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An antifungal protein purified from pearl millet seeds shows sequence homology to lipid transfer proteins

R. VELAZHAHAN*, R. RADHAJEYALAKSHMI, R. THANGAVELU and S. MUTHUKRISHNAN**

Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore-641 003, India
*Department of Biochemistry, Kansas State University, Manhattan, KS 66506-3702, USA***

Abstract

In the course of a search for antifungal proteins from plant seeds, we observed inhibition of mycelial growth of *Trichoderma viride* with extracts of pearl millet. We have identified several proteins with antifungal properties in the seeds of pearl millet. One of these proteins has been purified to homogeneity and characterized. The purified protein has a molecular mass of 25 kDa. The N-terminal sequence of the protein (25 residues) shows homology to non-specific lipid transfer proteins (LTPs) of cotton, wheat and barley. The purified LTP inhibited mycelial growth of *T. viride* and the rice sheath blight fungus, *Rhizoctonia solani in vitro*.

Additional key words: pathogenesis-related protein, *Pennisetum glaucum*, *Rhizoctonia solani*, *Trichoderma viride*.

Introduction

Many antifungal proteins have been detected in plant seeds. These proteins include chitinases (Anuratha *et al.* 1992, Swegle *et al.* 1992), β-1,3-glucanases (Leah *et al.* 1991), thionins (Carmona *et al.* 1993), permatins (Roberts and Selitrennikoff 1990, Vigers *et al.* 1991), ribosome-inactivating proteins (Roberts and Selitrennikoff 1986, Leah *et al.* 1991), defensins (Terras *et al.* 1992b) and non-specific lipid transfer proteins (Terras *et al.* 1992a, Cammue *et al.* 1995). It is suggested that these antifungal proteins may protect the seeds from the invading soil-borne plant pathogens (Cammue *et al.* 1995). The enzymes, chitinases and glucanases have the potential to hydrolyze chitin and β-1,3 glucan respectively, which are major components of fungal cell walls leading to direct inhibition of the growth of several fungi *in vitro* (Schlumbaum *et al.* 1986, Leah *et al.* 1991). Permatins, which are members of the PR-5 family of pathogenesis-related (PR) proteins have been shown to inhibit the growth of several fungi by permeabilizing fungal membranes (Vigers *et al.* 1991). Thionins show antifungal activity by permeabilizing fungal cell membranes probably by direct interaction with membrane phospholipids (Broekaert *et al.* 1997). Defensins exhibit antifungal activity via a receptor-mediated mechanism (Thevissen *et al.* 1996). Ribosome inactivating proteins (RIPs) inhibit protein synthesis in fungi by N-glycosidase action on specific purines of 28S rRNA (Girbes *et al.* 1996). Lipid transfer proteins (LTPs) of plants are small basic proteins with eight cysteines conserved in four disulfide bridges (Desormeaux *et al.* 1992). LTPs have been shown to inhibit the growth of fungi (Cammue *et al.* 1995) and bacteria (Molina *et al.* 1993, Segura *et al.* 1993) *in vitro*. A number of possible functions have been proposed for LTPs in plants including involvement in membrane biogenesis (Arondel *et al.* 1990), plant defense (Molina *et al.* 1993) and in transport of cutin monomers required for the biosynthesis of surface wax (Sterk *et al.* 1991). In the course of a search for antifungal proteins from plant seeds, we observed inhibition of mycelial growth of *Trichoderma viride* with extracts of pearl millet (Radhajeyalakshmi *et al.* 2001). In this paper we describe the purification of an antifungal protein from pearl millet (*Pennisetum glaucum* (L.) R. Br.) seeds. The N-terminal sequence of the purified protein shows homology to non-specific lipid transfer proteins of other plants.

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Abbreviations: LTP- lipid transfer protein, PDA- potato dextrose agar.
* Author for correspondence; fax: (+91) 422 431672, e-mail: velazhahan@hotmail.com

Materials and methods

Seeds of pearl millet [*Pennisetum glaucum* (L.) R.Br.] cv. Co-3 obtained from Millet Breeding Station, Tamil Nadu Agricultural University, Coimbatore, India were ground and the powder was suspended in 200 cm^3 5 mM sodium phosphate, 10 mM NaCl, 1 mM EDTA, pH 7.0, and then homogenized in a blender at 4 °C for 5 min. The homogenate was filtered through cheese cloth before centrifugation at 8 500 *g* for 15 min at 4 °C. The pellet was discarded and the supernatant was fractionated by the addition of $(NH_4)_2SO_4$, and the fraction that precipitated at 60 % saturation was collected by centrifugation at 8 500 *g* for 10 min at 4 °C. The precipitate was resuspended in 5 mM sodium phosphate buffer, pH 7.0, and dialyzed against the same buffer overnight. The sample was then applied to a *CM-Sephadex C-50* (*Pharmacia*, Uppsala, Sweden) column previously equilibrated with 50 mM sodium citrate-phosphate buffer (pH 5.2) and proteins bound to the column were eluted with a gradient of sodium chloride (0 - 200 mM) in the same buffer. The fractions were collected in 2 cm^3 volumes using a *Bio-Rad Automated Econo System* (*Bio-Rad*, USA). Amounts of protein in all samples were determined by the Bradford assay (Bradford 1976) using bovine serum albumin as standard. The peak fractions were analyzed on 12 % sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) using a *Mighty Small II* electrophoresis unit (*Hoefer*, USA) according to the method of Laemmli (1970). Gels were run at 20 mA for 2 h and stained for protein with Coomassie Brilliant Blue *R-250*.

N-terminal sequencing: Purified protein was subjected to 12 % SDS-PAGE and then the protein was electroblotted on to a PVDF membrane (*Bio-Rad*) using a *Trans-Blot SD* semi-dry transfer cell (*Bio-Rad*). The protein was subjected to automated Edman degradation using an *Applied Biosystems* sequencer at the Biotechnology Microchemical Core Facility, Kansas State University, Manhattan, KS, USA.

Fungal growth inhibition assay: All manipulations were carried out under sterile conditions. The protein fractions were sterilized using 0.2 μm filters (*Millipore*, USA) and used for assay of antifungal activity. A single sclerotium of *Rhizoctonia solani* was placed in the center of Petri dishes (90 mm in diameter) containing potato dextrose agar (PDA) medium (Vidhyasekaran *et al.* 1997). To allow initial mycelial growth, the plates were incubated for 24 h at room temperature (28 ± 2 °C). At this time, sterile filter paper discs (6 mm in diameter) were laid on the agar surface one cm away from periphery of the Petri dish and 0.02 cm^3 of the supernatant fractions (10 μg of protein) were applied to the each disc. The plates were further incubated at room temperature and photographed 72 h after the onset of treatment (Mauch *et al.* 1988).

For testing antifungal activity against *Trichoderma viride*, a spore suspension of *Trichoderma viride* (5×10^3 conidia cm^{-3}) was prepared and mixed with 20 cm^3 of molten PDA medium and poured onto the Petri dishes (90 mm in diameter). Sterile filter paper discs (6 mm in diameter) were laid on the agar surface one cm away from periphery of the Petri dish and 0.02 cm^3 of the supernatant fractions (10 μg of protein) were applied to the each disc. After the plates were incubated at room temperature for 48 h, inhibition of *T. viride* growth was observed.

Results

An antifungal protein was purified from pearl millet seeds by ammonium sulphate fractionation and cation exchange chromatography. The cation exchange chromatography on *CM-Sephadex* resulted in separation of a single peak at 0.1 M NaCl. SDS-PAGE of the proteins appearing at 0.1 M NaCl showed a single band corresponding to the molecular mass of 25 kDa (Fig. 1). The N-terminal sequence of the protein (25 residues) was determined by Edman degradation. By comparing this N-terminal sequence with other sequences in the GenBank data base by Blast search, we found substantial homology (above 50 %) with non-specific lipid transfer proteins of cotton, wheat and barley (Table 1). The LTP of pearl millet has 60 % identity with a *Gossypium hirsutum* non-specific LTP, 54 % identity with a *Triticum aestivum* LTP, 57 % identity with *Hordeum vulgare* LTP, 44 % identity with a rice and sweet cherry LTPs. Antifungal action of the purified 25 kDa LTP was assessed by hyphal growth inhibition assay. The purified LTP inhibited mycelial growth of *T. viride* and *R. solani*, the rice sheath blight fungus *in vitro* at 10 μg concentration (Fig. 2).

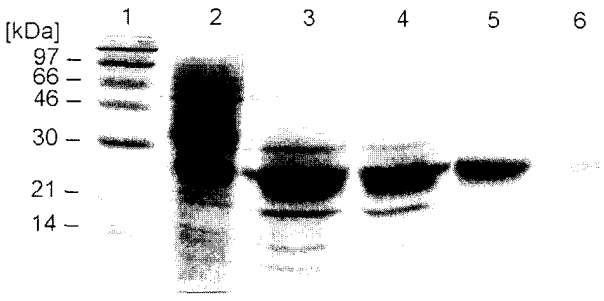

Fig. 1. Coomassie blue stained SDS-PAGE of pearl millet lipid transfer protein (LTP). *Lane 1* - molecular mass marker, *lane 2* - total protein from pearl millet seeds, *lanes 3 to 6* - peak fractions of *CM-Sephadex* column chromatography.

Table 1. N-terminal amino acid sequence alignment of the lipid transfer protein (LTP) of pearl millet and other LTPs. The N-terminal amino acid sequence of pearl millet LTP was compared with the sequences of cotton LTP (GenBank accession No. O24418), wheat LTP (GenBank accession No. AAB32997), barley LTP (GenBank accession No. CAA65680), rice LTP (GenBank accession No. Q42976) and sweet cherry LTP (GenBank accession No. AAF26449).

Species	N-terminal amino acid sequence alignment																							
Pearl millet	A	V	S	T	P	E	V	D	A	N	L	L	P	C	V	G	Y	V	T	G	K	N	A	S	P
Cotton	A	I	S	Y	D	Q	V	K	S	S	L	L	P	C	V	G	Y	V	R	G	N	N	A	R	P
Wheat	A	L	S	C	G	Q	V	D	S	K	L	A	P	C	V	A	Y	V	T	G	R	A	X	S	
Barley	A	L	S	C	G	Q	V	D	S	K	L	A	P	C	V	A	Y	V	T	G	R				
Rice	A	V	S	C	G	D	V	T	S	S	I	A	P	C	L	S	Y	V	M	G	R	E	S	S	P
Sweet cherry	A	L	T	C	G	Q	V	S	S	N	L	A	P	C	I	A	Y	V	R	G	G	G	A	V	P

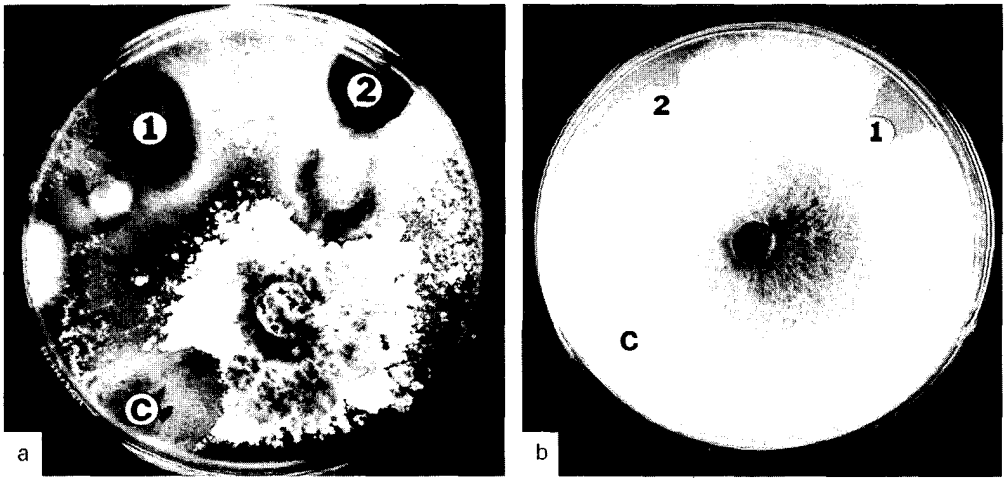

Fig. 2. Antifungal activity of the purified 25 kDa pearl millet lipid transfer protein (LTP) against *Trichoderma viride* (*a*) and *Rhizoctonia solani* (*b*). Water (C) or 10 µg of the purified pearl millet LTP (1 and 2) in 0.02 cm^3 of 5 mM sodium phosphate buffer, pH 7.0, was applied to the filter paper discs.

Discussion

LTPs have been isolated or cloned from a number of monocotyledonous and dicotyledonous plants including *Arabidopsis* (Clark and Bohnert 1999), barley (Breu *et al.* 1989), bean (Choi *et al.* 1996), *Brassica* (Sohal *et al.* 1999), carrot (Sterk *et al.* 1991), cotton (Orford and Timmis 2000), maize (Tchang *et al.* 1988), onion (Cammue *et al.* 1995), pepper (Jung and Hwang 2000), rice (Vignols *et al.* 1994, Garcia-Garrido *et al.* 1998), sorghum (Pelese-Siebenbourg *et al.* 1994), spinach (Bernhard *et al.* 1991), tobacco (Fleming *et al.* 1992), tomato (Trevino and O'Connell 1998) and wheat (Neumann *et al.* 1994). It has been demonstrated that LTP genes are induced in barley leaves after challenge inoculation with both virulent and avirulent strains of *Erysiphe graminis* f. sp. *hordei* (Molina and Garcia-Olmedo 1993). Since LTPs are induced in plants in response to infection by plant pathogens, these proteins have been included as one of the family member (PR-14) of pathogenesis-related (PR) proteins (Van Loon and Van Strien 1999). LTPs are also induced by external application of abscisic acid (Garcia-Garrido *et al.* 1998), NaCl (Garcia-Garrido *et al.* 1998) and by drought stress (Trevino and O'Connell 1998). Several LTPs have been purified from the seeds of barley (Davy *et al.* 1999), onion (Cammue *et al.* 1995), radish (Terras *et al.* 1992a) and rice (Poznanski *et al.* 1999). Terras *et al.* (1992a) purified a 9-kD LTP from radish seeds. Similarly, Davy *et al.* (1999) purified a 9.7 kDa LTP with four disulphide bridges from the barley grain. In these instances, the molecular mass of the protein ranges between 9 and 10 kDa. In the present study we purified a LTP from the pearl millet seeds to homogeneity. The purified LTP has a molecular mass of 25 kDa. The N-terminal sequence (25 amino acid residues) of the purified protein showed substantial homology (above 50 %) with LTPs of cotton, wheat and barley. To the best of our knowledge, this is the first report of purification of a LTP from pearl millet.

Not all LTPs show antifungal action against plant pathogens. For example, a LTP purified from onion seed

was highly inhibitory to a broad range of fungi, whereas a radish seed LTP was only moderately active and maize and wheat seed LTPs were inactive against most fungi tested (Cammue *et al.* 1995). In the present study the inhibitory action of pearl millet LTP on the mycelial growth of *R. solani* and *T. viride* has been demonstrated. This LTP may be potentially useful in enhancing the disease resistance of crop plants by genetic engineering. It has been demonstrated that transgenic tobacco and *Arabidopsis* plants over-expressing LTP gene of barley (LTP2) have enhanced resistance to bacterial pathogen, *Pseudomonas syringae* pv. *tabaci* and *Pseudomonas syringae* pv. *tomato* respectively (Molina and Garcia-Olmedo 1997). Recently, it has been demonstrated that constitutive, high level expression of a gene encoding LTP (*Ace*-AMP1) from onion in transgenic geranium (*Pelargonium* sp) can enhance resistance to *Botrytis cinerea* infection (Bi *et al.* 1999).

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