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Cloning and immunolocalization of an antifungal chitinase in jelly fig (*Ficus awkeotsang*) achenes

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Abstract

A 30-kDa protein extracted from the pericarpial portion of jelly fig (*Ficus awkeotsang* Makino) achenes has been identified as a thermostable chitinase based on its enzymatic activity. A cDNA fragment encoding the precursor protein (including a cleavable signal sequence) of this chitinase was obtained by PCR cloning, and subsequently confirmed by immunological recognition of its over-expressed protein in *Escherichia coli*. Homology modeling predicted that this thermostable chitinase in jelly fig achenes comprised a stable $(\beta\alpha)_8$ barrel fold with three pairs of disulfide linkage. Immunostaining indicated that this chitinase was exclusively localized in the pericarpial region but not in the seed cells where bulky protein bodies and massive oil bodies were accumulated. Spore germination of *Colletotrichum gloeosporioides*, a common post-harvest pathogen infecting ripening fruit of jelly fig and many other fruits, was inhibited by this chitinase purified from achenes. It is suggested that the biological function of the thermostable chitinase in the pericarp of jelly fig achenes is to protect the nutritive seeds from fungal attack during fruit ripening.

Keywords: Jelly fig (*Ficus awkeotsang*): Moraceae; Achene; Chitinase; *Colletotrichum gloeosporioides*; Pericarp

1. Introduction

Jelly fig (*Ficus awkeotsang* Makino) is a woody vine growing in the mountain areas of Taiwan, with the aqueous extract of jelly fig achenes used for preparation of jelly curd to make a popular summer drink in local markets (Huang et al., 1980). The gelation forms a three-dimensional structure constructed via ionic interactions between calcium ions and the carboxyl groups of pectin after de-methoxylation by pectin methylesterase (Lin et al., 1989). The major pericarpial protein extracted from jelly curd has been identified as a pectin methylesterase which is responsible for the gelation, and its corresponding cDNA clone has been sequenced (Ding et al., 2000). The second most abundant protein of 30 kDa has recently been purified and detected as an antifungal chitinase based on its enzymatic activity (Li et al., 2003), yet its corresponding gene has not been reported. So far, the biological functions of the proteins in the pericarp of jelly fig achenes have never been investigated.

Chitinases (E.C.3.2.1.14) catalyze the hydrolysis of chitin, a major structural component found in insect exoskeletons, crustacean shells, and fungal cell walls. They are present in all plants analyzed to date and many have been shown to inhibit fungal growth either in vitro (Schlumbaum et al., 1986; Leah et al., 1991) or when

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expressed in transgenic plants (Broglie et al., 1991; Jach et al., 1995). It is assumed that production of chitinases by plants is a part of defense mechanisms against fungal pathogens. Owing to their chitinolytic activities, chitinases have received substantial attention with regard to development of biocontrol agents and crop protection by genetic engineering (Rohini and Sankara Rao, 2001). Plants produce constitutively or inducibly a large number of chitinase isozymes that can be categorized according to their sequences into six classes (Patil et al., 2000). Among these chitinase isozymes, class III possesses a unique thermal stability (up to 60 °C) and shows no structural and sequence similarity with the other five classes (Hamel et al., 1997). Class III chitinase, also classified as a member of the glycosyl hydrolase family 18, forms a stable $(\beta\alpha)_8$ barrel fold termed the TIM barrel with three conservative intramolecular disulfide bonds (Terwisscha van Scheltinga et al., 1996).

In this study, we cloned a cDNA sequence encoding the 30-kDa jelly fig chitinase from maturing achenes by PCR. Immunolocalization of this chitinase in mature achenes was observed under a light microscope. Biological function of this chitinase in jelly fig achenes is proposed in view of the observation of its inhibition to *Colletotrichum gloeosporioides*, a fungus isolated from the jelly fig fruit.

2. Results

2.1. Cloning of a cDNA fragment encoding the 30-kDa chitinase in jelly fig achenes

To clone the corresponding gene of the 30-kDa chitinase in jelly fig achenes, the first 11 amino acid residues (AGGIAIYWGQN, counting from the N-terminus) of the purified 30-kDa chitinase determined by direct sequencing was used to design a degenerate primer. A full-length cDNA fragment (Accession No. AF497747) encoding the precursor protein of this chitinase was obtained by PCR cloning. The cDNA fragment comprises 977 nucleotides, consisting of a 3-nucleotide 5′ untranslated region, an open reading frame of 879 nucleotides, and a 95-nucleotide 3′ untranslated region. Aligned with two homologous chitinases from other species, the deduced amino acid sequence of the clone consists of a cleavable N-terminal signal sequence of 18 amino acid residues and a putative chitinase whose first N-terminal 11 residues are identical to the N-terminus of the 30-kDa chitinase purified from jelly fig achenes (Fig. 1). The deduced sequence of the putative chitinase comprises 274 amino acid residues in its mature protein with a molecular mass 29.6 kDa.

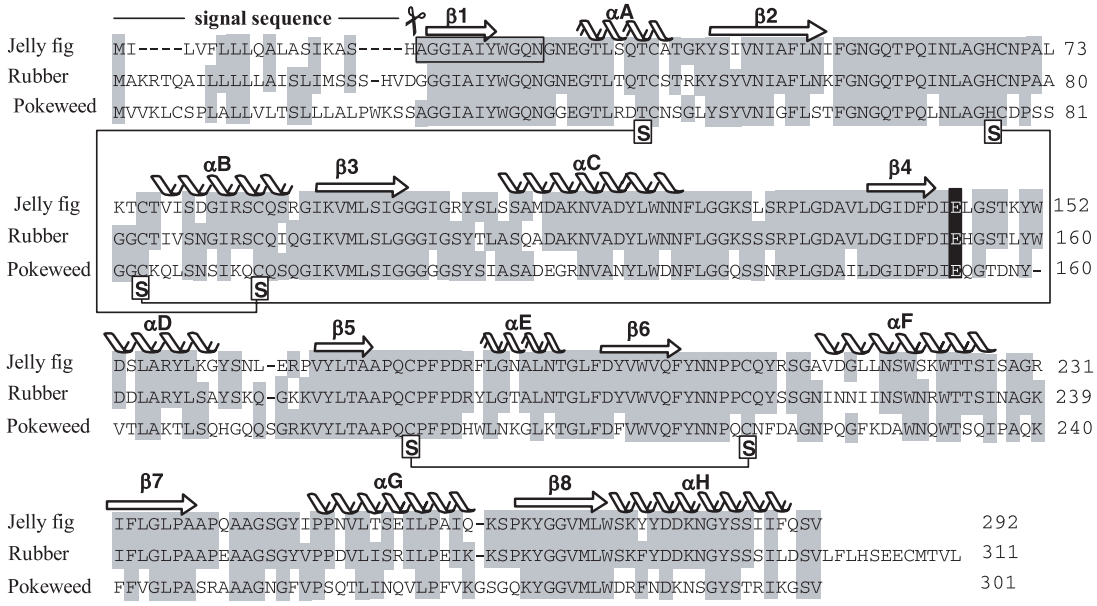

Fig. 1. Sequence alignment of the precursors of jelly fig, rubber tree, and pokeweed chitinases. The amino acid number for the last residue in each line is listed on the right for each species. Broken lines in the sequences represent gaps introduced for best alignment. The cleavage site of the N-terminal signal sequence is indicated by a scissors symbol. The N-terminal sequence obtained directly from amino acid sequencing is boxed. Six conserved cysteine residues are assumed to form intramolecular disulfide bonds. The catalytic glutamic acid residue responsible for chitinase activity is highlighted. Predicted secondary structures are indicated on tops of the sequences (see Fig. 3 for details). The locations of α-helices and β-strands in the predicted structure are indicated and labeled successively. The accession numbers of the aligned sequences are CAA07608 and BAB92957 for rubber tree and pokeweed, respectively.

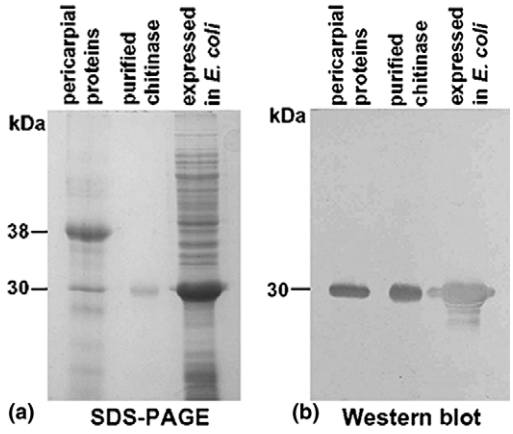

Fig. 2. SDS–PAGE and western blot of the purified 30-kDa chitinase from jelly fig achenes and the overexpressed chitinase in *E. coli*. (a) Along with the total pericarpial proteins of jelly fig achenes, the purified 30-kDa chitinase and the overexpressed chitinase in *E. coli* cell lysate, were resolved in SDS–PAGE. (b) A duplicate gel was transferred onto nitrocellulose membrane and then subjected to immunoblotting detected by antibodies against the 30-kDa chitinase. Labels on the left indicate the molecular masses of the 38-kDa pectin methylesterase and the 30-kDa chitinase.

2.2. Immunological recognition of overexpressed chitinase in E. coli

The cDNA fragment encoding the predicted mature chitinase (residues 19–292) was constructed in a non-fusion vector and then overexpressed in *E. coli*. As revealed by SDS–PAGE, the overexpressed polypeptide in *E. coli* lysate and the 30-kDa chitinase in jelly fig achenes migrated to the same position in the gel (Fig. 2(a)). In a western blot analysis, the overexpressed polypeptide in *E. coli* lysate was recognized by the antibodies raised against the 30-kDa chitinase purified from jelly fig achenes (Fig. 2(b)). These results confirm that the current clone encodes the precursor protein of the 30-kDa chitinase in jelly fig achenes, and suggest that no apparent post-translational modification, such as glycosylation, exists in its mature polypeptide.

2.3. Homology modeling of the 3D structure of the 30-kDa jelly fig chitinase

Sequence comparison reveals that the jelly fig chitinase is highly homologous to a rubber tree chitinase (M_r 29.5 kDa) whose three-dimensional structure has been determined by X-ray crystallography as a stable $(\beta\alpha)_8$ barrel fold with three pairs of disulfide linkage (Terwisscha van Scheltinga et al., 1996). Three-dimensional structure of the jelly fig chitinase was simulated by homology modeling using the crystal structure of the rubber tree chitinase as a template. Accordingly, eight alternating β-strands and α-helices were predicted in the jelly fig chitinase with six conserved cysteine residues presumably formed three pairs of disulfide bonds

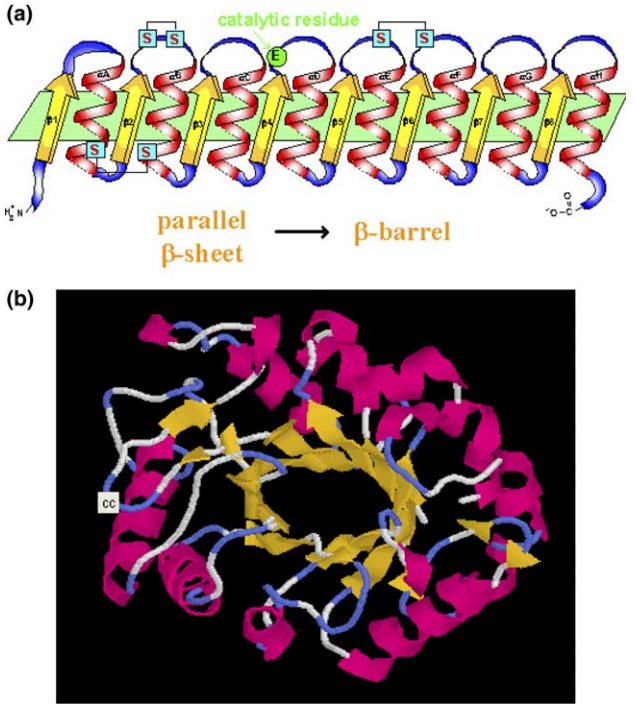

Fig. 3. Predicted structure of the jelly fig chitinase. (a) A secondary structural model of the 30-kDa jelly fig chitinase was proposed with eight alternate β-strands (yellow) and α-helices (red). Six conserved cysteine residues presumably forming three disulfide bonds are boxed (light blue). The catalytic glutamic acid residue responsible for chitinase activity is circled (green). (b) The 3D structure of the 30-kDa jelly fig chitinase was predicted using homology modeling (Lund et al., 1997).

(Fig. 3(a)). In addition, a catalytic glutamic acid residue responsible for chitinase activity was identified in the loop sequence connected to the 4th β-strand. Simulated 3D structure of the jelly fig chitinase comprises a stable barrel of eight parallel β-strands shielded by eight α-helices (Fig. 3(b)). The predicted active site is located in the loop regions connecting the carboxyl ends of the β strands and amino ends of α helices on top of the simulated 3D structure shown in the figure.

2.4. Immunolocalization of the 30-kDa chitinase in jelly fig achenes

For commercial purpose, achenes are harvested from jelly fig fruit of approximately 80% maturation, dried either under the sunlight or in an oven, and then detached from the fruit for making jelly curd (Fig. 4(a)–(c)). Inside the pericarp of a jelly fig achene, the seed cells with a thin layer of seed coat are filled with bulky protein bodies and massive oil bodies (Fig. 4(d), (e) and Fig. 5). No starch granule was observed in the seed cells. Immunostaining (blue color) showed that the 30-kDa chitinase was absent from the seed cells and exclusively localized in the pericarpial portions of jelly fig achenes (Fig. 6).

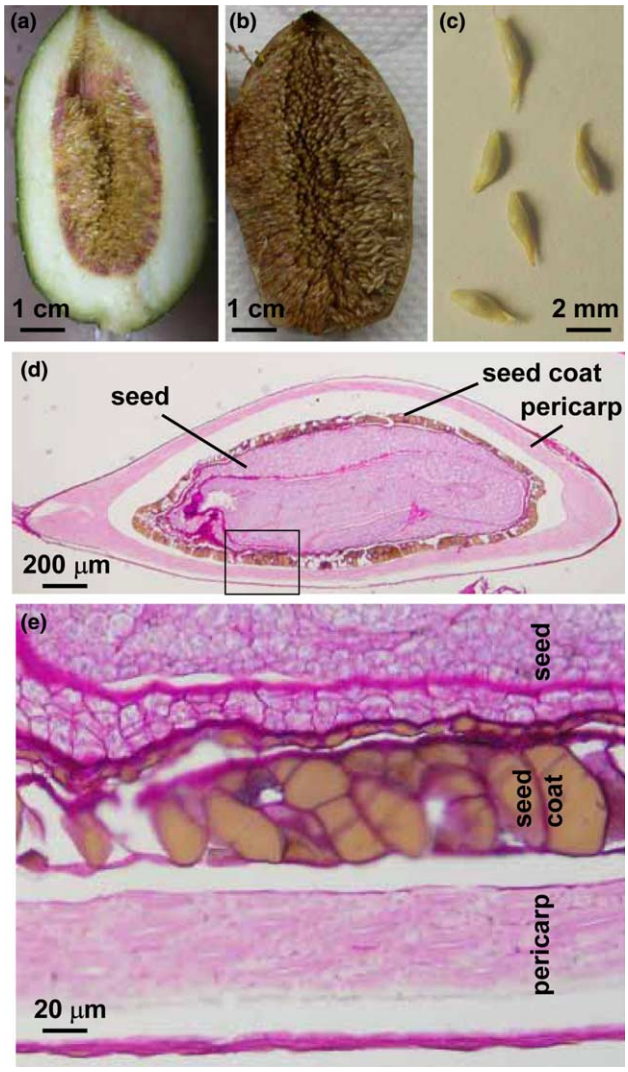

Fig. 4. Pictures of jelly fig fruit and achenes. Fresh fruit (a), dried fruit (b), and detached achenes (c) of jelly fig are photographed under the sunlight. Cellular structure of achenes is observed under a light microscope at different amplification (d and e).

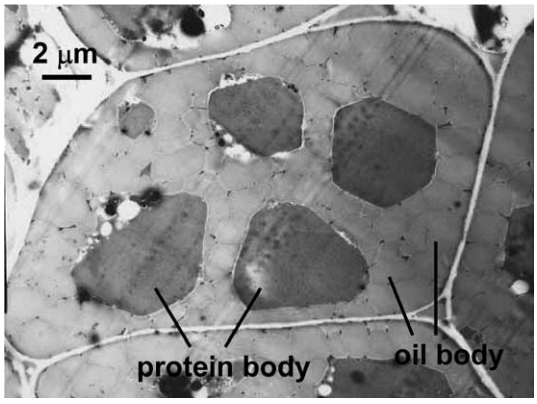

Fig. 5. Electron microscopy of mature jelly fig seed cells. Mature jelly fig achenes were fixed, and protein bodies and oil bodies in the seed cells of achenes were observed under an electron microscope.

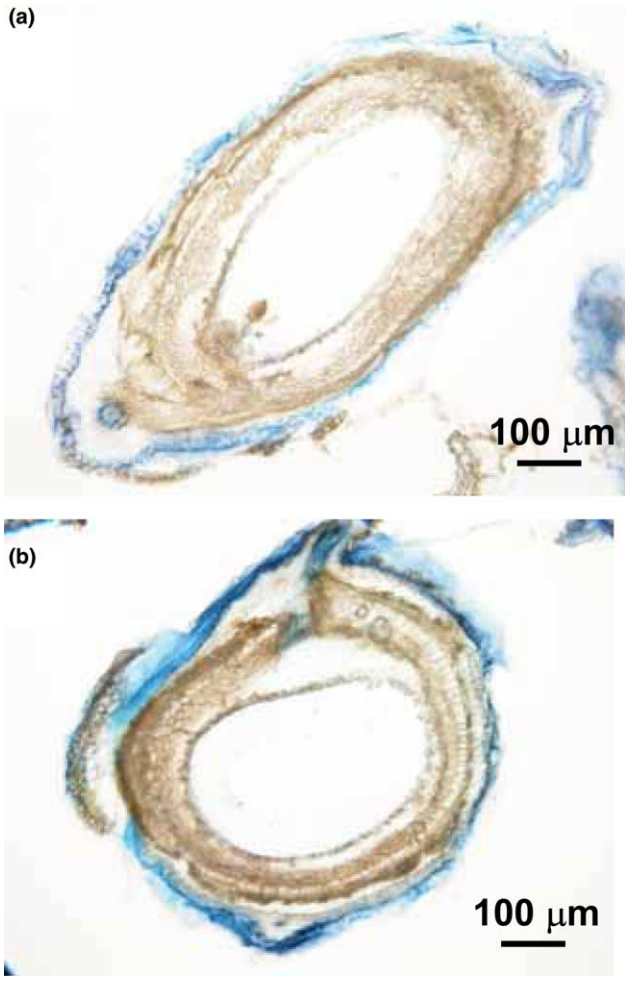

Fig. 6. Immunolocalization of the 30-kDa chitinase in jelly fig achenes. Longitudinal (a) and cross-sections (b) of mature jelly fig achenes were incubated with primary antibodies against the 30-kDa chitinase and then secondary antibodies conjugated with alkaline phosphatase. The localization of the 30-kDa chitinase was visualized (blue color staining) using BCIP as substrate.

2.5. Antifungal activity of the 30-kDa chitinase on a fungus isolated from jelly fig fruit

Colletotrichum gloeosporioides, an important post-harvest pathogen found in many fruits in Taiwan, was isolated from infected ripening fruit of jelly fig (Fig. 7(a) and (b)). Spore germination of this isolated fungus was significantly inhibited by the pericarpial chitinase purified from jelly fig achenes (Fig. 7(c)), and the inhibited spores remained inactive even after the removal of the chitinase. These fungal spores were presumably dead, based on the LIVE/DEAD two-color fluorescence staining of fungal viability (data not shown).

3. Discussion

In this study, a full-length cDNA fragment encoding the precursor protein of the thermostable chitinase in

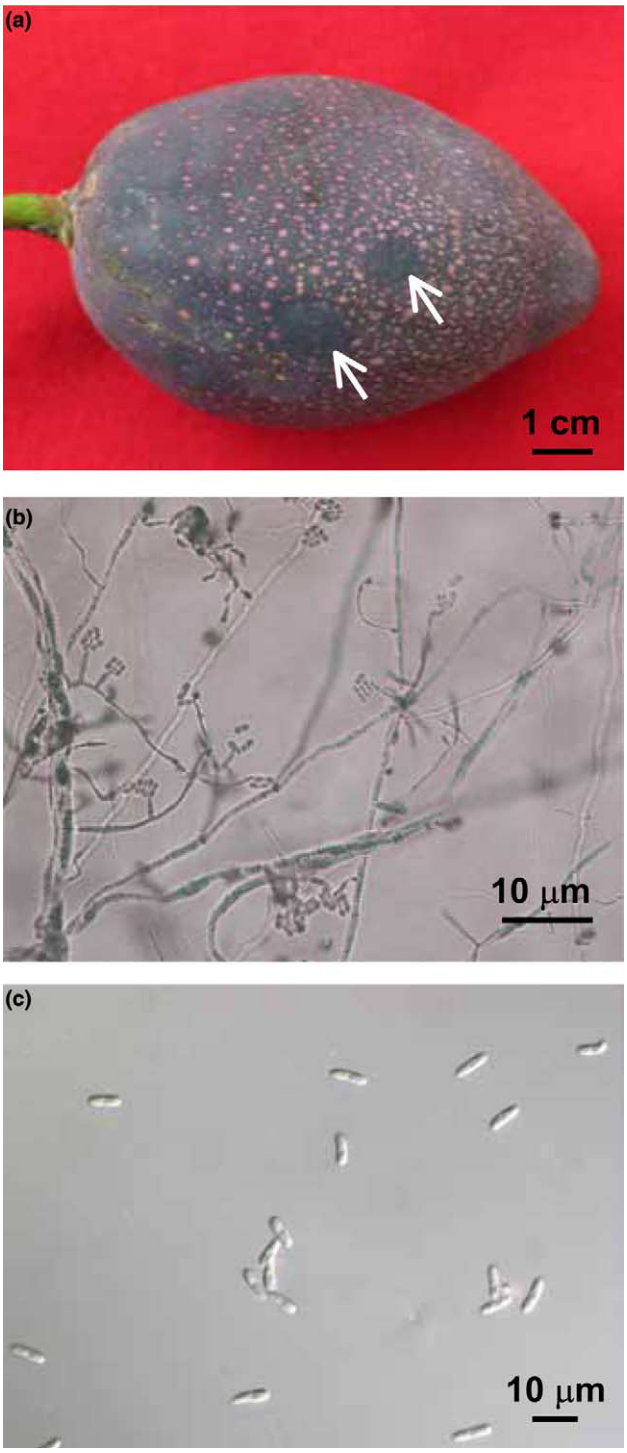

Fig. 7. Fungal infection of jelly fig fruit and antifungal activity of the 30-kDa chitinase. (a) Fungal infection was observed on a ripening fruit of jelly fig as pointed by arrows. (b) Spore germination of the fungus, *C. gloeosporioides* isolated from the infected jelly fig fruit. (c) Inhibition of spore germination of *C. gloeosporioides* isolated from the infected jelly fig fruit by the 30-kDa chitinase purified from jelly fig achenes.

jelly fig achenes was cloned and sequenced. Homology modeling of the deduced polypeptide predicted that the three-dimensional structure of this chitinase comprised a stable $(\beta\alpha)_8$ barrel structure accounting for its thermal stability. In addition, six conservative cysteine residues were found among homologous chitinases and proposed to form three specific pairs of disulfide linkage based on the crystal structure of a rubber tree chitinase (Terwisscha van Scheltinga et al., 1996; Bokma et al., 2002). The proposed formation of disulfide linkage was in agreement with the observation that the 30-kDa jelly fig chitinase could only be renatured in an SDS–PAGE gel when β-mercaptoethanol was removed from the sample buffer (Li et al., 2003).

Fruit pericarp protects seeds against mechanical damage and environmental fluctuations (Ozga et al., 2002). Several pericarpial proteins have been identified and proposed to play important roles in diverse physiological functions including fruit development and ripening, photosynthesis, starch synthesis, metabolism, and cell wall construction and degradation (Hood et al., 1993; Chen et al., 1998; Peters et al., 1998; Banik et al., 2001; Chung et al., 2002; Jones et al., 2002). Recently, a thermostable chitinase was purified from the pericarp of jelly fig achenes and proposed to protect seeds against fungal invasion or insect herbivory (Li et al., 2003). In the current study, we demonstrated that this chitinase was exclusively located in the pericarpial region of jelly fig achenes and capable of inhibiting spore germination of *C. gloeosporioides*, a common fungus causing anthracnose of jelly fig fruit as well as many other fruits in Taiwan. *C. gloeosporioides*, a broad-host-range phytopathogenic fungus, produces high levels of ammonia as a mechanism of tissue alkalinization that may be beneficial for fruit softening; the alkaline pH environment is necessary for the activities of some extracellular lyase enzymes responsible for fruit softening, a process associating with changes in the pectic fraction of middle lamella and cell wall (Payasi and Sanwal, 2003). It is suggested that the biological function of the thermostable chitinase in the pericarp of jelly fig achenes is to protect the nutritive seeds from fungal attack during fruit ripening.

Pectin methylesterase, the most abundant pericarpial protein extracted from jelly curd, has been identified as an N-linked glycoprotein with approximately 10% carbohydrate (Ding et al., 2002). The glycosylation is in agreement with the differences in the carbohydrate staining with an improved periodic acid-Schiff (PAS) reaction and in molecular weight of native pectin methylesterase (38 kDa) and the recombinant one (35 kDa) expressed in *E. coli*. In contrast, both the native chitinase from jelly fig achenes and the recombinant enzyme expressed in *E. coli* could not be stained in the PAS carbohydrate staining reaction (data not shown) and had the same molecular masses as observed in SDS–PAGE (Fig. 2). Evidently, this chitinase is neither glycosylated nor apparently modified in its mature form after the cleavage of its signal sequence for ER targeting. Unfortunately, the recombinant chitinase expressed in *E. coli* was predominately found in

the insoluble pellet after fractionation and lack of enzymatic activity in our experimental condition (data not shown), presumably due to a failure of correct formation of intramolecular disulfide bonds.

Protein bodies and oil bodies are subcellular organelles that serve as amino acid and energy sources for seed germination and seedling growth (Frandsen et al., 2001; Tai et al., 2001). Bulky protein bodies and massive oil bodies were observed in the seed cells of jelly fig achenes (Fig. 5). The presence of these two subcellular organelles in jelly fig seeds were also confirmed by cloning cDNA fragments encoding unique storage proteins found in protein bodies of dicotyledonous seeds and by isolation of oil bodies as well as their unique associated proteins, respectively (data not shown). It seems that the proteins and lipids in the residual jelly fig achenes after jelly curd making may be a nutritive source for generating useful by-products.

4. Experimental

4.1. Preparation of pericarpial proteins in jelly fig achenes

Jelly fig (*F. awkeotsang* Makino) achenes were purchased from local growers. Jelly curd was prepared according to the hand washing method developed by Ding et al. (2002). Pericarpial proteins of jelly fig achenes were extracted from jelly curd, and the insoluble pectin polymers were removed by centrifugation. The extracted pericarpial proteins were collected by ammonium sulfate precipitation at a final concentration of 85% saturation.

4.2. Partial amino acid sequencing of the 30-kDa chitinase in jelly fig achenes

The 30-kDa chitinase extracted from jelly fig achenes was eluted from SDS–PAGE gels and subjected to N-terminal sequencing. The protein was transferred onto a piece of PVDF membrane (Immobilon-P transfer membrane purchased from Millipore), and sequenced from N-terminus by the Applied Biosystems 476A Protein Sequencer in National Chung-Hsing University, Taiwan, as described by Li et al. (2003).

4.3. RNA isolation and cDNA library construction

Total RNA was extracted from maturing achenes of jelly fig approximately 70 days after flowering according to the phenol/SDS method (Wilkins and Smart, 1996). Poly(A)$^+$ RNA was isolated with Dynabeads (Dynal) following the manufacturer's instructions. cDNA was synthesized from poly(A)$^+$ RNA according to the protocol described in the manufacturer's instructions (cDNA synthesis, ZAP-cDNA synthesis, and ZAP-cDNA Gigapack III Gold Cloning kits purchased from Stratagene). A cDNA library of approximately 10^6 plaques was constructed with 10 μg poly(A)$^+$ RNA. The plaques were subjected to in vivo excision of the pBluescript phagemid from the Uni-ZAP XR vector following the manufacturer's instructions.

4.4. Cloning and sequencing of the 30-kDa chitinase

A degenerate primer, 5′-ATHGCNATHTAYTGGGGNCARAAY-3′, was designed according to eight residues in the N-terminal sequence of purified 30-kDa chitinase. PCR amplification was carried out using the designed primer and a poly(T) primer with the excised phagemids as templates. A PCR fragment of approximately 900 bp was harvested, ligated into the pGEM-T Easy Vector systems (Promega), and subjected to sequencing using the Sequence™ Version 2.0 DNA Sequence kit (USB). To obtain a complete cDNA clone encoding the precursor protein (including a signal sequence and the mature enzyme) of 30-kDa chitinase, a primer, 5′-CAAGCTCTATGTCAAAATCTAT-3′, was designed according to the sequence corresponding to the middle region of mature enzyme. Under the same strategy of PCR cloning using the designed primer and a vector primer, a DNA fragment of approximately 550 bp was obtained and sequenced. The complete cDNA clone of 977 bp was linked by PCR and sequenced from both directions.

4.5. Overexpression of the putative 30-kDa chitinase clone in E. coli

The putative cDNA fragment encoding the mature 30-kDa jelly fig chitinase was constructed in the non-fusion expression vector, pET29a(+)(Novagen), using an *Nde*I site and a *Xho*I site in the polylinker of the vectors. The recombinant plasmid was used to transform *E. coli* strain BL21 (DE3). Overexpression was induced by 1 mM IPTG in a bacteriophage T7 RNA polymerase/promoter system. After induction, the *E. coli* cells were harvested, lysed by heating at 95 °C for 10 min with the sample buffer, and then subjected to SDS–PAGE and western blot analyses.

4.6. SDS–PAGE and Western blotting

Proteins were resolved by SDS–PAGE using 12.5% acrylamide. The proteins were mixed with the sample buffer containing 62.5 mM Tris–HCl, pH 6.8, 2% SDS, 0.02% bromophenol blue, 10% glycerol, and 5% β-zmercaptoethanol according to the Bio-Rad instruction manual. Following electrophoresis, the gel was stained with Coomassie blue R-250. Polyclonal antibodies against the 30-kDa chitinase were raised in chickens as described previously (Li et al., 2003). In western blot

analysis, proteins resolved in the SDS–PAGE gel were transferred onto nitrocellulose membrane in a Bio-Rad Trans-Blot system according to the manufacturer's instructions. The membrane was subjected to immunodetection using secondary antibodies conjugated with horse radish peroxidase (Sigma), and then incubated with 4-chloro-1-naphthol containing H_2O_2 for color development.

4.7. Light microscopy and immunolocalization of jelly fig achenes

Dry jelly fig achenes were cut and placed immediately in fixative. The samples were fixed in 100 mM sodium phosphate buffer, pH 7 containing 3% paraformaldehyde and 0.25% glutaraldehyde. After incubation at 4 °C overnight, the specimens were washed in the phosphate buffer for three times. Dehydration was carried out using a series of aq. EtOH washings (50%, 70%, 85%, 95%, 99%, 100%, and 100% EtOH) and a final washing with EtOH for 24 h. After additional treatment with xylene for 2×9 and 1×24 h, and with paraffin for 2×24 h, the samples were embedded in paraffin (Cristensen et al., 1998). Longitudinal and cross-sections of 3–5 μm were made on a *Leica* 2145 microtome. The sections were counterstained with periodic acid Schiff (PAS) reagent.

Fresh jelly fig achenes were cut and mixed with cryomatrix embedding medium. Sections were prepared by *Shandon* cryotome, fixed with MeOH, rinsed with water, blocked with 3% goat serum in phosphate buffered saline (PBS) for 30 min at room temperature, and incubated with primary anti-chitinase antibodies or pre-immune antibodies. After washing with PBS, the samples were incubated with secondary antibodies, anti-chicken IgG conjugated with alkaline phosphatase (Niogret et al., 1996). Immunoreactivity was visualized with BCIP (5-bromo-4-chloro-3-indolyl-phosphate disodium salt) (Boehringer–Mannheim GmbH) as substrate.

4.8. Electron microscopy of jelly fig achenes

Jelly fig achenes were fixed in 2.5% glutaraldehyde in 100 mM sodium phosphate buffer, pH 7, at 4 °C overnight. After several rinses with the buffer, it was post-fixed in 1% OsO_4 in the buffer for 4 h. After dehydration in a graded ethanol series, the sample was embedded in LR white resin (London Resin Co.) as described by Peng and Tzen (1998). Sections of 80 nm were stained with uranyl acetate and lead citrate, and observed in a Hitachi H-300 electron microscope.

4.9. Inhibition of the 30-kDa chitinase on fungal spore germination

A fungus isolated from jelly fig fruit was identified as *C. gloeosporioides*, an important post-harvest pathogen of many fruits in Taiwan. This fungus was cultured in potato dextrose agar (DIFCO) for 2 weeks. After sporulation, the spores of the pathogen were suspended in 0.1% (w/v) glucose to a concentration of 10^4 spores/mL. The fungal spore stock (approximately 500 spores) was cultured in the glucose solution with or without 2 μg of the purified 30-kDa chitinase at 24 °C for 24 h. Inhibition of spore germination was examined under a light microscope at 100× amplification. Viability of fungal spores was detected by a two-color fluorescence staining using the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Molecular Probes) as described by Chen and Seguin-Swartz (2002).

Acknowledgments

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