Journal of Plant Biology, October 2006, 49(5) : 371-375

Differential Expression of Rice Lipid Transfer Protein Gene (*LTP*) Classes in Response to Abscisic Acid, Salt, Salicylic Acid, and the Fungal Pathogen *Magnaporthe grisea*

Tae Hyun Kim[1], Moon Chul Kim[1], Jong Ho Park[1], Seong Sook Han[2], Byung Ryun Kim[3], Byoung Yong Moon[4], Mi Chung Suh[5], and Sung Ho Cho[1]*

[1]Department of Biological Sciences, Inha University, Incheon 402-751, Korea
[2]Plant Pathology Division, National Institute of Agricultural Science and Technology, Suwon 441-857, Korea
[3]Biological Environment Division, Chungnam Agricultural Research and Extension Services, Yesan 340-861, Korea
[4]Department of Biotechnology and Biomedical Sciences, Inje University, Gimhae 621-749, Korea
[5]Department of Plant Biotechnology, Chonnam National University, Gwangju 500-757, Korea

Rice lipid transfer protein (*LTP*) genes belong to a complex multigene family and are differentially regulated. The organ specificity of expression for the different classes of rice *LTP* gene Subfamily I was investigated here. *LTPC1* (Class I) was highly expressed in flowers and stems, while *LTPC2* (Class II) showed higher expression in leaves and stems. *LTPC3* (Class III) showed higher expression in stems, whereas corresponding mRNA was barely detected in leaves. Treatment of 3-wk-old plants with ABA induced the expression of *LTPC1* and *LTPC3* after 1 d. Expression of all three *LTP* isoforms was induced in response to salicylic acid. Because some of these genes are reportedly induced by pathogens, we also investigated whether they might be responsive to infection by *Magnaporthe grisea*. Transcripts of *LTPC1* started to accumulate in the leaves at 8 h after incompatible inoculation, reaching a maximum level at 48 h. In contrast, the response was much slower and weaker after compatible inoculation.

Keywords: lipid transfer protein, *Magnaporthe grisea*, pathogen, rice, stress

Lipid transfer proteins (LTPs) are a family of proteins capable of moving various kinds of lipid molecules *in vitro* and *in vivo* (Kader, 1996). Initially, their suggested major role was the transport of phospholipid molecules synthesized in the endoplasmic reticulum either to other cell membrane compartments or between membranes. However, that functioning has never been demonstrated *in vivo* (Arondel et al., 2000). Instead, LTPs have now been revealed as being involved in many other biological functions. Because an LTP has a signal peptide indicative of a secretory protein, and is observed mainly in the cell walls and cuticle, the primary role of plant LTP could be the assembly of cutin and wax in the surface layers (Segura et al., 1993; Pyee et al., 1994; Cameron et al., 2006). LTP may also be responsive to environmental stresses, including salt, drought, abscisic acid (ABA), and cold treatment (Vignols et al., 1997; Arondel et al., 2000; Hong et al., 2001; Yubero-Serrano et al., 2003; Wu et al., 2004), as well as being protective against fungal and viral infections (Garcia-Olmedo et al., 1995; Blilou et al., 2000; Kristensen et al., 2000; Guiderdoni et al., 2002; Park et al., 2002). LTP is also involved in long-distance signaling during systemically acquired resistance in *Arabidopsis thaliana* (Maldonado et al., 2002). Moreover, a wheat LTP competes with elicitin for a receptor site on plasma membranes that participates in controlling plant defense responses, thereby suggesting a role for LTP as a signal mediator (Buhot et al., 2001; Blein et al., 2002).

The rice *LTP* gene family comprises at least 17 genes, which may be grouped into three subfamilies according to the structure of the LTP proteins (Vignols et al., 1994, 1997; Garcia-Garrido et al., 1998). Whereas, *LTP* genes of Subfamily I contain one intron, the other two subfamily members lack an intron. Subfamily I is further divided into five classes on the basis of the phylogenetic tree. The differential expression of three *LTP* gene classes for Subfamily I - Classes I, II and III - has been analyzed using germinated seeds of the rice 'Nipponbare' cultivar (Vignols et al., 1997). Furthermore, inoculation with the fungal agent of the rice blast disease, *Magnaporthe grisea*, causes the strong accumulation of *LTP* transcripts in two rice cultivars, 'Maratelli' and 'IRAT216' (Guiderdoni et al., 2002). Induction of the *LTP* gene is rapid in the case of a compatible host-pathogen interaction, which implies that, rather than serving in a systemic response, the LTP protein is involved in strengthening structural barriers and conferring organ protection against mechanical disruption and pathogen attack.

Here, we have analyzed the differential organ and environmental expression patterns of *LTP* gene classes in Subfamily I using the 'Dongjin' cultivar, a popular rice variety in Korea. We have also conducted further research into the pathogenic interaction between 'Dongjin' and two strains of *M. grisea*, KI-305 and KI-1117, to determine whether the LTP protein works as a signaling molecule or else establishes a physical barrier during pathogenic attacks.

MATERIALS AND METHODS

Growth of Rice Plants and Isolation of mRNA

Seeds of rice (*Oryza sativa* L. cv. Dongjin) were germinated for 2 d on moistened filter paper in sterile Petri dishes. Seedlings were then transferred to a greenhouse for further growth at 30°C under a 16-h photoperiod. Organ samples

*Corresponding author; fax +82-32-860-7696
e-mail shcho@inha.ac.kr

were collected into liquid N_2 and stored at -70°C. The RNeasy plant mini kit was used for mRNA isolation according to the manufacturer's instructions (Qiagen, USA).

Application of ABA, Salt, and Salicylic Acid Treatments

All treatments were performed on 3-wk-old plants. On three consecutive days, a solution of 10 μM ABA, 200 mM NaCl, or 10 mM salicylic acid (SA) was sprayed on their leaves (Yubero-Serrano et al., 2003; Shon et al., 2005). These experiments were repeated at least three times, producing an almost identical data set for each independent study.

Inoculation of Rice with *M. grisea*

Two strains of *M. grisea* - KI-305 and KI-1117 - were tested here because 'Dongjin' exhibits a resistant and incompatible interaction with KI-305 but is susceptible and compatible with KI-1117. Three-wk-old seedlings were inoculated by first spraying them with a suspension of spores in 0.05% Tween 20 solution (3×10^6 spores mL^{-1}) before leaving them in darkness for 24 h under 100% humidity. The plants were then transferred to normal growing conditions. These inoculation experiments were repeated twice, with almost identical data sets resulting from each independent study.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

With 2 μg total RNA as the template, the RT reaction was performed with M-MLV reverse transcriptase (Promega, USA) for 1 h at 42°C, followed by 10 min at 65°C. Afterward, 5 μL of the 20 μL RT reaction served as template for PCR. A forward primer, 5'-TGCTCCAGCGTGAACTAA-3', and a reverse primer, 5'-CATGTACATGCAGAAGTA-3', were used to synthesize a 287-bp fragment that corresponded to nucleotides from 268 to 554 (beginning to count from start codon) of the *LTPC1* cDNA. Other primers included: forward, 5'-GCTTCCATCGACTGCTCC-3' and reverse, 5'-GGCTCCACACATCGACC-3', to synthesize a 115-bp fragment corresponding to nucleotides from 335 to 449 of the *LTPC2* cDNA; and forward, 5'-TGCTCCAAGATCAACTAA-3' and reverse, 5'-TACATGTAAACATTGAAA-3', to synthesize a 162-bp fragment corresponding to nucleotides from 331 to 492 of the *LTPC3* cDNA. The PCR reaction mixture (25 μL) contained 500 ng template, 2.5 μL 10× PCR buffer [100 mM Tris-HCl (pH 8.0), 50 mM KCl, 1 mM EDTA, 0.1% Triton X-100, and 50% glycerol (v/v)], 50 μM primers, 200 μM dNTPs, and 25 units of Ex Taq polymerase (TaKaRa, Japan). Reactions were denatured for 40 s at 95°C, annealed for 30 s at 56.4°C (*LTPC1*), at 68°C (*LTPC2*), or at 51°C (*LTPC3*), and extended for 90 s at 72°C; 25 cycles were conducted in all instances. The PCR products were then sequenced and identified to match their corresponding *LTP* genes from 'Nipponbare'.

RESULTS AND DISCUSSION

Organ Specificity of *LTP* Expression

Three *LTP* genes, each representing individual classes of Subfamily I, were chosen for study. They are equivalent to the *b1* (Class I, accession number X83434), *a15* (Class II, accession number X83435), and *b21* (Class III, accession number X83433) cDNA clones of the rice 'Nipponbare' cultivar (Vignols et al., 1997), and have been renamed as *LTPC1*, *LTPC2*, and *LTPC3*, respectively, for the 'Dongjin' cultivar.

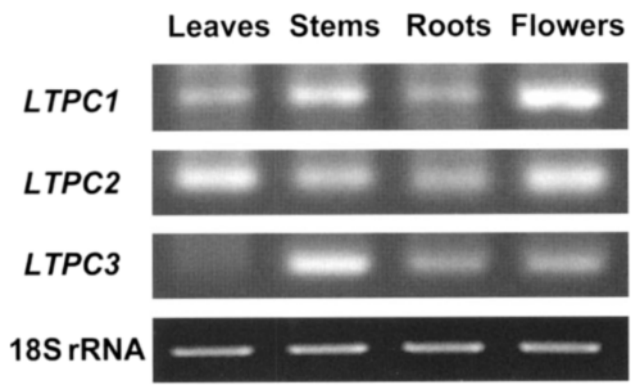

Figure 1. Expression of *LTPC1*, *LTPC2*, and *LTPC3* in different organs of rice seedlings. Total RNA was isolated, reverse-transcribed, and amplified by PCR with gene-specific primers.

Expression of *LTPC1*, *LTPC2*, and *LTPC3* was assessed in different organs from 3-wk-old seedlings as well as in flowers from mature plants (Fig. 1). Differential expression patterns were found for the isoforms in leaves, stems, roots, and flowers. Transcripts for *LTPC1* were detected in all organs analyzed, but were most prominent in the flowers and stems. *LTPC2* was also expressed in all organs, with expression being highest in the leaves and flowers. In contrast, *LTPC3* was barely detected in the leaves, but was highly expressed in the stems.

This differential expression pattern for 'Dongjin' closely resembles that shown in germinated seeds of 'Nipponbare' (Vignols et al., 1997). Whereas in the latter, transcripts of *b1* (Class I) and *b21* (Class III) were detected highly in the mesocotyl, *a15* (Class II) was mainly expressed in the leaves, while transcript of *b21* was not detected in leaves. However, because of differences in experimental design, the expression in seeds and flowers cannot be compared between these two cultivars. Nevertheless, organ specificity for *LTP* expression seems to be very conservative between them.

Effects of ABA, Salt, and SA Treatments on *LTP* Expression

Expression patterns for *LTPC1*, *LTPC2*, and *LTPC3* were compared in shoots under ABA, salt, or SA stress. Treatment of 3-wk-old plants with ABA induced the expression of *LTPC1* and *LTPC3* after 1 d, and their levels remained elevated until Day 3 post-treatment (Fig. 2). In contrast, *LTPC2* expression was not induced by ABA. Because this plant hormone is a mediator of tolerance to cold or water stresses, *LTPC1* and *LTPC3* could play a role upon such an environmental change.

When plants were treated with salt, the level of expression remained almost unchanged for all three genes (Fig. 3). Although a slight increase in transcripts was noticed for both *LTPC2* and *LTPC3*, it was not so conspicuous as seen with

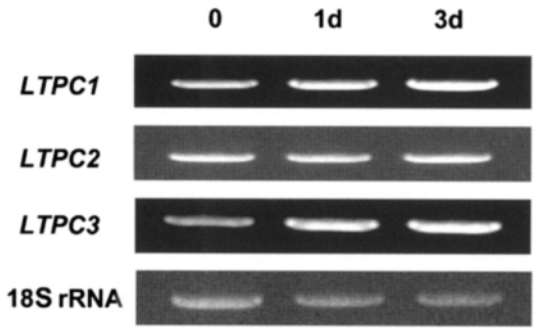

Figure 2. Effects of ABA treatment on expression of *LTPC1*, *LTPC2*, and *LTPC3* in leaves of 3-wk-old rice plants. A 10 μM ABA solution was sprayed onto leaves on 3 consecutive days. Total RNA was isolated, reverse-transcribed, and amplified by PCR with gene-specific primers.

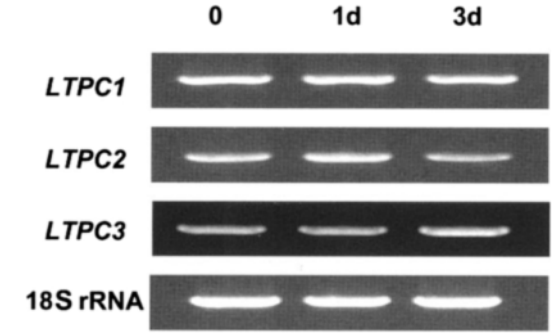

Figure 3. Effects of salt treatment on expression of *LTPC1*, *LTPC2*, and *LTPC3* in leaves of 3-wk-old rice plants. A 200 mM salt solution was sprayed onto leaves on 3 consecutive days. Total RNA was isolated, reverse-transcribed, and amplified by PCR with gene-specific primers.

the ABA treatment.

Expression of all three *LTP* isoforms was induced in response to SA (Fig. 4). While the induction of *LTPC1* and *LTPC2* was noticed at 1 d after SA treatment, *LTPC3* exhibited slower enhancement of the transcript. The level of *LTPC2* decreased on Day 3 after treatment. Because SA mediates the systemically acquired resistance to pathogens (Raskin, 1992), it is very likely that all three classes of LTP take part in defense mechanisms.

The responses of *LTP* gene classes to ABA and SA treatments were somewhat different from those observed in cotyledons of 'Nipponbare' (Vignols et al., 1997). In those tissues, ABA greatly enhances the level of *b21* transcripts (Class III) and SA promotes a similar increase in the expressions of *b1* (Class I) and *b21* (Class III). During plant growth, the expression of these different *LTP* classes is probably regulated to a high degree in different organs following stresses. In the case of salt, however, such treatment reduces the expression of all three gene classes in all organs tested (Vignols et al., 1997). That response in 'Nipponbare' against salt may explain why Japonica rice varieties are relatively resistant to that compound (Vignols et al., 1997).

Expression of *LTP* in Response to Infection by *M. grisea*

To infect the rice cultivar 'Dongjin', two strains of the blast pathogen *M. grisea*, KI-305 and KI-1117, were selected.

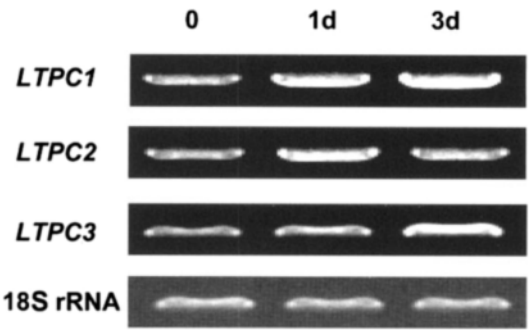

Figure 4. Effects of SA treatment on expression of *LTPC1*, *LTPC2*, and *LTPC3* in leaves of 3-wk-old rice plants. A 10 mM SA solution was sprayed onto leaves on 3 consecutive days. Total RNA was isolated, reverse-transcribed, and amplified by PCR with gene-specific primers.

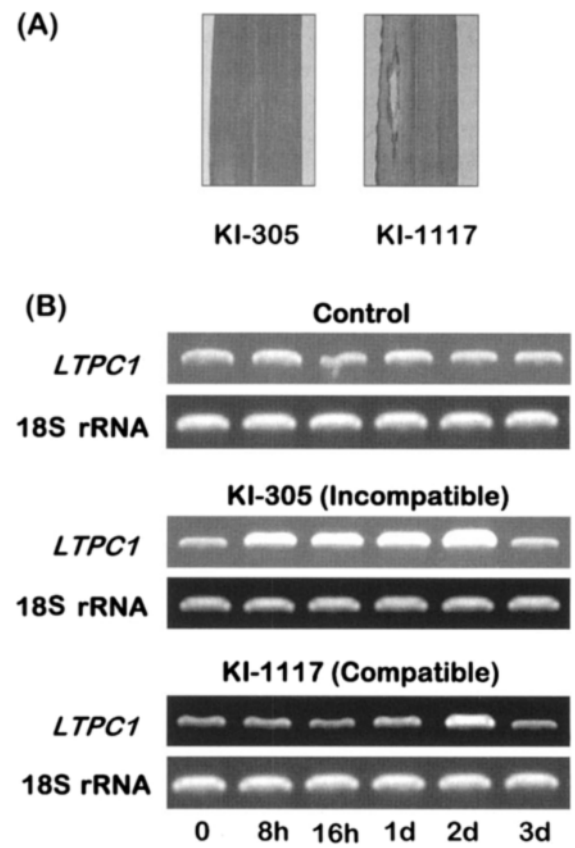

Figure 5. Pathogen inoculation of rice plants. (**A**) Leaves infected with *M. grisea* strains, KI-305 and KI-1117, at 7 d after inoculation. (**B**) Effects of *M. grisea* treatment on expression of *LTPC1* in leaves of 3-wk-old rice plants. Total RNA was isolated, reverse-transcribed, and amplified by PCR with gene-specific primers.

This cultivar exhibits a resistant and incompatible host-pathogen interaction with KI-305, but is susceptible to and compatible with KI-1117. Although no symptoms of the blast were detected in the case of the KI-305 inoculation, symptoms began to appear 5 d after inoculation with KI-1117, and lesions were conspicuous at Day 7, occupying almost 30% of the total leaf area (Fig. 5A).

Among the three classes in Subfamily I, only *LTPC1* was

induced in response to this incompatible inoculation, whereas *LTPC2* and *LTPC3* showed no changes in their expression for either incompatible or compatible interactions (data not shown). For the incompatible interaction with KI-305, *LTPC1* was very rapidly induced at 8 h after inoculation, compared with the control (Fig. 5B). That level of enhanced expression then increased until 2 d after inoculation, before decreasing again. Meanwhile, the induction of *LTPC1* was much slower and weaker for the compatible interaction with KI-1117. Its increased level of expression was observed only at Day 2 before returning to the basal level.

The rapid induction of *LTPC1* expression during the incompatible interaction between *M. grisea* and 'Dongjin' rice suggests that this gene might be involved in the plant defense against pathogen attacks. These results are contrary to those reported by Guiderdoni et al. (2002) for 'Maratelli' and 'IRAT 216' rice cultivars. The former, which is sensitive and exhibits compatible interactions with two strains of *M. grisea*, PH14 and CD142, quickly accumulates *LTP1* mRNA at 1 or 2 d after inoculation. This rapid induction is also noticed when CD142 is inoculated into 'IRAT216', which is sensitive to and compatible with CD142 but resistant to and incompatible with PH14. However, based on that previous study, induction of *LTP1* appears slower in the case of the incompatible interaction between 'IRAT216' and PH14, reaching its maximum at 3 d after inoculation. There, promoter assays have revealed that this induction is essentially restricted to the lesion area.

Nevertheless, several cases have been reported for various host-pathogen interactions in which the induction of *LTP* expression is accomplished in an incompatible manner. For example, when barley is infected with the fungal pathogen *Rhynchosporium secalis*, the incompatible, and not the compatible one, leads to an increase in *LTP4* mRNA and, to a lesser degree, in *LTP2* (Garcia-Olmedo et al., 1995). In hot pepper (*Capsicum annuum* L.), *CaLTP1* expression is induced in the incompatible interaction with an avirulent viral pathogen TMV-P_0 that induces a hypersensitive reaction, but is not induced in the compatible interaction with a virulent infection by TMV-P_{12} (Park et al., 2002). It is suggested that the discrepancy of *LTP* induction upon *M. grisea* infection between 'Dongjin' and two other cultivars may arise from the fact that their experiments focused on another *LTP* gene of Class I, but not *b1*, which is equivalent to *LTPC1*. Therefore, it is possible that the role of this *LTP* gene is to deliver the building materials for surface layers after the pathogenic invasion. However, a simpler explanation may reflect differences in the kinetics of induction. For Guiderdoni et al. (2002), the earliest they began recording their observations was 1 d after the inoculation, even though we have now shown that induction of *LTPC1* could be as soon as 8 h after inoculation.

The induction of *LTP* during incompatible interactions with pathogens may support the hypothesis that this gene plays a role in the systemic response against such attacks as well as in simply strengthening structural barriers and protecting organs. However, elucidation of the precise functioning for each *LTP* gene will need much more work in various aspects, both *in vivo* and *in vitro*. In this regard, it is interesting to note that purified LTP exhibits strong *in vitro* antifungal activity against *Cercospora beticola*, a pathogen in sugar beet, and inhibits fungal growth at very low concentrations (Kristensen et al., 2000). Likewise, the recent successful enhancement of antifungal activity and defense responses in transgenic wheat that overexpresses *LTP* genes (Roy-Barman et al., 2006) encourages us to develop a rice plant with antifungal activity against *M. grisea*. Based on those results, we have now also generated several lines of the 'Dongjin' cultivar that overexpress *LTPC1*, and are currently studying their molecular and physiological characteristics.

ACKNOWLEDGEMENTS

This research was mainly supported by the Korea Research Foundation (Grant No. KRF-2002-015-CP0397). It was also funded in part by grants from BioGreen 21 (Grant No. 20050401034593) and from the Interdisciplinary Research Program of KOSEF (Grant No. R01-2006-000-11056-0).

Received August 23, 2006; accepted September 9, 2006.

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