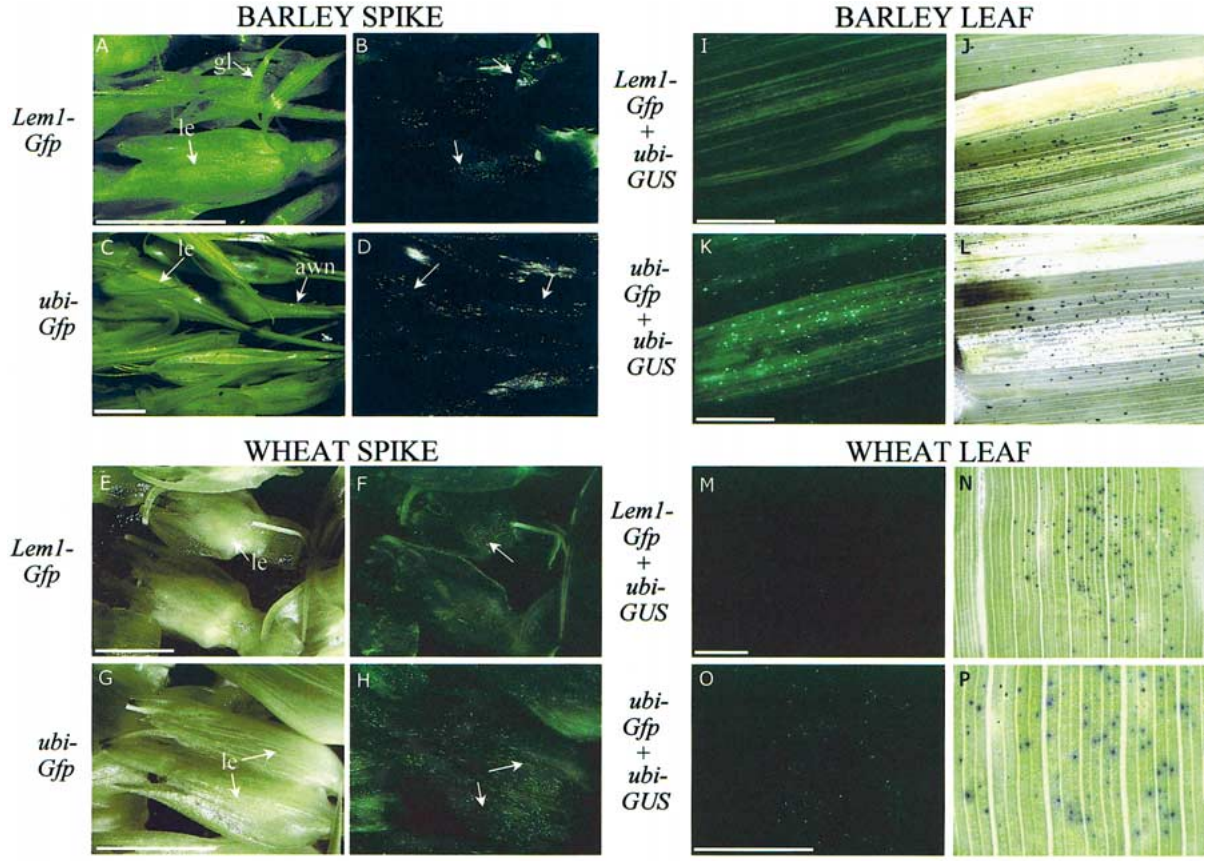

Figure 6. Transient expression analysis of *Lem1* promoter activity in barley and wheat spikes and flag leaves. Spikes (A–H) and leaves (I–P) were bombarded with promoter-reporter expression plasmid DNA attached to gold particles. After 72 h, green fluorescence from cells expressing the *Gfp* reporter gene was detected by illumination with short-wave blue light (B, D, F, H, I, K, M and O). The *Lem1* promoter drove reporter gene expression in spike tissues but not in leaves (B vs. I and F vs. M). After GFP photography, leaves were stained for GUS activity to ensure that they were competent to express reporter genes (J, L, N and P). *Lem1-Gfp*, *Lem1*promoter-*Gfp* reporter plasmid pUCD5SGFP; *Ubi-GUS*, *Ubiquitin* promoter-*GUS* reporter constitutive expression plasmid pAHC25; *Ubi-Gfp*, *Ubiquitin* promoter-*Gfp* constitutive expression plasmid pAHCSGFP; le, lemma; gl, glume (arrow tips point to corresponding positions in each photo). Paleas face downward on plates. Length bar = 5 mm.

after bombardment, while *Gfp* was strongly expressed under the control of the *Ubi* promoter in both spikes and leaves within 12 h (Figure 6D, K, H and O).

Deletion analysis of the promoter showed that the −3 to −80 sequence was sufficient to drive *Gfp* reporter gene expression in the lemma (Figure 7). All deletion constructs that positively expressed *Gfp* had a fluorescence intensity equal to that of the full 1227 bp promoter. Constructs that were negative had absolutely no visible GFP fluorescence. None of the deleted or full-length promoter constructs were able to drive *Gfp* expression in leaves. In addition, no activity resulted from the promoterless vector.

Discussion

These studies have identified a novel gene, *Lem1*, preferentially expressed in lemmas and paleas of young spikes, and have shown that its cloned promoter retains this specificity. The complete inability of *Lem1* to promote *Gfp* reporter gene expression in vegetative leaves demonstrates that elements restricting expression to the spike reside in the region upstream from the transcription start site. The ability of the constitutive ubiquitin promoter to drive *GUS* expression in leaves shows that the lack of *Lem1* activity in leaves does not simply reflect a general inability of leaf cells to support transient expression of introduced genes. It is possible that nuclear factors that interact with this promoter region reside in lemma/palea cells but

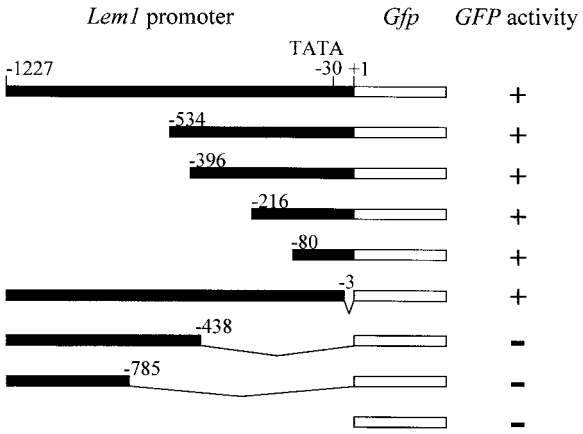

Figure 7. Lem1 promoter deletion analysis. Sequential 5′ and 3′ deletions of the Lem1 −1 to −1227 upstream region (black boxes, see Figure 3) were synthesized by PCR and ligated upstream of *Gfp* (open boxes). GFP was qualitatively analyzed, after transient expression in young spike tissues, and scored as active (+) or inactive (−). The putative TATA box starts at −33, and the mRNA start is denoted as +1. The connecting bars represent deleted sequences.

are absent in leaves. The mechanism of induction appears to be conserved in barley and wheat since the *Lem1* promoter functions in both. In addition, there is a strong temporal or developmental determinant associated with the promoter. This is indicated by the restriction of *Lem1* activity to elongating lemmas (and other elongating spike tissues) and by the inability of the promoter to direct *Gfp* reporter gene expression in spike tissues beyond the elongating stage. Since *Lem1* mRNA was not detected in leaves of 8-day old seedlings, it is not likely that this gene is active in all young elongating tissues. It is assumed that an organ-specific responsive element(s) in the *Lem1* upstream region interacts with upstream promoter-binding proteins that are produced as a consequence of a biochemical environment unique to the lemma. However, the promoter deletion studies showed that the first 80 bp of upstream sequence are sufficient to drive strong expression in the lemma. The strong expression seen in the −1227 to −3 construct indicated that sequences within the 5′-UTR of the native gene may not be necessary for promoter activity in the native gene. The −80 to −3 region contains a TATA box at −33 and elements homologous to other promoter motifs (Figure 3). The CACGTA sequence adjoining the TATA box has homology to the CACGTG G-box.

The proximal upstream regions of many genes have been shown to direct the expression of reporter gene fusions in a manner that reproduces the pattern of expression of the native gene. However, the existence of multiple promoter motifs in the more distal region (upstream from −80) indicates that *Lem1* could be controlled through interactions with multiple *trans*-acting factors. Such multiple interactions have been found in many plant promoters (Singh, 1998). Interactions between two or more promoter elements are required for induction by gibberellin (Rogers and Rogers, 1992), abscisic acid (Shen and Ho, 1995) and auxin (Ulmasov *et al*., 1995). An auxin-responsive element (ARE), TGTCTC, occurs at −93 (Figure 3A; Ulmasov *et al*., 1995). *Lem1* expression occurred primarily in the elongating phase of lemma/palea development, and this suggested that *Lem1* may be an auxin-responsive gene. However, the ARE motif was deleted in the −80 to +1 construct, which has strong promoter activity. In addition, an ethylene-responsive element was found further upstream at −615 (GCCGCC; Ohme-Takagi and Shinshi, 1995). A sequence (TCTGTTG) homologous to a gibberellin-responsive element (TCTGTTA) occurs at −158 (Sutliff *et al*., 1993). The upstream sequence also contains a CGTCA motif (−171) involved in methyl jasmonate responsiveness of the lipoxygenase 1 gene in barley (Rouster *et al*., 1997). However, although the associated TGACG inverted repeat (necessary for bZIP factor binding) occurs three times, they are probably too far upstream to be functional. In addition, the dinucleotide CA is repeated 18 times from −19 to 61. This is a common feature in the promoters of hydrolytic enzyme genes induced by gibberellin in cereal grains (Skadsen, 1998) and is known to promote DNA curvature (Qian *et al*., 1996).

The role and subcellular location of LEM1 are unknown. It has no homology to other known proteins other than a slight homology to an anther-specific protein found in the tapetum of rice (Figure 3B). The role of this protein and its subcellular location are also unknown (T. Hodges, personal communication). *Lem1* may be involved in organ formation, maintenance of organ identity or non-morphogenic roles. Its expression in the lemma/palea only during the elongating stage, but after floral initials have already been formed, argues against being a meristem identity gene, such as *APETALA1* or *LEAFY* in *Arabidopsis*. Its expression may also come too late to function as an organ identity gene, such as *APETALA2* and 3, *PISTILATA* or *AGAMOUS* in *Arabidopsis*. However, Ogihara *et al.* (1998) have noted that cereal homologues of these genes are expressed far longer than in dicots. *Lem1* has no homology with other known regulatory genes. The strong possibility that the 1–29 peptide region of

LEM1 is a transit peptide and the existence of several possible transmembrane domains suggests that it is a membrane-associated protein. This also argues against it having a transcriptional regulatory role.

Ogihara *et al.* (1998) classified genes expressed in spikelets into two groups, one specifying the differentiation of cells or tissues for gamete formation and one involved in maintaining the activity of flower organs. They identified four genes expressed in both the lemma/palea and in the pistil and suggested that the lemma/palea may be part of the flower organ. The expression of *Lem1* in the lemma, its (low) expression in anthers, and its partial homology with the rice anther-specific *RTS2* gene also suggests this association.

The development of tissue-specific gene promoters is essential for the targeted expression of genes to improve such traits as grain quality and pathogen resistance. This is preferable to constitutive expression, which could have agronomically undesirable effects or possibly hasten the development of resistant pathogens or pests. The above finding may aid in the targeting of specific proteins to the spike tissues of cereals.

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