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Resistance Function of Rice Lipid Transfer Protein LTP110

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Plant lipid transfer proteins (LTPs) are a class of proteins whose functions are still unknown. Some are proposed to have antimicrobial activities. To understand whether LTP110, a rice LTP that we previously identified from rice leaves, plays a role in the protection function against some serious rice pathogens, we investigated the antifungal and antibacterial properties of LTP110. A cDNA sequence, encoding the mature peptide of LTP110, was cloned into the Impact-CN prokaryotic expression system. The purified protein was used for an *in vitro* inhibition test against rice pathogens, *Pyricularia oryzae* and *Xanthomonas oryzae*. The results showed that LTP110 inhibited the germination of *Pyricularia oryzae* spores, and its inhibitory activity decreased in the presence of a divalent cation. This suggests that the antifungal activity is affected by ions in the media; LTP110 only slightly inhibited the growth of *Xanthomonas oryzae*. However, the addition of LTP110 to cultured Chinese hamster ovarian cells did not retard growth, suggesting that the toxicity of LTP110 is only restricted to some cell types. Its antimicrobial activity is potentially due to interactions between LTP and microbe-specific structures.

Keywords: Antimicrobial activity, Expression, Lipid transfer protein, Pathogen, Rice

Introduction

Lipid transfer proteins (LTPs) in plants are encoded by members of different gene families and are unevenly distributed in a variety of plant tissues and organs (Kader, 1996 and 1997). Due to their *in vitro* ability to bind and transfer lipids between biomembranes, LTPs were originally thought to play a role in facilitating the cytoplasmic lipid transfer between organelles, such as mitochondria and chloroplasts (Kader, 1975). However, subsequent studies revealed that LTP molecules are synthesized as pre-peptides with putative signal sequences and targeted to the cell wall of epidermal cell layers in plants (Thoma *et al.*, 1994), suggesting that these proteins do not play cytoplasmic roles. Therefore, other physiological functions for them were supposed, such as participating in cuticle formation, protecting plants against pathogen attack, and acting as membrane cryoprotectin against freezing injury (Thoma *et al.*, 1993; Cammue *et al.*, 1995; Hollenbach *et al.*,1997; Hincha *et al.*, 2002).

Different LTPs share some common characteristics, such as basic isoelectric point, low molecular weight, and 8 cysteines at conserved locations that are engaged in forming four disulfide bridges. Three-dimensional structures have been determined for several LTPs, revealing the presence of a flexible hydrophobic tunnel within the molecule that is capable of binding lipids of different sizes (Lerche *et al.*, 1998; Charvolin *et al.*, 1999).

Previously, we reported two rice LTP cDNA sequences (Zhan *et al.*, 1997; Ge *et al.*, 1999; Liu *et al.*, 1999). One of them, LTP110, is a new isoform. Our research goal was to understand whether LTP110 is involved in rice protection against pathogens. In most areas of the world, rice is an important field crop and rice pathogens have caused serious production loss. It is very significant in agronomic application to seek molecules that can be used in bioengineering for improving the resistance property of rice. To investigate the antimicrobial activity of LTP110, we expressed this protein in *E. coli* and used it for *in vitro* inhibition tests. The expressed-rice LTP110 protein inhibits the growth and proliferation of rice fungal and bacterial pathogens, but does not affect the growth of cultured hamster ovarian cells. This suggests that LTP110 is not a common lipidic membrane destroyer, and that inhibitory activity is related to the specific structure of the microbes.

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Materials and Methods

Materials The rice seeds, *Oryzae Sativa* L.SSP.*indica* var. Guanglu'ai No. 4, were obtained from the Chinese National Institute for Rice Research. The rice pathogens, *Pyricularia oryzae* and *Xanthomonas oryzae*, were provided by the Shanghai Agricultural College.

Construction of recombinant plasmids and protein expression Plasmid containing the cDNA sequence of the rice lipid transfer protein LTP110 (Liu *et al.*, 1999) was used to amplify the sequences encoding the mature peptide of LTP110. The upstream primer is 5'-GGTGGTTGCTCTTCCAACGCGGTTAGCTGCG GCGAC-3' (*Sap*I site is underlined) and the downstream primer is 5'-TCAGAATTCCTATTAGTTGATCTTGGAGCAG-3' (*Eco*RI site is underlined). The polymerase chain reaction resulted in an expected 0.3 kb fragment. This fragment was recovered from the agarose gel and double-digested with *Sap*I and *Eco*RI, then subcloned into the *Sap*I and *Eco*RI double-restricted pTYB11 vector (New England Biolabs Inc., Beverly, USA), forming the recombinant plasmid pTYB11-LTP110. The sequence encoding LTP110 was confirmed by automatic sequencing.

Protein expression Recombinant plasmid pTYB11-LTP110 was transformed into the host strain ER2566 and selected by 100 μg/ml ampicillin on LB media. A single colony was inoculated into 2 ml LB with 100 μg/ml ampicillin and cultured overnight at 37°C. The overnight culture was then used to inoculate 250 ml liquid LB media (also with 100 μg/ml ampicillin) and grown at 37°C until A_{600} reached 0.8, then the growth temperature was lowered to 30°C. IPTG was added to the final concentration of 0.1 mM to induce expression. After 5 h induction, bacteria were collected, suspended in a column buffer (20 mM TrisHCl, 500 mM NaCl, 1 mM EDTA, pH 8.0), and stored at −70°C until use.

Purification of recombinant LTP Aliquots of the previously mentioned suspension were thawed at room temperature, supplemented with PMSF (phenylmethanesulfonyl fluoride) to 1 mM and Triton X-100 to 0.1%, and sonicated on ice. The mixture was centrifuged at 15,000 × *g* for 20 min. The supernatant was passed through a 0.45 μm filter membrane and loaded onto a chitin column (New England Biolabs Inc.), which had been pre-equilibrated with a column buffer. The column was then washed with a column buffer until A_{280} of the flow-through liquid was less than 0.02. The cleavage buffer (20 mM TrisHCl, 500 mM NaCl, 1 mM EDTA, 50 mM DTT, pH 8.0) of three-fold the bed volume was passed quickly through the column. The column was then incubated at 4°C for 36 h to induce self-cleavage. The released target protein was eluted with a column buffer, dialyzed overnight (molecular weight cutoff 8 kDa) against 10 mM NH_4AC, and freeze-dried. Before use, the protein was solved in deionized water and sterilized by filtration through a 0.22 μm sterilized membrane. Lipid-binding activity was checked using 1-pyrenedodecanoic fatty acid, as described by Zachowski *et al.* (1998).

In vitro* inhibition test: *Pyricularia oryzae *Pyricularia oryzae* were grown on PDA (potato dextrose agr) media until spores were abundantly produced. The spores were collected and counted by a haemocytometer under a microscope. Their concentration was adjusted to 2×10^4 spores/ml with sterile water. Microtiter plates were used to test the antifungal activity of LTP110. Each well contained 10 μl spores (200 spores), 20 μl protein of various protein concentrations, and 120 μl PD (potato dextrose) liquid media. After incubation at 28°C for 36 h, the growth of *Pyricularia oryzae* was quantified by measuring A_{490} with an ELISA Reader. Growth inhibition at each protein concentration was tested three times. As a negative control, BSA was added instead of LTP.

In vitro* inhibition test: *Xanthomonas oryzae A single colony of *Xanthomonas oryzae* was inoculated into 2 ml liquid media (sucrose 2%, Bacto-tryptone 0.5%, K_2HPO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.025%, pH 7.2-7.4) and grown overnight in a 28°C shaker before the culture was used to inoculate 25 ml of the media. Once the bacteria reached the logarithmic growth phase, various concentrations of purified LTP110 were added to 0.5 ml aliquots of the bacterial culture. After continuous culture for an additional 18 h, A_{600} was measured. As a negative control, BSA was added instead of LTP.

Addition of LTP to Chinese hamster ovarian cells Chinese hamster ovarian (CHO) cells were grown in Dulbeccos modified Eagle's medium (DMEM, GIBCO, Detroit, USA) that was supplemented with 10% fetal bovine serum (FBS). Next, 100 μl cells with a concentration of 10^4 cells/ml were inoculated into 96-well tissue culture plates and grown in a 37°C incubator until 50-60% confluence. Different amounts of purified LTP110 were then added into the wells. BSA was used as a negative control. After a continuous culture for an additional two days, the growth conditions of the cells were observed under a microscope, and the living cells were counted.

Results and Discussion

Expression and purification of LTP110 In order to obtain a sufficient number of active proteins for the inhibition test, LTP110 (amino acid sequence refers to Fig. 1) was expressed in *E. coli* by the Impact-CN Expression System. The expression vector encodes a chitin-binding domain (CBD) before the foreign gene-cloning region, which allows the expression of a CBD-fused protein as well as the protein being purified by affinity chromatography through binding to the chitin beads. The expressed fusion protein was induced to self-cleavage in a column, releasing the foreign protein while the fusion tag still remained on the chitin beads. Therefore, 1-2 mg recombinant LTP110 can be obtained from each liter of the bacterial culture (Fig. 2). To check if the expressed protein was suitable for the following inhibitory assays, the purified protein was subjected to a lipid-binding activity analysis. It showed the typical curve of the lipid transfer proteins when using 1-pyrenedodecanoic fatty acid as the fluorescent probe to check its activity.

Antifungal activity To study whether the recombinant LTP110 exhibits antifungal activity, we tested its effect on the

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          1        10        20        30        40        50
LTP110    AVSCGDVTSS IAPCLSYVMG RESSPSSSCC SGVRTLNGKA SSSADRRTAC
            *         *         **                          *
                  60        70        80        90
LTP110    SCLKNMASSF RNLNMGNAAS IPSKCGVSVA FPISTSVDCS KIN
            *                   *         *
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Fig. 1. Mature peptide sequence of LTP110. Asterisks show the conserved cysteine residues.

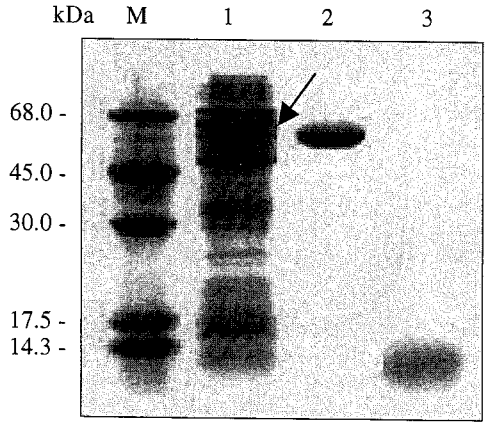

Fig. 2. Purification of LTP110. M, marker; 1, total soluble protein of ER2566 expressing CBD-LTP110 fusion protein; The arrow indicates the fusion protein. 2, CBD fusion tag remaining on chitin column after self-cleavage; 3, LTP110 released from chitin column after self-cleavage.

germination of *Pyricularia oryzae* spores, which causes rice blast in the field (Fig. 3). Spore germination was strongly inhibited by LTP110 when the concentration was as low as 27 μg/ml, but when the protein concentration reached 133 μg/ml, the inhibitory effect dropped slightly, possibly due to protein aggregation at high concentrations.

LTP110 slowed down the elongation of hyphae, but did not induce morphological distortions. The new growing hyphae showed stunted hyphal elongation. This phenomenon has also been observed for many defensive proteins, but the mechanisms may be different. The mechanisms for each protein still need to be studied. Moreover, when divalent cations, such as 1 mM Ca^{2+}, were added into the sample wells, the antifungal activity of LTP110 was drastically reduced, even at the highest protein concentrations. This suggests that divalent cations antagonize the inhibitory activity of LTP110 (Fig. 4). Among the antimicrobial peptides that have been studied, most of them show reduced antifungal activities in the presence of cations, such as Mg^{2+}, Ca^{2+}, and Ba^{2+}(Broekaert *et al.*, 1997). A low ionic strength environment is necessary for them to exert inhibitory activities. This is in accord with the cell wall environment of LTP under normal physiological conditions.

Antibacterial activity The antibacterial activity of LTP110 to the pathogen *Xanthomonas oryzae*, which causes the most

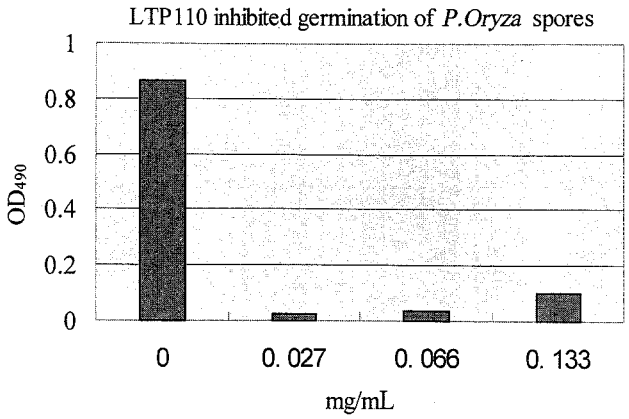

Fig. 3. Effect of LTP110 on the germination of *Pyricularia oryzae* spores.

devastating blight disease in rice, was studied. We found that *Xanthomonas oryzae* grew slowly in the presence of LTP110 (Fig. 5). The inhibitory effect was proportionate to the protein concentration across a range of 27 μg/ml to 133 μg/ml. However, overall, the antibacterial activity to *Xanthomonas oryzae* was weaker than the observed antifungal activity to *Pyricularia oryzae*. This shows that LTP110 is not an active inhibitor to *Xanthomonas oryzae*. We also checked its activity to other bacterial pathogens, such as *Pseudomonas syringae*. The results demonstrated that the antibacterial activity of LTP110 is strongly pathogen-dependent, since its inhibition ability varied when the different kinds of bacteria were checked. For example, complete inhibition to the growth of *Pseudomonas syringae* was observed when the concentration was only 27 μg/ml (results not shown).

Influence of LTP110 on cultured animal cells Most antimicrobial peptides exert their effects by migrating through the pores of microbial cell walls and interacting with the microbial plasma membranes, thereby causing the physical alteration of the cell membrane, such as aggregation and leakage. If this is also the case for LTP110, it should then also affect the growth of mammalian cells since there is no cell wall and only plasma membranes surround the cytoplasm of animal cells. Moreover, LTP110 is a lipid transfer protein, it is reasonable to postulate that it might destroy the intact structure of a plasma membrane by binding and moving some lipidic molecules away. However, we found no significant effect on the growth of cultured Chinese hamster ovarian cells when LTP110 was added into the media (data not shown). This suggests that the antimicrobial activity of LTP110 may be related to the specific inherent structures of microbes themselves, rather than being generally damaging to plasma membranes.

This inherent structure might be a receptor or ligand in the cell walls or on the cell membranes of microbes. Park *et al.* (Park *et al.*, 2000) reported that a nsLTP-like protein in the extracellular matrix of lily styles is correlated with the

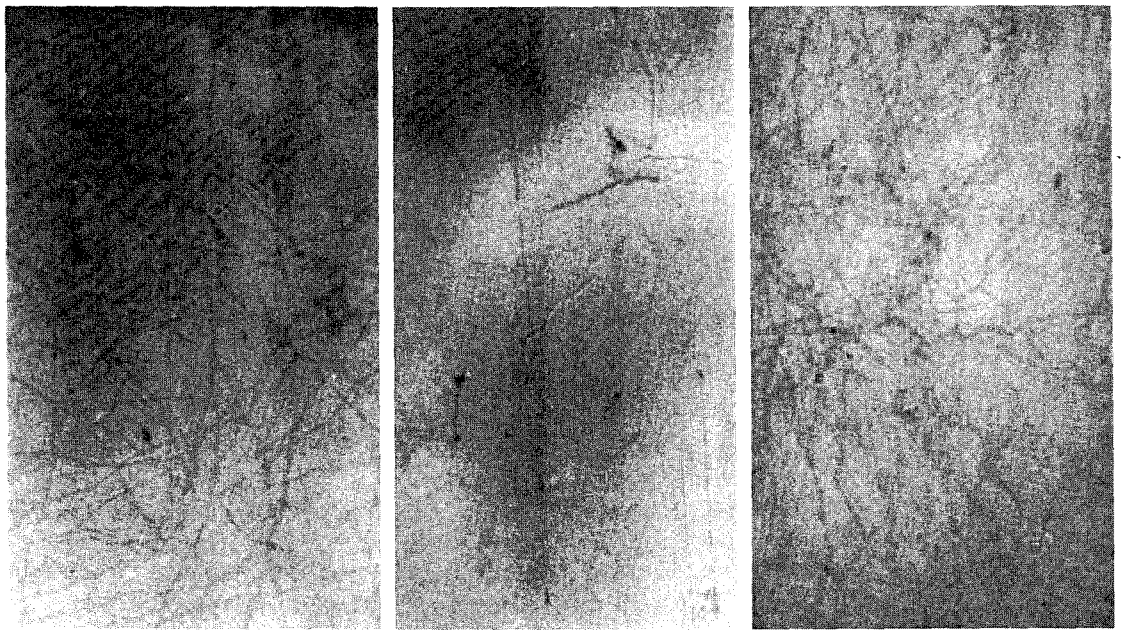

Fig. 4. Comparison of the growth of *Pyricularia Oryzae* in media with BSA (control), LTP110, LTP110 and 1 mM $CaCl_2$. The protein concentrations for each sample are all the same (66 μg/ml).

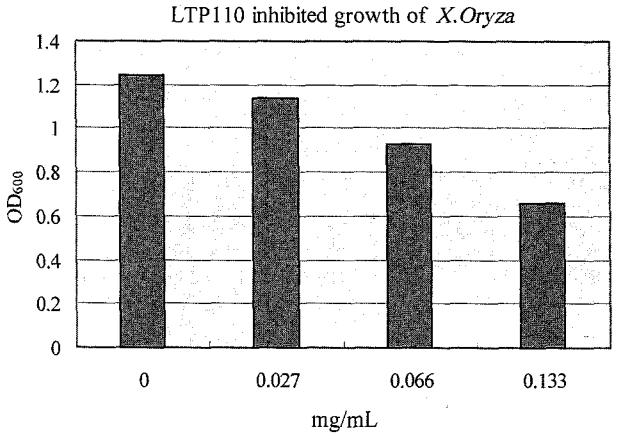

Fig. 5. Effect of LTP110 on the growth of *Xanthomonas oryzae*.

adhesion between pollen tubes and stigmas, where it might act as a ligand or a signal molecule. Buhot and Blein *et al.* (Buhot *et al.*, 2001; Blein *et al.*, 2002) also showed that LTPs could compete with elicitins to bind common receptors on tobacco plasma membranes. These findings suggest that LTP may interact with ligands within the cell walls or on the plasma membranes to affect the relevant physiological processes.

Furthermore, the influence of ions on the inhibitory activity of LTP110 might also imply its molecular mechanism. LTP110 binding to target molecules might be regulated by these ions. A recent report (Maldonado *et al.*, 2002) revealed that a LTP-like protein in Arabidopsis acts as a co-signal to bind with an unknown movable signal molecule to induce SAR (Systematic Acquired Resistance). Therefore, there is a need for further research on the biological ligands of LTPs under physiological conditions.

Our research has shown that rice LTP110 has a resistance function to two of the most serious rice pathogens, *Pyricularia oryzae* and *Xanthomonas oryzae*, though the efficiency of resistance is pathogen-dependent. Surprisingly, LTP110 does not affect the growth of animal cells, which is contrary to its potential activity to transfer membrane lipids. In agricultural engineering, in order to improve rice resistance, this characteristic is beneficial since it does no harm to human cells.

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