Chloroplast-localized nonspecific lipid transfer protein with anti-fungal activity from rough lemon

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Abstract

A full-length cDNA from rough lemon that encodes a nonspecific lipid transfer protein (nsLTP) was isolated by rapid amplification of cDNA ends (RACE) and designated *RlemLTP*. Subcellular localization analysis of *RlemLTP* fused to a green fluorescence protein gene using particle bombardment in tobacco cells indicated that RlemLTP is located in the chloroplast. Transcripts of *RlemLTP* were detected in young rough lemon leaves and seeds but not in stems and roots. The transcription of *RlemLTP* was higher in young leaves than in old leaves. Wounding and fungal inoculation induced *RlemLTP* expression, and transcription levels were increased 6 to 15 times by these treatments. RlemLTP protein obtained using an *E. coli* expression system showed anti-fungal activity against *Alternaria alternata* and *Fusarium oxysporum*.

Key words: *Citrus jambhiri*, nonspecific lipid transfer protein, anti-fungal protein, *Alternaria alternata*, *Fusarium oxysporum* f. sp. *lycopersici*.

Abbreviations: GFP, green fluorescent protein; IPTG, isopyl-1-thio-b-D-galactoside; LB medium, Luria-Bertani medium; nsLTP, nonspecific lipid transfer protein; ORF, open reading frame; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; ROS, reactive oxygen species; UTR, untranslated region.

1. Introduction

Nonspecific lipid transfer proteins (nsLTPs) are small basic proteins in higher plants [1, 2]. They share several structural features: a conserved pattern of disulfide bridge formation with a conserved eight-cysteine motif [1]. Three-dimensional structure analyses of several nsLTPs demonstrated that the protein has four α-helices cross-linked by four disulfide bridges at the conserved eight-cysteine motif sites [3, 4]. Hydrophilic positive-charged amino acids are exposed on the surface of the protein, while hydrophobic residues line the internal cavity around the motif sites in all nsLTPs [1, 3, 4]. Lipids bind to this internal cavity of nsLTP, and the intermembrane exchange or transfer of lipid molecules is enhanced [1].

Two main families of nsLTPs have been characterized in plants, nsLTP1 with a molecular mass of about 9 kDa and nsLTP2 with a molecular mass of about 7 kDa [2, 3, 5]. The significant differences found between nsLTP1 and nsLTP2 families are their molecular weights and the patterns and locations of disulfide bonds at the LTP motif in their protein structures [2, 5]. In the sequences of the nsLTP1 family, the disulfide bridges are formed between the first and sixth cysteine residues and between the fifth and eighth cysteine residues [2, 3], while the disulfide bridges are formed between the first and fifth cysteine

residues and between the sixth and eighth residues in nsLTP2 [2, 3].

It has been known that nsLTPs play a certain role in plant responses to biotic, abiotic, and environmental stresses. The expressions of nsLTP genes were induced by pathogen attacks and fungal elicitor treatments [6, 7], and nsLTPs showed anti-microbial activities in some plants [6, 8, 9]. Grape nsLTP inhibited fungal growth and onion Ace-AMP1, which shows sequence homology to plant lipid transfer proteins but no lipid transfer activity, exhibited anti-fungal and anti-bacterial activity [6, 10]. Furthermore, overexpression of nsLTPs in barley and Arabidopsis enhanced the resistance to pathogen attacks [11-13]. The expression of nsLTPs was also induced by abiotic and environmental stresses, such as plant hormones, drought, high salinity, low temperature, and wounding [6, 7, 11, 12]. High tolerance to NaCl stress and dehydration was observed in nsLTP-transgenic Arabidopsis [11]. Some nsLTPs are reported to localize to protein storage vesicles, lipid-containing vesicles, and glyoxysomes [14, 15], but nsLTPs are generally known as extracellular proteins and localized in the cell wall [8, 16, 17], which is the first barrier to pathogens in plant tissues. Thus, nsLTPs have been thought to play important roles in several defense responses under pathogen attack in plants, but these roles of nsLTP in plant defense have never been published in any fruit tree.

In the present study, the cDNA encoding nsLTP from the citrus cultivar rough lemon was cloned, and the subcellular localization of the nsLTP, induction patterns of *RlemLTP* mRNA under biotic and abiotic stress, and anti-fungal character of RlemLTP protein were investigated to examine the role in citrus defense mechanisms.

2. Materials and methods

2.1. Plant and fungal materials

Seeds of rough lemon (*Citrus jambhiri* Lush) were germinated in vermiculite and grown as seedlings until they were about 10–15 cm high in a greenhouse with a minimum

temperature of 15ºC. The seedlings were then transferred to pots and maintained under greenhouse conditions. Young leaves (midrib length 2–3 cm), mature leaves (midrib length 6–7 cm), young green stems, and roots were collected from rough lemon plants at 2–5 years old with average heights of 100–150 cm.

The following *Alternaria alternata* strains were used in inoculation of rough lemon leaves and anti-fungal activity tests: the tangerine pathotype of *A. alternata* (strain SH20), which produces the host-selective ACT-toxin and is pathogenic to tangerine or mandarin species and its hybrids but not to rough lemon [18-21] and non-pathogenic strain O-94 of *A. alternata* [18, 19, 22], which does not produce a host-selective toxin and can germinate on the leaf surface and form appressoria but cannot penetrate the living leaf of any plants examined. *Fusarium oxysporum* f. sp. *lycopersici* (strain CK3-1) were also used in anti-fungal activity tests.

2.2. Cloning of rough lemon nonspecific lipid transfer protein gene

cDNA of the rough lemon nonspecific lipid transfer protein gene was isolated based on a polymerase chain reaction (PCR)-cloning strategy. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) from young leaves of rough lemon harvested 2 h after inoculation with a non-pathogenic strain (SH20) of *A. alternata*. This total RNA was converted into double-stranded cDNA using the Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA). To obtain a full-length coding sequence of the nonspecific lipid transfer protein cDNA, 5′ RACE and 3′ RACE were performed using the Marathon cDNA Amplification Kit (Clontech) as described previously [23-26]. For 5′ and 3′ RACE of *RlemLTP*, PCR was performed with the double-stranded cDNA attached to an adapter and gene specific primers designed from the sequences of the subtractive PCR products [25]; 5′ RACE-nsLTP-1st

(5′-CGGACCTTAGAGCAGTCAGTGGAGGTGC-3′) and 3′ RACE-nsLTP-1st (5′-TAACATGTGGGCAGGTGAGTAGCGCATT-3′) following the manufacturer's instructions. The full sequence was obtained by RT-PCR using primer sets designed from 5′ and 3′ untranslated regions (UTR) obtained by sequencing RACE products. The primer pair used for RT-PCR was LTP sense full (5′-ATCTCAAATGGCTGCCCTC-3′) and LTP anti-sense (5′-CTACTCCGTCAACTTAAATC-3′) for amplification of full size *RlemLTP*. The RACE and RT-PCR products were subcloned into a pT7Blue-2 T-vector (Novagen, Madison, WI), and the sequence of the products was obtained from both strands by the dideoxy chain termination method using an ABI Prism Dye Termination Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) and an automated fluorescent DNA sequencer (Model 310, Applied Biosystems). DNA and deduced amino acid sequences were aligned, and a phylogenetic tree was generated under default parameters using the ClustalW program [27]. Sequence analysis was performed using PSORT program [28], ChloroP program [29], and Genetyx-Mac (Software Development Ltd., Tokyo, Japan). Homology analysis was performed using BLAST at the DNA Data Bank of Japan (DDBJ).

2. 3. Transient localization assay

RlemLTP-GFP fusion proteins were expressed using particle gun-mediated DNA delivery to tobacco epidermal cells and imaged by epifluorescence microscopy with a CCD digital camera system DP70-SET-A outfitted with a green fluorescent protein (GFP)-specific filter unit U-MGFPHQ and by differential interference contrast microscopy using an Olympus BX51 system with a BX51-34-FD microscope (Olympus, Tokyo, Japan). About 100 cells/construct were examined for GFP-localization in at least three independent experiments. The entire ORF of *RlemLTP* without the stop codon was prepared by subcloning the full ORF in a pT7Blue-2 T-vector by PCR using the forward primer pBI

121 LTP full forward (5′-GGGTCGACATGGCTGCCCTCAAGC-3′) containing an adapted *Sal*I site and the reverse primer pBI 121 LTP full reverse (5′-GGCCATGGACCGGACCTTAGAGCA-3′) containing an adapted *Nco*I site, and subcloned into the pCaMV35S-sGFP(S65T)-NOS3′ vector [30], which adapted GFP into the C-terminus of RlemLTP. Tissue bombardment was performed by the method of Chiu et al. [30] with some modifications. Tobacco epidermal cell layers were removed, and plasmid DNAs with about 1.5 μg/mg gold particles (1 μm diameter) were introduced using a particle gun (BioRad: PDS-1000/He) at 900 psi. After the bombardment, the tissues were incubated for 24 h at 24ºC in the dark, and samples were observed on glass slides [30].

2. 4. Accumulation of RlemLTP transcripts in different tissues and induction of expression by microbial attack and wounding treatments

Total RNA was isolated from microbe-inoculated, wounded, water-sprayed, or untreated young rough lemon leaves, old leaves, young stems, roots, and seeds using the RNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. A conidial suspension (10^5 spores/ml) of a non-pathogenic strain (O-94 or SH20) of *A. alternata* was sprayed on detached young leaves (midrib length 2–3 cm) of rough lemon as described previously [18, 22-26]. For wounding treatment, leaves were wounded using the method of Gomi et al. [23-26]. Briefly, young leaves (midrib length 2–3 cm) were wounded at four places using the edge of a spatula to crush a line across the lateral veins of each leaflet without making any visible breaks in the tissue [23-26]. The PCR probe for Northern blots was prepared using a PCR DIG Probe Synthesis Kit (Roche, Mannheim, Germany) following the manufacturer's instructions. The primer sets used for synthesis of a DIG-labeled PCR probe were LTP sense full (5′-ATCTCAAATGGCTGCCCTC-3′) and

LTP anti-sense (5′-CTACTCCGTCAACTTAAATC-3′) to amplify the full sequence of *RlemLTP*.

Total RNA from microbe-inoculated, wounded, water-sprayed, or untreated leaves of rough lemon were loaded on 1% agarose-formaldehyde gels (5 or 10 μg/lane) and transferred to Hybond N^+ membrane (GE Healthcare, Princeton, NJ) by capillary transfer. Total RNA on the membrane was stained with 0.02% (w/v) methylene blue in 0.3 M sodium acetate (pH 5.2) and destained in 10% (v/v) ethanol. The rRNAs were stained with methylene blue to confirm equal RNA loading. Hybridization was performed at 68ºC overnight with the DIG-labeled PCR probe. Hybridizing bands were detected and visualized by treating the membrane with anti-DIG antibody-alkaline phosphatase and CSPD (Roche) using an LAS-3000 (Fuji Film, Tokyo, Japan) version 2.2 system with a 3,200,000-pixel cooled CCD camera (Fuji Film). The chemiluminescence signals on the blots were also captured directly by the LAS-3000 system, and quantification of the signals within the linear range was carried out using Image Gauge software (Fuji Film) version 4.23. The relative intensity of each signal was calculated against that obtained from untreated control leaves.

2. 5. Expression and purification of RlemLTP recombinant protein

The open reading frame (ORF) of *RlemLTP* was prepared from a subcloned full ORF in a pT7Blue-2 T-vector by PCR using a set of the forward primer pCold Xho LTP no signal F (5′-CCGCTCGAGCATGGGATAACATGTGGGCAG-3′) containing an adapted *Xho*I site and the reverse primer LTP 2 pGEX asp (5′-GGAATTCCTCACCGGACCTTAGAGCAGT-3′) containing an adapted *Eco*RI site and subcloned into the frame of the pCold II vector (Takara, Shiga, Japan). Overexpression of *RlemLTP* was performed with *E. coli* BL21 containing a chaperone

plasmid pGro7 (Takara) in Luria-Bertani (LB) medium containing ampicillin (50 μg/ml) and L-arabinose (0.5 mg/ml). Cultures were shaken (200 rpm) at 37ºC overnight. These cells were harvested by centrifugation and resuspended in LB medium with ampicillin (50 μg/ml) and further incubated to the logarithmic phase (OD_{600} of 0.4). These cells were incubated at 15ºC for 30 min. Isopropyl-1-thio-b-D-galactoside (IPTG) was then added to a final concentration of 1 mM, and the cells were incubated for different periods up to 6 h at 15ºC with shaking (200 rpm). Expression products of *RlemLTP* were purified using the HisTrap™ HP Kit (GE Healthcare) according to the manufacturer's instructions. The extracted proteins were refolded by dialysis against four changes of 50 mM Tris-HCl buffer (pH 7.5). The dialysate was then used for different assays. Protein was analyzed by SDS-PAGE using 12% polyacrylamide gels and stained with Coomassie brilliant blue R-250 as described previously [31].

2. 6. Anti-fungal activity

The anti-fungal activity of RlemLTP was tested on germination of fungal spores in 12-well culture plates (Becton Dickinson, Franklin Lakes, NJ). Conidia of *A. alternata* (strain O-94) and *F. oxysporum* f. sp. *lycopersici* (strain CK3-1) were incubated in 0.5 ml of 100 mM glucose solution, and treated with/or without RlemLTP at final concentrations of 10-100 μg/ml. After 24 h incubation, germinating conidia were counted using a microscope (Olympus DP70). The bioassays were repeated three times with microscopic observations of more than 100 spores each, and the relative rate (%) of spore germination was calculated by comparison to the growth of spores without any addition.

3. Results

3. 1. Isolation of rough lemon cDNA encoding nonspecific lipid transfer protein

Using the partial sequence of nsLTP obtained by subtractive PCR in our previous study [23], we first synthesized the gene-specific primers of 5′ RACE-nsLTP-1st and 3′ RACE-nsLTP-1st (Fig. 1A), and 5′ and 3′ RACEs were performed. From the sequences of the RACE products, two additional gene-specific primer sets (LTP sense full and LTP anti-sense) were designed with each encompassing the 5′ and 3′ UTR regions of nsLTP, and the full sequence of nsLTP cDNA, designated *RlemLTP,* was identified by RT-PCR using the primer set (Fig. 1A) (data bank accession number AB437259). The internal region of the amplified *RlemLTP* sequence was identical to the sequence of the subtractive PCR product [25]. *RlemLTP* consists of a 345 bp ORF with a deduced amino acid sequence of 115 amino acids, and this primary amino acid sequence exhibited typical signatures of plant nsLTPs, including the conserved cysteine residues, at positions 27, 37, 51, 52, 72, 74, 97, and 111 (Fig. 1A). The calculated molecular weight of RlemLTP is 9.4 kDa. Hydropathy plot analysis indicates a possible transmembrane-spanning domain encoded by amino acids No. 1-24, and this region has a potential N-terminal signal peptide, and the putative subcellular localization was identified as the cell wall by the PSORT program [28] (Fig. 1A). The ChloroP program [29] did not find any signal sequence indicating transport to chloroplasts. Phylogenetic analysis indicated that RlemLTP is classified into the plant nsLTP1 family (Fig. 1B).

3. 2. Subcellular localization of RlemLTP

For the cellular localization analysis of RlemLTP, we translationally fused RlemLTP to GFP [30] and transiently expressed the fusion product in tobacco cells. As shown in Figure 2, RlemLTP was localized to the chloroplast of transformed cells. GFP alone was localized to the nucleus and cytoplasm (Fig. 2), as described by others [30-32].

3.3. Expression of RlemLTP in different tissues and induction of transcription by microbial attack or wounding

Northern blot analysis of total RNA isolated from rough lemon did not detect transcription of *RlemLTP* in stems or roots, but weak expression was detected in leaves and seeds (Fig. 3A). The *RlemLTP* transcripts were higher in young leaves than in old leaves (Fig. 3A). After wounding treatment, the expression of *RlemLTP* was highly induced in young leaves and reached a maximum at 4 h during the incubation period (Fig. 3B, C). The transcript level of *RlemLTP* in the wounded leaves was increased about 15-fold compared to that in unwounded leaves (Fig. 3B, C). After inoculation with spores of *A. alternata* (strain O-94), the expression of *RlemLTP* was also induced, and the induction of *RlemLTP* was faster and stronger than that induced by wounding. Fungus-induced expression reached a maximum at 4 h after treatment, and the transcript level of *RlemLTP* in the fungal inoculated leaves was increased about 6-fold compared to that in water-sprayed leaves (Fig. 3B, C).

3. 4. Anti-fungal activity of RlemLTP

To determine whether *RlemLTP* encodes a functional protein, the ORF region of *RlemLTP* was subcloned into a pCold II vector and expressed in *E. coli*. The expressed RlemLTP protein was purified by an affinity purification system, and the purified RlemLTP protein with a molecular mass of 9.4 kDa on SDS-PAGE (Fig. 4A) was tested for its anti-fungal activity. RlemLTP at concentrations of 10-100 μg/ml was added to fungal spore suspensions of *A. alternata* or *F. oxysporum*, and incubated at 24ºC for 24 h. The germination rate of fungal spores (*A. alternata* and *F. oxysporum*) was decreased in a dose-dependent manner by the addition of different concentrations of RlemLTP (Fig. 4B). RlemLTP at the concentration of 25 μg/ml showed inhibitory activity on spore germination

of both *A. alternata* and *F. oxysporum*, and the rate of inhibition increased in a dose-dependent manner (Fig. 4B). RlemLTP at the concentration of 100 μg/ml reduced spore germination of *A. alternata* to 51.6% and *F. oxysporum* to 38.4% (Fig. 4B).

4. Discussion

A full-length cDNA encoding nsLTP was identified from total RNA of rough lemon leaves by a RACE-based PCR strategy using the partial sequence information from our previous subtractive PCR study [25], and the cloned gene was designated *RlemLTP*. The deduced amino acid sequence showed 62% similarity to carrot nsLTP, and the nsLTP motif with eight conserved cysteine residues was well conserved in the identified *RlemLTP* sequence. Phylogenetic analysis indicated that RlemLTP is a member of the plant nsLTP1 family (Fig. 1B), and the calculated molecular weight from the deduced amino acid sequence and molecular weight identified by SDS-PAGE of 9.4 kDa also indicate that RlemLTP has a similar size as nsLTP1.

nsLTPs generally contain a signal peptide sequence for the secretory pathway, and the proteins can be found in the cell wall and extracellular space [1, 2, 16, 17]. For example, carrot nsLTP was detected extracellularly in embryogenic cell cultures [16], and immunocytochemical analysis revealed that the nsLTP was localized in the cell wall [17]. Pepper nsLTP localization was investigated with GFP-fused nsLTP, and GFP fluorescence was observed at the cell wall [33]. In the deduced amino acid sequence of RlemLTP, the putative signal peptide was found at the position of 1-24, and was thought to secrete extracellularly. PSORT analysis also predicted that RlemLTP was likely to be an extracellular protein, but, unexpectedly, localization analysis of RlemLTP using GFP as the reporter demonstrated that RlemLTP localized at the chloroplast. There are also a few reports describing the intracellular localization of nsLTPs, for example cowpea and castor

bean nsLTPs localized in the cytoplasm, mitochondria, microsomes, and glyoxysomes [14, 15]. However, as far as we know, RlemLTP is the first nsLTP identified to locate at the chloroplast (Fig. 2).

The principal role of nsLTP in chloroplast is not known. However, many fatty acids, glycerolipids, sulfolipids, monogalactosyldiacylglycerol, digalactocyldiacylglycerol, and phosphatidylglycerol are synthesized in the plant chloroplast [34-37]. The precursor of diacylglycerol derives from phosphatidylcholine, and oxylipins from hydroperoxides of polyunsaturated fatty acids are also known to be synthesized in plant chloroplasts. Thus, nsLTP in chloroplasts might be involved in synthesis and transport of these products. nsLTP is also thought to remove acyl-CoA thioesters, which inhibit activity of membrane-associated enzymes and damage the membranes by their detergent properties [1, 38]. Thus, acyl-CoA thioesters should be rapidly removed from the membrane and transported to the endoplasmic reticulum [38]. These lines of evidence for synthesis and transport of lipids and removal of acyl-CoA thioesters suggest that nsLTP may help membrane biosynthesis, protection, and repairing in chloroplasts.

Further, fungal inoculation and wounding are known to induce reactive oxygen species (ROS) production and ROS-related enzymes such as lipoxygenase, which catalyzes hydroperoxidation of plant membrane lipids [23, 39, 40]. Because the lipoxygenase gene of rough lemon, *RlemLOX*, is induced rapidly by inoculation of *A. alternata* and wounding [23], chloroplast membrane lipids are easily damaged by oxidation, and RlemLTP might act as a transporter facilitating the replacement of the impaired membrane lipid. *RlemLTP* expression was higher in young leaves than old leaves, and wounding or fungal inoculation induced the expression of *RlemLTP,* which support the hypothesis that the putative role of RlemLTP is in biosynthesis, protection,

and recovery of the chloroplast membrane.

RlemLTP expression was induced by both wounding and fungal inoculation in Northern blot analysis, and RlemLTP showed anti-fungal activity. These results suggest that RlemLTP may be involved in some of the defense responses in rough lemon. Similar to the putative role of other nsLTPs possessing anti-fungal activity, a central function of RlemLTP is likely to be membrane permeabilization of fungal cells and leakage of cell contents [41, 42] if RlemLTP locates at the cell wall, where it is the first barrier against pathogen attacks. However, the potential role of RlemLTP is probably different from that of nsLTPs because RlemLTP locates in chloroplasts. Other proteins have been found that are located in chloroplasts and possess anti-microbial activity, such as tomato AP24, which is homologous to osmotin and induced by systemic acquired resistance [43], indicating that chloroplast-localized anti-microbial proteins play a defensive role against pathogens in plants. Although the exact role of RlemLTP in chloroplasts in rough lemon defense is still ambiguous, further studies of RlemLTP might provide clues to the meaning of the localization of LTP in chloroplasts and plant defense.

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Figure Legends

Fig. 1. Rough lemon cDNA sequence encoding a nonspecific lipid transfer protein. A) Nucleotide and its deduced amino acid sequences of rough lemon *RlemLTP* cDNA. The nucleic acid sequence is presented on the top line, and the derived

one-letter amino acid sequence is shown below. The putative ORF is in upper case letters, and the stop codon is marked with an asterisk. The eight conserved cysteine residues were boxed. The putative signal peptide is underlined. Primer sites for RACEs were arrowed. The DDBJ/EMBL/GenBank accession number of the *RlemLTP* gene is AB437259. B) Phylogenetic tree of RlemLTP and other LTPs from various plants. The lengths of branches are proportional to number of base substitutions indicated by the scales below. The numbers following the plant name indicate the accession numbers of the corresponding LTPs.

Fig. 2. Subcellular localization of GFP-tagged RlemLTP in plant cells. Epidermis layers of tobacco leaves were bombarded with particles coated with constructs to express GFP alone (A), or the entire region of RlemLTP fused with GFP (B). Green fluorescence corresponding to the localization of the proteins was detected by epifluorescence microscopy. Bars = 50 μm.

Fig. 3. Northern blot analysis of *RlemLTP* in different organs of rough lemon, and induction of mRNA accumulation of *RlemLTP* in leaves by several treatments. A) Accumulation of *RlemLTP* transcripts in different organs. Symbols at each lane: YL, young leaves; OL, old leaves; St, stems; Ro, roots; and Se, seeds. 28S and 18S rRNA stained with methylene blue were used for verification of equal RNA loading (10 μg) in each lane. B) Induction of *RlemLTP* transcription by wounding and fungal inoculation. Conidial suspension (10^5 spores/ml) of non-pathogenic *A. alternata* (strain O-94) was sprayed onto the underside of rough lemon leaves. Wounded and fungal-inoculated leaves were incubated in a moist chamber at 24ºC for up to 12 h in the dark. Water-sprayed leaves incubated in the same way served as the control.

Total RNA was extracted at different times after wounding or fungal inoculation, and total RNA (5 μg) of each sample was subjected to RNA blot analysis. Numbers above each blot indicate the time (h) after respective treatments. 28S and 18S rRNA stained with methylene blue are shown for verification of equal RNA loading in each lane. C) Quantification of *RlemLTP* mRNA accumulation in wounded, fungus-inoculated, and water-treated rough lemon leaves. Gray, black, and white bars indicate wounding, fungus-inoculated, and water-sprayed fractions, respectively. mRNA quantification was determined by LAS-3000 (Fuji Film), and the relative increase of each signal intensity was calculated against that obtained from untreated control leaves.

Fig. 4. Heterologous expression and determination of anti-fungal activity of RlemLTP. A) RlemLTP protein was expressed in an *E. coli* expression system, purified by HisTrap™ HP Kit (GE Healthcare), and run on SDS-PAGE. Protein extracts from *E. coli* without induction (lane 1), with induction (lane 2), and purified RlemLTP (lane 3) were resolved on 12% acrylamide gel followed by Coomassie brilliant blue staining. The numbers on the left indicate the positions of protein size markers in kDa. B) Effects of RlemLTP on germination rate of fungal spores (*A. alternata* and *F. oxysporum*) were determined, and relative values (%) of germination rates with different concentrations of RlemLTP were calculated by comparison to the germination rate without any protein addition. Each data bar represents the mean of three independent replicates of more than 100 spore observations, and error bars represent standard deviations of the mean.

A

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1   atctcaaATGGCTGCCCTCAAGCTTGTTTCAGCTTTGGTTCTGTGCATGTTGGTGACTGG  60
          M  A  A  L  K  L  V  S  A  L  V  L  C  M  L  V  T  G

                                  3' RACE-nsLTP-1st
61  TCCCCTGAGTGCTCAGGCCATAACATGTGGGCAGGTGAGTAGCGCATTGGCACCATGCAT  120
    P  L  S  A  Q  A  I  T  C  G  Q  V  S  S  A  L  A  P  C  I

121 CTTTTACTTGAGAGCGGGCGGCCCTATACCTGTGCCATGCTGCAACGGCGTCAGGTCTCT  180
     F  Y  L  R  A  G  G  P  I  P  V  P  C  C  N  G  V  R  S  L

181 CAACGCTGCCGCCAGAACCACACCTGACCGCCAAACTGCATGCAACTGCTTGAAGCAAGC  240
     N  A  A  A  R  T  T  P  D  R  Q  T  A  C  N  C  L  K  Q  A

241 CGCCGGATCAATCCCTAACCTCAACCCTAATAATGCTGTTGGTCTTCCACGAGCTTGTGG  300
     A  G  S  I  P  N  L  N  P  N  N  A  V  G  L  P  R  A  C  G

                                  5' RACE-nsLTP-1st
301 AGTCAGCATTCCTTACAAGATCAGCATCTCCACTGACTGCTCTAAGGTCCGGtgaacagg  360
     V  S  I  P  Y  K  I  S  I  S  T  D  C  S  K  V  R  *

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B

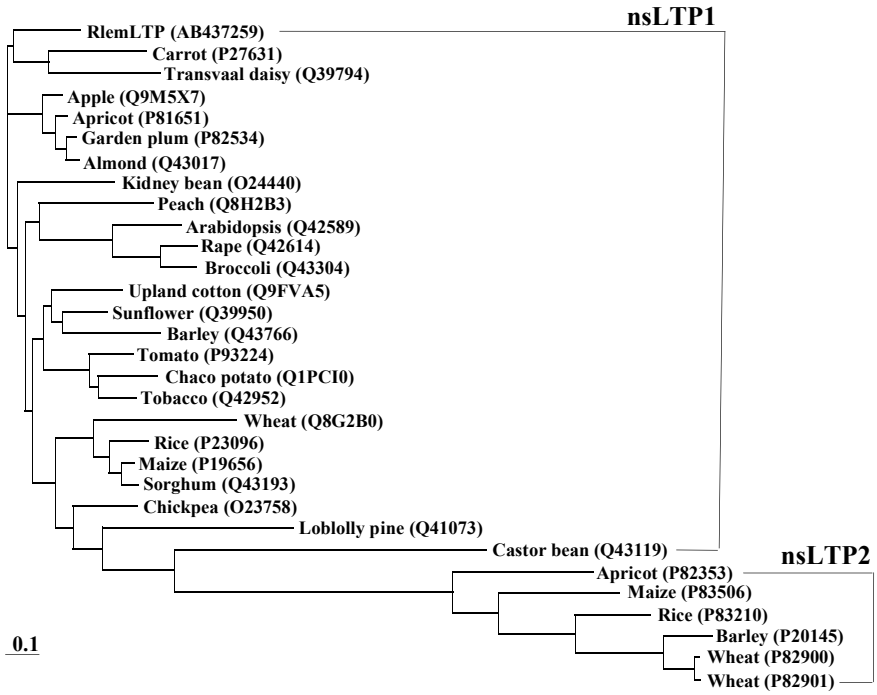

Figure 2 Nishimura

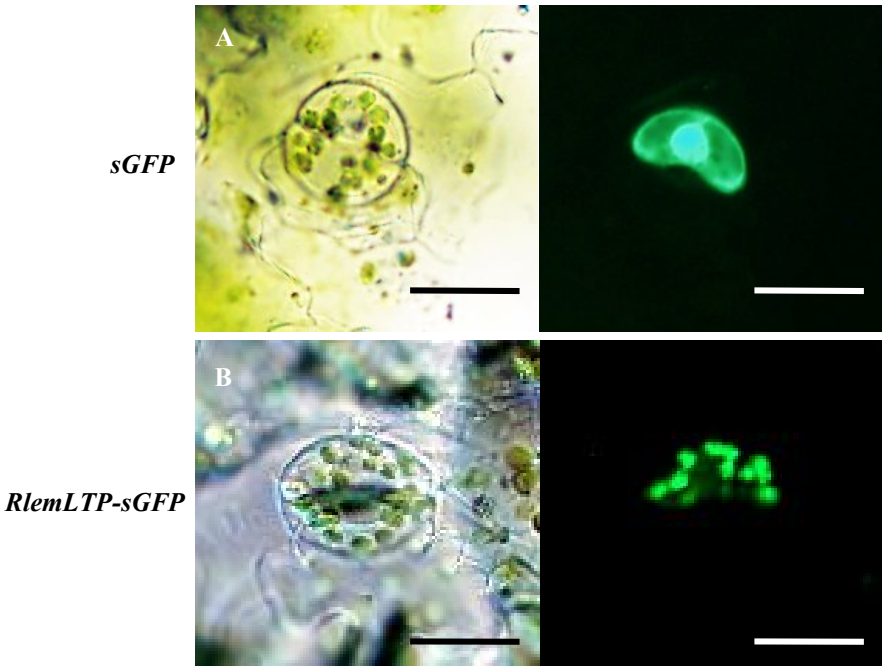

Figure 3 Nishimura