Characterization of a gene encoding an abscisic acid-inducible type-2 lipid transfer protein from rice

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Abstract The cloning and sequence analysis of a novel gene that encodes a type 2 non-specific lipid transfer-like protein (LTP) from rice is reported. Sequence analysis revealed an ORF encoding a protein showing characteristics of the LTP proteins. However, rice LTP2 is more similar to heterologous LTPs than to rice LTP1, supporting the existence of two distinct families of plant LTPs. *Ltp2* mRNA is accumulated only in mature seeds. In vegetative tissues, mRNA was only detected after treatment with abscisic acid (ABA), mannitol or NaCl. Transient expression experiments that the 61 nucleotides upstream of the TATA box, containing two ACGT boxes and the motif I, are sufficient for ABA responsiveness of the *Ltp* gene.

Key words: Lipid transfer protein; Abscisic acid; Abscisic acid responsive element

1. Introduction

Plant lipid transfer proteins (LTPs) have been identified by their ability to facilitate the movement of lipids between membranes in vitro, and thus have been initially assumed to participate in membrane biogenesis [1]. Following purification of LTP proteins from various plants, cDNA clones have been isolated from maize [2] and a number of other plants (reviewed in [3]). Sequence analysis of these clones revealed the presence of a signal peptide, presumably targeting the protein to the cell wall and outside the cell, as well as homology with the amylase/protease inhibitor family. In most species several *Ltp* genes exist, which are often differentially regulated. However, its role has not yet been elucidated even though some *Ltp* genes are good markers of epidermal differentiation and some LTP proteins have antifungal and antibacterial activities [4–7].

While most *Ltp* genes are highly conserved, an additional class of clones encoding an LTP-like protein has been isolated in barley and named LTP2 [8]. Other *Ltp2* related clones have been isolated from *Vigna* [9] and *Zinnia* [10], in addition to barley. As for LTP1, the exact function of LTP2 is not known. However, various studies indicate that *Ltp* genes are responsive to environmental factors like water and salt stress [11,12] or cold treatment [13]. Other *Ltp* genes, like *Ltp1* from *Arabidopsis thaliana* [14], are potential candidates to be regulated by environmental or biotic factors like stress-regulated genes of the phenylpropanoid biosynthetic pathway. These reports suggest that promoters of *Ltp* genes might be useful for engineering plant response to biotic and/or abiotic stresses.

To this end we investigated the *Ltp* gene family in rice and found that *Ltp1* genes do indeed constitute a complex multigene family with at least seven members grouped into three, possibly four, differentially regulated subfamilies [3]. We previously isolated one genomic *Ltp1* clone [15] which is responsive to abscisic acid (ABA) and wounding. Continuing this analysis, we isolated a *Ltp2* genomic clone using the zB11E barley *Ltp2* cDNA as probe [8]. In this report we characterize rice *ltp2* gene and we analyzed its expression and its promoter.

2. Materials and methods

2.1. Plant material

Rice (*Oryza sativa*, var. IR36) seeds were surface sterilized in 10% calcium hypochlorite (w/v) solution and then immersed in water for 2 h at room temperature. Rice seeds were germinated on moistened filter paper at 28°C in the dark.

Maize (W64A line of *Zea mays* L.) immature embryos were obtained from seeds of ears collected 20 or 25 days after pollination (DAP) from greenhouse grown plants.

2.2. Isolation of the rice Ltp2 gene

An *Oryza sativa* (var. IR36) genomic library (kindly provided by Dr. Steven Kay) was constructed in lambda-DASH with 15–25 kbp DNA fragments from *Sau*3A partially digested genomic DNA. The library was screened with a ^{32}P-labeled zB11E fragment of the barley *Ltp2* gene encoding a LTP type 2 [8–16]. Among several positive clones analyzed, a common hybridizing 2.2 kbp *Sac*I fragment was subcloned into pGEM-7Zf(+) vector (Promega) and the resulting plasmid was named pSSOA.

2.3. Southern analysis

DNA was isolated from rice leaves essentially as described by Dellaporta et al. [17]. Genomic DNA was digested with endonucleases, subjected to electrophoresis in 0.8% agarose and transferred to Hybond N membranes (Amersham) following standard procedures. Hybridizations with ^{32}P-labeled specific probes of the *Ltp2* gene were carried out at 65°C overnight, according to Sambrook et al. [18]. Washes of filters were performed 2 times in 2×SSC, 0.1% SDS for 20 min at room temperature and 65°C respectively, and finally once in 0.2×SSC, 0.1% SDS for 20 min at 65°C.

2.4. Northern analysis

Three days after germination, seedlings growing on moistened filter papers were subjected to different treatments by partially drying the filter paper and moistening it with NaCl, ABA or mannitol solutions. Treated and control seedlings were harvested at the appropriate times after treatment. Roots and shoots were separately immersed in liquid N_2 and stored at −80°C until RNA extraction. Total RNA was obtained from frozen tissues by phenol/chloroform extraction [19] and electrophoresed in formaldehyde/agarose gels that were blotted onto

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Hybond N membranes following standard procedures. Hybridizations with ^{32}P-labeled specific probes of the *Ltp2* gene were carried out at 42°C in a 50% formamide-based solution [18]. Final washes of filters were performed 2 times in 0.2×SSPE, 0.1% SDS for 20 min at 65°C.

2.5. Sequence analysis

DNA sequencing was performed on both strands by the dideoxy method [20] on doubled stranded DNA with fluorescent primers in an ABI 373A automatic DNA sequencer (Applied Biosystems Industries) using the Taq Dye Primer Cycle Sequencing Kit (Applied Biosystems), according to the manufacturer's instructions. Sequence data and sequence similarity analyses were carried out with DNA Strider and CLUSTAL V programs, respectively.

The sequence of the rice *Ltp* gene, type 2, has been submitted to EMBL under accession number N16721.

2.6. DNA constructs

A *Bam*HI-*Sma*I fragment containing 1114 bp of the 5′ *Ltp2* gene sequence (numbered with respect to the ATG) was produced by the polymerase chain reaction (PCR) utilizing pSSOA DNA as template, T7 (5′-TAATACGACTCACTATA-3′) as upstream primer and a specific oligonucleotide as downstream primer (5′-CACCCGGG-CATCCTCCAGCTACTGATCG-3′). This primer contains a *Sma*I site at the 5′-end followed by 20 nucleotides of the transcribed strand, beginning at the complementary sequence to the ATG initiation codon. Plasmid p1114*Ltp2*GUS (p1114) was constructed by inserting the fragment *Bam*I-*Sma*I containing 1114 bp of the *Ltp2* promoter and 5′ untranslated sequence into plasmid pGUS3, derived from pBI101 plasmid [21] in which the promoterless GUS cassette had been transferred to pUC18 (made by Dr. J. Topping, Department of Botany, Leicester University, UK). The correctness of the construct was checked by restriction mapping and DNA sequencing.

To produce the 5′ regulatory sequences of constructs p591*Ltp2*GUS (p591) and p140*Ltp2*GUS (p140) by PCR, two specific oligonucleotides (5′-CGAAGCTTCTGAAACGGGTTCGAATGGG-3′ and 5′-CGAAGCTTCTGACGCGTGGCGTCGTACG-3′) were designed as upstream primers, respectively. These primers contain a *Hin*dIII site at the 5′-end followed by 20 nucleotides of the *Ltp2* promoter

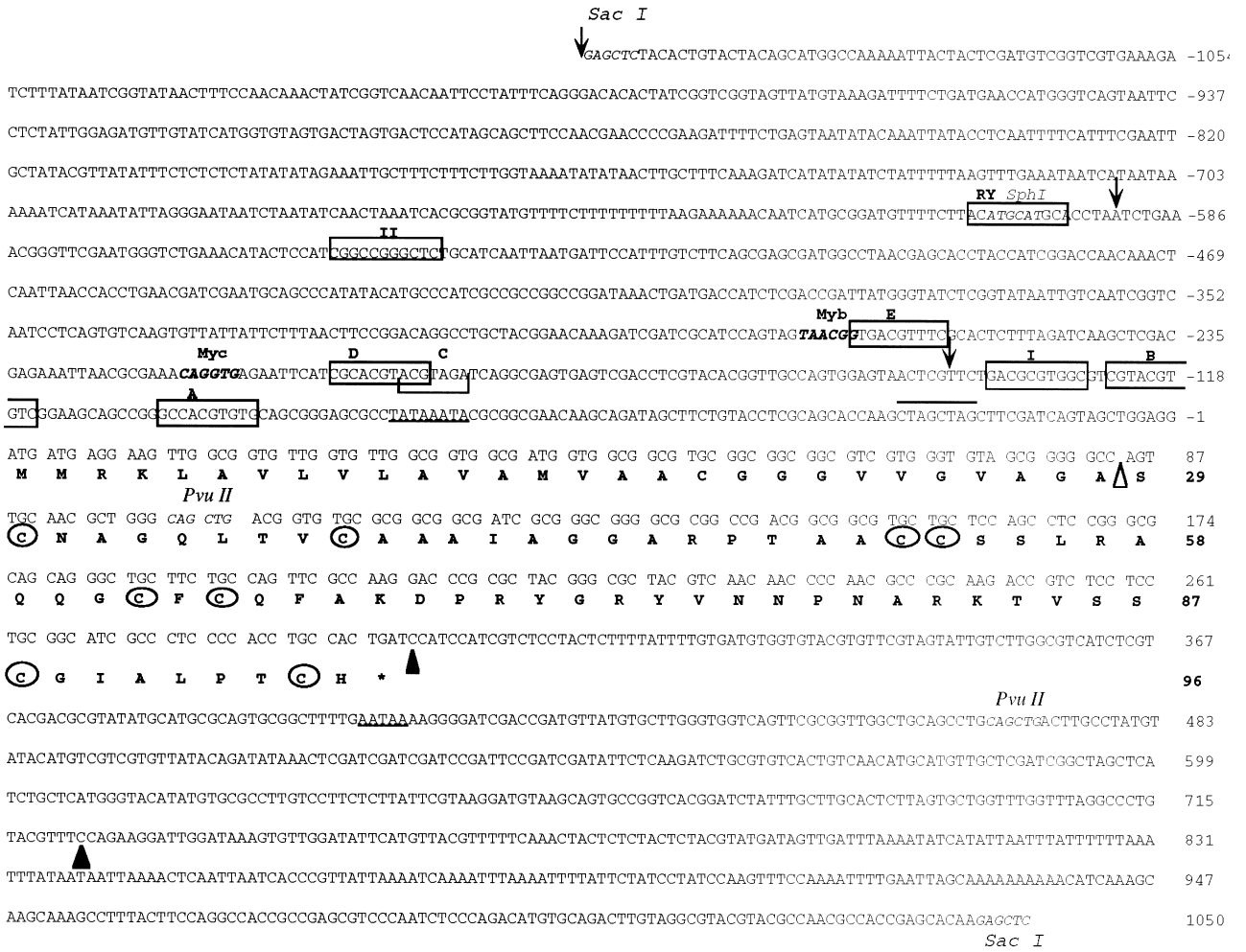

Fig. 1. The nucleotide sequence of the rice *Ltp2* gene. Nucleotides are numbered from the A of the translation start codon (+1). The deduced aa sequence for LTP2 is shown below the nucleotide sequence as single letter code, and the eight cysteines characteristic of LTPs are circled. The most likely cleavage site for the signal peptide in LTP2 is indicated by a vertical arrow. The stop codon is marked by an asterisk. The putative TATA box in the promoter and the polyadenylation signal in the 3′ untranslated region are underlined. Putative ABRE motifs having the typical ACTG central core of these sequences are boxed, in the proximal part of the promoter, and called A, B, C, D and E. An ABRE-like sequence GAC**GCGT**GGC with GCGT as central core, named box I, the sequence CGGCCGGGCTC, called box II, similar to the consensus sequence motif II of rice *Rab* genes and a RY motif, including a *Sph* box, which is found in many seed specific promoters, are also boxed. Consensus mammalian *myb* (C/TAACG/TG) and *myc* (NCANNTGN) binding sites and relevant restriction enzyme sites are indicated by their name and highlighted by italics. The eight nucleotide palindromic sequence CTAGCTAG found in the leader of several *Ltp* genes is overlined. The vertical arrows indicate the 5′-ends at positions −1114, −591 and −140 of the three promoter deletions used for the DNA constructs shown in Fig. 5. The boundaries of the fragment amplified by PCR to be used as specific probe (Fig. 4) are indicated by arrowheads at the 3′ untranslated region of the gene.

coding strand, defining the start point of the 591 and 140 deletion constructs, respectively. The template and the downstream primer for PCR amplification of these 5′ regulatory sequences were the same as for the p1114 plasmid. Finally, the PCR generated *Hin*dIII-*Sma*I fragments, containing 591 and 140 bp respectively, were inserted into plasmid pGUS3.

2.7. Particle bombardment, manipulation of samples and detection of reporter gene expression

Rice seedlings were bombarded immediately after transferring them onto moistened filter paper in Petri dishes. Maize immature embryos were excised under aseptic conditions from immature seeds of 20 or 25 DAP sterilized ears and transferred to Petri dishes containing solid MS medium [22] (MS salts and vitamins, 30 g/l sucrose, 2.4 g/l Gelrite, pH 5.8). The embryos were kept with the axis in contact with the medium at 28°C in the dark for 24 h and then transferred, axis up, onto fresh MS medium before bombardment.

A more detailed protocol of the procedure used in particle bombardment technique has been described elsewhere [23]. Particle bombardment was performed with the PDS1000/He device (DuPont-Bio-Rad) using rupture disks of 900 psi. The distances selected were 9.5 mm between the rupture disk and the macrocarrier, 6 mm between the macrocarrier and the stopping screen and 10 cm between the stopping screen and the sample. For each shot, one per sample, 8 μl of gold particles coated with DNA were applied onto the macrocarrier and left to dry in a flow hood for 5 min before bombarding.

After bombardment, samples were incubated at 28°C under the appropriate conditions and kept in the dark for 24 h. The histochemical detection of GUS activity was performed on maize immature embryos or rice seedlings by incubation with X-Gluc solution (0.1 M sodium phosphate buffer pH 8.0, 0.5 mg/ml X-Gluc (Biosynt AG), 0.1% Triton X-100, 10 mM EDTA, 5 mM potassium ferrocyanide and 5 mM potassium ferricyanide) for 24 h at 28°C in the dark.

GUS expression in maize immature embryos was quantified using the fluorometric method of detection. Upon bombardment, the embryos were appropriately treated, collected after 24 h and immediately frozen until the GUS fluorometric assay was performed. This was essentially as described by Jefferson [21], except for the inclusion of 25% (v/v) methanol in the enzyme reaction buffer, in order to suppress endogenous GUS activity [24]. The GUS activity was measured in a TKO 100 Hoeffer fluorometer and expressed in pmol of 4-methylumbelliferone (MU) produced per min of enzymatic reaction and per mg of protein in the sample extract (with the fluorometer calibrated to 500 fluorescence units equivalent to 100 pmol of MU).

A

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RLTP2     MMRKLAVLVLAVAMVAACGGGVVGVAGASCNAGQLTV--CAAAIAGGARPTAA
RLTP1     .A.AQL...AL..AALLLA.PHTTM.AI..GQVNSA.SP.LS-Y.R.L..S..
          * *   ***  **      +*    +*+ **   + +*  * +  * * **+**

RLTP2     CCSSLRA-----------QQGCFCQFAKDPRYGRYVNNPNARKTVSSCGIAL
RLTP1     ...GV.SLNSAASTTADRRTA.N.-LKNVAGSISGL.AG..ASIP.K..VSI
          ***++*+            + +* * + + +     +*  **    * **+++

RLTP2     P-------TC---H
RLTP1     .YTISPSID.SSVN
          *        *   +
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B

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RLTP2     M-------MRKLAVLVLAVAMVAACGGGVVGVAGASCNAGQLTVCAAAIAGG
WLTP2                                    A.Q.S..A...S..LS.
BLTP2     .AMAMGMA...E.AVAVMMV..VTLAA.ADAG...A.EPA..A...S..L..
VLTP2     .TMKMKMK.SVVCAV.VVALFLIDV.PVAEA.T---..PTE.SS.VP..T..
          *        *     + +    +   +    + +   *++ +*+ * +** +*

RLTP2     ARPTAACCSSLRAQQGCFCQFAKDPRYGRYVNNPNARKTVSSCGIALPTCH
WLTP2     .K.SGE..GN..........Y.......Q.IRS.H..D.LT...L.V.H.-
BLTP2     TK.SGE..GN.......L..YV...N..H..SS.H..D.LNL...PV.H.-
VLTP2     SK.SST...K.KV.EP.L.NYI.N.SLKQ...S.G.K.VL.N..VTY.N.-
          ++*++ **+ *+ *+ *+*++ *+*   +*+ +* *+  +  **+  * *
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C

	RLTP1	WLTP2	BLTP2	VLTP2
RLTP2	27/45	-/-*	48/78	34/67
RLTP2mP	26/45	60/90	56/88	43/78

Fig. 2. Comparison of amino acid sequence of rice LTP2 with rice LTP1 and other plant LTP2. Alignments were done with the CLUSTAL V program. Gaps (indicated by dashes) have been introduced to obtain optimal alignment. Points indicate identity with rice LTP2 (RLTP2) aa sequence and only different aa are shown. Asterisks and plus signs under the sequence represent identical and similar amino acids in all the sequences. A: Alignment of the rice *Ltp2* ORF deduced aa sequence with rice LTP1 (RLTP1) [15]. Eight conserved cysteines are highlighted in bold face. B: Alignment of the rice LTP2 aa sequence with wheat (WLTP2) [26], barley (BLTP2) [16] and *Vigna unguiculata* (VLTP2) [9] LTP type 2 sequences. The eight conserved cysteines are highlighted in bold face. C: Percentage identity and similarity between the complete rice *Ltp2* ORF deduced aa sequence (RLTP2) or its putative mature protein (RLTP2mp) and the aa sequences of other LTPs from A and B either in their entire (first line) or mature protein versions (second line). Numbers above the diagonal are the percentage identity and numbers below it the percentage of similar amino acids. *The sequence of complete WLTP2 is not available. Sequence from WLTP2 mature protein was experimentally determined [26]. BLTP2 amino acid sequence was deduced from the barley *Ltp2* gene [16], and VLTP2 amino acid sequence was deduced from the *Vigna Ltp2* cDNA [9].

3. Results

3.1. Cloning and sequence analysis of Ltp2 gene

The nucleotide sequence presented in Fig. 1 corresponds to the rice genomic clone pSSOa. The sequence has an open reading frame, which encodes a 96 aa LTP type 2 like protein. The protein has an estimated molecular weight of 9600 and contains the typical eight Cys residues whose position is highly conserved in all plant LTPs [1]. The protein contains a putative signal peptide, according to the criteria of von Heijne [25], and exhibits a high degree of identity (60, 56 and 43%) and similarity (90, 88 and 78%) with the LTP2 mature proteins of wheat, and predicted barley and *Vigna* LTP2 proteins respectively (Fig. 2B). Following the criteria of von Heijne [25], one of the predicted cleavage sites for the signal peptide of rice LTP2 protein is located between alanine 27 and serine 28. Although the proposed site for the barley LTP2 protein [16] is between two alanines, they are coincident when their animo acid sequences are aligned (Fig. 2B). Moreover, the amino acid sequence of mature 7 kDa wheat protein has been experimentally determined [26] and could be aligned with a large number of matches to the previous ones from rice and barley LTP2. Its N-terminal residue alanine coincides with the proposed alanine in barley LTP2 and with serine 28 in rice LTP2. Therefore we tentatively propose serine 28 as the N-terminal residue of rice LTP2. In contrast to the high degree of identity among LTP2 from different species, rice LTP1 [15] shows only 26% identity with rice LTP2, even though the eight Cys residues conserved among plant LTPs match in the alignment shown in Fig. 2A.

In contrast to the presence of one intron in the coding region of the rice *Ltp1* gene and most other *Ltp* genes [1], there is no intron in the rice *Ltp2* gene. This has been deduced by comparing the rice *Ltp2* ORF nucleotide sequence and its deduced amino acid sequence with the barley *Ltp2* cDNA and genomic sequences and their deduced amino acid sequence. The barley *Ltp2* gene lacks introns [16]. The coding sequence is flanked by two unusually AT-rich stretches (77% between −869 and −603 and 80% between 764 and 954) which contrasts with the GC content (72%) of the coding sequence.

Careful inspection of the 5′ upstream region of the *Ltp2* gene revealed the presence of the TATA box and other putative relevant motifs (Fig. 1). Among these, the RY repeat, the *Sph*I motif, the MYB and MYC binding sites and several ABA responsive elements (ABREs) are considered interesting in relation to the properties of the gene. The RY repeat, which is conserved in many seed specific promoters [27], was found at position −606. This sequence, or the *Sph*I motif that partially overlaps with it in the 3′-end (GCATGC), has been involved in ABA independent transactivation by the transcriptional activator VP1 of the wheat ABA induced *Em* gene [28] and the maize anthocyanin regulatory *C1* gene [28].

The rice *Ltp2* promoter contains putative recognition sites for animal MYB and MYC transcription factors (Fig. 1). These sequences have been identified in numerous plant genes, including the barley *Ltp1* and *Ltp2* genes [16,30] and a gene from *Arabidopsis thaliana* whose induction by dehydration is mediated by ABA [31].

The 5′ untranslated region of the rice *Ltp2* gene also contains several motifs that are present in promoters of ABREs from different plants. Box II element shows high similarity to the consensus sequence motif II in the rice *Rab16* gene [32],

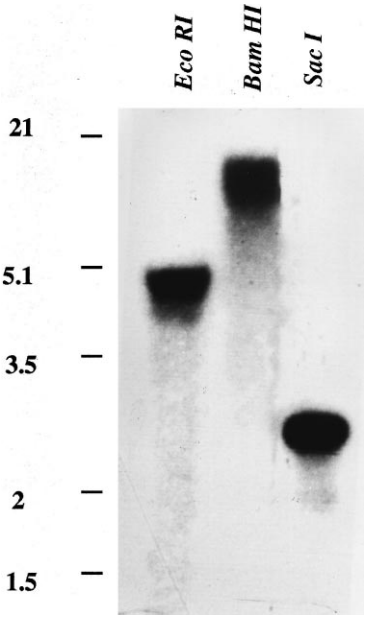

Fig. 3. Southern blot of the IR36 rice genomic DNA digested with *Eco*RI, *Bam*HI, or *Sac*I (S). The Southern membrane was hybridized with the labeled *Pvu*II DNA fragment corresponding to the C-terminus of the protein and part of the 3′ untranslated region of the *Ltp2* gene (Fig. 1), as described in Section 2. The location and size of molecular weight markers (in kb) are indicated on the left.

and boxes A, B, C, D and E contain the ACGT element which is characteristic of defined or putative ABREs in other plant ABA-responsive gene promoters [33]. Box I (GACGCGTGGC) is located at position −136 of the rice *Lpt2* promoter and is defined here as an ABRE-like sequence, because it has a similar central core (GCGT) to the consensus ACGT central core of ABREs. Box I has nine of 10 identical nucleotides to the *Hex-3* synthetic element [34] and eight out of 10 to the coupling element *CE3* as defined by Shen et al. [33]. Both the *Hex-3* and *CE3* elements mediate the response to ABA [33,34].

Two additional relevant motifs are present in the putative untranslated regions of the *Ltp2* gene: the putative polyadenylation signal sequence (AATAAA), which is identical to the canonical consensus, is present 115 nt downstream of the stop codon and the eight-nucleotide palindromic sequence CTAGCTAG located at −28 nt upstream of the ATG start codon (Fig. 1). The latter motif has been found in the leader sequence of a number of *Ltp* genes [35].

3.2. Southern analysis and copy number estimation of the Ltp2 gene

Southern hybridization analysis of the *Ltp2* gene was performed using the *Pvu*II fragment (100–470, Fig. 1) as a probe. This probe hybridized strongly, under high stringency conditions of hybridization and washing, to a single band of DNA digested with the restriction enzymes *Eco*RI, *Bam*HI, or *Sac*I (Fig. 3). These results show the *Ltp2* gene does not cross-hybridize with any *Ltp1* gene member, although the probe contained the most conserved region of *Ltp* genes. The *Sac*I hybridizing fragment is the same size (2.2 kb) as the insert in the pSSOA clone, which comprises the *Ltp2* gene and flanking sequences, indicating that there is only one copy of *Ltp*, type 2 gene in the rice genome.

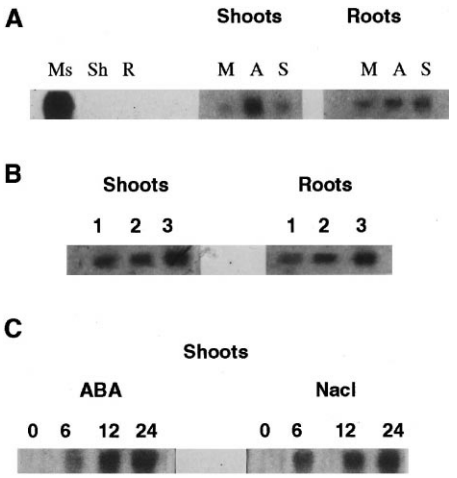

Fig. 4. Northern hybridization analysis of the *Ltp2* mRNA from rice tissues under normal conditions or treated with ABA or osmotic stress producing agents. 15 μg of total RNA was loaded per lane. The probe used was obtained by conventional PCR procedures using two specific oligonucleotides: 5′-CCATCCATCCATCGTCTC-3′ as upper primer and 5′-GAAACGTACAGGGCCTAAACC-3′ as lower primer respectively (see Fig. 1) and the plasmid pSSOA as template. The single hybridizing transcript of 600 bases was shown. In all the cases, the membranes were stained with methylene blue [18] to monitor the load of RNA, that was roughly the same in all the lanes (not shown). A: *Ltp2* mRNA accumulation in mature seeds (MS), shoots (Sh) and roots (R) 3 days after germination, and in shoots and roots incubated 24 h with 300 mM mannitol (M), 10 μM ABA (A) or 300 mM NaCl (S). The 24 h untreated controls of Northern blots corresponding to shoots and roots are not given any signal corresponding to an ARN band (not shown). B: Effect of salt concentration on the *Ltp2* mRNA levels in roots and shoots of rice seedlings incubated 24 h with 100 mM (1), 200 mM (2) or 300 mM (3) NaCl. C: Time course of accumulation of the *Ltp2* mRNA in 3 days after germination rice shoots incubated for 0, 6, 12, and 24 h with 10 μM ABA or 300 mM NaCl.

3.3. Expression of rice Ltp2

The expression of the *Ltp2* gene in various organs of rice seedlings and in response to ABA and osmotic stress generating agents was examined by Northern hybridization. As a probe, a DNA fragment from the 3′ untranslated region of the *Ltp2* gene was obtained by PCR amplification using the primers described in the legend of Fig. 4.

High levels of *Ltp2* mRNA accumulation can be seen in dry seeds (Fig. 4A), suggesting that it is present as long-lived mRNA in the resting grain. The long-lived *Ltp2* mRNA in dry rice seed must be rapidly turned over at the onset of germination as no transcript was detected in roots and shoots of seedlings (Fig. 4A). However, accumulation of this mRNA can be resumed by incubating the germinated seedlings with ABA, NaCl or mannitol (Fig. 4A). These results indicate that ABA and osmotic stress producing agents (NaCl or mannitol) increase the level of *Ltp2* mRNA. The accumulation of the mRNA transcript was dependent on salt concentration. In both roots and shoots the maximum levels of mRNA were detected at 300 mM NaCl (Fig. 4B). The time course of induction in response to NaCl was similar to that obtained with ABA (Fig. 4C), with maximum levels of accumulation obtained after 12–24 h. This time of maximum accumulation is similar to other rice ABA responsive genes [36] and to the tomato NaCl induced *Ltp* gene [12].

3.4. Promoter functional analysis

The ABA responsiveness of the *Ltp2* gene prompted us to search, among the putative *cis*-elements of the promoter (Fig. 1), for those sequences reported previously to be involved in the ABA response. For this purpose a series of DNA constructs consisting of progressive deletions of the *Ltp2* promoter translationally fused to the *uidA* coding region were produced. In the beginning, we used the longer construct (p1114) to assay the GUS activity in rice coleoptiles after bombardment and the appropriate treatment. The activity detected, by the histochemical assay of GUS, was low but clearly higher in ABA treated than in water treated coleoptiles (results not shown). However, this activity was not enough to accurately quantify, fluorometrically, the rate of GUS induction in ABA treated coleoptiles. In contrast, the use of bombarded immature maize embryos to study transient gene expression [23] allowed us to quantify the activity of *Ltp2* promoter upon ABA induction, due to the ease of the system and to the high GUS activity attained after 24 h incubation with 50 μM ABA. This experimental system was used to search putative *cis*-elements of the promoter to be involved in ABA response, using the *Ltp2* constructs p591 and p140 in addition to p1114 and the p35S construct (Fig. 5A). The GUS activity driven by the shorter deletion of the promoter (p140) in water treated embryos was greatly reduced compared to those driven by either the 1114 or 591 bp long *Ltp2* promoter (Fig. 5B). Upon ABA treatment the three promoter deletions were able to drive considerably higher GUS activity than water treated embryos (Fig. 5B). No significant increase in GUS activity was observed in ABA versus water treated embryos transformed with the p35S construct (Fig. 5B). This system behaves as expected for an ABA non-responsive promoter, as is the case with cauliflower mosaic virus 35S promoter. Surprisingly, the shorter promoter (p140) produced the higher ratio of increment of GUS activity in response to ABA (25 times) than p1114 (about 5 times) or p591 (Fig. 5C). These

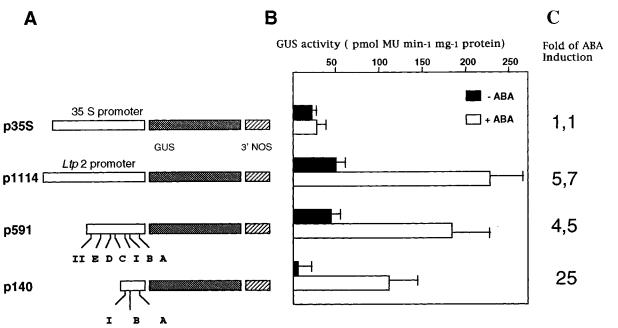

Fig. 5. Functional analysis of the *Ltp2* promoter in maize immature embryos in response to ABA. Twenty DAP maize embryos were bombarded with the DNA constructs indicated in A. After bombardment, embryos were transferred, with the axis in contact with solid MS0 medium with or without 50 μM ABA, for 24 h. A: Schematic representation of p35S and *Ltp2* promoter deletion series GUS gene constructs used. Numbers and letters over the proximal part of the *Ltp2* promoter refer to DNA sequence motifs described in Fig. 3. B: Bars represent the mean of GUS activity values from at least three different bombardment experiments and the error bars the S.E. C: Fold ABA induction is the mean of the GUS activity values in the presence of ABA divided by the corresponding figure in the absence of ABA. All these experiments were carried with the same groups of embryos and the same day for +ABA and −ABA in order to be able to compare the results.

results indicate, on the one hand, that 140 nucleotides of promoter in p140 are sufficient to confer ABA inducibility to this construct. Therefore, a *cis*-acting element (or elements) involved in ABA inducibility must be present in the promoter downstream of nucleotide −140. On the other hand, the enhancing effect of ABA on promoter activity is more pronounced in the latter construct than in any of the enhancing others.

4. Discussion

The rice gene, fished out with a barley *Ltp2* probe, does indeed encode an LTP-like predicted protein with eight cysteine residues at conserved locations, a putative signal peptide and of small size, as in all other LTPs [1]. However, its amino acid sequence shows weak similarity to the corresponding rice LTP1 [15], besides the eight cysteines and a few other amino acids (Fig. 2A). In addition, the new LTP is smaller than LTP1, with a molecular mass of 9.6 kDa versus 11.6 kDa of LTP1 and 6.5 versus 8.6 kDa for the putative mature proteins of the rice LTPs. In contrast, this new rice protein is more similar in terms of amino acid sequence to LTP2 from wheat, barley and *Vigna unguiculata*, especially in the sequence corresponding to the mature protein, ranging from 60% identity for rice and wheat to 43% for rice and *V. unguiculata* LTP2 mature proteins (Fig. 2C). However, the signal peptide sequence of rice LTP2 shows lower similarity to other LTP2 signal peptide sequences than to the rice LTP1. These LTP2 and LTP1 signal peptides display 35% identity, compared to the whole proteins which show 26% identity. In addition, the *Ltp2* gene does not cross-hybridize with any rice *Ltp1* genes and is present only once in the rice haploid genome (Fig. 4). Our results confirm one of the hypotheses proposed by Kalla et al. [16] on the existence of a distinct family or subfamily of LTPs, when only the sequences of barley and wheat LTP2 were available, taking into account that LTP activity was shown in vitro for wheat LTP2. We extend this family of LTPs with two additional members, *V. unguiculata* LTP2, taken from Krause et al. [9] and rice LTP2 from our work. In support of LTP2 as a distinct family is the absence of introns in the two known genomic sequences from barley [16] and rice (this work) in contrast to the presence of one intron in barley [30] and rice [15] *Ltp1* genes. Based on this evidence, we suggest that *Ltp1* and *Ltp2* originated after a duplication event before the divergence of monocotyledonous and dicotyledonous plants taking into account the lower similarity displayed by rice LTP1 and LTP2 than, for example, the latter with *V. unguiculata* LTP2. Nevertheless, some species specific features are conserved in the putative signal peptides of rice LTPs. In spite of plant LTPs having been traditionally classified into a single family, we propose that LTP2 should be clearly considered a distinctive family or sub-family of LTPS, perhaps with a different function or target that awaits for experimental demonstration.

The pattern of expression of *Ltp2* mRNA accumulation shares with LEA (late embryogenesis abundant) genes like *Rab17* [36] an mRNA accumulation in dry seeds and gene induction in response to ABA and osmotic stress producing agents. At this point it is important to emphasize that, in spite of the high similarity between rice and barley LTP2, the expression patterns of rice and barley *Ltp2* genes are different. The barley gene is specifically expressed in the aleurone cells shortly after the initiation of aleurone differentiation. The accumulation of barley *Ltp2* mRNA increases until grain mid-maturity, but the mRNA is absent in mature grains [16].

Rice *Ltp2* gene expression is seed specific under normal developmental conditions. However, on treatment with ABA or osmotic stress provoking agents such as NaCl or mannitol, accumulation of *Ltp2* transcripts can also be observed in roots and shoots. In this respect, the *Ltp2* expression pattern is also distinct from those of the various members of the *Ltp1* multigene family [3], as rice *Ltp1* genes are not, normally, expressed at high levels in seeds or roots. In addition, none of the *Ltp1* gene members are induced by salt, and only two members, which are expressed under normal developmental conditions in coleoptiles, are induced by ABA treatment.

Although the spatial pattern of *Ltp2* expression has not been determined during rice seed development, the *Ltp2* promoter was found to be active in 25 DAP maize embryos and inactive in 3 day old rice coleoptiles, in transient expression experiments. These results argue in favor of relatively high endogenous ABA levels, known to be present in mid-mature maize embryos [37], being responsible for *Ltp2* activity, as has been reported for the ABA responsive gene *Rab16B* in rice [38] and for the osmotin gene in tobacco [39]. As the 1114 bp *Ltp2* promoter fragment, used in the transient expression experiments, is able to drive GUS activity in both maize embryo and rice coleoptile cells in response to ABA, regulatory *cis*-elements that respond to ABA must be present in that promoter. This possibility is further supported by the presence, in the *Ltp2* gene promoter, of several conserved DNA boxes present in other ABA induced genes, identified as ABREs [32].

All three deletions tested, comprising 1114, 591 and 140 bp, respectively, from the ATG start codon, were able to drive GUS activity in the absence of exogenous ABA and to increase promoter activity upon exogenous ABA treatment, in contrast to the CaMV 35S promoter whose activity was not increased after ABA treatment (Fig. 5). Both p1114 and p591 *Ltp2* constructs are about twice as efficient as the 35S promoter in directing GUS activity in the absence of exogenous ABA. These results suggest that endogenous ABA levels present in 20 DAP maize embryos are already high enough [37] to keep relatively high levels of *Ltp2* promoter activity in the absence of an exogenous supply of ABA. Removal of the RY/*Sph* element in p591 does not affect the level of relative activity of p1114 and p591 in the presence or absence of exogenous ABA. The RY/*Sph* element is frequently found in the promoters of seed expressed genes and has been involved in regulation of the C1 maize gene by ABA and the Vp1 transactivator [29]. However, in other ABA and VP1 regulated seed genes, such as the wheat *Em* gene, such a box is not involved in ABA regulation [28]. Whether the rice Vp1 homologous gene plays a role in the regulation of the *Ltp2* gene through an ABRE element (ACGT core containing G-box element) still present in p591 and p140 is currently under investigation. When a further 451 bp of promoter are deleted from p591, to give rise to p140, a reduction of the basal expression by a factor of 10 is observed (Fig. 5). This suggests that some of the elements deleted may play a quantitative role in the activity of the promoter in non-treated embryos. The fragment contains: box II, putative ABREs E, D, C and putative MYB and MYC binding sites. MYB and MYC binding sites are important for regulation of the maize *Bz1* gene and they have been proposed as important regulatory elements for

the barley *Ltp* genes [16,30]. This seems not to be the case for the rice *Ltp2* gene, at least with respect to the ABA response.

Upon exogenous ABA treatment of bombarded embryos, around a 5-fold increment in the level of GUS activity is achieved with both p1114 and p591. Surprisingly, the highest level of induction upon ABA treatment, 25-fold, was attained with the p140 construct. Similar levels of induction to those reported above for p1114, p591 and p140 have been observed in embryos of 15, 25 and 30 DAP (data not shown). First, these results agree with the fact that embryos of 15–30 DAP have constant levels of endogenous ABA [37], and addition of exogenous ABA is needed to reach higher levels of *Ltp2* gene expression. Secondly, all the *cis*-acting elements necessary for the ABA response must be present in 140 bp upstream of the ATG initiation codon, presumably upstream of the TATA box, located at 79 bp to the start codon (Fig. 1). Finally, the differential rate of induction by ABA observed for p591 and p1114 (around 5-fold) and p140 (around 25-fold) could be explained by the loss in p140 of one or more quantitative *cis*-acting elements independent of ABA, or alternatively, these elements may be responsive to low levels of ABA. It is also possible that *cis*-acting elements present in the first 140 bp of all the constructs may only be responsive to high levels of ABA.

The proximal 140 region of the *Ltp2* promoter contains three candidates for the ABRE motif, box A (GCCACGTGTG), box B (CGTACGTGTC) and what we have called element I (GACGCGTGGC). Both A and B ACGT boxes have been shown to be involved in regulation by ABA of several cereal genes [33–40]. Therefore, any of these boxes could, in principle, mediate the ABA response of the *Ltp2* gene. This could also be the case for motif I taking into account the high degree of similarity with *Hex3* and *CE3* elements and the data published for them. *Hex3* is a synthetic promoter sequence that confers seed expression and transcriptional enhancement in response to water stress and ABA in transgenic tobacco [34]. The *CE3* element has been shown to be necessary, bur not sufficient, for ABA inducibility in a minimal promoter of *HvA1* gene in barley [33]. The individual involvement of those putative *cis*-acting elements of the *Ltp2* gene in ABA responsiveness is currently being evaluated by functional dissection of the p140 promoter region.

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