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

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ABSTRACT

A full-length cDNA (*RlemLTP*) from rough lemon that encodes a nonspecific lipid transfer protein was isolated. Results of subcellular localization of *RlemLTP* fused to a green fluorescence protein indicated that *RlemLTP* is located in the chloroplast. Transcripts of *RlemLTP* were detected in 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 6–15 times by these treatments. *RlemLTP* obtained using an *E. coli* expression system showed anti-fungal activity against *Alternaria alternata* and *Fusarium oxysporum*.

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].

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.

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.

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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 15 °C. The seedlings were then transferred to soil in pots 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

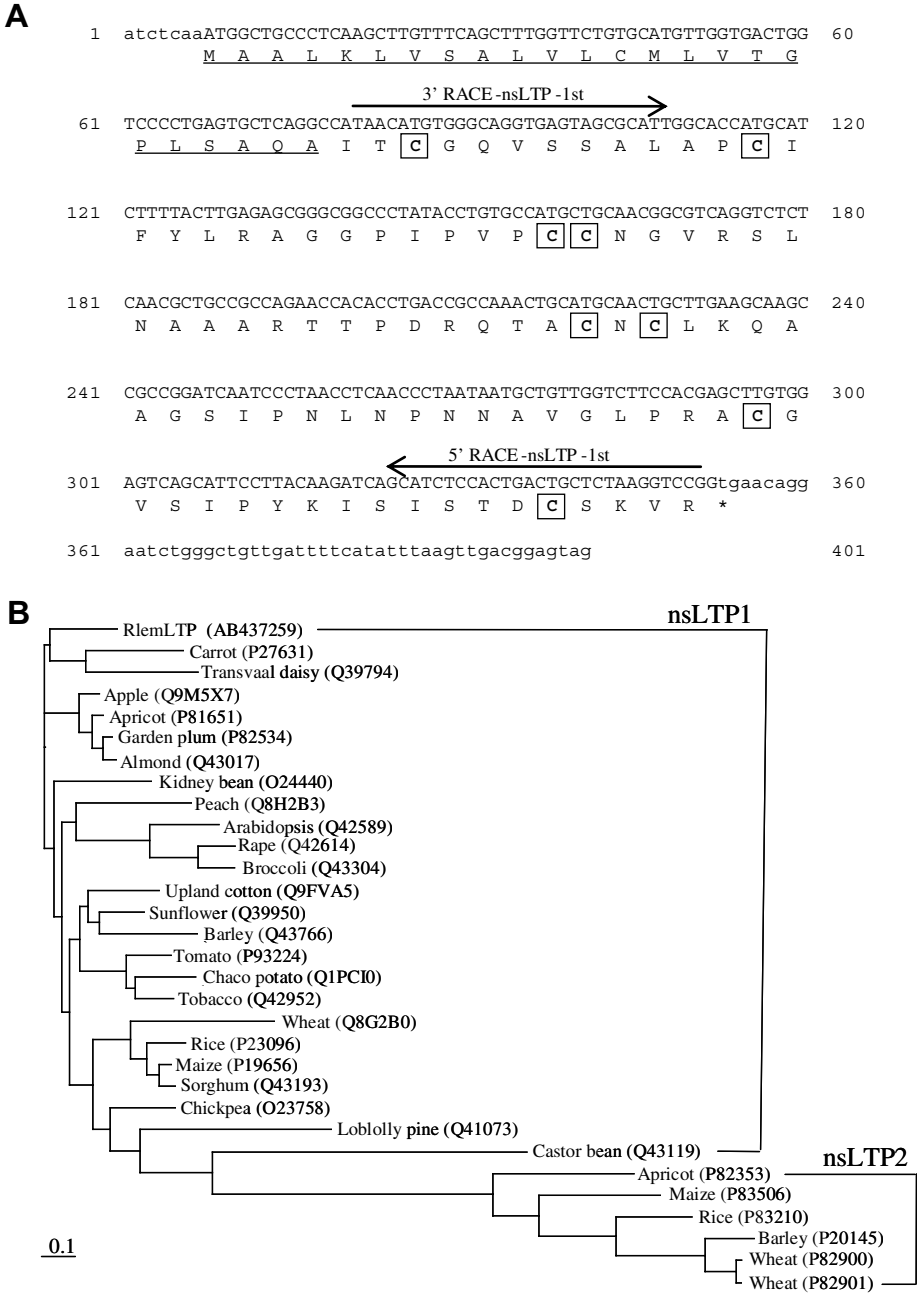

Fig. 1. Rough lemon cDNA sequence encoding a nonspecific lipid transfer protein. (A) Nucleotide and its deduced amino acid sequences of rough lemon *RlemLTP* cDNA. The nucleic acid sequence is presented on the top line, and the derived one-letter amino acid sequence is shown below. The putative ORF is 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) Neighbor-joining 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.