The LP2 leucine-rich repeat receptor kinase gene promoter directs organ-specific, light-responsive expression in transgenic rice

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Received 24 April 2009; revised 23 July 2009; accepted 6 August 2009.

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GenBank Accession No.: FJ831442.

Keywords: plant biotechnology, GUS, GFP, leucine-rich repeat-receptor kinase.

Summary

Biotechnologists seeking to limit gene expression to nonseed tissues of genetically engineered cereal crops have only a few choices of well characterized organ-specific promoters. We have isolated and characterized the promoter of the rice Leaf Panicle 2 gene (LP2, Os02g40240). The LP2 gene encodes a leucine-rich repeat-receptor kinase-like protein that is strongly expressed in leaves and other photosynthetic tissues. Transgenic rice plants containing an LP2 promoter-GUS::GFP bifunctional reporter gene displayed an organ-specific pattern of expression. This expression corresponded to transcript levels observed on RNA blots of various rice organs and microarray gene expression data. The strongest β-glucuronidase activity was observed in histochemically stained mesophyll cells, but other green tissues and leaf cell types including epidermal cells also exhibited expression. Low or undetectable levels of LP2 transcript and LP2-mediated reporter gene expression were observed in roots, mature seeds, and reproductive tissues. The LP2 promoter is highly responsive to light and only weak expression was detected in etiolated rice seedlings. The specificity and strength of the LP2 promoter suggests that this promoter will be a useful control element for green tissue-specific expression in rice and potentially other plants. Organ-specific promoters like LP2 will enable precise, localized expression of transgenes in biotechnology-derived crops and limit the potential of unintended impacts on plant physiology and the environment.

Introduction

Plant biotechnology has the potential to significantly improve agricultural crop productivity and utilization properties, and to facilitate production of novel products and optimize biomass for biofuels production. An important component in the implementation of biotechnological crop improvement is the use of various gene expression control elements (i.e. promoters) that direct the expression of the introduced genes. A diverse array of promoters has been identified from model and crop plant systems. These promoters are either (1) constitutive, that is they confer expression in all or most tissues under most conditions; (2) inducible, where expression is elicited only under certain conditions; or (3) organ- or tissue-specific, where expression is delimited spatially within certain plant organs or cell types (Potenza et al., 2004). Relatively few of these control elements have been demonstrated to perform well in crop plants and fewer still have been utilized in the currently deployed genetically engineered crops (AgBios; http://www.agbios.com/dbase.php). Since most of the well-characterized promoters that are currently available confer constitutive expression, novel promoters with reliable organ-specific expression in transgenic plants are needed. The availability of a series of organ-specific promoters with diverse specificities would allow precise spatial control of transgene expression for the biotechnological improvement of crop plants.

In the grasses, the maize Ubi1 (Christensen and Quail, 1996), rice Act1 (McElroy et al., 1990; Zhang et al., 1991)
and the CaMV 35S promoters (Benfey et al., 1989) are commonly used to constitutively express transgenes. Several other strong monocot promoters have been characterized more recently including OsCc1 (Jang et al., 2002), RUBQ2 (Wang et al., 2000; Wang and Oard, 2003), RUBia3 (Sivamani and Qu, 2006; Lu et al., 2008a,b; Samadder et al., 2008) and Act2 (He et al., 2009), all derived from rice. Most of these promoter constructs include 5′ introns and untranslated regions (UTR), which contribute significantly to the strength and/or specificity of expression observed in transgenic plants. In the case of the 35S promoter, addition of an intron has been shown to enhance transgene expression in various monocot species (Tanaka et al., 1990; Maas et al., 1991; Takumi et al., 1994; Li et al., 1995). These promoters are useful tools for over-expressing transgenes in most or all the tissues of rice and other monocot plants.

Only a modest number of promoters with organ- or tissue-specific expression have been thoroughly examined in transgenic grasses. In transgenic rice plants, the rbcS (ribulose-bisphosphate carboxylase), Pdk (pyruvate orthophosphate dikinase), LHCPII (light harvesting chlorophyll a/b binding protein of photosystem II), D540 (photosystem II 10 kDa polypeptide) and cyFBPase (cytosolic fructose-1,6-bisphosphatase) photosynthetic-related promoters have been shown to activate expression in green tissues (Tada et al., 1991; Lu and Bogorad, 1992; Kyozuka et al., 1993; Jang et al., 1999; Nomura et al., 2000; Si et al., 2002, 2003; Cai et al., 2007). These promoters primarily confer mesophyll cell-specific expression in leaves, with weak or no expression in the epidermal or other leaf cell types. The rice sps1 (sucrose phosphate synthase1) promoter activates expression in seed scutellum tissue and pollen, in addition to leaf mesophyll cells (Chavez-Barcenas et al., 2000; Martinez-Trujillo et al., 2004). Although the above described promoters are useful tools for transgene expression, they either confer expression in multiple diverse cell types (sps1) or are relatively specific to mesophyll cells. Thus a promoter which confers expression in the other cell types of leaves, and other green tissues is needed.

To isolate such a promoter, we utilized in-house and publicly available gene expression data to identify a candidate gene that is expressed only in leaves and panicles of rice plants. We tested the approximately 2.2 kbp region upstream of this gene, including a 5′ intron and the UTR, for its ability to confer organ-specific transgene expression in rice. We describe the expression properties of this promoter and discuss its potential uses for precisely controlling transgene expression in genetically engineered rice and other monocot plants.

Results

Identification and isolation of a rice gene expressed primarily in leaves and other green tissues

To identify rice promoters that have organ- or tissue-specific expression patterns of potential use in crop biotechnology, transcript profiling approaches were used to examine gene expression. Using a rice cDNA microarray (http://www.life.uiuc.edu/bohnert/ricearray/), we identified an Expressed Sequence Tag (EST; GenBank accession: CA753991) that exhibited substantial levels of expression in Nipponbare rice seedling leaf and flowering panicle samples (data not shown). This same microarray element exhibited little or no detectable expression in seedling root, immature panicle (nongreen tissue dissected from the boot), stamen, and developing seed samples. Based on the CA753991 EST sequence, primers were designed and a 744bp partial cDNA was amplified with reverse transcriptase-polymerase chain reaction (PCR) using Nipponbare leaf RNA as template. The amplified CDNA was cloned, sequenced and compared with the publicly available rice genome sequence. The cloned cDNA sequence matched the 5′-end of a novel leucine-rich repeat (LRR)-receptor kinase-like gene (LOC_Os02g40240). We named this gene Leaf Panicle 2 (LP2) based on its organ-specific expression pattern. Several other rice genes had modest nucleotide sequence similarity to the cloned LP2 cDNA fragment, but each contained <70% overall nucleotide identity and thus were unlikely to have cross-hybridized with this sequence.

The steady-state transcript levels of LP2 in various tissues were further examined using RNA blot analysis. Using the 744 bp LP2 cDNA as a probe, a transcript approximately 3.5 kb in length was detected in seedling leaf and flowering panicle RNA samples (Figure 1a). Little or no transcript was detectable in RNA from seedling roots, stamens (prior to anthesis), developing seeds at the soft-dough stage, undifferentiated callus tissue grown in the dark, and immature panicle tissue prior to emergence from the boot. The leaf and flowering panicle samples used for RNA blot analysis included chlorophyll-containing tissues (leaf blade or the lemma, palea and rachis respectively), while the other samples did not, suggesting that LP2 was specifically expressed in photosynthetically active green tissues.
To further validate the organ-specific expression pattern of the LP2 gene, we also examined global transcriptional profiling data that had recently become publicly available. The steady state LP2 transcript levels detected on the rice Affymetrix GeneChip™ arrays were visualized using the Genevestigator Metaprofile Analysis tool (http://www.genevestigator.ethz.ch/). Using the anatomy analysis scatterplot visualization feature, significant signal for the probe set representing the LP2 gene (Os11890.1_S1_at) is exclusively detected in the leaf and shoot samples (Figure 1b). Only background levels of signal are observed in the other organ/tissue types currently represented in the Genevestigator database. The green-tissue expression specificity of the LP2 gene was similarly supported from analysis performed with the rice gene expression atlas (RiceGE; http://signal.salk.edu/cgi-bin/RiceGE) and Massively Parallel Signature Sequence tags (MPSS; Nobuta et al., 2007; http://mpss.udel.edu/rice/; Figure S1). Interestingly, it became clear from these analyses that LP2 transcript expression was only detected in leaf and shoot samples of japonica rice, but not in samples from indica rice (Figure S1a,b). Genome sequence analysis suggests that the region of chromosome 2 containing the LP2 gene is absent or highly divergent in indica rice (Figure S1c), thus explaining the failure to detect LP2 expression in the indica samples. Overall, these results support the conclusion that the LP2 gene is strongly and specifically expressed within leaves and other photosynthetic shoot tissues of japonica rice plants.

The observed size of the LP2 transcript on the RNA blot is consistent with expectations based on the available full-length cDNA and partial EST sequences present in the database. A full length cDNA (GenBank accession: AK065018) 3475 bp in length has been cloned and sequenced (Kikuchi et al., 2003), but sequences from other partial cDNA clones suggest that a shorter LP2 transcript 3420 bases in length is also expressed. These two LP2 transcripts differ in length by 55 bases because of alternative splicing of an intron within the 5’ UTR upstream of the translational start site (Figure 2a). Determining whether one or both of these two LP2 transcripts are detected via RNA blot hybridization was difficult since the difference in length is small relative to the total transcript size and thus unlikely to be resolved on the gel. The shorter LP2 transcript is represented by 54 EST sequences in the database, while the longer transcript is supported by only 24 EST (http://compbio.dfcii.harvard.edu/cgi/; Quackenbush et al., 2001). These EST abundances suggest that the shorter 3420 base transcript accumulates to higher levels, at least in those samples used to generate the various EST libraries. The predominant transcription start site, designated +1 in Figure 2a (323 bp upstream of the translation start codon) was deduced based on alignment of the guanine capped, full length cDNA (AK065018) and the available 5’ EST sequences with the rice genome sequence.

Isolation of the LP2 promoter and generation of reporter gene fusion constructs

To examine the ability of the LP2 promoter to control organ/tissue-specific transgene expression in rice, we used PCR to amplify a 2221 bp region of sequence upstream of the translation start codon. Initially, we made a translational fusion of this fragment (from −1807 to +414 numbered relative to the transcription start site) to the GUS::mGFP5...
The LP2 promoter and intron sequence and the structure of the T-DNA regions of the pC1303-LP2, pGPro1-LP2, and pGPro1-CaMV35S binary vectors. (a) The 2272 bp sequence of the LP2 promoter and coding sequence from +1936 to +336 is shown (GenBank accession: FJ831442). Direct repeat sequences are highlighted in light and dark grey. The LP2 transcription start site is designated +1 and the direction of transcription is shown with an arrow. The putative TATA box is boxed in bold text. The pyrimidine-rich “Y patch” is boxed lower case text. The larger 5’ intron sequence is shown in lower case letters with the alternatively spliced smaller intron shown in bold italic lowercase text. The LP2 start codon is underlined. (b) The pC1303-LP2 binary vector is a derivative of pCAMBIA1303, while the pGPro1-LP2 and pGPro1-CaMV35S binary vectors are derivatives of the pGreen binary vector pGPro1. Both vectors contain a GUS::GFP gene fusion which encodes a bifunctional reporter protein. RB Right Border, LB Left border (the pGPro1 constructs have two left borders in tandem), PLP2 rice Lleaf Panicle2 promoter, Pe35S double enhanced CaMV35S promoter, Peact1 rice Actin1 promoter, T35S CaMV35S terminator, Tnos nopalene synthase terminator, hptII hygromycin phosphotransferase II gene, GUS β-glucuronidase gene, mGFP5 modified green fluorescent protein gene, eGFP-enhanced green fluorescent protein gene. Transcription start sites are designated +1 and the direction of transcription is shown with an arrow. The LP2 promoter (grey), 5’ UTR (hashed box) is translationally fused to the reporter gene (the partial LP2 coding region is shown as a black rectangle). The 5’ LP2 and Actin1 introns are drawn as diagonal lines. The locations of unique restriction sites used for promoter cloning and genomic DNA digestion for DNA blot hybridization are shown. The 496 bp GUS region used as a probe for DNA and RNA blot hybridization is shown as a black stippled box.
typical location of a TATA box (Figure 2a). It also contains a ‘Y patch’ (C/T pyrimidine-rich sequence at +97 to +108; Figure 2a), which is present in the 5’ UTR of many rice transcripts (Civan and Svec, 2009). Another feature of the LP2 promoter is a 353 bp directly repeated sequence (Figure 2a). The two repeat sequences are 93% identical and A + T rich (70% A + T), but do not match other sequences in the rice genome and are not transposon-derived repeat elements. The structure of the resulting binary vector (pC1303-LP2) was confirmed by DNA sequencing and the plasmid was introduced into Agrobacterium tumefaciens strain AGL1. A diagram of the pC1303-LP2 T-DNA region is shown in Figure 2b.

Recently, independent research groups have observed an undesirable interaction between several dicot promoters with tissue/organ specificity and the CaMV35S enhancer used in many common binary vector plasmids to control the expression of the selectable marker (Yoo et al., 2005; Zheng et al., 2007; Gudynaite-Savitch et al., 2009). It is clear from these results that the CaMV35S enhancer exhibits promiscuous bidirectional enhancer activity causing transgene expression in transformed plants that is inconsistent with the known specificity of the other promoters present within the T-DNA. To avoid a potential interaction between the CaMV35S double enhancer and the expression conferred by the rice LP2 promoter in the pC1303-LP2 construct, we constructed pGPro1, a novel binary vector well suited for promoter analysis in monocot plants (Thilmony et al., 2006). A 2272 bp LP2 promoter and partial coding region from −1936 to +336 (Figure 2) was amplified and translationally fused to the GUS::eGFP bifunctional reporter gene in the pGPro1 binary vector. This LP2 promoter fragment includes 13 bp of coding sequence, the entire 323 bp 5’ UTR including the alternatively spliced 5’ intron and 1936 bp of upstream promoter sequence (Figure 2a). The pGPro1-LP2 vector was sequenced to confirm its structure and introduced into Agrobacterium tumefaciens strain AGL1 (Lazo et al., 1991). A diagram of the T-DNA region of the pGPro1-LP2 binary vector is shown in Figure 2b. A positive control binary vector (pGPro1-CaMV35S) carrying the double enhanced CaMV35S promoter fused to the GUS::eGFP reporter gene (Figure 2b) was also constructed.

**Generation of LP2 promoter-reporter transgenic rice**

Transgenic rice plants were generated using the pC1303-LP2 and pGPro1-LP2 constructs via Agrobacterium tumefaciens transformation (see Experimental procedures). A total of 23 pC1303-LP2 plant lines and ten pGPro1-LP2
plant lines were regenerated and grown to maturity in the greenhouse. Reporter gene expression was examined in the leaves of the T₀ plants using histochemical staining; 27 of the 29 lines tested exhibited β-glucuronidase (GUS) activity. The two transgenic pC1303-LP2 plant lines that lacked detectable activity in leaves were not characterized further. Southern blot hybridization analysis was performed on seven T₁ plant DNAs from lines, five pC1303-LP2 and two pGPro1-LP2 lines (Figure 3). Genomic DNA was digested with either Ncol or KpnI restriction enzymes, which each cut only once within the T-DNA. Genomic DNA from pC1303-LP2 transgenic lines digested with Ncol or KpnI and hybridized with a GUS probe (shown in Figure 2b) are expected to produce bands >2.9 kb or 5.1 kb respectively for intact T-DNAs. Likewise, genomic DNA from pGPro1-LP2 transgenic lines that contain complete T-DNAs are expected to produce bands >5.7 kb for the Ncol digest or > 5.6 kb for the KpnI digest when hybridized with a GUS probe. The blot hybridization results illustrate that the seven lines contain one to four copies of the T-DNA (Figure 3). Each transgenic line contains one or more GUS hybridizing bands of different sizes larger than the expected T-DNA fragment, indicating that each line is independent with a likely intact reporter transgene cassette(s) integrated at a unique genomic location(s).

LP2 promoter drives high expression in leaves of transgenic rice seedlings

The plant lines examined by Southern blot analysis shown in Figure 3 were fully fertile, and thus were selected for further characterization of reporter gene expression in the T₁ and T₂ generations. Detection of β-glucuronidase activity allowed easy and reliable documentation of reporter gene expression, but we also evaluated whether we could utilize visualization of fluorescence in live plant tissues mediated by the GUS::Green Fluorescent Protein bifunctional reporters fused to the LP2 promoter in the pC1303-LP2 and pGPro1-LP2 vectors. Several T₁ hygromycin-resistant seedlings for each line were examined under ultraviolet and blue light to visualize GFP fluorescence. No green fluorescence was detected in seedling roots of either the pC1303-LP2 or pGPro-LP2 construct lines (data not shown) suggesting the LP2 promoter, as expected, does not confer detectable levels of GFP expression in seedling roots. Unfortunately, GFP fluorescence in seedling leaves was somewhat difficult to detect using the available visualization systems because it was masked by chlorophyll (red) autofluorescence (data not shown). We have found that detection of GFP fluorescence in leaves of other transgenic rice lines expressing the bifunctional reporters controlled by strong constitutive promoters (e.g. CaMV35S and rice Act1) was also challenging (data not shown). Only a few LP2 and 35S promoter transgenic lines with unusually high levels of expression reproducibly exhibited levels of GFP fluorescence that were distinguishable from chlorophyll autofluorescence in green tissues and were clearly different from wild-type controls. For these reasons, we chose to utilize detection of β-glucuronidase activity to further examine and document LP2 promoter-mediated reporter gene expression in subsequent experiments.

Seedlings of the seven lines shown in Figure 3 were grown for 2 weeks in sand and then histochemically stained

Figure 4 Rice seedlings histochemically stained for β-glucuronidase activity. Two-week-old rice seedlings germinated in sand in the growth chamber were stained for GUS activity. Following an overnight incubation in X-Gluc substrate at 37 °C, the seedlings were destained in 95% ethanol to remove chlorophyll. (a) wild-type nontransgenic Nipponbare seedling (b) T₂ homozygous pC1303-LP2 line #62 seedling (c) T₂ homozygous pGPro1-CaMV35S line #53 seedling and (d) T₂ homozygous pGPro1-LP2 line #3 seedling.
for GUS activity. Representative results for two of the LP2 promoter transgenic lines compared with nontransgenic and CaMV35S control plants are shown in Figure 4. Both roots and leaves of the seedling containing the pGPro1-CaMV35S promoter construct stained strongly for β-glucuronidase activity, while no background staining was observed in wild-type Nipponbare seedlings under our staining conditions. GUS staining observed in both the pC1303-LP2 and pGPro1-LP2 transgenic lines was the strongest in the leaf blade with weaker staining in the leaf sheath and little or no staining visible in the roots (Figure 4). Similar results were observed for the other LP2 transgenic lines, and although the strength of staining varied among the lines, the five pC1303-LP2 and two pGPro1-LP2 independent transgenic lines all exhibited the same organ-specific pattern of expression shown in Figure 4. The two pGPro1-LP2 lines (#2 and #3) tended to exhibit quantitatively darker staining in the seedling leaf blade and sheath compared with the pC1303-LP2 lines (#7, 32, 51, 55 and 62). The pC1303-LP2 line #7 had the weakest staining in the seedling leaf blade and exhibited little or no GUS staining in the leaf sheath. These results demonstrate that both the pC1303-LP2 and pGPro1-LP2 binary vector constructs generated multiple transgenic rice lines with organ-specific reporter gene expression despite containing different T-DNA components in different orientations relative to the borders and the selectable marker expression cassette (Figure 2b).

**LP2 promoter exhibits green tissue-specific expression in adult transgenic rice plants**

The organ-specific expression pattern conferred by the LP2 promoter was further characterized spatially and temporally throughout plant development. β-glucuronidase activity was examined in various tissues of pC1303-LP2 and pGPro1-LP2 transgenic rice plants grown in the greenhouse. Histochemical staining was strong in both seedling and mature leaves, but exhibited distinctly different spatial GUS staining patterns depending on leaf maturity.

**Figure 5** Spatial and temporal β-glucuronidase (GUS) activity in LP2 transgenic rice. T2 transgenic pGPro1-LP2 line #2 samples were stained for GUS activity. (a) seedling leaf (b) mature leaf (c) immature panicle (d) spikelet prior to anthesis (e) mature leaf blade cross-section near the cut edge of a stained leaf (f) hard-dough seed with the pericarp partially removed to show the endosperm (g) imbibed mature seed cross-section (h) adult plant roots (i) seedling root cross-section and (j) embryogenic callus. A, anther; B bulliform cells; E epidermal cells; En endosperm; Em embryo; Le lemma; M mesophyll cells; P phloem; Pa palea; Per pericarp; Ped pedicel; X xylem.
Since the native promoter is light-induced in seedling leaves (Figure 5a, b). Two-week-old seedling leaves stained evenly throughout the blade, while mature leaves from greenhouse grown plants typically stained most strongly at the cut edges and along the longitudinal veins. GUS staining away from the cut edges was usually much weaker than near the cut edges. This staining pattern in mature leaves is likely an artifact of the failure of the X-Gluc substrate solution to evenly penetrate the hydrophobic surfaces of the mature rice leaves. We attempted to improve the uniformity of staining by vacuum infiltrating the GUS staining solution, but even with these additional measures, staining of LP2 and CaMV35S control transgenic lines was typically weak or not detected more than a few millimeters from the cut edge of the leaf as shown in Figure 5b. In mature leaf blade cross-sections, the darkest GUS staining occurs within the mesophyll cells while lighter staining was visible in the other cell types of the leaf including the epidermal, xylem, phloem and bulliform cells (Figure 5e).

β-Glucuronidase activity was detected histochemically also in leaf sheaths, and nonreproductive green tissues of the panicle in both the pC1303-LP2 and pGPro1-LP2 transgenic lines. The lemma, palea, awn and pedicel each exhibited GUS activity with the strongest staining typically observed in the pedicel and along the ribs of the lemma (Figure 5c, d). The stamen and pistil reproductive structures do not show detectable GUS activity (Figure 5d). The (green) pericarp of immature seeds also showed GUS activity (Figure 5f), but the immature embryo or endosperm did not stain. Imbibed mature seeds did not display detectable β-glucuronidase activity in the embryo, endosperm or the pericarp (Figure 5g). Whole roots from greenhouse grown plants neither exhibit GUS staining activity (Figure 5h) nor contain any visibly blue cells when examined following sectioning (Figure 5i). Hygromycin-resistant callus was generated from the T1 seed of several of the transgenic lines and then examined for β-glucuronidase activity. Most of the calli pieces did not exhibit blue staining, but occasionally some sectors of individual calli had weak GUS activity (Figure 5j).

**LP2 promoter is light-induced in seedling leaves**

Since the native LP2 gene exhibited expression in photosynthetic tissues and the LP2 promoter conferred reporter gene expression in these same locations, we further investigated whether expression was regulated in response to light. Homozygous T2 seed of control and transgenic lines were germinated in sterile sand in the dark or under a 16L : 8D light regime in the growth chamber at 28 ℃. After 2 weeks, individual seedlings were either stained for β-glucuronidase activity or the leaf and root tissues were harvested for RNA or protein extraction. The pattern of histochemical GUS staining of representative light and dark grown seedlings is shown in Figure 6a arrows. Dark grown LP2 seedlings, compared with the light grown individuals, had much lower levels of GUS staining in the aerial parts and reproducible staining was observed only in the tips of the coleoptile and first true leaves (Figure 6a arrows). In contrast, GUS activities in the light and dark grown pGPro1-CaMV35S plants were similar to one another, although the leaves and the roots of the light grown seedlings tended to stain more darkly than the dark grown plants (Figure 6a).

β-Glucuronidase enzyme activity was also measured using a fluorimetric substrate to quantify the histochemical staining results (Figure 6b). CaMV35S and LP2 promoters exhibited similar levels of activity in light-grown seedling leaves, while leaves of the LP2 dark grown seedlings exhibited detectable activity at levels approximately seven-fold lower (Figure 6b). The GUS activity in the LP2 seedling was more than 150-fold higher in the light grown leaves than the roots, which had levels of activity essentially indistinguishable from background. The CaMV35S promoter also exhibited a modest light-responsive induction (~2- to 3-fold) in leaves (Figure 6b).

Reporter gene transcript levels in the light and dark grown seedlings were also examined using RNA blot analysis. RNA transcript was not detected in light or dark grown seedling roots or dark grown seedling leaves of the LP2 transgenic lines, while approximately 40 fold higher levels of transcript were detected in light grown LP2 transgenic leaves compared with the background signal detected on the blot for the dark grown leaves (Figure S2a), consistent with the observed reporter gene activity. This level of transcript was similar to that observed in light grown leaves of a CaMV35S transgenic line. Negligible activity was detected in nontransgenic leaf and root samples as well as root samples from LP2 expression lines.

The light-responsive expression mediated by the LP2 promoter could be dependent on light-stimulus in a diurnal fashion or potentially regulated via the plant circadian clock. We further examined the light-dependent pattern of expression of the LP2 gene using the Diurnal website (http://diurnal.cgrb.oregonstate.edu; Mockler et al., 2007). The LP2 gene exhibited diurnal cycling with the normalized transcript levels varying three to fourfold between day and night in the ‘LDHH’ (Light, Dark, High temp, High temp) and ‘LDHC’ (Light, Dark, High temp, Cold temp)
samples (Figure S2b). The LDHH samples were grown under a 12L : 12D with a constant temperature of 31 °C, while the LDHC samples were grown under a 12L : 12D with a day-time temperature of 31 °C and a night-time temperature of 20 °C (T. Mockler, personal communication). Surprisingly, the peak expression levels were detected at midnight, while the lowest transcript levels were observed from dawn to midday in the diurnal time-course experiments (Figure S2b).

**Discussion**

We have demonstrated that the LP2 promoter confers strong organ-specific reporter gene expression in leaves and other green tissues of transgenic rice plants. LP2-mediated reporter gene expression was either weak or not detected in roots, seeds (with the exception of the green pericarp during seed development) or the nongreen reproductive structures. Multiple independent transgenic plants containing either the pC1303-LP2 or pGPro1-LP2 T-DNAs, located in different genomic positions with different copy numbers, displayed a consistent pattern of organ-specific expression despite the differences between the composition and structure of the two constructs. These results suggest that the CaMV35S enhancer present in the pCAMBIA derived vector construct did not alter the pattern of expression mediated by the LP2 promoter, as has been observed with several dicot organ-specific promoters (Yoo *et al.*, 2005; Zheng *et al.*, 2007). It is possible the 35S enhancer either does not alter the expression conferred by nearby promoters in rice as it does in *Arabidopsis* and tobacco, or possibly the LP2 promoter is inherently
insensitive to the CaMV35S enhancer’s effects. It will be intriguing to examine whether other rice promoters analysed in transgenic plants exhibit this insensitivity, suggesting a general difference in the CaMV35S enhancer’s function between monocot and dicot species.

The pattern of expression conferred by the LP2 promoter in transgenic rice plants was entirely consistent with the expression pattern of the native LP2 gene. This suggests that the important control elements responsible for organ-specific and light-responsive expression of the LP2 gene are present within the approximately 2.2 kb upstream fragment fused to the reporter genes in our transformation constructs.

The LP2 sequence in the transformation constructs tested included approximately 1 kb of promoter sequence (upstream of the transcription start site) containing two 353 bp direct repeats, the entire 5' UTR, the 5' intron, and a portion of the first coding exon translationally fused to the reporter gene (Figure 2). When this portion of sequence was analysed for the presence of cis regulatory elements using various web-based analysis programs (see Experimental procedures), numerous motifs were found that are consistent with the LP2 promoter exhibiting light-responsive expression. The sequence contains 20 different putative light-responsive cis elements (Figure S3). These 20 elements occur 37 times within the LP2 sequence because of multiple appearances of some sequences. Several of these elements are seven, eight or nine nucleotides in length and are exact matches for known motifs (Figure S3). For example, the LP2 promoter contains exact matches for the nine nucleotide ATC-motif (start position -490), the eight nucleotide AE-box (start position -421) and I-box (start position -81) elements.

Each of these elements is associated with light-responsive-ness in numerous plant species (Arguello-Astorga and Herrera-Estrella, 1996; Park et al., 1996). The 5' intron also contains three light responsive cis elements including an exact eight nucleotide match for the chs-CMA2a light response element (Arguello-Astorga and Herrera-Estrella, 1996) at position +135, and two seven nucleotide matches for the Box-I element (Kuhlemeier et al., 1988) at positions +180 and +213 (Figure S3). Sequences further upstream, although not identical to the known light-responsive motifs, match 10/11 nucleotides of the GATA-motif (Lam and Chua, 1989), 10/11 nucleotides of 3-AF1 binding site (Lam et al., 1990), 9/10 nucleotides of the ATCT-motif (Conley et al., 1994) and 11/13 nucleotides of the LPSE2 motif (Cai et al., 2007; Figure S3). The LPSE2 motif is present in the rice D540 promoter and was shown to have positive regulatory function for leaf expression and negative regulatory function for root expression (Cai et al., 2007).

The LP2 RNA transcript displays light-responsive diurnal changes in transcript abundance with peak levels detected in the middle of the dark cycle. The significance of this light-regulated cycling with peak expression at midnight is unknown, but it is not surprising that the LP2 transcript cycles, since 89% of reliably detected Arabidopsis transcripts have been shown to cycle under at least one environmental condition (Michael et al., 2008). Consistent with the diurnal expression pattern of the LP2 gene, sequences similar to the PBX protein box (Michael et al., 2008) and TBX telo-box motifs (Tremousaygue et al., 2003) are present twice and seven times, respectively, within the promoter sequence (Figure S3). The PBX box is identical in sequence to the FORC^A response element which is associated with light signalling and defense responses in Arabidopsis (Evrard et al., 2009).

The LP2 5' intron sequence was examined via bioinformatics to estimate the likelihood that it contributes to intron-mediated enhancement (IME) of expression (Rose et al., 2008). Despite the modest size of the larger intron (230 bases), it contains two exact matches for the six nucleotide IME motif which is typically present within rice introns that enhance expression (Rose et al., 2008; Figure S3). When this intron is analysed using the IMEter tool (http://korlab.ucdavis.edu/cgi-bin/web-imeter.pl), it achieves a 10.8 score. Positive IMEter scores correlate with IME capability. For example, the IMEter score was 218 for the 462 base rice Act1 first intron and 51 for the 535 base maize Adh1 first intron, both expression-enhancing introns. In contrast, the IMEter score was -14 for the 89 base nonenhancing rice glutelin first intron (Rose et al., 2008). Thus, IMEter predicts that the LP2 first intron is a modest enhancer of gene expression. It will be interesting in the future to determine experimentally whether the 5' intron, the direct repeats, the 5' UTR, or specific cis elements in the promoter sequence are responsible for the observed expression pattern.

The LP2 gene encodes a LRR-receptor kinase-like protein of the LRR-XII subfamily (Dardick and Ronald, 2006). The N-terminus of the protein is predicted to contain a signal sequence targeting the protein for secretion. Bioinformatics analyses predict that the LP2 protein is plasma membrane localized with an extracellular LRR domain, a single transmembrane domain and an intercellular kinase domain (http://localizome.org/; Lee et al., 2006; Figure S4a). The LP2 protein contains a cysteine residue in place of the con-
served arginine residue in kinase domain VI, classifying LP2 as a non-RD receptor-like kinase (Figure S4b). Non-RD receptor kinases of known function in plants are pathogen recognition receptors involved in innate immunity (Dar-dick and Ronald, 2006), suggesting a potential involvement of LP2 in plant-pathogen interactions. Indeed, the LP2 protein is 41% identical and 58% similar to the Xa21 (Song et al., 1995), and 33% identical and 50% similar to the Xa26/Xa3 (Sun et al., 2004; Xiang et al., 2006) bacterial blight resistance proteins (Figure S4b). The LP2 protein also contains conserved Ser686 and Ser689, but not the Thr688 (residues numbered based on Xa21 sequence), autophosphorylation sites required for Xa21 protein stability and disease resistance (Xu et al., 2006; Figure S4b). Recently, two potential downstream targets, a MYB-like DNA-binding protein (Os01g74020) and a NAD-dependent epimerase/dehydratase-like protein (Os02g54890) have been shown to interact with the LP2 kinase domain in the yeast two-hybrid system (Ding et al., 2009).

Although the LP2 transcript is abundantly expressed in Nipponbare leaves, its expression appears to be further enhanced in leaves upon challenge with virulent and avirulent Magnaporthe oryzae and Xanthomonas oryzae pv. oryzae (Xoo) pathogens which cause blast and bacterial blight of rice respectively (Figure S1b; Shimono et al., 2003). Probenazole, a chemical inducer of disease resistance, also enhanced LP2 expression in leaves (Shimoto et al., 2003; sequence ID S12429). Interestingly, the LP2 gene is transcriptionally induced approximately fivefold in Nipponbare rice roots four days after inoculation with the parasitic plant Striga hermonthica compared with the mock-inoculated control (Swarbrick et al., 2008). Although these results show that Nipponbare plant roots that are resistant to Striga parasitism have significantly increased LP2 expression compared with the control, the induced expression level is still approximately 100-fold lower than the levels detected in green leaf tissue samples (Swarbrick et al., 2008; and data not shown). The number of LP2 MPSS tags also increases two- to threefold in water weevil (Lissorhoptrus oryzophilus) damaged leaves 24 h after exposure compared with untreated leaves (Figure S1b).

The LP2 transcript also has also been shown to be responsive to abiogenic stresses. Leaves from salt-stressed rice plants six days after treatment exhibited a fivefold down-regulation of LP2 expression (Kim et al., 2007; sequence ID AK065018). Rice plant leaves exposed to cold stress (4 °C for 24 h) generated no MPSS tags for the LP2 gene, suggesting a substantial reduction in transcript abundance elicited by cold treatment.

Consistent with LP2 regulation by abiogenic and biotic stresses, its promoter sequence contains five putative stress or pathogen response elements (Figure S3). There are two 9/10 matches for the ABRE (Abscisic Acid Response Element; Ono et al., 1996) at positions −1248 and −883, one 9/10 match for a tricarboxylic acid TCA salicylic acid response element (Pastuglia et al., 1997) at position −724, and a perfect match for the seven nucleotide tobacco EIRE (Elicitor Response Element; Shah and Klessig, 1996) located at position −604 (Figure S3). Taken collectively, our experimental and bioinformatics results, and the published data demonstrate that the LP2 transcript accumulates specifically in leaves and other green tissues, and is responsive to various biotic and environmental stresses in Nipponbare rice plants.

Research examining epigenetic inheritance in japonica rice variety ‘Yamada-nishiki’ identified the LP2 locus (Akimoto et al. named the gene Xa21G) as a highly methylated region with silenced gene expression (Akimoto et al., 2007). This is somewhat surprising since Nipponbare (also a japonica variety) exhibits strong gene expression. Akimoto et al. used treatment with 5-aza-deoxycytidine (an inhibitor of DNA cytosine methylation) to develop hypomethylated rice plants. They showed that a region upstream of the LP2 gene exhibited a dramatic loss in cytosine methylation in the progeny of these hypomethylated plants. This loss of methylation corresponded with activation of gene expression and surprisingly, an increase in resistance to Xanthomonas oryzae pv. oryzae race PR2 (Akimoto et al., 2007). Although this result supports the hypothesis that LP2 is a functional Xoo resistance gene, it is possible that other 5-aza-deoxycytidine-induced changes present in their hypomethylated rice progeny are responsible for the increased resistance. Indeed, the LP2 gene is strongly expressed in Nipponbare rice plants and yet, this genotype is fully susceptible to multiple strains of Xanthomonas (Nino-Liu et al., 2006; P. Ronald, personal communication). Despite the wealth of information available on LP2, its biological function remains unknown. The LP2 expression pattern and sequence homology strongly suggest a role in pathogen defense within leaves and other photosynthetic tissues of japonica rice plants.

Although other promoters from rice (rbcS, Pdk, LHCP II, D540 and cyFBPase) have been demonstrated to confer green-tissue specificity, they were derived from genes whose functions were photosynthetic related and were primarily expressed in leaf mesophyll cells (Tada et al., 1991; Luan and Bogorad, 1992; Kyozuka et al., 1993; Iang et al., 1999; Nomura et al., 2000; Si et al., 2002, 2003; Cai et al.,
2007). The LP2 promoter is also green-tissue specific and clearly confers strong expression in leaf mesophyll cells as well, but uniquely it also directs reporter gene expression in multiple other cell types present in the leaf including the epidermal and vascular cells. This aspect of leaf cell type specificity distinguishes and broadens the usefulness of the LP2 promoter as a transgene expression control element relative to other available promoters. Thus, if expression is desired in leaf epidermal cells or the leaf vascular tissue (in addition to leaf mesophyll cells), for example to confer foliar disease resistance, the LP2 promoter provides organ-specific expression capability in those cell types. In summary, we have shown that the 2.2 kb LP2 promoter exhibits consistent organ-specific light-responsive expression specificity in multiple independent transgenic rice plants, demonstrating that this promoter will be a useful tool for rice biotechnology and potentially other crop plants.

**Experimental procedures**

**Plant materials, growth conditions and transformation**

Nipponbare rice (Oryzae sativa, japonica type, GSOR #100) was obtained from the Genetic Stocks—Oryza Collection at the Dale Bumpers National Rice Research Center in Stuttgart, Arkansas. Rice plants were grown in a greenhouse environment under a mean temperature of 28 °C and supplemented with 16 h/day of sodium lamp light. Seeds and transplanted seedlings were potted in Sunshine mix #1 (SunGrow Horticulture Distribution, Bellevue, WA, USA) with slow release fertilizer Osmocote Plus 15-9-12 plus micronutrients (Scotts-Sierra Horticultural Products, Marysville, OH, USA) added. The six inch pots were partly submerged in trays of water and Peter’s liquid fertilizer 20-20-20 plus micronutrients (Scots-Sierra Horticultural Products) was added once each week to the twice daily watering regime.

Rice was transformed via Agrobacterium-mediated transformation according to a method derived from Sallaud et al. (2003) and Yang et al. (2004) as previously described (Thilmony et al., 2006). Regenerated T0 plants were transferred to soil and grown in the greenhouse as described above. Harvested T1 and T2 transgenic rice seed was dried for 5 days at 50 °C, de-hulled and then surface sterilized (placed in 70% ethanol for 5 min, transferred to a solution of 30% bleach with 0.1% Triton X-100 for 20 min, and then rinsed five times with sterile water) prior to sowing. The seed was then either sown in sterilized sand, or placed on germination media containing 4.33 g/L of MS basal salts, 2.6 g/L of Phytagel, 0.5 mg/L of 6-benzylaminopurine and 40 mg/L of hygromycin and then incubated in a growth chamber at 28 °C under a 16-h light/8-h dark cycle. Seedlings were scored for antibiotic resistance after 2–3 weeks of growth.

Young leaves and root tissue for RNA analysis were harvested from approximately two-week-old sand-grown seedlings. Mature leaf tissue and reproductive tissues were harvested from greenhouse plants. For the light-dark expression analysis, seed was grown in sterile sand in a growth chamber either exposed to the 16-h light/8-h dark cycle with an approximate light intensity of 250 µmol/m²/s provided by fluorescent and incandescent bulbs or kept in complete darkness within a light-tight container. Both leaf and root tissues were harvested approximately two weeks after germination. All plant material was immediately frozen in liquid nitrogen, then stored at −80 °C.

**LP2 cloning and vector construction**

A partial LP2 cDNA was amplified from rice leaf RNA using Reverse Transcriptase-PCR with the following primers: OligoT32V (an anchored oligo-dT primer for reverse transcription, V = A, C or G), Os5867f1 5’-AGGTAACATGGCCCTACG-3’ and Os5867r1 5’-GGCAACCATAGACAAGCCAACCG-3’. The 744 bp PCR product was cloned using the Invitrogen TOPO TA cloning kit (Carlsbad, CA, USA) and sequenced. The cloned LP2 cDNA was 99.6% identical to the rice Os2pg40240 gene, and has <70% nucleotide identity with other genes in the rice genome.

The LP2 upstream promoter, 5’ intron and 5’ portion of the first coding exon (−1807 to +414) was amplified from Nipponbare genomic DNA with high fidelity polymerase using the following primers: Os5867f2_BamH I 5’-CGCGGATCCCGACCGACCCGCTTTGAGG-3’ and Os5867r2_Nco I 5’-CGCCCATGGAGATCGCGTGGCAATCCCA-3’. This 2220 bp LP2 genomic fragment was digested and cloned into the BamH I and Nco I sites of pCAMBIA1303 (http://www.cambia.org/daisy/cambia/585.html; GenBank accession: AF234299) binary vector making a translational fusion to the gusA::mGFP5 reporter gene (see Figure 2b for T-DNA map of the pC1303-LP2 vector). The LP2 upstream promoter, 5’ intron and 5’ portion of the first coding exon (−1936 to +336) was amplified from Nipponbare genomic DNA with high fidelity polymerase using the following primers: Os5867f3_SacI 5’-CCGGGTATGGAAATCCATGAGGATGTCGG-3’ and Os5867r3_NcoI 5’-CCATGAGCGTGACCCGATGTTACCCCA-3’. This 2271 bp LP2 genomic fragment was cloned into the SacI and NcoI sites of the pGPro1 (Thilmony et al., 2006) binary vector making a translational fusion to the GUS::eGFP reporter gene (see Figure 2b for T-DNA map of the pGPro1-LP2 vector). The pGPro1-35S vector was constructed by inserting a 819 bp double-enhanced CaMV 35S promoter fragment from pCAMBIA1303 into the upstream promoter, 5’ intron and 5’ portion of the first coding exon (−1936 to +336) was amplified from Nipponbare genomic DNA with high fidelity polymerase using the following primers: Os5867f3_SacI 5’-CCGGGTATGGAAATCCATGAGGATGTCGG-3’ and Os5867r3_NcoI 5’-CCATGAGCGTGACCCGATGTTACCCCA-3’. This 2271 bp LP2 genomic fragment was cloned into the SacI and NcoI sites of the pGPro1 (Thilmony et al., 2006) binary vector making a translational fusion to the GUS::eGFP reporter gene (see Figure 2b for T-DNA map of the pGPro1-LP2 vector). The pGPro1-35S vector was constructed by inserting a 819 bp double-enhanced CaMV 35S promoter fragment from pCAMBIA1303 into the SacI and NcoI restriction sites of the pGPro1 (see Figure 2b for T-DNA map of the pGPro1-CaMV35S vector). After DNA sequencing to confirm their structures, each binary vector plasmid was isolated with the Fermentas GeneJet Plasmid Miniprep Kit (Gemini Burnie, MD, USA), electroporated into Agrobacterium tumefaciens strain AGL1 (Lazo et al., 1991) and used to generate transgenic Nipponbare rice plants.

**DNA and RNA gel blot analyses**

RNA genomic DNA was isolated from leaves of greenhouse grown plants using a miniprep procedure (Dellaporta et al., 1983). Ten micrograms of rice genomic DNA was digested with Ncol or KpnI, separated on a 0.8% agarose gel and blotted onto Hybond N+ (Amersham Biosciences, Piscataway, NJ, USA) positively charged nylon membrane using 0.4 M sodium hydroxide. RNA was isolated from rice callus, leaf, root and reproductive tissues using Trizol
reagent (Invitrogen) with chloroform washes. Isopropanol and sodium acetate were used to pellet the RNA. The pellet was rinsed with 70% ethanol and resuspended in RNase-free water. Three micrograms of each RNA or 2 μg of light or dark grown leaf and root RNA were separated on an 0.8% agarose gel and transferred onto Hybond N+ (Amersham Biosciences) membrane using 20× SSC buffer (Sambrook et al., 1989). DNA and RNA blots were cross-linked using the UV Stratalinker 2400 (Stratagene, La Jolla, CA, USA).

A 496 bp gusA DNA fragment called GUSS (shown in Figure 2b) was used as a probe in DNA and RNA blot analyses. The 744 bp LP2 partial cDNA was used as a gene-specific probe in RNA blot hybridization analysis. Probes were radioactively labelled using α32P-dCTP with the Rediprime II Random Prime Labeling System and purified through ProbeQuant G-50 microcolumns from Amersham Biosciences. Blot hybridizations were performed using the Sigma PerfectHyb™ Plus hybridization buffer (Sigma-Aldrich, St Louis, MO, USA) as recommended by the manufacturer. Hybridized blots were washed to 1× SSC 0.1% sodium dodecylsulphate at 50 °C and exposed to X-ray film.

The radioactive signal present on the light-dark RNA blot was quantified using storage phosphor autoradiography with a Molecular Dynamics Storm 820 Phosphoimager™ (Sunnyvale, CA, USA). Image Quant software (Molecular Dynamics, Sunnyvale, CA, USA) measured the radioactive signal intensity using equal sized boxes placed over the regions of the blot containing the RNA transcript for each of the samples. The background signal was calculated by averaging the signal for the four nontransgenic samples shown in Figure S2. The signal intensity measured for each sample was then divided by this background value to generate the quantified transcript levels.

Detection of β-glucuronidase activity

Histochemoical staining for β-glucuronidase activity was performed as previously described (Jefferson, 1987; Rueb and Hensgens, 1989). Briefly, the samples were prewashed in 0.07% (v/v) Liqui-Nox soap (ALCONOX, Inc. New York, NY, USA) in 0.1 M phosphate buffer and vacuum-infiltrated in GUS staining solution for approximately ten min to promote substrate penetration (Rueb and Hensgens, 1989). The GUS staining solution contains 0.1 M sodium phosphate pH 7.0, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 1.5 g/L of X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) and 0.5% (v/v) Triton X-100. The plant tissue samples were typically incubated in staining solution at 37 °C for approximately 12 h. Occasionally, incubation was performed at 55 °C (Hansch et al., 1995) instead of 37 °C if background staining was observed in nontransgenic control tissue samples at the lower temperature. When incubation was performed at 55 °C, background staining in wild-type control samples was not observed, allowing the histochemical detection of reporter gene-mediated β-glucuronidase activity.

Fluorometric analysis of β-glucuronidase activity in leaf and/or root tissues was performed using the TKO 100 DNA Fluorometer (Hoefer Scientific Instruments, San Francisco, CA, USA). β-glucuronidase enzymatically converts the substrate MUG (4-methylumbelliferone) to the fluorescent product MU (4-methylumbellif erone). Crude protein extract was prepared from ground tissue samples according to Serres et al. (1997). The fluorometer was calibrated with 0.5 pmol of MU (Sigma-Aldrich) equal to 1000 U and 2 μL of protein extract was added to 500 μL of 1 mM MUG (Sigma-Aldrich) assay buffer. Aliquots of the reaction were sampled every 15 min and the quantity of MU produced over a 60 min reaction per microgram of crude protein was calculated. Protein extract quantification was performed using the Bio-Rad Protein Micro Assay (Bio-Rad Laboratories, Hercules, CA, USA).

Microscopy, sectioning and photography

Glucuronidase reporter gene expression was visualized under bright field conditions using an Olympus BX51 microscope system, and images were documented using the attached DP70 digital camera (Olympus, Melville, NY, USA). Microscopic images between 2x and 10x were documented using a Leica MZ16F stereomicroscope (Leica Microsystems, Bannockburn, IL, USA) with attached Retiga 2000R FAST Cooled Color 12 bit digital camera (Q Imaging, Pleasanton, CA, USA). GFP fluorescence was examined with the addition of an Xcite 120 Fluorescence Illumination System (EXFO Life Sciences, Mississauga, Ontario, Canada) to the Leica stereomicroscope with the following fluorescence filter set: excitation: 450–490 nm and barrier/long pass 515 nm. Five- to 10 μm thin sections of GUS stained rice leaves and roots were produced using a Leica CM3000 (Leica Microsystems) cryomicrotome at −18 °C. The sections were adhered to glass slides and examined for cell-localized GUS expression.

Sequence analysis and cis element identification

Sequences were examined using the Gramene website BLAST search and Contig Viewer (http://www.gramene.org/; Ware et al., 2002). Putative promoter cis regulatory elements were analysed using the Plant Cis Acting Regulatory Element search for CARE tool (PlantCARE, http://bioinformatics.psb.ugent.be/webtools/plantcare/html/; Lescot et al., 2002), the Plant Promoter Analysis Navigator (PlantPAN, http://plantpan.mbc.nctu.edu.tw/index.php; Chang et al., 2008), and the Database of Plant cis-acting Regulatory DNA Elements (PLACE, http://www.dna.affrc.go.jp/PLACE/; Higo et al., 1999). The presence of several known cis elements that were not included within the above websites’ databases, i.e. LPSE2 and other motifs present in the rice green tissue specific D540 promoter (Cai et al., 2007), the PBX and TBX elements (Regad et al., 1994; Tremousaygue et al., 2003; Michael et al., 2008), and the rice IME motif (Rose et al., 2008) were queried and annotated manually within the LP2 sequence.

Distribution of materials

The LP2 promoter and all the plasmid vectors described will be distributed upon request. Contact the corresponding author for more information.

Acknowledgements

The authors thank Dawn Chiniquy for technical assistance in generating the transgenic rice lines and John Vogel and
Yong Gu for critical reading of the manuscript. This research was supported by USDA Agricultural Research Service CRIS project 5325-21000-002-00D. Mention of trade names or commercial products is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

References


A rice organ-specific, light-responsive promoter


Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 LP2 transcript abundance and genomic organization.

Figure S2 LP2 light-regulated expression.

Figure S3 Putative cis regulatory motifs within the LP2 sequence.

Figure S4 LP2 protein analysis.

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Supporting Information

**Figure S1.** \(LP2\) transcript abundance and genomic organization. (a) A bar graph of the \(LP2\) transcript levels generated by the Rice Functional Genomic Express Database (RiceGE [http://signal.salk.edu/cgi-bin/RiceGE](http://signal.salk.edu/cgi-bin/RiceGE)) is shown. The x-axis displays the Gene Expression Omnibus (GEO) accession number and the experimental treatment descriptions provided by RiceGE. The normalized microarray intensity levels present on the Affymetrix arrays are shown on the y-axis. Yellow shading denotes samples derived from indica rice, while a white background indicates japonica samples. Green arrows mark samples derived from leaves or other green tissues. (b) The normalized number of the 20bp and 17bp Massively Parallel Signature Sequence (MPSS) tags detected for the \(LP2\) transcript are shown. The background color corresponds to the MPSS tag abundances as shown on the color scale from 0 to 300. Each column in the display represents one of the 70 MPSS libraries. A brief description of each library is shown; see the Rice MPSS website ([http://mpss.udel.edu/rice/](http://mpss.udel.edu/rice/)) for more information. The colored bars on the display identify if the library is from japonica, indica or indica x japonica \(F_1\) rice plants. (c) Rice MPSS Chromosome Viewer display for the japonica rice genomic region containing the \(LP2\) gene (blue box). Annotation of the display indicates the portion of the \(LP2\) genomic region that is a sequence/alignment gap within the indica genome. For more information on the display see the rice MPSS website ([http://mpss.udel.edu/rice/](http://mpss.udel.edu/rice/)).

**Figure S2.** \(LP2\) light-regulated expression. (a) RNA blot hybridization of seedling root and leaf samples germinated in the light or dark is shown. A radioactively labeled GUS probe (shown in Figure 2b) detected the \(GUS::eGFP\) transcript in the CaMV35S samples and the \(LP2\) light-exposed leaf samples. The radioactive signal on the blot was quantified and the fold signal relative to background (which was set=1.0) is shown below each lane. The \(LP2\) \(GUS::eGFP\) transcript is expected to be either 122 or 67 bases longer (depending on 5’ intron splicing) than the CaMV35S \(GUS::eGFP\) transcript. (b) The normalized \(LP2\) transcript abundance detected in two 12 hour day-night diurnal experiments (LDHH and LDHC) using Nipponbare plants is shown. The graph was generated by the Diurnal website ([http://diurnal.cgrb.oregonstate.edu](http://diurnal.cgrb.oregonstate.edu)) and shows the 48 hour time-course along the x-axis and the normalized microarray signal intensity along the y-axis. The day-night regime is depicted along the top of the graph. See the text for description of the LDHH and LDHC experiments.

**Figure S3.** Putative \(cis\) regulatory motifs within the \(LP2\) sequence. The 2272 bp sequence of the \(LP2\) promoter, intron and coding sequence from -1936 to +336 (GenBank Accession FJ831442) is shown. Direct repeat sequences are highlighted in light and dark gray backshading. The \(LP2\) transcription start site is designated +1 and the direction of transcription is shown with an arrow. The larger 5’ intron sequence is shown in lower case letters with the alternatively spliced smaller intron shown in bold italic lowercase text. The \(LP2\) start codon is boxed in bold text. Putative \(cis\) elements are underlined in colored text or highlighted in color with backshading (any \(LP2\) nucleotides that do not match the known \(cis\) element sequence remain uncolored). Each \(cis\) element is listed in the table in the same color as shown in the \(LP2\) sequence. The location of each element relative to the +1 transcription start site, its strand orientation, matrix score, sequence, and corresponding description (including the abbreviation of the species of origin) is shown. The \(cis\) elements that match the negative DNA strand (-) are highlighted as the reverse complement in the positive strand promoter sequence shown. The matrix score equals the total number of nucleotides that match the known \(cis\) element sequence. *A. Arabidopsis thaliana*, B.o. *Brassica oleracea*, L.e. *Lycopersicon esculentum*, O.s. *Oryza sativa*, P.c. *Petroselinum crispum*, P.s. *Pisum sativum*, N.t. *Nicotiana tabacum*, S.o. *Spinacia oleracea*, S.t. *Solanum tuberosum*, T.e. *Triticum aestivum*, and Z.m. *Zea mays*.

**Figure S4.** \(LP2\) protein analysis. (a) Predicted \(LP2\) protein localization and topology. The image was generated by the Localizome website ([http://localizome.org/](http://localizome.org/)) (Lee et al., 2006). The LRR domain is predicted to be extracellular, separated from an intercellular kinase domain by a single transmembrane domain. (b) Alignment of the \(LP2\), Xa21 (Accession U37133) and Xa29/Xa3 (Accession ABD36512) protein sequences. The sequences were aligned with ClustalW and visualized based on the percent identity using JalView (Clamp et al., 2004). The Serine residues in Xa21 which are autophosphorylation sites are denoted with red asterisks and the non-RD kinase domain VI is highlighted in the green box.
Figure S1a
Figure S1b
Thilmony et al. A rice organ-specific, light-responsive promoter

(c) Rice MPSS: Chromosome Viewer

Gap Information
- Sequence gap: missing sequence from Indica genome
- Alignment gap: sequence present, but gap in alignment (indel)

Figure S1c
(a) 

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(b) 

transcript level

![Graph](image5.png)

Figure S2
Thilmony et al.  

A rice organ-specific, light-responsive promoter

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Thilmony et al.  A rice organ-specific, light-responsive promoter

Figure S4