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Molecular chaperone function of *Arabidopsis thaliana* phloem protein 2-A1, encodes a protein similar to phloem lectin

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ABSTRACT

Although several phloem sap proteins have been identified from protein extracts of heat-treated *Arabidopsis* seedlings using FPLC gel filtration columns, many of the physiological roles played by these proteins remain to be elucidated. We functionally characterized a phloem protein 2-A1, which encodes a protein similar to phloem lectin. Using a bacterially expressed recombinant protein of AtPP2-A1, we found that it performs dual functions, showing both molecular chaperone activity and antifungal activity. mRNA expression of the *AtPP2-1* gene was induced by diverse external stresses such as pathogens, and other signaling molecules, such as ethylene. These results suggest that the AtPP2-A1 molecular chaperone protein plays a critical role in the *Arabidopsis* defense system against diverse external stresses including fungal pathogenic attack and heat shock.

1. Introduction

The phloem provides an efficient pathway for long-distance communication and the trafficking of macromolecules [1] and constitutes a strategic location for the mounting of defenses against pathogens [2]. PP2 is a phloem lectin conserved in plants [3,4] and is known to play a role in the phloem-specific defense mechanism induced by insect attacks [5–7] and other stresses, such as wounding [5,7,8] and oxidative stress [6]. Pumpkin PP1 and PP2 hetero-complex have covalent bonds which make high molecular weight complex [5–7] and this disulphide bond is induced by oxidative stress [6]. Phloem protein plugging, callose closure of sieve pores, and callose coagulation on sieve plates are thought to play a major role as a physical barrier to prevent the insect from phloem-feeding activity [7]. Nevertheless, there is a paucity of study for the defense mechanism of phloem proteins in pathogenic attacks. In cucurbit, a reactive oxygen species (ROS) enhances formation of the PP2–PP1 hetero-polymers [6]. This ROS is a conserved response in *Arabidopsis* treated with harpins [9–11]. *Myzus persicae* infestation induces an elevation of the ethylene level [9,12] and triggers modest induction of ethylene-dependent responses [12,13].

Only a few functions have been elucidated for the *Arabidopsis* phloem sap proteins that represent the largest group of these proteins identified by sequencing. During isolation and characterization of *Arabidopsis* heat shock-resistant proteins that are involved in a variety of cellular processes such as protein folding, trafficking, maturation, degradation and cell stress protection, we identified a thermo-stable AtPP2-A1. Expression of the *AtPP2-A1* gene was changed by external stresses, such as pathogens, and a signaling molecule, ethylene. We characterized AtPP2-A1 chaperone function and its functional roles in plant defense.

2. Materials and methods

2.1. Materials

Arabidopsis thaliana seedlings were used for the screening of heat-stable proteins. *Arabidopsis* plants (ecotype Columbia) were grown under a 16/8 h light/dark cycle at 22 °C and 70% humidity. Malate dehydrogenase (MDH) and citrate synthase (CS) were obtained from Sigma. Fast protein liquid chromatography (FPLC) column Superdex 200 10/300 GL was from Amersham Biosciences.

2.2. Isolation of heat-stable protein from Arabidopsis thaliana seedlings

To isolate and characterize heat-stable proteins that are involved in a variety of cellular processes, such as protein folding, degradation, signal transduction and cell stress protection, soluble proteins from 2-week old *Arabidopsis* seedlings heat-treated at 60 °C for 30 min were extracted by using CelLytic P cell lysis buffer (Sigma). From the supernatant, several novel heat-stable proteins

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have been identified by using 2-D gel PAGE and MALDI-TOF techniques (data not shown).

2.3. RT-PCR analysis

To analyze expression level of *AtPP2-A1* in *Arabidopsis* whole plants treated with 2 ppm ethylene or fungal pathogen (see below), RT-PCR analysis was performed using specific primers. DNA using the following primers: *AtPP2-A1* forward (5′-ATGAGCAAGAAACATTGCTC-3′) and *AtPP2-A1* reverse (5′-CGTGATGTCGAGCCAACAAA-3′). The template was cDNA made from total RNA [14]. The leaves of 4 week old wild-type *Arabidopsis thaliana* plants were infiltrated with 108 cfu/ml *Pseudomonas syringae* pv. *tomato* DC3000 and harvested. ACTIN2 (At3g18780) was used as the internal reference as described previously [14].

2.4. Histochemical localization of GUS activity

The pCAMBIA1381-GUS plasmid was used for GUS activity of *AtPP2-A1* promoter (−1550 to +30 relative to the ATG translation start codon) [15]. The plasmid was introduced into *Agrobacterium* GV3101 and transformed into Arabidopsis plants using the floral dip method [16]. Histochemical localization of GUS activity was performed as described by Jefferson et al. [15]. Transgenic Arabidopsis tissues were infiltrated in 50 mM potassium phosphate buffer (pH 7.0), 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide, and 0.2% Triton X-100 containing 1 mM X-GlcA. The tissues were incubated in the dark at 37 °C with agitation for more than 12 h and, subsequently, removed the chlorophylls with 70% ethanol solution. GUS staining analysis is the representatives of at least five independent transgenic lines.

2.5. Cloning of AtPP2-A1 and expression in E. coli

AtPP2-A1 gene was cloned from an *Arabidopsis* cDNA library by polymerase chain reaction (PCR) and the *AtPP2-A1* BamHI/XhoI cDNA construct was confirmed by a sequencing analysis. For expression of AtPP2-A1 in *Escherichia coli*, the *AtPP2-A1* gene was cloned into the pGEX vector. The resulting construct, pGEX-*AtPP2-A1*, was introduced into *E. coli* BL21 (DE3) and grown in LB medium. The GST fusion protein was isolated from the cytosolic fraction of the *E. coli* by glutathione Sepharose affinity gel filtration. The AtPP2-A1 was used for the analysis of its biochemical properties.

2.6. Chaperone activities of AtPP2-A1 protein

Holdase activity of AtPP2-A1 protein was measured by using MDH and CS, as substrates [17]. Turbidity due to substrate aggregation was monitored in a DU800 spectrophotometer (Beckmann, USA) equipped with a thermostatic cell holder.

2.7. Assay for antifungal activity

To investigate the antifungal activity of the isolated *Arabidopsis* protein, we performed a radial growth inhibition assay and a 96-well microdilution assay for evaluation of effective concentration [18]. The following fungal strains were used: *Aspergillus flavus* (KCTC 6905), *Didymella bryoniae* (KACC 40,669), *Fusarium moniliforme* (KACC 40,386), *Fusarium solani* (KCTC 6326), *Rhizoctonia solani* (KACC 40,138), *Trichoderma harzianum* (KCTC 6043) and *Trichoderma viride* (KCTC 6047). These strains were obtained from the Korea Collection for Type Cultures or the Korea Agricultural Culture Collection.

3. Results and discussion

Considering the fact that AtTrx-h3 changed its protein structure from low molecular weight (LMW) to high molecular weight (HMW) complexes by heat shock and its original disulfide reductase function of the HMW AtTrx-h3 was changed to a molecular chaperone function [19], we decided to screen heat-stable HMW proteins that have a similar molecular chaperone function with the AtTrx-h3. To isolate new heat stable proteins and analyze their functions, wild type *Arabidopsis thaliana* seedlings were heat shocked at 60 °C for 30 min. Soluble proteins were extracted using high-speed centrifugation (15,000 rpm) and fast protein liquid chromatography (FPLC) (Fig. 1A). Other heat stable proteins such as α-crystallins or small heat shock proteins, function as molecular chaperones [20–23]. We therefore decided to characterize the chaperone functions of our isolated proteins. First, we examined the holdase chaperone activity of three major fractions of soluble proteins by assessing their ability to inhibit the thermal aggregation of the model substrate, MDH at 45 °C for 15 min. Fraction 1 protein effectively suppressed thermal aggregation of the substrate

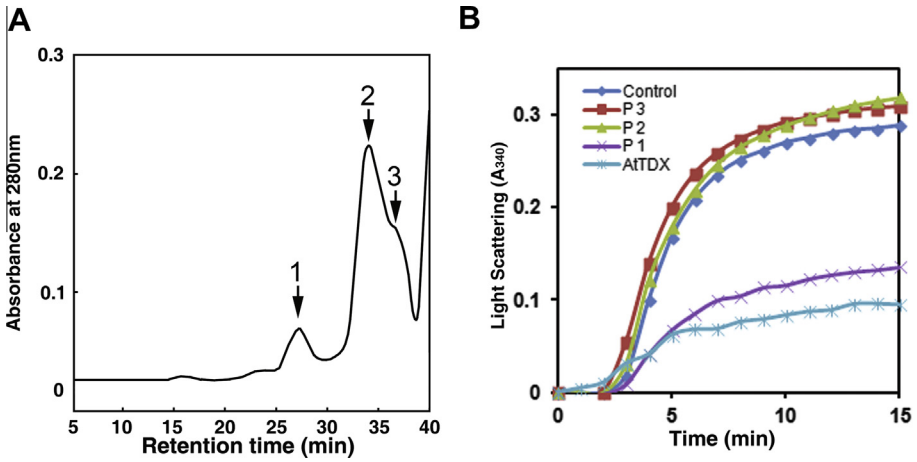

Fig. 1. Purification of chaperone proteins from heat-treated *Arabidopsis* seedlings. (A) FPLC gel filtration chromatography with a Superdex 200 column was performed to investigate the chaperone activity with three major peaks. (B) Holdase chaperone activity. Thermal aggregation of 1 μM MDH was examined for 20 min in the absence (Control) or presence of isolated proteins; fraction 1 (P1), fraction 2 (P2), and fraction 3 (P3). In addition, AtTDX was analyzed as a positive molecular chaperone protein.

(Fig. 1B; P1). However fraction 2 and 3 did not show any MDH protection activity (Fig. 1B; P2 and P3).

Using MALDI-TOF/MS analysis, we determined that the amino acid sequence of the purified protein was identical to the amino acid sequence of a hypothetical *Arabidopsis* protein recorded in GenBank (accession No. At4g19840.1). To determine whether the protein can prevent unstable MDH from heat treatment, we used PCR to clone the full-length cDNA encoding the hypothetical protein from an *Arabidopsis* seedling cDNA library and we expressed the protein in *E. coli* by fusing the cDNAs with a pGEX expression vector. After the fusion protein was purified using GSH-agarose affinity gel chromatography, the GST-portion of the proteins was removed by thrombin treatment and we thus obtained the native form of the AtPP2-A1 protein (Fig. 2A). To verify the chaperone activity of the recombinant protein, we investigated the holdase activity of the recombinant AtPP2-A1 protein. The protein showed a highly-potent holdase chaperone activity which was about 40% lower than that of *Arabidopsis* TDX (Fig. 2B) [17]. The same results could be obtained by using another substrate, CS (data not shown). Previous study suggested that PP2-A1 is involved in virus transmission by aphids and stabilizes virus particles [24]. This is consistent with our result in which AtPP2-A1 functions as a chaperone. PP2 is abundant in the phloem and is transported from companion cells to sieve elements. Therefore, it would be abundant enough to protect many glycoproteins from this trafficking against severe environmental stresses or pathogenic attacks.

Previous studies showed that phloem sap proteins are induced by biotic and abiotic stresses and they are functionally related to defense responses [5–8]. We investigated the transcript level of the *AtPP2-A1* gene with signal molecules or environmental stress-treated *Arabidopsis* whole plants (Fig. 3). Ethylene (ET), salicylic acid (SA) and jasmonic acid (JA) participate in the transcriptional activation of pathogen-response genes [14]. Such genes are thought to be responsible for the induced defense mechanism in plants. As shown in Fig. 3A, the *AtPP2-A1* transcript level was significantly induced by ethylene within 3 h of treatment, but decreased within 48 h. In contrast, when two week old seedlings were treated with SA or JA, the *AtPP2-A1* transcript level did not differ significantly from untreated seedlings (data not shown).

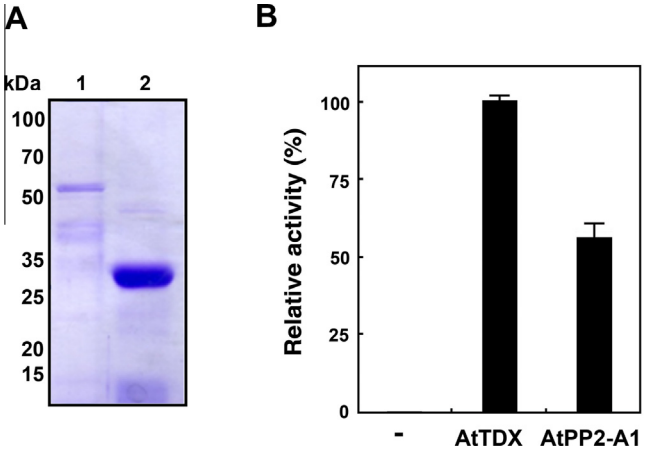

Fig. 2. SDS–PAGE of proteins isolated by affinity chromatography. (A) 12% polyacrylamide gel was loaded with proteins obtained from the GST-AtPP2-A1 fusion protein purified by affinity chromatography with a glutathione-agarose affinity column (lane 1). A portion of GST was removed by thrombin treatment (lane 2). Numbers indicate molecular standards. The proteins were stained with Coomassie blue. (B) Holdase chaperone activity of AtPP2-A1. Relative activity of holdase chaperone function was analyzed. The activities of both fractions were compared to AtTDX, whose activity was set to 100%.

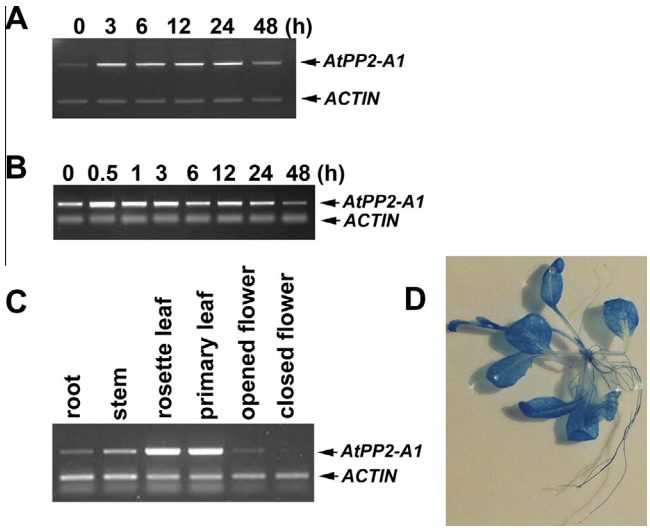

Fig. 3. Expression levels of *AtPP2-A1* mRNA in *Arabidopsis* treated with various stress signals. Total RNA was extracted from *Arabidopsis* plant tissues after treatment with 2 ppm ethylene (A) or 108 cfu/ml *Pseudomonas syringae* (B) for indicated times. The mRNA expression level in untreated cells was used as a control (0 h). (C) *AtPP2-A1* gene expression in young and adult *Arabidopsis* plant tissues. mRNA level of *ACTIN2* was used to confirm equal amounts or RNA were used in each lane. (D) GUS expression in transgenic *Arabidopsis* plants carrying the GUS coding region fused to the *AtPP2-A1* promoter. Histochemical assay show *AtPP2-A1* promoter::GUS expression pattern in representative GUS expression from 14-day-old plants grown under normal growth condition.

The induction of *AtPP2-A1* by ethylene suggests that it may be expressed in response to pathogens. To test this hypothesis further, we treated seedlings with *Pseudomonas syringae*. Compared to untreated seedlings, the *AtPP2-A1* gene was quickly induced within 30 min of treatment (Fig. 3B).

Finally, we measured mRNA levels of *AtPP2-A1* in various plant tissues. We found *AtPP2-A1* was expressed highest in primary and rosette leaves, and was also present in roots, stems, and unopened flowers (Fig. 3C). To confirm the expression pattern of *AtPP2-A1* gene, T3 *AtPP2-A1* promoter::GUS transgenic plants were grown and then subjected to histochemical staining for GUS activity. *AtPP2-A1* was continuously expressed in the primary and rosette leaves, and GUS activity was also observed in roots. This GUS activity assay clearly shows that *AtPP2-A1* expression was strong in the leaves (Fig. 3D). Altogether, these results support a hypothesis that the *AtPP2-A1* gene-product may function in an induced defense mechanism against various environmental stress and pathogenic damage.

Although plant lectins can not bind to the membrane of the fungal cell, several plant lectins produce antifungal activity by binding to carbohydrates on the fungal cell wall [25,26]. Fungal growth inhibition of chitin-binding proteins is related to their chitin-binding activity. It remains unclear whether AtPP2-A1 presents such properties. In order to investigate the antifungal function of the purified protein in more detail we tested the antifungal activity of AtPP2-A1 against various fungal strains including *A. flavus*, *Fusarium moniliforme*, *Fusarium solani*, *Rhizoctonia solani* and *Trichoderma harzianum*. We found that the growth of *Fusarium solani* tested was especially inhibited by the AtPP2-A1 protein (Fig. 4). Interestingly, AtPP2-A1 displayed a significant difference in antifungal activity against a variety of fungi (Table 1).

To our knowledge, this is the first report of the molecular chaperone function of AtPP2-A1. It is likely that AtPP2-A1 may play diverse cellular functions that are critical for cell survival. The identification of a novel function of AtPP2-A1 as a molecular chaperone and an antifungal protein may ultimately enhance the

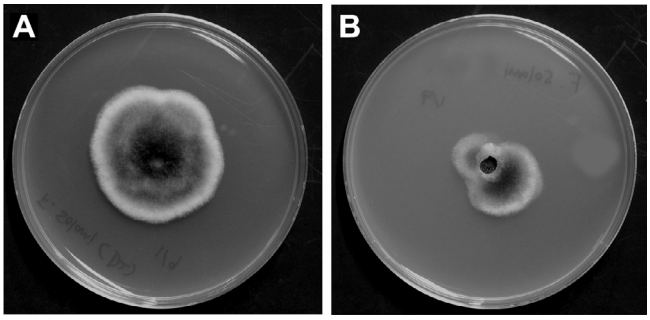

Fig. 4. Antifungal activity of recombinant AtPP2-A1 protein against a fungus. Recombinant AtPP2-A1 was subjected to radial growth inhibition tests with *Fusarium solani*. 100 μg AtPP2-A1 protein was loaded onto a plate (B). 25 mM Tris–HCl buffer (pH 7.2) was loaded as a negative control (A).

Table 1
Inhibitory effects of recombinant AtPP2-A1 protein against various strains of filamentous fungi.

Pathogens	Protein (EC_{50})[a] (μM)
Fungi	
Aspergillus flavus	NA
Fusarium moniliforme	60
Fusarium solani	20
Rhizoctonia solani	20
Trichoderma harzianum	40

NA, not active at concentration <160 μM.
[a] EC_{50} Effective concentration for 50% inhibition.

resistance of crop plants against phloem-feeding insects and helps our understanding of the uncharacterized cellular functions of AtPP2-A1 in plant cells.

Acknowledgements

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