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Purification, Cloning, and Identification of Two Thaumatin-like Protein Isoforms in Jelly Fig (*Ficus awkeotsang*) Achenes†

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Jelly curd used for a popular summer drink in Taiwan is prepared by extracting the pericarpial portion of jelly fig (*Ficus awkeotsang* Makino) achenes. The two most abundant proteins found in jelly curd have been identified as a pectin methylesterase and a chitinase. A method was developed to purify the next abundant protein by 40% ammonium sulfate precipitation and flowing through Mono Q chromatography. In sodium dodecyl sulfate−polyacrylamide gel electrophoresis analyses, the purified protein migrated as a polypeptide of 20 kDa in the absence of β-mercaptoethanol but split into a minor polypeptide of 20 kDa and a major polypeptide of 27 kDa in the presence of this reducing agent. Two cDNA fragments encoding precursor polypeptides of two putative thaumatin-like protein isoforms were obtained by polymerase chain reaction cloning and subsequently overexpressed in *Escherichia coli* to generate recombinant proteins for antibody preparations. Immunological detection and mass spectrometric analyses indicated that the two split polypeptides were thaumatin-like protein isoforms encoded by the two cloned cDNA fragments.

KEYWORDS: Achene; jelly fig (*Ficus awkeotsang*); mass spectrometric analyses; pericarp; thaumatin-like protein

INTRODUCTION

Jelly fig (*Ficus awkeotsang* Makino), a member of the family Moraceae, is a unique evergreen woody vine distributed rampantly in tropical and subtropical regions of Taiwan. Flowers pollinated by a specific symbiotic wasp (*Blastophaga pamila* Hill) produced inside the fruit tiny seeds termed achenes, which have long been used to prepare a popular summer drink in the form of jelly curd. A three-dimensional structure constructed via the ionic interaction between the calcium ion and the carboxyl group of pectin after demethoxylation is responsible for the gelation (*1*). The major pericarpial protein extracted from the jelly curd has been identified as a pectin methylesterase involved in the gelation process (*2*), and its corresponding cDNA has been cloned (*3*) and functionally expressed in yeast (*4*). The second most abundant protein has been purified and characterized as an antifungal chitinase (*5*), and its corresponding cDNA has also been cloned (*6*). Besides these two abundant proteins, there are several unidentified proteins in the pericarp of jelly fig achenes.

Thaumatin-like proteins (TLPs), belonging to the pathogenesis-related protein 5 family, play an important role in plant defense against pathogen invasions (*7*). These antifungal proteins, active against a broad spectrum of pathogens (*8*−*13*), possess a high degree of sequence similarity with thaumatin, a sweet protein found in *Thaumatococcus danielli* (*14*). Although the precise mechanism is not fully understood, the antifungal activity of TLPs is assumed to be a consequence of increasing membrane permeability of fungal cells by forming transmembrane pores that cause an influx of water into the cells followed by osmotic rupture (*8*, *15*). In addition to antifungal activity, TLPs may also be involved in various developmental processes, such as flower formation and fruit ripening (*16*, *17*). Other unexpected functions, such as antifreeze activity, β-1,3-glucan binding, and endo-β-1,3-glucanase activity, have also been found in some TLPs (*18*−*20*).

The objective of this study is to identify the third abundant protein in the pericarp of jelly fig achenes. In sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) analyses, this protein migrated as a single polypeptide of 20 kDa in the absence of β-mercaptoethanol (β-ME) but split into two polypeptides of 20 and 27 kDa in the presence of this reducing

† The nucleotide sequences reported in this paper have been submitted to the GenBank database with accession numbers DQ277011 and DQ277012 for thaumatin-like protein isoforms 1 and 2, respectively.

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agent. Here, we report the purification, cloning, and identification of these two split polypeptides.

MATERIALS AND METHODS

Purification of P3 (the Third Abundant) Protein in the Pericarp of Jelly Fig Achenes. Jelly fig (*Ficus awkeotasang* Makino) achenes were purchased from local growers, and jelly curd was prepared by extracting 50 g of achenes with 600 mL of water according to a modified method developed by Ding et al. (*21*). Pericarpial proteins of jelly fig achenes were extracted from jelly curd, and the insoluble pectin fraction were removed by filtration after centrifugation at 15000*g* for 10 min. The extracted pericarpial proteins were collected by ammonium sulfate precipitation at a final concentration of 40% saturation. After centrifugation, the ammonium sulfate precipitate was dissolved in 50 mM Tris-HCl at pH 8.0 and dialyzed against the same buffer at 4 °C overnight. The supernatant of 1 mL was applied to an anion-exchange column (7 × 25 mm Mono Q column, Amershan Pharmacia Biotech) previously equilibrated with 50 mM Tris-HCl at pH 8.0. The flow-through fraction of the Mono Q column was collected for further analyses.

Analysis of Protein Contents in SDS−PAGE. The protein samples were heated with the reducing or nonreducing sample buffer (125 mM Tris-HCl at pH 6.8, 10% glycerol, and 0.02% bromophenol blue, with or without 0.5% β-ME) at 95 °C for 5 min and resolved in a 15% SDS−PAGE gel (Hoefer Scientific Instruments, Amersham Pharmacia Biotech) according to the method of Laemmli (*22*). After electrophoresis, the proteins in the gel were stained with 0.125% Coomassie brilliant blue R-250 in 50% methanol and 10% acetic acid and then destained. To estimate the protein amount and recovery yield in purification, protein samples as well as a serial dilution of known bovine serum albumin concentrations were resolved by SDS−PAGE. The gel image was captured with a CCD camera, and the band intensity was quantified by Multi Gauge V3.0 software.

RNA Isolation and cDNA Library Construction. Total RNA was prepared from 2 g of maturing achenes by grounding the material in liquid nitrogen using the phenol/SDS method (*23*). The poly(A)$^+$ mRNA was purified from total RNA using the oligo dT magnetic beads (Dynabeads, Dynal) following the instructions of the manufacturer. A cDNA library was constructed in Lambda Uni-ZAP XR Vector using Stratagene's ZAP-cDNA synthesis kit according to the protocol described in the instructions of the manufacturer (cDNA synthesis, ZAP-cDNA synthesis, and ZAP-cDNA Gigapack III Gold cloning kits were purchased from Stratagene). After *in vivo* mass excision of the jelly fig cDNA library, the resulting plasmid bank was used as templates for the following polymerase chain reaction (PCR) cloning.

Cloning of Two TLP Isoforms. To clone cDNA fragments encoding TLPs, a specific primer, TLPR (5′-AAGCTTTCAAGGGCAGAACACGA-3′; the *Hin*dIII site is underlined), designed according to a partial clone of putative TLP accidentally found previously and a specific T3 primer (5′-ATTAACCCTCACTAAAGGGA-3′) were used for PCR amplification using the cDNA library as templates. Two partial cDNA fragments of 801 and 797 bp encoding two TLP isoforms were obtained. To complete these two cDNA clones, two specific primers, TLPF-1 (5′-ACTATGGGCTCCTTAGCC-3′) and TLPF-2 (5′-AGCATGGGTCTTCTCAAG-3′), were designed according to the sequences of the two partial cDNA fragments. Two fragments of 1028 and 970 bp containing the complete coding sequences were obtained by PCR reactions using the two designed primers and a specific T7 primer (5′-TAATACGACTCACTATAGGG-3′), with the cDNA library as templates.

Overexpression of the Two TLP Isoforms in *Escherichia coli*. The two cDNA clones encoding jelly fig TLP isoforms were constructed in the fusion expression vector, pET21b(+) (Novagen), and thus, both recombinant TLPs were expected to possess a C-terminal fusion of the His tag. The resulting plasmids were used to transform *E. coli* strain Rosetta-gami(DE3)pLysS. Cells were grown to 0.5 OD_{600} at 37 °C, and then the expression of recombinant TLPs was induced by adding 1 mM isopropyl-β-D-thiogalactoside (IPTG). After induction for 4 h, *E. coli* cells were harvested by centrifugation for further analyses.

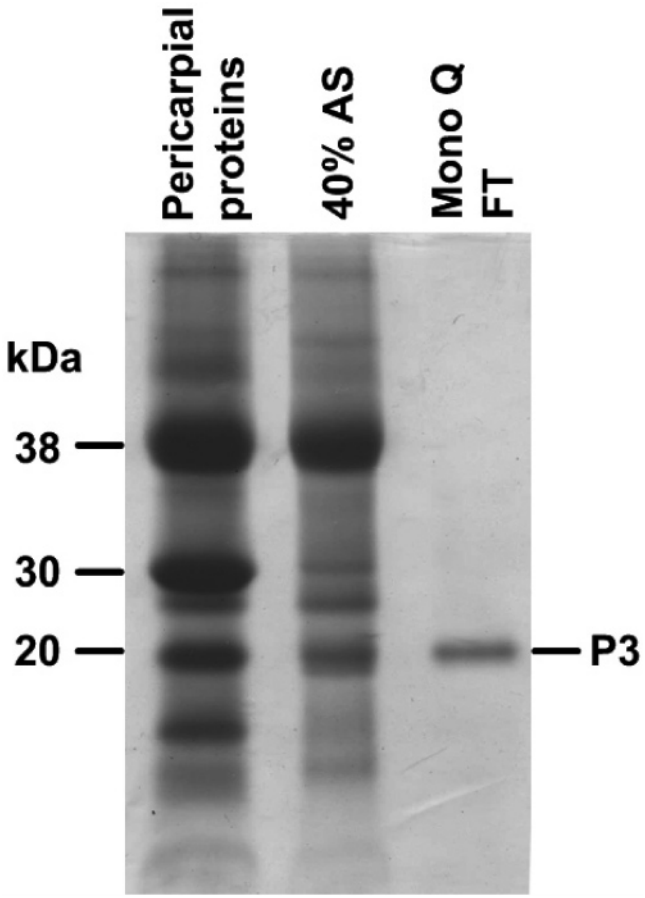

Figure 1. SDS–PAGE analysis of protein contents after different steps of purification for the preparation of the third abundant protein (P3) in the pericarp of jelly fig achenes. To compare the relative purity and recovery yield of P3 after different steps of purification, the loaded protein amount of each sample was adjusted to represent the content extracted from an equal quantity of pericarpial proteins (~80 μg) from jelly fig achenes. Labels on the left indicate the molecular masses of pectin methylesterase (38 kDa), chitinase (30 kDa), and P3 (20 kDa).

Preparation of Antibodies. Recombinant jelly fig TLPs, rTLP-1 and rTLP-2, in *E. coli* cells were resolved in SDS−PAGE gels, eluted according to a modified gel extraction procedure (*24*), emulsified with an equal volume of complete Freund's adjuvant (Life Technologies), and injected separately into chicken to raise polyclonal antibodies. Multiple subcutaneous injections into chicken were performed at intervals of 7 days using incomplete Freund's adjuvant (Life Technologies) in subsequent boosters, and titers of antibodies were also monitored. The eggs were collected 28 days after immunization. Immunoglobulins were purified from egg yolks according to the method developed by Polson (*25*).

Western Immunoblot Analysis. For Western immunoblot analysis, proteins separated by SDS−PAGE were transferred to an Immobilon polyvinylidene difluoride (PVDF) membrane (Millipore) in a Bio-Rad Trans-Blot system according to the instructions of the manufacturer. The PVDF membrane was incubated with 5% (w/v) nonfat dry milk in TBS (50 mM Tris-HCl and 100 mM NaCl at pH 7.9) for 1 h to fill in the unoccupied sites and treated with an appropriate dilution of the polyclonal antibodies in TBS at 4 °C overnight. The membrane was then washed 3 times with TBS plus 0.1% Tween 20 (TBST) for 5 min each time and incubated with alkaline phosphatase-conjugated goat anti-chicken IgG (Jackson ImmunoResearch) in TBS for 2 h. After three washings with TBST and three washings with TBS, the immunoblotting membrane was visualized with 5-bromo-4-chloro-3-indolylphosphate−nitroblue tetrazolium (KPL).

Identification of TLP Isoforms by Mass Analyses. Bands of putative jelly fig TLP isoforms excised from SDS−PAGE gels were

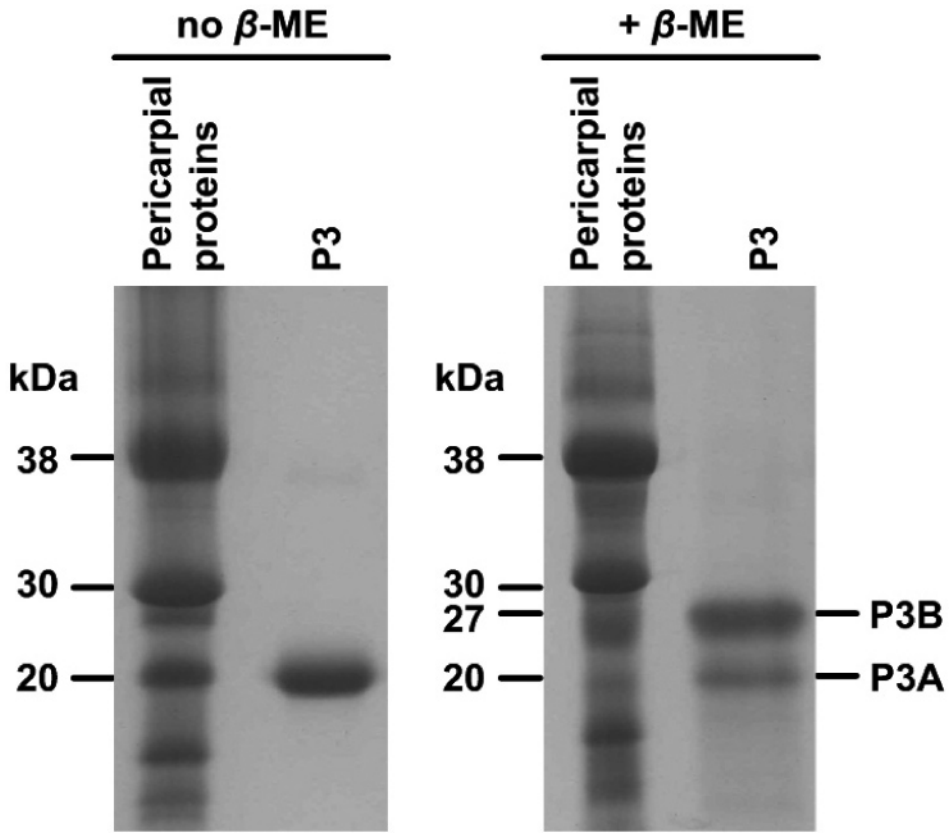

Figure 2. SDS–PAGE of P3 from the pericarp of jelly fig achenes in the absence and presence of β-ME. Total pericarpial proteins (~80 μg) and purified P3 incorporated with the sample buffer with or without β-ME were resolved in SDS–PAGE. Labels on the left indicate the molecular masses of pectin methylesterase (38 kDa), chitinase (30 kDa), P3 (20 kDa), P3A (20 kDa), and P3B (27 kDa).

subjected to reduction and alkylation at 56 °C for 45 min in 10 mM dithiothreitol and 55 mM iodoacetamide in 25 mM ammonium bicarbonate. After dehydration in acetonitrile, the protein bands were dried and digested with 0.1 μg of TPCK-treated modified porcine trypsin (Promega) overnight at 37 °C. The resulting tryptic peptides were subjected to matrix-assisted laser desorption/ionization mass spectrometry (MALDI−MS) and MALDI−tandem mass spectrometry (MALDI−MS/MS) analyses. All data were acquired by quadrupole time-of-flight (Q-TOF) hybrid mass spectrometers (Micromass Q-Tof Ultima, Manchester, U.K., and Applied Biosystems QSTAR, Foster City, CA). The matrix used was α-cyano-4-hydroxycinnamic acid. The low-energy collision-induced dissociation MS/MS product ion spectra acquired from Q-Tof Ultima and QSTAR were analyzed using Micromass ProteinLynx Global Server 2.0 and Applied Biosystems BioAnalyst data processing software, respectively. MS/MS data were also subjected to search algorithms against a Swiss-Prot protein sequence database using MASCOT software (Matrix Science Ltd., London, U.K.).

RESULTS

Purification of P3 Protein in the Pericarp of Jelly Fig Achenes. To purify P3, the third abundant protein in the pericarp of jelly fig achenes, total pericarpial proteins were extracted from jelly curd made of jelly fig achenes. After the P3 protein was precipitated by 40% saturation of ammonium sulfate and flowing through Mono Q chromatography, it was purified with a recovery yield of approximately 40% as revealed in SDS−PAGE (**Figure 1**). The purified P3 migrated as one polypeptide of 20 kDa in the absence of β-ME but split into a minor polypeptide of 20 kDa (P3A) and a major polypeptide of 27 kDa (P3B) in the presence of this reducing agent in SDS−PAGE analyses (**Figure 2**). Mass spectrometric (MALDI−MS) analyses showed that some tryptic fragments of P3A and P3B matched those of thaumatin-like (osmotin-like) proteins from *Solanum tuberosum*, *Actinidia deliciosa*, *Gossypium hirsutum*, and *Fagus sylvatica* (data not shown), suggesting that P3A and P3B are putative TLPs.

Cloning of Two cDNA Fragments Encoding TLP Isoforms in Jelly Fig Achenes. Two full-length cDNA fragments, *TLP-1* and *TLP-2* (accession numbers DQ277011 and DQ277012), were obtained from the cDNA library of jelly fig achenes by PCR cloning. Sequences of *TLP-1* and *TLP-2* contained 992 and 926 nucleotides with open-reading frames encoding 244 and 228 amino acid residues, respectively. Sequence analyses showed that both deduced TLP-1 and TLP-2 precursor polypeptides contained a cleavable N-terminal signal sequence and 16 conserved cysteine residues putatively responsible for the formation of 8 disulfide bonds (**Figure 3**). TLP-1 and TLP-2 are highly homologous isoforms sharing 66% sequence identity, with the main distinction of an extra C-terminal extension of 17 amino acids in TLP-1.

Overexpression and Immunological Cross-Recognition of TLP-1 and TLP-2. Recombinant TLP-1 (rTLP-1) and TLP-2 (rTLP-2) with a C-terminal fusion of the His tag were overexpressed in *E. coli* after IPTG induction (**Figure 4**). The molecular masses of rTLP-1 and rTLP-2 fusion polypeptides were 25.3 and 23.4 kDa theoretically calculated from their deduced sequences but were 28 and 29 kDa estimated on the reducing SDS−PAGE gel. rTLP-1 and rTLP-2 eluted from SDS−PAGE gels were used as antigens to raise antibodies, namely, anti-rTLP-1 and anti-rTLP-2, respectively. Immunodetection indicated that both anti-rTLP-1 and anti-rTLP-2 not only recognized their own original antigens but also adequately cross-recognized the other recombinant TLP (**Figure 5**). In terms

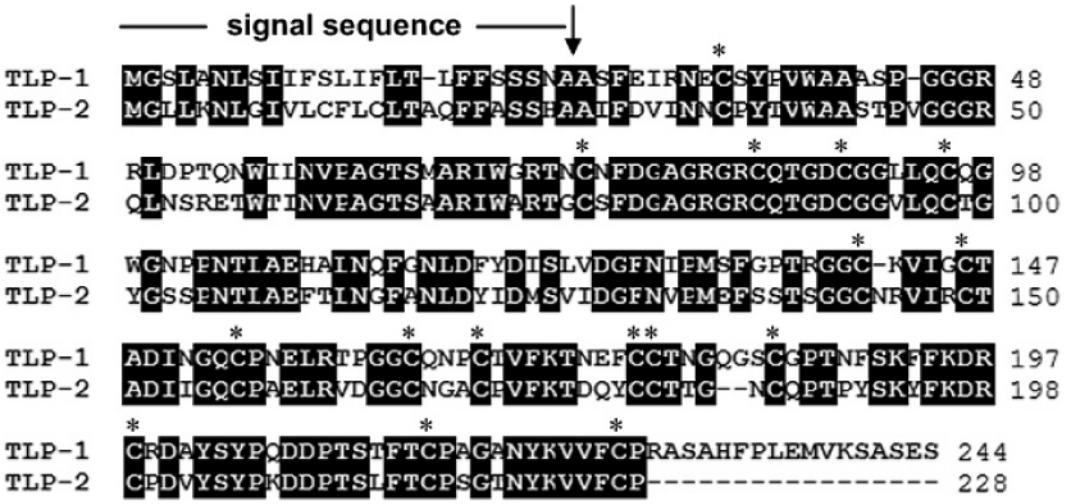

Figure 3. Sequence alignment of the deduced TLP-1 and TLP-2 sequences. The amino acid number for the last residue in each line is listed on the right. Broken lines in the sequences represent gaps introduced for best alignment. Identical residues in these two sequences are highlighted. The cleavage site of the N-terminal signal sequence is indicated by an arrow. A total of 16 conserved cysteine residues presumably forming 8 disulfide bonds are indicated by asterisks. The accession numbers of the aligned sequences are ABB86297 and ABB86298 for TLP-1 and TLP-2, respectively.

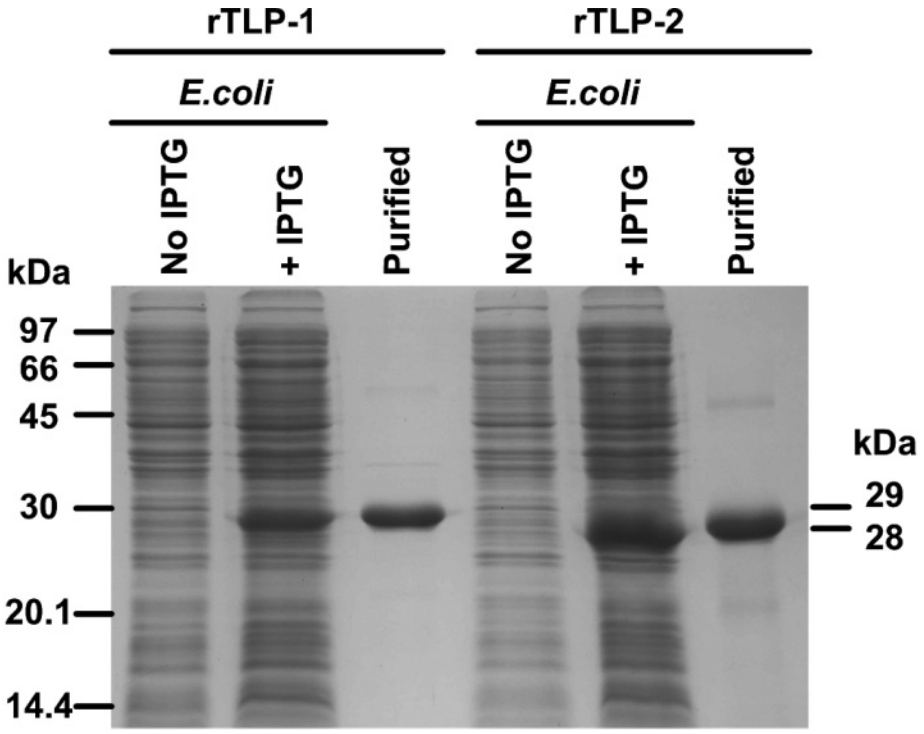

Figure 4. SDS–PAGE of the overexpressed rTLP-1 and rTLP-2 in *E. coli*. Along with gel-purified rTLP-1 or rTLP-2, total cellular proteins (~50 μg) of *E. coli* cells harvesting *TLP-1* or *TLP-2* before and after IPTG induction were resolved in SDS–PAGE. The molecular masses of commercial marker proteins are indicated on the left. Labels on the right indicate the molecular masses of rTLP-1 (29 kDa) and rTLP-2 (28 kDa).

of relative antibody binding, the cross-recognition of anti-rTLP-1 to rTLP-2 was stronger than that of anti-rTLP-2 to rTLP-1.

Immunodetection of P3A and P3B from Jelly Fig Achenes. To examine if P3A and P3B in the pericarp of jelly fig achenes were TLP isoforms, purified P3A and P3B were resolved on SDS−PAGE in the presence of β-ME and then subjected to immunoblotting by anti-rTLP-1 and anti-rTLP-2, respectively (**Figure 6**). The results showed that anti-rTLP-1 clearly recognized both P3A and P3B at comparable efficiencies, while anti-rTLP-2 adequately recognized P3B but only scarcely recognized P3A. Thus, P3A and P3B could be taken as putative TLP isoforms.

Identification of P3A and P3B as TLP-1 and TLP-2 by MALDI−MS/MS. For further identification, protein bands of P3A and P3B cut from SDS−PAGE gels were in-gel-digested with trypsin and then subjected to MALDI−MS and MALDI−MS/MS analyses. A MASCOT homology search revealed that four tryptic peptides of P3A or P3B matched those of the deduced TLP-1 or TLP-2, and the sequences of these tryptic peptides were further confirmed by MALDI−MS/MS analyses (**Table 1**). These results clearly indicate that P3A and P3B were encoded by *TLP-1* and *TLP-2* clones, respectively.

DISCUSSION

Several pericarpial proteins have been proposed to play important roles in diverse physiological functions including fruit

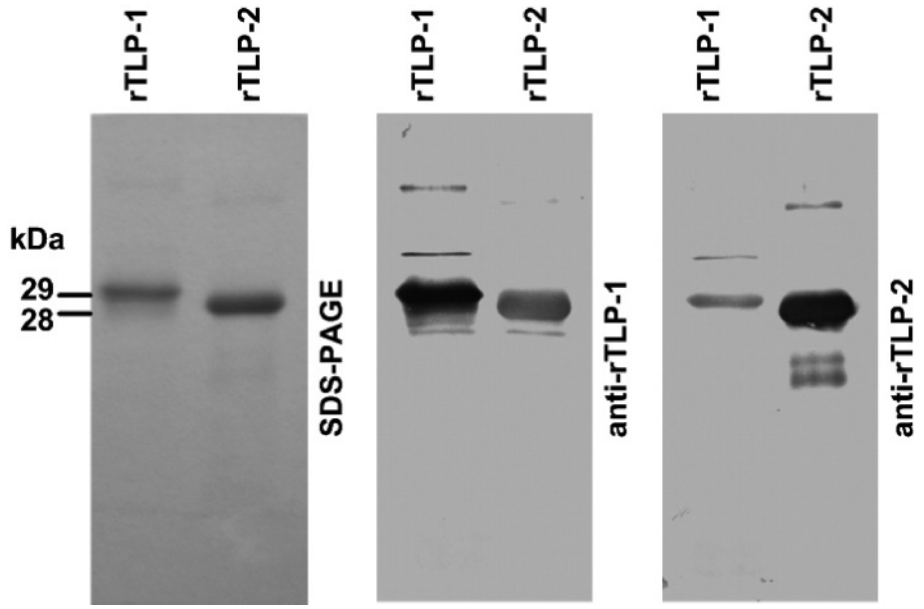

Figure 5. SDS–PAGE and Western blotting of rTLP-1 and rTLP-2. Gel-purified rTLP-1 and rTLP-2 were resolved in SDS–PAGE. Two replicate gels were transferred onto the PVDF membrane and then subjected to immunodetection using anti-rTLP-1 and anti-rTLP-2, respectively. Labels on the left indicate the molecular masses of rTLP-1 (29 kDa) and rTLP-2 (28 kDa).

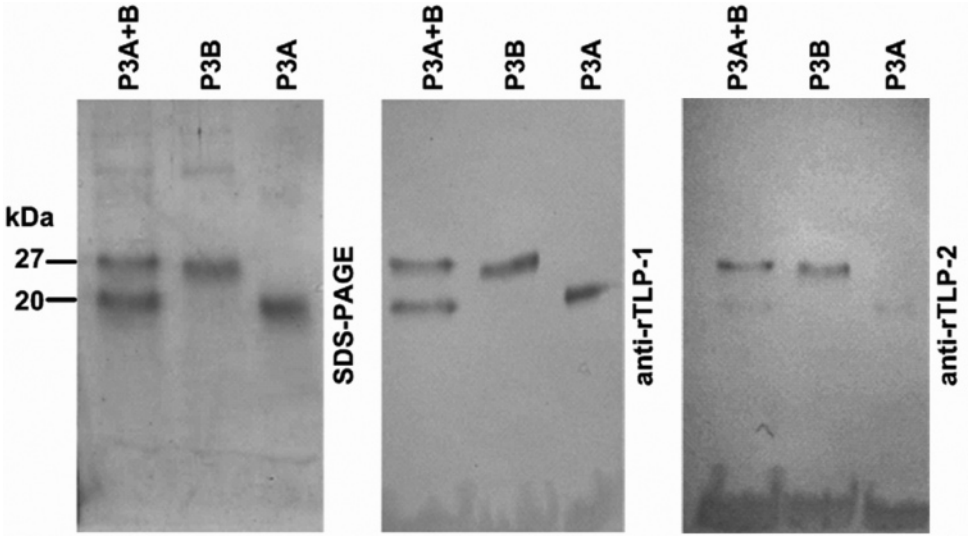

Figure 6. SDS–PAGE and Western blotting of P3A and P3B from jelly fig achenes. Gel-purified P3A and P3B were resolved in SDS–PAGE. Two replicate gels were transferred onto the PVDF membrane and then subjected to immunodetection using anti-rTLP-1 and anti-rTLP-2, respectively. Labels on the left indicate the molecular masses of P3A (20 kDa) and P3B (27 kDa).

Table 1. Fragments of Thaumatin-like Protein Isoforms Identified by MALDI–MS and MALDI–MS/MS Analyses

isoform	residue	M_r (Da)	sequence
	32–48	1779.1007	NECSYPVWAAASPGGGR
P3A	143–159	1916.1013	VIGCTADINGQCPNELR
(TLP-1)	160–172	1465.4534	TPGGCQNPCTVFK
	173–192	2266.5050	TNEFCCTNGQGSCGPTNFSK
	56–70	1572.6723	ETWTINVPAGTSAAR
P3B	149–162	1603.1913	CTADIIGQCPAELR
(TLP-2)	199–223	2912.2976	CPDVYSYPKDDPTSLFTCPSGTNYK
	207–223	1802.2599	DDPTSLFTCPSGTNYK

development and ripening, photosynthesis, starch synthesis, metabolism, and cell-wall construction and degradation (*26*−*31*). Similarly, a thermostable chitinase purified from the pericarp of jelly fig achenes has been demonstrated to protect seeds against fungal invasion (*5*). In the current study, another class of antifungal proteins, TLPs, was also identified in the pericarp of jelly fig achenes. The coexistence of chitinase and the putatively antifungal TLPs in the pericarp may synergistically enhance their protection of jelly fig achenes against fungal pathogens.

The 8 disulfide bonds formed by the 16 cysteine residues of TLPs are essential for the overall folding and antifungal activity of these proteins as well as their resistance to protease degradation and denaturation in harsh environments, such as extreme pH conditions and heat (*32*). The anomalous migration of TLPs in SDS−PAGE has also been attributed to their unusually high number of disulfide bonds (*33*, *34*). The overestimated molecular weight of TLPs in the presence of β-ME presumably resulted from a conversion of the native compact structure to a loose and enlarged structure of irregular

shape because of the breakdown of disulfide bonds. In this study, we identified two TLP isoforms in jelly fig achenes and confirmed that they were encoded by two independent cDNA fragments rather than resulting from artifacts in SDS−PAGE by using two methodologies, immunological cross-recognition with antibodies against the two recombinant TLPs and MALDI−MS/MS. Interestingly, these two highly homologous TLPs migrated concurrently in the absence of β-ME but separately in the presence of this reducing agent in SDS−PAGE, although both proteins putatively possessed a equivalent number of disulfide bonds (**Figure 2**). The main distinction of these two deduced TLPs is the extra C-terminal extension of 17 amino acids in TLP-1 (**Figure 3**), and it is uncertain if the different migration patterns of TLP-1 and TLP-2 were related to this C-terminal extension. A similar C-terminal extension has also been observed and demonstrated to serve as a vacuolar-sorting motif in some TLPs (*35*, *36*). It remains to be studied whether the C-terminal extension in TLP-1 serves as a vacuolar-sorting motif that leads to different cellular locations of TLP-1 and TLP-2 in the pericarpial cells of jelly fig achenes.

More than half of the jelly fig achenes in the markets are harvested from the wild in mountain areas in Taiwan. When jelly fig fruits ripen and dehisce in the summer time, the achenes are exposed to abiotic stresses and pathogen infection in the hot and humid environment. In a viewpoint of evolution, the pericarpial enzymes and polypeptides that presumably protect jelly fig achenes against the adverse environments (abiotic stresses and pathogen infection) after fruit ripening should be thermally stable and thus may be used as natural preservatives to prolong the storage period of diverse food products. Thus far, the pectin methylesterase and chitinase identified in the pericarp of jelly fig achenes have been demonstrated thermostability. Presumably, the two TLP isoforms identified in this study are also as thermostable as those found in other species (*37*). It remains to be seen if other thermostable antifungal or antibacterial enzymes and peptides are present in the pericarp of jelly fig achenes.

ABBREVIATIONS USED

IPTG, isopropyl-β-D-thiogalactoside; MALDI−MS, matrix-assisted laser desorption/ionization mass spectrometry; MS/MS, tandem mass spectrometry; PVDF, polyvinylidene difluoride; Q TOF, quadrupole time-of-flight; rTLP, recombinant thaumatin-like protein; TLP, thaumatin-like protein.

ACKNOWLEDGMENT

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