

STUDIES OF PATHOGENESIS-RELATED PROTEINS IN THE STRAWBERRY  
PLANT: PARTIAL PURIFICATION OF A CHITINASE-CONTAINING PROTEIN  
COMPLEX AND ANALYSIS OF AN OSMOTIN-LIKE PROTEIN GENE

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## ABSTRACT

Plant chitinases and osmotin-like proteins (OLPs) are both pathogenesis-related (PR) proteins, which are implicated in plant responses to pathogen attacks and environmental stresses. In this dissertation, a chitinase-containing protein complex was purified to near homogeneity from strawberry leaf extracts. This protein complex contained at least five different chitinase molecules as revealed by activity gel assays. A previous study showed that winter rye leaves contain seven protein complexes, which consist of various combinations of a chitinase, two glucanase-like proteins (GLPs) and a thaumatin-like protein (TLP). Western blot analysis of the strawberry chitinase complex, however, did not detect the presence of any GLP or TLP in the complex.

The second part of this dissertation research dealt with studies of strawberry OLP genes. A genomic clone containing an OLP gene, designated *FaOLP2*, was isolated and completely sequenced. *FaOLP2* contains no intron, and has a potential to encode a precursor protein of 229 amino acid residues with a 27-amino acid signal peptide at the N-terminus. Southern blot analysis showed that *FaOLP2* represents a small multi-gene family. The expression of *FaOLP2* in different strawberry organs was analyzed using real-time PCR. The result showed that *FaOLP2* expressed at different levels in leaves, crowns, roots, green fruits and ripe red fruits. Furthermore, the expression of *FaOLP2* under different abiotic stresses was analyzed at different time points. All of the three tested abiotic stimuli, abscisic acid, salicylic acid and mechanical wounding, triggered significant induction of *FaOLP2* within 2-6 h post-treatment. Comparing the three stimuli, *FaOLP2* was more prominently induced by salicylic acid than by abscisic acid or mechanical wounding. The positive responses of *FaOLP2* to these stress factors

suggested that *FaOLP2* may be involved in the protection of strawberry against pathogen attacks and against osmotic-related stresses. In addition to *FaOLP2*, the expression of a previously cloned OLP gene (*FaOLP1*) upon fungal infection was examined at different time points post-infection. Each of the two tested fungal species, *Colletotrichum fragariae* and *Colletotrichum acutatum*, triggered a substantial induction of *FaOLP1* at 24-48 h post-inoculation, indicating that *FaOLP1* could be involved in strawberry defense against fungal infection.

# CHAPTER 1

## LITERATURE REVIEW

### 1.1 Pathogenesis-Related (PR) Proteins

Higher plants have developed various defense mechanisms against biotic and abiotic stresses, such as pathogen invasions, wounding, exposure to heavy metal, salinity, cold, and ultraviolet rays. These defense mechanisms include: physical strengthening of the cell wall through lignification, suberization, and callose deposition; production of phytoalexins which are secondary metabolites, toxic to bacteria and fungi; and synthesis of pathogenesis-related (PR) proteins such as  $\beta$ -1,3-glucanases, chitinases and thaumatin-like proteins (Bowles, 1990).

PR proteins were first observed in tobacco plants infected with tobacco mosaic virus (TMV) (van Loon and van Kammen, 1970), and they were subsequently identified in many other plants species. Based on their primary structures, immunologic relationships, and enzymatic properties, PR proteins are currently grouped into seventeen families (PR-1 through 17) (Van Loon, 1999; Görlach et al., 1996; Okushima et al., 2000; Christensen et al., 2002). The PR-1 family consists of proteins with small size (usually 14-17 kD) and antifungal activity. The PR-2 family consists of  $\beta$ -1,3-glucanases, which are able to hydrolyze  $\beta$ -1,3-glucans, a biopolymer found in fungal cell walls. The PR-3, -4, -8 and -11 families consist of chitinases belonging to various chitinase classes (I – VII). The substrate of chitinases, chitin, is also a major structural component of fungal cell walls. The PR-5 family consists of thaumatin-like proteins and osmotin-like proteins. Other PR families include proteinase inhibitors, endoproteinases, peroxidases,

ribonuclease-like proteins, defensins, thionins, lipid transfer proteins, oxalate oxidases, and oxalate oxidase-like proteins.

A defensive role of PR proteins in plant systems has been suggested based on the induction of their synthesis upon pathogen infection, and on their *in vitro* and *in vivo* antifungal activities. PR proteins may also function to alleviate the harmful effects to cells and organisms caused by natural stresses, such as cold, drought, osmotic stress, UV light, and metal toxicity. In addition, some PR proteins, for example,  $\beta$ -1,3-glucanases, chitinases and thaumatin-like proteins, have been implicated in regulating various developmental processes such as flower formation, fruit ripening, seed germination, and embryogenesis (van Loon, 1999).

## **1.2 Plant Chitinases**

Chitin is a structural component of the cell wall of many fungi, as well as insects and nematodes, which are major pathogens and pests of crop plants (Collinge et al., 1993). Chitinases (E.C. 3.2.1.14) are ubiquitously distributed in bacteria, fungi, animals and plants. They hydrolyze the  $\beta$ -1,4-linkage between N-acetylglucosamine residues of chitin.

Plant chitinases usually have a wide range of optimum pH (pH 4-9), and they are generally stable at temperature up to 60 °C (Collinge et al., 1993). These enzymes usually have a molecular weight ranging from 25,000-35,000. Some chitinases undergo chemical modifications such as glycosylation and prolyl-hydroxylation. As demonstrated in other PR protein families, there are acidic and basic isoforms of chitinases. Basic chitinases are usually in the vacuole and have antifungal activity, while acidic chitinases are usually extracellular and show little antifungal activity. It seems that extracellular chitinases are

involved in generation of signal and transfer of information about infection, whereas vacuolar chitinases take part in repressing pathogen growth (Collinge et al., 1993).

### **1.2.1 Classification of Chitinases**

Based on the presence of a chitin-binding domain and the amino acid sequence homology, plant chitinases have been classified into seven classes, class I through VII (Neuhaus, 1999).

#### **1.2.1.1 Class I and II Chitinases**

Class I and II chitinases belong to the PR-3 family of PR proteins. Class I chitinases have a cysteine-rich chitin-binding domain (CBD) at the N-terminus. The CBD is linked to the catalytic domain by a spacer region which is rich in proline and glycine but variable in length and composition. Class I chitinases are synthesized as precursor proteins, with an N-terminal signal sequence directing them to the secretory pathway; most of them also contain a C-terminal signal sequence, which is required for targeting to the vacuole (Neuhaus et al., 1991a). Class II chitinases do not contain the N-terminal CBD domain and the spacer region, but have high amino acid sequence homology to the catalytic domain of class I chitinases. They usually are secreted to the extracellular space due to the lack of vacuolar target sequence at the C-terminus.

It has been suspected that the CBD domain is not essential for chitinolytic activity or antifungal activity though it does contribute to both activities. Recombinant tobacco class I chitinases (CHN A) were constructed with deletion of the CBD alone or in combination with the spacer region (Suarez et al., 2001). Both truncated chitinases retained 53% of the hydrolytic activity, while the antifungal activity was reduced by about 80%. It is proposed that the CBD might help anchor the catalytic domain to the

surface of polymeric substrates (e.g. pathogen cell wall), and, hence, allow the hydrolysis of many neighboring chitin strands (Neuhaus, 1999). This could explain the weaker enzymatic activity of class II chitinases compared to class I chitinases.

The crystal structures of a barley seed class II chitinase and a jack bean class II chitinase have been determined (Hart et al., 1995; Hahn et al., 2000). Both chitinases are mostly composed of  $\alpha$ -helices and form a globular structure. They resemble lysozymes at the active site region. Two active site glutamate residues have been identified in the crystal structure of the barley chitinase at amino acid positions 67 and 89. Jack bean chitinase has the activity site glutamate residues at similar positions. Mutations of either glutamate residue in the barley chitinase or in a tobacco class I chitinase caused a great loss of activity (Andersen et al., 1997; Iseli-Gamboni et al., 1998). In addition, mutation of Tyr 123 of a *Zea mays* chitinase and a similar tyrosine of an *Arabidopsis* chitinase in the active site motif, NYNY, which is highly conserved in most class I chitinases, also caused greatly reduced chitinase activities (Verburg et al., 1992 and 1993).

### **1.2.1.2 Class III Chitinases**

Class III chitinases belong to the PR-8 family of PR proteins. They generally have lysozyme activity, and do not display any sequence similarities to either class I or II chitinases. In addition, all plant chitinases of this class have highly similar sequences, but their isoelectric points differ widely.

One of the major latex proteins of *Hevea brasiliensis*, hevamine, was identified as a dual lysozyme and chitinase (Jekel et al., 1991). The crystal structures of hevamine and its complex with the inhibitor allosamodin has been determined (Terwisscha van Scheltinga et al., 1994, 1995, 1996). Despite the low sequence similarity, the structure of

hevamine resembles that of a bacterial family18 chitinase, both containing a  $(\alpha/\beta)_8$  barrel fold. These enzymes contain a substrate-binding cleft located at the C-terminal end of the  $\beta$ -strand in the barrel structure. At the active site of hevamine, residue Glu127 is the catalytic residue, whereas the neighboring Asp125 contributes to widen the catalytic pH range. The class III chitinase from *Tulipa bakeri* has the two active site residues at similar positions. Mutation of the glutamate residue completely abolished the enzyme activity of the *Tulipa* chitinase, while mutation of the aspartate residue decreased the enzyme activity (Suzukawa et al., 2003).

#### **1.2.1.3 Class IV, V, VI, VII Chitinases**

Class IV chitinases share low degrees of homology (41-47%) to class I chitinases. Although class IV chitinases contain a CBD and a catalytic domain resembling those of class I chitinases, they are significantly smaller due to one deletion in the CBD and three deletions in the catalytic domain.

Class V chitinase was initially represented only by a chitinase from *Urtica dioica*, which has two CBDs in tandem (Lerner and Raikhel, 1992). Yet this protein probably does not have catalytic activity, since the two catalytic glutamate residues are not present. Later, a chitinase named BjCHI1 was isolated from *Brassica juncea* by Zhao and Chye (1999). It too has two CBDs and structurally resembles the *Urtica dioica* chitinase. However, these investigators claimed that BjCHI1 should be classified as a new class of chitinase, since it has only 36.9% sequence identity with the *Urtica* chitinase. On the other hand, this enzyme shares high degrees of sequence identity with many class I chitinases.

Class VI and VII chitinases have unique structures and each is represented by one example so far. The lone example of class VI chitinases was isolated from sugar beet (Berglund et al., 1995). This chitinase features a heavily truncated CBD and a long proline-rich spacer. Class VII chitinase is represented by a rice chitinase, which has a catalytic domain homologous to that of class IV chitinases but without a CBD (Truong et al., 2003).

## **1.2.2 Functions of Plant Chitinases**

### **1.2.2.1 Antifungal Activity**

The fact that chitin is a structural component in cell walls of many fungi rapidly led to the proposal of chitinases as a defensive protein against pathogens. Various *in vitro* studies have demonstrated the inhibitory effect of chitinases against fungal growth (e.g. Broekaert et al., 1988; Huynh et al., 1992; Kim and Huang, 1996; Yun et al., 1996). Mauch et al. (1988) found that while a purified pea chitinase alone only inhibited the growth of one fungus, the combination of this chitinase and a  $\beta$ -1,3-glucanase inhibited the growth of all fungi tested, showing a synergism in activities. Similar results were subsequently observed in a number of different plant species, such as tobacco (Sela-Buurlage et al., 1993; Melchers et al., 1994) and cucumber (Ji and Kuc, 1996). In addition, chitinase has been shown to act synergistically with thaumatin-like proteins or other compounds that can alter the membrane structure or permeability (Hejgaard et al., 1991; Lorito et al., 1996).

However, not every chitinase is effective in inhibiting fungal growth. It has been proposed that only specific chitinases are able to inhibit specific fungi (Sela-Buurlage et al., 1993). For instance, a tobacco class I chitinase caused the lysis of the hyphal tips of

*Fusarium solani*, while a tobacco class II enzyme exhibited no antifungal activity against the same fungus. A chitinase from *Arabidopsis* effectively inhibited the growth of *Trichoderma reesei*, but it did not affect the growth of several other fungi (Verburg and Huynh, 1991).

Transgenic plants have been produced to express chitinases in a constitutive manner, with the goal to enhance plants' disease resistance. In the first successful report, transgenic tobacco or *Brassica napus* plants that constitutively expressed a bean vacuolar chitinase showed delayed disease development caused by the necrotrophic pathogen, *Rhizoctonia solani* (Broglie et al., 1991). Since then many successful results have been reported. Transgenic cucumber plants harboring a rice chitinase gene exhibited enhanced resistance against gray mold (Tabei et al., 1998). Transgenic rice plants over-expressing a rice chitinase showed significantly higher resistance against the rice blast pathogen *Magnaporthe grisea* (Nishizawa et al., 1999). Furthermore, co-expression of chitinase and  $\beta$ -1,3-glucanase genes in transgenic plants has been shown to synergistically enhance the resistance of plants against pathogens. For example, transgenic tomato plants expressing either a tobacco chitinase or glucanase had no protection against *Fusarium oxysporum* f.sp. *lycopersici*, whereas simultaneous expression of both enzymes reduced the disease severity by 36-58% (Jongedijk et al., 1995). However, there were also studies showing that transgenic plants exhibited no resistance to fungal infection (Neuhaus et al., 1991b; Nielsen et al., 1993). These observations suggest that not all chitinases are effective as defense mechanisms against pathogens, and that even those chitinases with the defensive capability can not be universally effective against all chitin-containing fungi species.

### **1.2.2.2 Nodule Formation**

During the development of nitrogen-fixing symbiosis between legumes and rhizobacteria, Nod factors, the oligosaccharides structurally resembling chitin, are released by the bacterial partner, and trigger the plant program for nodule formation. The involvement of chitinase in nodulation has been reported in a number of studies. In the alfalfa–*Rhizobium meliloti* interaction, several chitinases were activated once the first nodule primordia was formed, which was proposed as a feedback response of the plant to limit the infection caused by the bacteria and therefore to regulate nodulation (Vasse et al., 1993). A class III chitinase from *Sesbania rostrata* was induced in the early stage of nodulation and accumulated around the developing nodule (Goormachtig et al., 1998). It also displayed Nod factor degradation activity. These results suggest that this chitinase can regulate the intensity of root nodule formation by limiting the action of Nod factors. In a more recent study, another *Sesbania rostrata* class III chitinase induced by nodulation bacteria was found to be localized to the outermost cortical cell layers of the developing nodules (Goormachtig et al., 2001). However, this enzyme lacks the active site glutamate residue, which renders it to a chitin-binding lectin. It has been suggested that this protein functions as a Nod factor-binding protein which would protect, concentrate, or facilitate the interaction of Nod factor with a receptor protein.

### **1.2.2.3 Embryogenesis**

An unexpected function of chitinase as a differentiation factor in embryogenesis was demonstrated by studies on carrot somatic embryogenesis. A class IV chitinase was found to be able to rescue the somatic embryo of a mutant carrot cell line (De Jong et al., 1992; Kragh et al., 1996). Later, a potential substrate for this activity was identified by

van Hengel et al. (2001), namely arabinogalactan proteins (AGPs) that are required in carrot embryogenesis and contain chitinase-sensitive oligosaccharides. In the same study, chitinase-treated AGPs were demonstrated to have enhanced embryo-promoting activity compared to untreated molecules. Furthermore, chitinases were shown to be able to increase somatic embryogenesis from wild-type protoplasts. Taken together, these data suggest a general role for chitinases in plant embryogenesis.

Furthermore, a unique receptor-like kinase was identified in tobacco (Kim et al., 2000). This kinase, named CHRK1, harbors an extracellular chitinase-like domain, a membrane segment and cytosolic kinase domain. The essential Glu residues required for chitinase activity are mutated in the chitinase-like domain, and thus the protein is assumed to be devoid of hydrolytic activity. Further study suggested that CHRK1 is involved in a developmental signaling pathway regulating cell proliferation or differentiation and the endogenous cytokinin levels in tobacco (Lee et al., 2003).

#### **1.2.2.4 Other Functions**

Plant chitinases might also be involved in other developmental processes which can be illustrated by the presence of chitinases in selective parts of flowers (Lortan et al., 1989; Neale et al., 1990), and the appearance of chitinases during leaf senescence (Hanfrey et al., 1996). Recently, it was reported that *Arabidopsis* mutants of a chitinase-like protein are cellulose deficient with phenotypes indicative of weak primary cell walls (Mouille et al., 2003). Furthermore, two cotton chitinase-like proteins were shown to be expressed preferentially during secondary wall deposition (Zhang et al., 2004). These observations suggest that some chitinases might be essential for cellulose synthesis in primary and secondary cell walls. In addition, chitinases from the apoplast of cold-

adapted winter rye leaves have been shown to retard the growth of ice crystal, demonstrating antifreeze activity (Hon et al., 1995).

### **1.2.3 Chitinase Gene Structures**

A large number of cDNAs clones but relatively fewer genomic clones have been isolated for plant chitinases, and most of the gene information is related to class I, II, and III chitinases. Thus, only these three classes of chitinase genes will be discussed in this section.

#### **1.2.3.1 Class I and II Chitinase Genes**

The available genomic sequences for class I and II chitinases show that most genes of these two classes contain two introns. The first intron is usually located after the position corresponding to the conserved catalytic site motif SHETTG, whereas the second intron is located just before the conserved motif NYNY. The introns are usually small, ranging in size from approximately 50-200 bases. On the other hand, some class I or II chitinase genes contain no introns for example in wheat (Liao et al., 1994) or potato (Gaynorl and Unkenholz, 1989), while and some contain one intron, for example, in *Brassica napus* (Hamel and Bellemare, 1995).

Class I and II chitinase genes have various genomic structures, represented by a single copy to a multi-gene family. For instance, potato class I chitinase genes (Ancillo et al., 1999), maize class I genes (Wu et al, 1994) and two strawberry class II genes (Khan and Shih, 2004) exist as one or two copies per haploid genome, whereas potato class II chitinase genes show complex genomic organization with a minimum of 5 copies per haploid genome (Stanford et al., 1989).

### 1.2.3.2 Class III Chitinase Genes

Similar to class I and II chitinase genes, the exon/intron structure of class III chitinase genes also displays variability. Genes encoding class III chitinase in strawberry (Khan et al., 1999), cucumber (Lawton et al., 1994), *Vitis vinifera* (Ano et al., 2003) and *Benincasa hispida* (Shih et al., 2001) are intronless. In comparison, class III chitinase genes from soybean and *Arabidopsis* contain one intron and two introns, respectively (Watanabe et al., 1999; Samac et al., 1990).

Class III chitinase genes in *Arabidopsis* (Samac et al., 1990), *Sesbania rostrata* (Goormachig et al., 2001), *Beta vulgaris* (Nielsen et al., 1993), *Lupinus albus* (Regalado et al., 2000), and *Cucurbita sp.* (Kim et al., 1999) exist as single-copy genes. On the other hand, soybean class III chitinase genes and heveamine from *H. brasiliensis* are encoded by a small multi-gene family (Bokma et al., 2001).

### 1.2.4 Regulation of Chitinase Genes

In healthy plants, some forms of chitinases, both vacuolar and extracellular, are synthesized constitutively. Class I chitinases were found to be constitutively expressed at high levels in the roots of many plants (e.g.: Samac et al., 1990; Neale et al., 1990; Hamel et al., 1995). Class III chitinase transcripts are constitutively present in the leaf vascular tissues, hydathodes and guard cells of *Cucumis sativus* and *Arabidopsis* (Lawton et al., 1994; Samac and Shah, 1991). Moreover, it was observed that constitutive expression of chitinase genes increased with the plant's age (Samac et al., 1990; Lawton et al., 1994). Generally, higher chitinolytic activity is detected in older leaves than in young leaves.

The expression of some chitinases is developmentally and organ-specifically regulated. For example, the presence of a class III chitinase was detected during the seed

development of *Lupinus albus* (Regalado et al., 2000). The expression of a class IV chitinase and a class III chitinase increased markedly during grape and banana ripening, respectively (Robinson et al., 1997; Peumans et al., 2002). A tobacco class I chitinase was found to be highly expressed during flower formation (Neale et al., 1990).

Induction of chitinase gene expression by pathogen attack is reported in numerous studies (as reviewed in Collinge et al., 1993, and Neahauss, 1999). It has often been observed that different chitinase genes within a single plant are differentially regulated in response to a specific pathogen. For instance, in barley leaves infected with powdery mildew, only one of the three chitinases investigated was significantly induced (Kragh et al., 1993). In strawberry plants inoculated with *Colletotrichum fragariae* or *Colletotrichum acutatum*, one class II chitinase gene was induced within 2-6 h post-inoculation, while another class II chitinase gene did not respond until 24-48 h post-inoculation (Khan and Shih, 2004). Furthermore, the induction of chitinases can be systemic or local. It depends on the infecting pathogen, its virulence or the particular chitinase class. When parsley leaf buds were infected with *Phytophthora sojae*, one class II chitinase was induced rapidly, strongly, and locally around infection sites, whereas the other class II chitinase was induced slowly and systemically throughout the infected leaves and even the whole organism (Ponath et al., 2000). A class III chitinase in *Vitis vinifera* was first induced in the leaf inoculated with *Plasmopara viticola*, and induced later in the upper-stage healthy leaf; in contrast, the expression of a class I chitinase remained negligible under experimental conditions in the study (Busam et al., 1997).

Pathogen attacks lead to an increase in the endogenous salicylic acid, jasmonic acid or ethylene content in plants, which act as secondary signaling molecules to activate

both local and systemic defenses (Thatcher et al., 2005). Thus, it is rational to expect that exogenous application of these signal molecules would stimulate the expression of chitinases, which actually has been demonstrated in various plant species (e.g.: Ishige et al., 1993; Buchter et al., 1997; Davis et al., 2002; Ding et al., 2002; Wu and Bradford, 2003). In general, different chitinase genes in the same plant often show differential response upon treatment with these compounds. Moreover, one chitinase gene often shows distinct expression patterns in response to different signal molecules.

In addition to the factors described above, the expression of chitinase genes can be induced by other external stimuli, e.g., wounding, drought, cold, ozone, heavy metals, salinity and UV light. Wounding stimulated chitinase gene expression in a number of different plants, such as maize (Bravo et al., 2003), *Brassica napus* (Hamel et al., 1995) and pea (Chang et al., 1995); some chitinases were even induced in a systemic manner (e.g., Parsons and Gordon, 1989; Standford et al., 1990). Cold acclimation and dehydration induced the expression of one class II chitinase gene in Bermuda grass (de los Reyes et al., 2001). A pumpkin chitinase was induced by osmotic stress (Arie et al., 2000). Ozone treatment caused a rapid increase in intracellular chitinases in tobacco plants (Schraudner et al., 1992).

Several chitinase promoters have been fused to the reporter genes and introduced in plants, in order to identify the *cis*-elements and the *trans*-acting factors involved in regulation of chitinase expression. The promoter of a tobacco class I chitinase gene (*CHN48*) was fused to a  $\beta$ -glucuronidase (GUS) reporter gene, and this chimeric gene construct was introduced to tobacco plants (Shinshi et al., 1995). The DNA sequence between positions -480 and -410 relative to the transcription start site was found to be

absolutely necessary for ethylene-responsive transcription of *GUS*. This 71-base DNA fragment contains two copies of the GCC box element, which was originally identified as an ethylene responsive element in the promoter of several tobacco basic PR genes (Hart et al., 1993). Gel mobility-shift assays showed the presence of nuclear factors that interact with the ethylene-responsive region.

In addition, a series of promoter constructs of the tobacco chitinase *CHN50* fused to the *GUS* gene was introduced into cultured tobacco cells (Fukuda and Shinshi, 1994; Fukuda, 1997). Promoter deletion analysis revealed that the DNA region between positions -788 and -345 from the transcription initiation site was required for induction by fungal elicitor. It was also found that a nuclear factor(s) bound specifically to the sequence motif GTCAGAAAGTCAG between positions -533 and -521. This sequence motif includes a TGAC core sequence of the W box element on the complementary strand. W boxes have been shown to mediate pathogen and/or elicitor induced gene transcription via the W box-binding WRKY transcription factors (Ruston et al, 1996). A W box related sequence element was also identified within the region between -125 and -69 of *CHN48* (Yamamoto et al., 2004). The DNA fragment corresponding to the -125 and -69 region was then fused to a luciferase reporter gene. The expression of the reporter gene in transgenic tobacco was induced by treatment with fungal elicitor. Furthermore, the tobacco WRKY homologs were shown to be able to bind to the W box of *CHN48* and stimulate the W box-mediated transcription of a luciferase reporter gene in transient expression assays. These results suggested the involvement of tobacco WRKYs and the W box element in elicitor-responsive transcription of tobacco chitinase genes.

### **1.2.5 Existence of Chitinase-Containing Protein Complex**

All plant chitinases examined thus far exist as single-chain polypeptide molecules, except a chitinase present in the apoplastic space of cold-adapted winter rye leaves (Yu and Griffith, 1999). The apoplastic fluid from cold-acclimated winter rye leaves contained nine native proteins (NPs), seven of which were found to be protein complexes consisting of multiple polypeptides. Western blot analysis revealed that all these complexes are composed of various combinations of one 35-kDa chitinase-like protein (CLP), two  $\beta$ -1,3-glucanase-like proteins (GLP, 32 kDa and 35 kDa), one 25-kDa thaumatin-like protein (TLP), and other unidentified proteins. One of the NP complexes was isolated using affinity chromatography, and was shown to contain the 35-kDa CLP, the 35-kDa GLP, and two unknown proteins. The gene encoding the 35-kD CLP was subsequently cloned, and the sequence of the gene indicated that the protein is indeed a chitinase (Yeh et al. 2000).

A more recent study by Stressmann et al (2004) showed that repeated cycles of freeze-thaw treatments or certain cations could affect the structure and organization of the winter rye protein complexes. Specifically, the study showed that the complexes were partially unfolded or rearranged after freezing and thawing, which led to the exposure of new  $\text{Ca}^{++}$ -binding sites. Binding of  $\text{Ca}^{++}$  to these sites caused inhibition of the antifreeze and chitinase activities of these complexes.

### **1.3 The PR-5 Family: Thaumatin-Like Proteins/Osmotin-Like Proteins**

Members of the PR-5 family were originally described from tobacco when induced upon TMV infection. The amino acid sequences of PR-5 proteins share a high degree of homology with thaumatin, the sweet-tasting protein that accumulates in the

fruit of *Thaumatococcus daniellii* plants, and, thus, they are often referred to as thaumatin-like proteins (TLPs). In addition, osmotin, which was originally identified as the predominant protein in salt-adapted tobacco cells, is related to thaumatin in amino acid sequence and therefore belongs to the PR-5 family as well (Singh et al., 1985).

### **1.3.1 Physicochemical Properties of TLPs**

The TLPs are generally resistant to proteases and pH- or heat-induced denaturation. The molecular masses of TLPs fall into two size ranges. One group of proteins has a size ranging from 22 to 26 kDa, while the other group comprises proteins of 16 kDa, due to an internal deletion of 58 amino acids. No glycosylation has been observed in any TLP so far.

The TLPs have a wide range of pI values, varying from very acidic to very basic (pI 3.4-12). Similar to other PR families, the extracellular TLPs tend to be acidic, while the vacuolar TLPs tend to be basic. It is not clear at the present time whether there is any biological significance to this observation. PR-5 proteins are synthesized as precursor proteins with an N-terminal signal sequence, with a highly conserved alanine residue at the cleavage site. Basic PR-5 proteins have an additional signal peptide at the C-terminus which is required for their targeting to the vacuole (Melchers et al., 1993).

The three-dimensional structure of thaumatin has been determined using X-ray crystallography (Ogata et al., 1992). Thaumatin is composed of three domains, domains I through III. There is a so-called thaumatin loop within domain II, the structure that is speculated to be responsible for the sweetness of thaumatin. Moreover, there are 16 cysteine residues within thaumatin, which form 8 disulfide bonds. Disruption of these disulfide bonds will result in loss of the tertiary structure of the thaumatin molecule, and

loss of sweetness (Van der Wel and Loeve, 1972). The locations of the 16 cysteine residues are highly conserved in the higher-molecular-weight TLPs.

The crystal structures of maize zeamatin, tobacco PR-5d protein and tobacco osmotin have also been determined (Batalia et al., 1996; Koiwa et al., 1999; Min et al., 2004). Their tertiary structures closely resemble that of thaumatin. However, the thaumatin loop is absent from domain II of all the three PR-5 proteins, which probably explains why other PR-5 proteins do not have a sweet taste. Another most notable structural difference between the three PR-5 proteins and thaumatin lies in a cleft region that is formed between domains I and II. The cleft region of the three PR-5 proteins is highly acidic, whereas thaumatin mainly has a basic surface in the cleft region. The acidic residues involved in the formation of the acidic cleft are three aspartate residues and one glutamate residue, and they are present at similar positions in all the three PR-5 proteins. This is an important feature, because zeamatin, PR-5d and osmotin are all antifungal proteins, but thaumatin is not. This suggests that this acid cleft could be involved in the antifungal activity of PR-5 proteins.

### **1.3.2 Biological Functions of TLPs**

#### **1.3.2.1 Antifungal Activity**

Although they lack hydrolytic enzyme activity, purified TLPs have been shown to inhibit fungal growth *in vitro*. For instance, both tobacco and tomato AP24 caused sporangial lysis of *Phytophthora infestans* (Woloshuk et al., 1991). Grape osmotin exhibited inhibitory activities against hyphal growth of *Guignardia bidwellii* and *Botrytis cinerea* (Salzman et al., 1998). A TLP from the flower buds of Chinese cabbage caused a rapid release of cytoplasmic materials from the fungal hyphal tips of *Neurospora crassa*,

and inhibited conidial germination of *Trichoderma reesei*, *Fusarium oxysporum* and *B. cinerea* (Cheong et al., 1997).

It has been observed that TLPs exhibit some degrees of specificity toward the fungi species upon which they act. In a study conducted by Vigers et al. (1992), the antifungal activities of three different TLPs (maize zeamatin, tobacco osmotin, tobacco PR-S) were compared. Among the three TLPs, PR-S was the most effective against *Cercospora beticola*. On the other hand, PR-S failed to inhibit the growth of *Trichoderma viride*, *Candida albicans*, and *N. crassa*, while both zeamatin and osmotin completely inhibited the growth of these three fungi. Furthermore, Abad et al. (1996) demonstrated that tobacco osmotin could inhibit *Bipolaris*, *Fusarium*, and *Phytophthora* species, but had no effect on *Aspergillus*, *Macrophomina*, and *Rhizoctonia* species.

The *in vitro* antifungal activity of TLPs indicated that this protein family could play an important role in plant defense against pathogen invasions. In the view of this possibility, transgenic plants over-expressing PR-5 proteins have been produced for several plant species. In many cases, the transgenic plants exhibit enhanced disease resistance. For example, over-expression of tobacco osmotin in transgenic potato plants led to enhanced resistance to *P. infestans*, the potato late blight pathogen (Liu et al., 1994). Transgenic wheat plants with constitutive expression of a rice TLP exhibited delayed development of wheat scab caused by *Fusarium graminearum* (Chen et al., 1999). In a more recent study, two transgenic carrot lines that constitutively expressed a different rice TLP were developed (Punja, 2005). Both lines showed significantly fewer disease symptoms when inoculated with six different pathogens.

The molecular mechanism that accounts for the antifungal activity of PR-5 proteins, however, is still not clear. The mechanism may involve interactions with specific plasma membrane component(s) of the fungal target and/or destabilizing the fungal plasma membrane. Abad et al. (1996) demonstrated that tobacco osmotin could cause membrane leakage and dissipated the pH gradient across the cell wall/membrane of sensitive fungal species. In addition, the species specificity of osmotin suggested the existence of membrane receptors. Using *Saccharomyces cerevisiae* as a model system, several studies have found that the antifungal activity of PR-5 protein was mediated by the composition of fungal cell wall (Coca et al., 2000; Ibeas et al., 2001). In particular, fungal cell wall phosphomannans were shown to facilitate the toxic activity of PR-5 proteins (Ibeas et al., 2000; Salzman et al., 2004). Furthermore, recently, a seven transmembrane domain receptor-like protein was found to be an osmotin-binding plasma membrane protein, and this protein was required for the osmotin-induced apoptosis in *S. cerevisiae* (Narasimhan et al., 2001; Narasimhan et al., 2005).

### **1.3.2.2 Antifreeze Activity**

The antifreeze activity of a TLP was first described in winter rye (Hong et al., 1995). The apoplast of cold-acclimated winter rye leaves contains several distinct proteins, which are found to be homologs of PR proteins, including a TLP. The accumulation of these PR proteins in the apoplast upon exposure to cold temperature is correlated with the increase in freezing tolerance. In addition, a cryoprotective protein was purified from the stem of bittersweet nightshade and identified as an OLP (Newton and Duman, 2000). This protein was subsequently expressed in *Escherichia coli*, and the

partially purified protein in the supernatant fraction of the culture medium showed cryoprotective activity.

### **1.3.2.3 Roles in Developmental Processes**

Various TLPs have been observed to accumulate during the fruit ripening of bananas (Clendennen and May, 1997), cherries (Fils-Lycaon et al., 1996), grapes (Salzman et al., 1998), and tomato (Pressy, 1997). Tobacco osmotin was highly expressed in explants during de novo flower formation (Neale et al., 1990). In addition, the mRNAs of one barley and one oat TLP accumulated in an unusual bimodal pattern during seed development (Skadsen et al., 2000). The mRNA was highly abundant around the time of pollination, and then decreased rapidly to near-zero, and a second peak appeared in the doughy stage of development. Osmotin-like protein was also identified as one of the PR proteins related to the somatic embryogenesis of *Cichorium* (Helleboid et al., 2000). These studies demonstrate that TLPs play a role in various developmental processes.

### **1.3.2.4 Beta-1,3-Glucanase Activity**

In the study conducted by Trudel et al. (1998), seven purified TLPs from corn, pea and barley, either constitutively expressed or stress-induced, were shown to bind to polymeric glucan. In a more recent study (Grenier et al., 2001), six TLPs from barley, tomato, cherry, and tobacco were shown to exhibit glucanase activity on polymeric glucan. The cherry fruit TLP and two tobacco TLPs can even hydrolyze crude cell wall preparations from *Saccharomyces cerevisiae*, indicating that these TLP enzymes could act on complex fungal  $\beta$ -1,3-glucans. Furthermore, analysis of hydrolyzed products by thin-layer chromatography revealed that all six active TLPs acted as endo- $\beta$ -1,3-

glucanases. This unexpected activity of some TLPs brings new insight into the mechanisms accounting for the antifungal activity of TLPs: destabilizing target membrane on one hand, binding and/or hydrolyzing fungal  $\beta$ -1,3-glucan on the other hand.

### **1.3.3 TLP Gene Structure and Regulation**

A large number of cDNAs, but fewer gene sequences, have been isolated for TLPs. To date, all known genomic sequences of TLPs, except one, contain no introns, e.g., in tobacco (Velazhahan et al., 1999), potato (Castillo Ruiz et al., 2005), strawberry (this study and Wu et al., 2001), *Benincasa hispida* (Shih et al., 2001) and black nightshade (Campos et al., 2002). The lone exception is a pistil-specific TLP gene from Japanese pear which contains one intron of 351 bp (Sassa et al., 2002). The genomic organization of TLP genes shows variability; TLP genes are represented by a single-copy gene to a large multi-gene family. For example, OLP genes from *Arabidopsis* and *Benincasa hispida* have one copy per genome (Capelli, 1997; Shih et al., 2001). In contrast, black nightshade shows a complex organization of OLP genes with at least 8 members (Campos et al., 2002).

Recently, a bacterial artificial chromosome (BAC) library containing about 50,000 clones was constructed from an interspecific hybrid between two cultivated potato species (Castillo Ruiz et al., 2005). The BAC library was screened with a tobacco PR-5 cDNA probe or a potato osmotin probe. Positive BAC clones were characterized by southern hybridization, sequence analysis and genetic mapping. The results revealed that four acidic PR-5 homologous genes were localized to a 45-kb segment on potato chromosome XII. In addition, nine basic PR-5 homologous genes were found to be

organized at two loci: eight genes in a 90-kb cluster on chromosome VIII and one single gene on chromosome XI. To my knowledge, this is the only report demonstrating that PR-5 protein genes could appear as gene clusters. Yet the frequency of PR-5 protein genes to exist as clusters in the plant kingdom is unknown.

TLPs normally are either not expressed or are expressed at very low levels in the leaves of young healthy plants. But they could accumulate to high levels in response to biotic or abiotic stress. The highest levels of TLPs or their mRNAs were frequently found in roots (e.g., Neale et al., 1990; Zhu et al., 1995; Hong et al., 2004), presumably due to constant exposure to soil-associated microbes. In addition, as described in a previous section, high expressions of TLPs were observed in flower tissues, overripe fruits, as well as seeds of several cereals, indicating TLP genes can be developmentally regulated.

The induction of TLPs in response to microbial infection has been observed in various plant species. For example, inoculation of *Arabidopsis* with turnip crinkle virus induces acidic PR-5 protein genes (Dempsey et al., 1993). Three potato PR-5 protein genes were strongly expressed 4 days after infection by *P. infestans* (Zhu et al., 1995). When oat seedlings were challenged with stem rust fungus, four distinct TLPs genes were induced, some as early as 24 h after infection (Lin et al., 1996). Ward et al. (1991) studied the gene expression profile during TMV-induced systemic acquired resistance (SAR). They found that PR-5 protein genes were not only induced in locally infected tobacco leaves, but also in the secondary uninfected leaves of the same plant. Similarly, the systemic expression of the PR-5 gene was found to be activated in *Arabidopsis* plants infected with *Pseudomonas syringae* (Van Wees et al., 1999;) and pepper plants infected with *Xanthomonas campestris* pv. *vesicatoria* (both virulent and avirulent strains) or

*Pseudomonas fluorescence* (Hong et al., 2004), suggesting the involvement of PR-5 proteins in SAR.

Wounding-induced expression of OLP genes was also demonstrated in various plant species (e.g. Fredo et al., 1992; Zhu et al., 1995; Ruperti et al., 2002). Interestingly, wounding appeared to be able to trigger the systemic expression of OLPs as well. In a study by Neale et al. (1992), the level of tobacco osmotin mRNA in unwounded leaves was elevated to a similar level as that in wounded leaves. In addition, a pepper OLP was induced in both wounded and intact pepper leaves (Hong et al., 2004). These results suggest that a systemic signal might be involved in PR-5 gene regulation.

Selective members of the PR-5 family can be induced by osmotic-related stress. Tobacco osmotin and a tomato TLP (NP24) were shown to accumulate in salt stressed tobacco cells and tomato cells, respectively, and were the most abundant protein in the cell (Singh et al., 1985; King et al., 1988). Furthermore, the expression of OLP genes from potato and pepper were activated by low temperature, high salinity and drought (Zhu et al., 1995; Hong et al., 2004).

In addition, the expression of OLP genes can be stimulated by exogenous application of plant hormones, including abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA), and ethylene. These hormones can serve as secondary signal molecules in plant signaling pathways in response to biotic and abiotic stress. The induction of OLP genes by ABA, SA, JA or ethylene has been observed in various plant species, such as *Arabidopsis* (Uknes et al., 1992), tobacco (La Rosa et al., 1992; Xu et al., 1994), potato (Zhu et al., 1995), pepper (Hong et al., 2004) and strawberry (this study). In addition, Xu et al. (1994) showed that combinations of JA and ethylene, or JA and SA

were more potent than individual compounds. On the other hand, results from several studies suggested that the induction and accumulation of OLP mRNAs by these compounds might not lead to a corresponding increase of proteins, indicating additional regulation at the translational level. For instance, ABA induced the accumulation of tobacco osmotin mRNA, but not the protein (LaRosa et al., 1992). Similarly, ABA and SA resulted in the induction of three potato OLPs only at the mRNA level (Zhu et al., 1995). In contrast, the combination of ethylene and methyl jasmonate caused both tobacco osmotin mRNA and protein accumulation (Xu et al., 1994). Several TLPs in wheat were induced by SA or JA treatment at both the mRNA and protein levels (Jayaraj et al., 2004).

The promoter of tobacco osmotin gene has been extensively studied using osmotin promoter-*GUS* fusions (Ragothama et al., 1993; Ragothama et al., 1997). The region of the promoter between -248 to -108 from the transcription start site was found to be essential for the gene activity, and responsible for regulation by ABA, ethylene, salt, desiccation and wounding. Within this region, a DNA sequence resembling the ABA-responsive sequence (CACGTG) was identified; this element was shown to bind nuclear factors presumably involved in transcription regulation. In addition, this region contains a GCC box element (AGCCGCC), which is the ethylene responsive element conserved in the promoters of a number of PR genes. This GCC box has been shown to be necessary and sufficient to confer ethylene-induced transcription of tobacco osmotin gene.

Similarly, the promoter of one tobacco OLP contains two copies of GCC box sequences (Sato et al., 1996). Transgenic tobacco plants with wild type or mutated OLP promoter-*GUS* fusion showed that mutation in the GCC box caused loss of ethylene-inducible GUS

expression. Furthermore, EREBP2, a nuclear protein factor that specifically binds to the GCC box of a tobacco  $\beta$ -1,3-glucanase gene, was shown to bind to this sequence element in the tobacco OLP gene. This observation suggests that ethylene-induced expression of this OLP is regulated by the binding of this factor to the GCC box sequence.

## **1.4 Defense Signaling Pathways in Plants**

Plants defend themselves against pathogen invasions through a combination of preformed and induced defense mechanisms. Preformed structural barriers such as cell wall lignification and callose deposition, and antimicrobial compounds (e.g. phytoalexin), can provide non-specific protection. On the other hand, the induced defense mechanisms are more sophisticated, starting with plant-pathogen recognition, which is based on the interaction between pathogen elicitors and plant receptors. This interaction as a signal is subsequently transmitted through different signaling pathways, and eventually leads to the expression of plant defense genes. A number of secondary signaling molecules are involved in the downstream signal transduction, including SA, JA and ethylene. Each of these signal molecules leads to a different signal pathway, induces a different subset of plant defense genes, and eventually provides resistance against specific pathogens.

### **1.4.1 SA-Dependent Signaling Pathway**

Salicylic acid has been shown to play central roles in a plant's local defense against pathogen invasion, and is required for the establishment of systemic acquired resistance (SAR) (Uknes et al., 1992). SAR is associated with an increase of SA initially locally and then systemically through out the plant, which leads to expression of defense genes including PR protein genes. SAR results in a long-lasting, systemic resistance to subsequent infection by a broad range of pathogens (Ryals et al., 1996).

A key regulatory protein in SA signaling is NPR1 (nonexpressor of PR genes), which is required for the development of SAR and induction of PR genes (Cao et al., 1994). NPR1 appears to act downstream of SA and has been shown to translocate to the nucleus at the onset of SAR (Dong, 1998). It is also involved in the feedback regulation of SA biosynthesis during SAR (Cao et al., 1997). NPR1 is an ankyrin-repeat containing protein, a domain often involved in protein-protein interactions. A subclass of basic region/leucine zipper (bZIP) transcription factors called TGAs have been shown to specifically interact with NPR1 through the ankyrin repeat domain (Zhang et al., 1999; Zhou et al., 2000). Furthermore, TGA2 was shown to bind to a so-called as-1-like motif within the PR-1 gene promoter, a motif essential for SA-induced PR-1 gene expression (Lebel et al., 1998; Zhang et al., 1999). These results suggest TGA transcription factors can take part in regulating SA-responsive expression of PR genes.

Another important transcription factor family involved in SA-mediated defense response is the plant-specific WRKY family, which binds to the W box motif containing a TGAC core (Eulgem et al., 2000). A number of pathogen inducible genes contain the W box in the promoter, suggesting a role for this element in pathogen-induced gene expression (Rushton and Somssich, 1998). The *NPR1* promoter contains several copies of the W box, and mutations of the W box were shown to abolish its expression, indicating that WRKY transcription factors are crucial for *NPR1* expression (Yu et al., 2001). In addition, analysis of *PR-1* co-regulated genes, which were induced during SAR, revealed an over-representation of W box or W box-like motifs in their promoters (Maleck et al., 2001). Therefore, WRKY transcription factors might also be involved in the regulation of SA-responsive defense-related genes.

### 1.4.2 JA-Dependent Signaling Pathway

Both JA- and ethylene-mediated signaling are required for the development of another type of systemic resistance termed induced systemic resistance (ISR), which is independent of SA signaling (Pieterse et al. 1998). Certain strains of non-pathogenic rhizosphere bacteria can initiate ISR. These bacteria are referred to as plant growth-promoting rhizobacteria (PGPR) due to their ability to stimulate plant growth and to improve plant stand under stress conditions (Kloepper et al., 1980). These PGPR have been shown to be able to protect plants from pathogen infection through induction of systemic resistance (i.e., ISR) in plants, without provoking any symptoms themselves (Van Loon et al., 1998). In ISR, a different subset of *PR* genes, including the defensin (*PR-12*) and thionin (*PR-13*) genes, are induced (Dong, 1998).

Jasmonic acid signaling can be activated by wounding, insects, microbial pathogens and abiotic stresses (Turner et al., 2002). These stimuli activate JA biosynthesis. A mitogen-activated protein kinase (MAPK) named *WIPK* was identified in tobacco, and was shown to be required for wound-induced JA biosynthesis (Seo et al., 1999). On the other hand, in *Arabidopsis*, a different MAPK, *ATMPK4*, appears to regulate JA perception or response, since the *mpk4* mutant failed to express the JA-regulated defensin and thionin after treatment with JA (Petersen et al., 2000). Mutational studies reveal two additional regulators in JA signaling, *JAR1* and *COI1*. The *JAR1* gene encodes a protein similar to adenylate-forming enzymes, suggesting that *JAR1* may control JA signaling by metabolizing JA molecules (Staswick et al., 2002). The *COI1* gene encodes a leucine-rich-repeat-containing F-box protein, which might function by

recruiting transcriptional repressors and targeting them for removal by ubiquitination (Xie et al., 1998).

### **1.4.3 Ethylene-Dependent Signaling Pathway**

Ethylene-mediated signaling is the most extensively studied signaling pathway. Ethylene signaling can be activated by pathogens, wounding and various abiotic stresses. The ethylene receptors have been identified in *Arabidopsis* and other plant species. They are membrane-localized proteins, and are homologs of the bacterial two-component histidine kinases involved in sensing environmental changes (Wang et al., 2002). Interestingly, it appears that ethylene receptors negatively regulate the ethylene response (Hua and Meyerowitz, 1998). Furthermore, mutational analyses identified another negative regulator, CTR1, in ethylene signaling (Kieber et al., 1993). CTR1 appears to work downstream of the ethylene receptor, and is a member of the Raf family of Ser/Thr protein kinases which initiate MAPK signaling cascades in mammals. Wang et al. (2002) propose that in the absence of an ethylene signal, ethylene receptors activate CTR1, which in turn negatively regulates the downstream ethylene signaling, possibly via a MAPK signaling cascade. They also propose that binding of ethylene results in the deactivation of the receptors as well as CTR1, allowing downstream signaling events to occur.

In addition, EIN2, a novel integral membrane protein, has been shown to be an essential positive regulator and acts downstream of CTR1 and upstream of EIN3 (Ethylene-Insensitive 3) in ET signaling (Alonso et al., 1999). EIN3 is a nuclear-localized component in ET signaling. In *Arabidopsis*, it was able to bind directly to the promoter region of Ethylene-responsive-factor 1 (ERF1), suggesting its role as a transcriptional

regulator of ERF1 (Cao et al., 1997; Solano et al., 1998). ERF1 is a member of plant specific transcription factors referred to as ethylene-response-element binding proteins (EREBPs). ERF1 binds to the GCC box promoter element to activate expression of defense genes, such as defensin and chitinase (Solano et al., 1998), while the GCC box is often associated with ET- and pathogen-induced gene expression and is conserved in many pathogen-responsive genes.

#### **1.4.4 Cross-Talk between Signaling Pathways**

The SA-dependent and JA-dependent pathways often seem to act antagonistically. For example, SA has been shown to inhibit JA biosynthesis and JA-responsive gene expression (Penninckx et al., 1996; Gupta et al., 2000). The NahG mutant that is unable to accumulate SA exhibited increased JA level and JA-responsive gene expression in response to pathogens, suggesting that pathogen-induced SA biosynthesis could suppress JA accumulation and JA-responsive gene expression (Spoel et al., 2003). JA also can negatively regulate SA signaling. The *mpk4* mutant, impaired in JA signaling, constitutively expressed SA-mediated gene expression (Petersen et al., 2000). On the other hand, some studies show that SA and JA can work synergistically. For example, in a gene expression profiling study, 55 genes were induced by both SA and JA (Schenk et al., 2000). Interestingly, NPR1, the essential protein in SAR, is also required for the establishment of ISR (Pieterse et al., 1998). Several studies indicate that NPR1 is a central regulator which coordinates different plant defense responses including SAR, ISR, and SA/JA interaction (Durant and Dong, 2005).

The ET- and JA-dependent pathways generally act together. For instance, in a microarray study, most genes induced by ET were also induced by JA (Schenk et al.,

2000). Furthermore, both JA and ET were required for the induction of a defensin gene (Penninckx et al., 1998), and the establishment of ISR requires JA and ET signaling (Pieterse et al., 1998).

The plant hormone abscisic acid (ABA) is mainly known as the regulator of the signaling pathway involved in plant responses to abiotic stresses such as salinity, drought and coldness, as well as plant growth and development. Recently, ABA was shown to have a negative effect on JA or ET mediated defense gene expression, while ET had a negative effect on ABA regulated gene expression (Anderson et al., 2004). Furthermore, mutants defective in ABA signaling exhibited increased JA or ET regulated defense gene expression. These results, taken together, demonstrate the degree of complexity in the regulation of defense signaling pathways.

## **1.5 Study of Strawberry's Pathogenesis-Related Genes / Proteins**

Strawberry is a member of the *Rosaceae* family, which is a large family consisting of 100 genera and more than 3,000 species (Baumgardt, 1982). The *Rosaceae* family includes many economically important fruit crops such as apples, pears and blueberries. Strawberry is a major economic fruit crop in the southern United States. Florida is one of the largest producers of strawberry, accounting for 12% of the annual domestic production, but 100% of the winter crop. In Louisiana, strawberry has always been an important horticultural crop. However strawberry production has been declining consistently in recent years, mainly due to strawberry diseases. One of the most severe diseases of strawberry is anthracnose disease, which causes crown, fruit, and root rot and damages petiole and runners. The agents responsible for this disease are the fungal species *Colletotrichum fragariae*, *C. acutatum*, and *C. gloeosporioides*. Currently there is

no fungicide approved that can be safely applied for disease control. The only precaution farmers can take is to obtain plants which are fungus free. Bioengineering of strawberry plants to generate plants with enhanced disease resistance, therefore, is an attractive alternative. Such goals for other crop species have been achieved. While transfer of foreign genes such as chitinases or  $\beta$ -1,3-glucanases under control of a strong promoter, such as CaMV 35S RNA, has been achieved in strawberry, it is still necessary to obtain knowledge of strawberry's pathogenesis-related proteins to better understand the plant defense as a whole. A former student in Dr. Shih's lab found that a major acidic chitinase isoform in strawberry leaves could be a protein complex. This is an interesting observation since such a chitinase-containing complex has only been reported in cold-acclimated winter rye leaves. Thus, one of the goals of this dissertation is to purify and characterize this protein complex. In addition, among all the PR proteins, chitinases and  $\beta$ -1,3-glucanases have been studied extensively. On the other hand, the PR-5 protein family is less understood, especially the regulation of these genes. Therefore a second goal of this dissertation is to characterize the osmotin-like protein genes in strawberry and to study their expression under abiotic and biotic stress.

## CHAPTER 2

### PARTIAL PURIFICATION OF A CHITINASE-CONTAINING PROTEIN COMPLEX IN THE STRAWBERRY PLANT

#### 2.1 Introduction

Plant chitinases are pathogenesis-related (PR) proteins, which are implicated in plant defense mechanisms against pathogens. Chitinases catalyze the hydrolysis of chitin, a  $\beta$ -1,4-linked homopolymer of N-acetylglucosamine, which is a major structural component of the cell walls of many pathogenic fungi. Most plant species contain multiple chitinase isozymes. Plant chitinases are currently divided into seven classes (classes I through VII) based on their structural properties and amino acid sequence homologies (Neuhaus, 1999). The majority of these hydrolytic enzymes identified thus far belong to classes I through IV.

Plant chitinases are generally expressed constitutively at low levels. However, their synthesis could increase upon viral, bacterial, or fungal infection (Neuhaus, 1999). Some plant chitinases have been shown to inhibit fungal growth *in vitro* by causing lysis of the hyphal tips (Sela-Buurlage et al., 1993; Melchers et al., 1994). Transgenic plants constitutively expressing chitinases, alone or in combination with a second PR protein such as  $\beta$ -1,3-glucanase, have been shown to exhibit higher levels of resistance to fungal infection or delayed development of disease symptoms (Jach et al. 1995; Datta et al. 2001).

All plant chitinases examined thus far exist as single-chain polypeptide molecules with the exception of a chitinase present in the apoplastic fluid of winter rye leaves. A study reported by Yu and Griffith (1999) showed that fractionation of the apoplastic fluid

prepared from cold-acclimated winter rye leaves on a native polyacrylamide gel resulted in the separation of nine proteins. When each of these proteins, designated as native proteins (NPs) 1 through 9, was isolated from the native gel, denatured, and examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), seven of the NPs were found to be protein complexes consisting of multiple polypeptide chains. For example, NP4, NP5 and NP6 contained seven, eight, and five polypeptides, respectively. In addition, western blot analyses revealed that all these complexes are composed of various combinations of a 35- kDa chitinase-like protein (CLP), two  $\beta$ -1,3-glucanase-like proteins (GLP), a thaumatin-like protein (TLP), and other unidentified proteins. Interestingly, all the protein complexes except NP1 exhibited antifreeze activity. One of the NPs, NP3, was further purified using affinity chromatography. Analysis of this protein revealed that it contains the 35-kDa CLP, one GLP, and two unknown polypeptides. The gene encoding the 35-kD CLP was subsequently cloned, and the sequence of the gene indicated that the protein is indeed a chitinase (Yeh et al. 2000). A more recent study by Stressmann et al (2004) showed that repeated cycles of freeze-thaw treatment or certain cations could affect the structure and organization of the winter rye protein complexes. Specifically, the study showed that the complexes underwent structural changes during the cycles of freezing and thawing, which leads to the exposure of new  $\text{Ca}^{++}$ -binding sites. Binding of  $\text{Ca}^{++}$  to these sites caused inhibition of the antifreeze and chitinase activities of these complexes.

Strawberry is a member of the *Rosaceae* family, which consists of more than 3000 species (Baumgardt, 1982). This plant family includes many important fruit crops such as apples, pears and raspberries. Thus far, relatively few studies have been reported

on PR proteins or PR protein genes of the *Rosaceae* family. Our laboratory has previously reported the nucleotide sequences of a strawberry class III chitinase gene and two class II chitinase genes, and the expression of the two class II chitinase genes upon fungal infection (Khan et al., 1999; Khan and Shih, 2004). In this chapter, I report the partial purification of a strawberry protein complex consisting of at least five chitinase isoforms. To our knowledge, this protein complex represents the second example of a chitinase-containing protein complex in higher eukaryotic species. Furthermore, this strawberry chitinase complex appeared to be structurally different from protein complexes present in the winter rye leaves.

## **2.2 Materials and Methods**

### **2.2.1 Plant Materials**

Dormant strawberry (*Fragaria ananassa* Duchesne) plantlets were purchased from Nourse Farms (Deerfield, MA). The plantlets were planted into 9 cm<sup>2</sup> containers (Kord, Ontario, Canada) that contained a soil mix [bark, peat moss, and perlite (7:2:1, v/v/v)] mixed with dolomitic lime (4.7 kg m<sup>-3</sup>). Approximately 5 g of Osmocote-plus fertilizer (15-9-12; Scotts-Sierra, Marysville, OH) was spread on top of each container. The plants were grown in Percival growth chambers (Percival Scientific, Boone, IO, USA, Model AR-60L) at 26/18°C (day/night) and an 11-h photoperiod. General Electric (T32T8SP41) lamps were used delivering irradiance of 8 W m<sup>-2</sup>. The relative humidity was kept at 60% to 70%. The plants were watered with distilled water approximately every other day. Field-grown strawberry plants were collected during the months of May and June at the Burden Research Station of the Louisiana State University Agriculture Center.

### **2.2.2 Preparation of Protein Extracts**

Strawberry leaves or other organs were ground to fine powders in liquid nitrogen. The ground samples were homogenized at 4 °C with extraction buffer [25 mM Tris-HCl, pH 8.5, 5% polyvinylpyrrolidone, 14 mM  $\beta$ -mercaptoethanol, and 30 uL/g fresh tissue weight of a plant protease inhibitor cocktail (Sigma, St. Louis, MO)]. Four ml of the extraction buffer was used for every gram of fresh tissue. The homogenate obtained was centrifuged at 12,000 x g for 10 min at 4°C and the supernatant was filtered through one layer of Miracloth (BD Biosciences Clontech, Palo Alto, CA). The crude extract was stored at -20°C.

### **2.2.3 Detection of Chitinase Isoforms on Native Gels**

Acidic chitinase isoforms were separated and detected according to the method described by Trudel and Asselin (1989) with minor modifications. Gel electrophoresis was carried out using the Hoeffer SE250 Mighty Small mini-gel apparatus (Amersham Biosciences, Piscataway, NJ). The gels were prepared according to Laemmli (1970) with the resolving gel being 12.5% and the stacking gel being 5%, except that sodium dodecylsulfate (SDS) was omitted. The protein samples were prepared in a sample buffer lacking SDS and any reducing agent. Samples were applied to the gels without heating. The gels were run at 4°C at a constant voltage of 150 V. Once the protein samples compacted in the gel, voltage was turned to 200 V.

After electrophoresis, the gel was incubated in 100 mL of 0.1 M sodium acetate buffer (pH5) for 10 min. This gel was then overlaid on a substrate (glycol-chitin)-containing acrylamide gel. The gels were sandwiched between two glass plates and incubated at 37 °C under moist conditions for 2-4 hr. A weight of about 100 g was placed

on top of this assembly. After incubation, the overlay gel was stained with 0.01% (w/v) fluorescent dye, Calcofluor White M2R (Sigma), in 0.5 M Tris-HCl buffer (pH8.8) for 5 min in the dark. Following extensive destaining with water in the dark, the acidic chitinase isoforms appeared as dark bands against a fluorescent background when the gel was viewed on a UV-transilluminator.

#### **2.2.4 Detection of Chitinase Isoforms on SDS Gels**

Gel electrophoresis was carried out using the Hoeffer SE250 Mighty Small minigel system. Resolving (12.5%) and stacking (5%) gels were prepared according to Laemmli (1970). Glycol-chitin (0.01%) was included in the resolving gel solution. Protein samples were prepared in a sample buffer lacking any reducing agent, and the samples were applied to the gel without heating. The gel was run at 16°C at a constant voltage of 150 V. Once the protein samples compacted, voltage was increased to 200 V. After electrophoresis, the gel was incubated in 200 mL of 0.1 M sodium acetate buffer (pH5.0) containing 1% (v/v) Triton X-100 (USB, Cleveland, OH) at 37°C with mild agitation in an incubator-shaker (Model G-25, New Brunswick Scientific Co., Edison, NJ) for 16 h. This incubation slowly released SDS from the gel, while promoting protein renaturation. The gel was subsequently stained with Calcofluor White M2R, destained, and viewed as described above.

#### **2.2.5 Partial Purification of Chitinase-Containing Protein Complex**

Protein extract was prepared in large scale from leaves of field-grown strawberry plants. Crude extract was prepared as described above. Ammonium sulfate was then added to the crude extract to 50% saturation and stirred at 4°C overnight. The precipitate

was collected by centrifugation and resuspended in buffer A (25 mM Tris-HCl, pH8.5, 14 mM  $\beta$ -mercaptoethanol) and dialyzed against the same buffer for 4 h.

The dialyzed sample (approximately 70 mg of proteins) was loaded onto a DEAE-Sepharose anion-exchange column (2.5 x 15 cm, Amersham Biosciences) equilibrated with buffer A. The column was first washed with 0.1 M NaCl in buffer A at a flow rate of 2 mL/min until the absorbance (A<sub>280</sub>) returned to near the base line. The bound proteins were then eluted with 0.4 M NaCl in buffer A at the same flow rate. The eluted proteins were dialyzed and concentrated by freeze-drying.

The eluted protein samples from several preparations of DEAE-Sepharose batch-wise chromatography were combined; and a total of approximately 11 mg of proteins were loaded onto a HiPrep Sephacryl S-200 HR column (2.6 x 60 cm; Amersham Biosciences) equilibrated with buffer A containing 0.15 M NaCl. Proteins were eluted with 320 mL of the same buffer at a flow rate of 1 mL/min controlled with a ÄKTA FPLC system (Amersham Biosciences). The size of each collected fraction was 1.5 mL. Chitinase activity of the collected fractions was determined using [<sup>3</sup>H]-labeled chitin as a substrate. Fractions containing the chitinase complex were identified using the native gel system. Appropriate fractions were pooled and concentrated by freeze-drying.

This concentrated sample (approximately 0.7 mg of proteins) was further fractionated on a Tricorn Mono Q GL column (5 x 50 mm; Amersham Biosciences) equilibrated with buffer A. Proteins were eluted with a linear gradient of 0-0.5 M NaCl in buffer A in a total volume of 60 mL. The flow rate was 1 mL/min and controlled with the same FPLC system mentioned above. The size of each collected fraction was 1 mL. Chitinase activity of the collected fractions was determined. The isoform patterns of

chitinase-containing fractions were examined on acidic native gel, and appropriate fractions were pooled.

All the fractions from each chromatographic step were stored at  $-20^{\circ}\text{C}$  before analysis or further fractionation.

### **2.2.7 Radiometric Chitinase Activity Assay**

Total chitinase activity was determined using a radiometric method described by Cabib (1988) with some modifications (Shih et al., 2001). Each assay reaction mixture contained 50  $\mu\text{L}$  of 0.1 M sodium acetate buffer (pH5.0), 20  $\mu\text{L}$  of [ $^3\text{H}$ ]-chitin (approximately  $3 \times 10^5$  CPM), various amounts of protein samples, and de-ionized distilled water in a final volume of 200  $\mu\text{L}$ . The reaction mixtures were incubated in 1.5 mL microcentrifuge tubes at  $37^{\circ}\text{C}$  for 2 h, with shaking at 200 rpm in an incubator-shaker. After incubation, the reactions were stopped by addition of 200  $\mu\text{L}$  of 10% trichloroacetic acid. The undigested substrate was pelleted by centrifugation at 15,000 g for 10 min. Avoiding the pellet, 220  $\mu\text{L}$  of supernatant was withdrawn from each reaction mixture, and filtered through a 0.45  $\mu\text{m}$  Millipore Ultrafree-MC filter (Millipore Corp., Bedford, MA) by centrifugation at 15,000 g for 15 s. A 200  $\mu\text{L}$  aliquot from each filtrate was withdrawn and added to 2 mL of the Lquiscient<sup>TM</sup> scintillation cocktail (National Diagnostics, Atlanta, GA) and counted in a Beckman LS 60001 C Scintillation counter (Beckman Instruments, Inc., Fullerton, CA).

### **2.2.8 Silver Staining**

Silver staining was performed with GelCode Color Silver Stain Kit (Pierce, Rockford, IL) according to the manufacture's protocol.

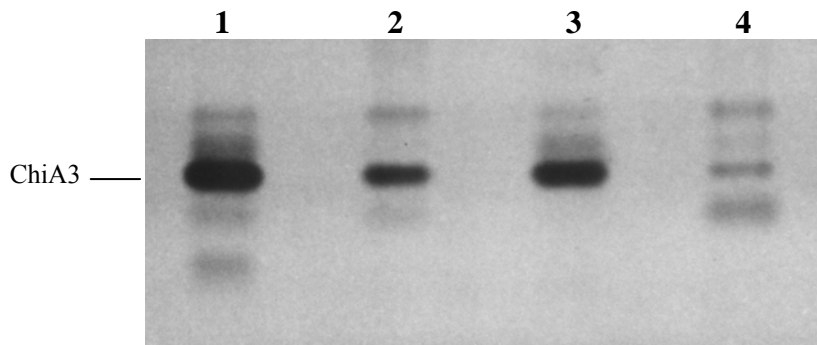
### **2.2.9 Immunoblotting**

Proteins were separated by SDS-PAGE using a 12.5 % gel, according to Laemmli (1970). The proteins in the gel were then blotted onto Hybond ECL nitrocellulose membrane (Amersham Biosciences) by using a Hoefer electronic transfer unit (Model TE 52X, Amersham Biosciences). The membranes were then incubated with blocking solution (5% nonfat dry milk powder in PBS buffer with 0.05% Tween-20) at room temperature for 2 h, and followed by incubating overnight with antiserum against a barley  $\beta$ -1,3-glucanase or against a rice TLP (dilution, 1:250) (The antisera were obtained from Professor Muthukrishnan, Kansas State University). The immuno-reactions were detected by using horseradish peroxidase conjugated to goat anti-rabbit IgG (Pierce) with SuperSignal West Pico chemiluminescent substrate kit (Pierce) according to the manufacturer's protocol.

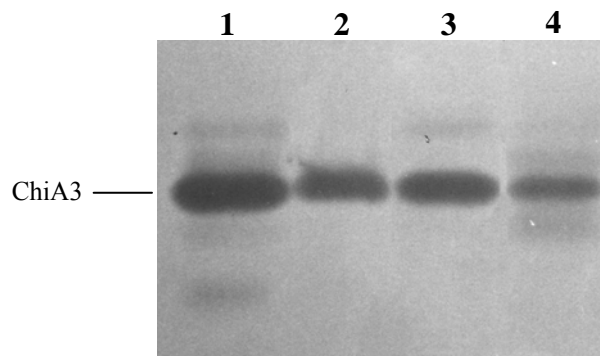
## **2.3 Results**

### **2.3.1 Analysis of Strawberry Chitinase Isoform Patterns**

To obtain information on strawberry chitinase activity, the isoform patterns of the enzyme in different strawberry organs were analyzed. Protein extracts were prepared from leaf, root, crown, and fruit samples collected from field-grown strawberry plants. Fig. 2.1A shows the activity band patterns of acidic chitinase isoforms. These zymograms revealed that leaves, roots and crowns (lanes 1 through 3) contained a predominant isoform, designated as Chi3A, plus two to four minor isoforms. Fruits also contained ChiA3, but the enzyme band was less prominent than those observed with the other three organs (lane 4). ChiA3 was also the predominant isoform in the root and crown samples prepared from plants grown in the growth chamber (Fig. 1B, lane 3 and 4). However,



A



B

Figure 2.1 The acidic isoform patterns of different strawberry organ extracts. Five  $\mu\text{g}$  of total protein was loaded in each lane. (A) Plants grown in the field. Lane 1, leaf extract; lane 2, root extract; lane 3, crown extract; lane 4, fruit extract. (B) Plants grown in growth chambers. Lane 2, leaf extract; lane 3, root extract; lane 4, crown extract. For comparison, leaf extract made from field-grown plants grown is shown in lane 1.

ChiA3 was the only isoform detectable in the leaf sample from plants grown in growth chamber (Fig. 2.1B, lane 2). Analysis of the organ extracts on basic native polyacrylamide gels revealed that leaf extracts contained two basic chitinase isoforms while other organs contained either one or two basic isoforms (data not shown).

The native activity gel results showed that leaves from growth chamber-grown plants contained only one acidic chitinase isoform and two basic chitinase isoforms. However, when the same extract was fractionated on a glycochitin-containing SDS-

polyacrylamide gel (referred to hereafter as SDS activity gel), at least six chitinase activity bands were observed after proteins were renatured by removing SDS from the gel following electrophoresis (Fig.2.3, lane 2 and 3). In view of the existence of chitinase-containing antifreeze protein complexes in winter rye leaves, a possible explanation of this discrepancy is that one of the isoforms resolved on the native gel is a protein complex consisting of multiple chitinases.

Since ChiA3 is the predominant isoform in most tested strawberry organs, we chose ChiA3 to investigate the possibility of it being a protein complex. First, the leaf extract was incubated under different conditions and fractionated in a higher concentration native gel (20 % instead of 12.5%) to determine whether ChiA3 still remained as a single band. To this end, the leaf extract was diluted four-fold to decrease the total buffer concentration, thus the ionic strength, of the extract solution to 6.25 mM Tris. The diluted extract was heated at 40°C or 55°C for 30 min in the presence of 0 or 10 mM of additional mercaptoethanol, and fractionated on a 20% native gel. The result in Fig.2.2 clearly shows that ChiA3 remained as a single band under these conditions, although some degrees of enzyme inactivation occurred when the extract was incubated at 55°C in the presence of mercaptoethanol. These results indicated that ChiA3 is not a non-specific aggregate or a fortuitous result of several chitinases migrating together in the 12.5% gel.

Next, ChiA3 was isolated from the native gel and run on a SDS activity gel. Three major chitinase activity bands along with two minor bands were detected (Fig.2.3, lane 1), which represent a subset of the activity bands observed in the total leaf protein extract (lane 2 and 3). This result indicates that ChiA3 is probably a protein complex consisting

of several different chitinases. With regard to the total number of detected activity bands on the SDS gel, it should be pointed out that the observed SDS activity gel pattern may not necessarily account for all the chitinase molecules present in the extracted ChiA3 or the unfractionated leaf protein extract, since some chitinase may not be able to renature under the conditions used. On the other hand, it is highly unlikely that the appearance of multiple activity bands was the result of proteolytic degradation, since protease inhibitors were included in the extraction buffer and chitinases in general are resistant to protease degradation (Collinge, 1993).

### **2.3.2 Partial Purification of ChiA3**

To further ascertain that ChiA3 is a protein complex, we attempted to purify ChiA3. Leaf extracts prepared from field-grown plants were concentrated by ammonium sulfate precipitation and then a DEAE-Sepharose batch-wise elution step was carried out. The main purpose for including this batch-wise elution step was to remove some sticky materials present in the leaf extract which made the extract viscous. These sticky materials would seriously affect protein resolution in subsequent fractionation steps if not sufficiently removed.

Following the batch-wise elution step, the protein sample was fractionated on a HiPrep Sephacryl S-200 HR column connected to a FPLC system. Fig. 2.4 shows the elution profile. A chitinase activity peak appeared around fraction 100, whereas a large portion of contaminant proteins were eluted near the end of the elution. The isoform patterns of the chitinase-containing fractions were determined on native activity gels, and the patterns of four fractions from the peak area (fractions 97, 99, 101, and 103) are shown in the inset of Fig. 2.4 (lanes 1 through 4). The gel results revealed that all these

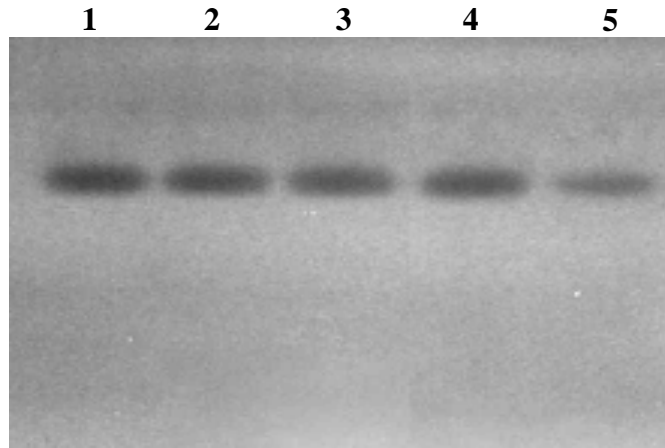


Figure 2.2 The stability of ChiA3 after treatments at different conditions. Electrophoresis of a diluted leaf extract incubated at 40 or 55°C in the presence or absence of additional mercaptoethanol on a 20% native polyacrylamide gel. Each lane was loaded with 5  $\mu$ g of total protein. Lanes 1, untreated control; lanes 2 and 3, diluted extract incubated at 40°C in the presence of 0 and 10 mM additional mercaptoethanol, respectively; lanes 4 and 5, diluted extract incubated at 55°C in the presence of 0 and 10 mM additional mercaptoethanol, respectively.

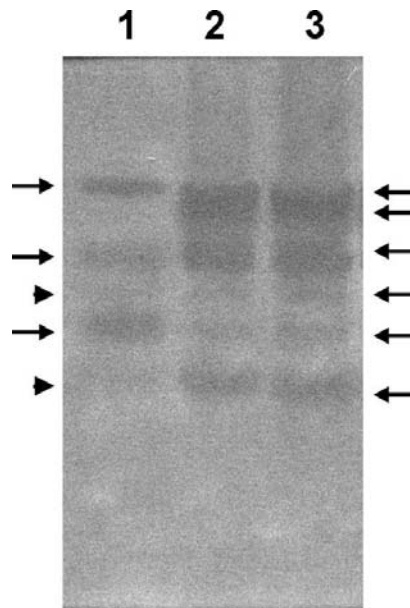


Figure 2.3. Fractionation of isolated ChiA3 on a glycol chitin-containing SDS polyacrylamide gel. Lane 1, extracted ChiA3; lanes 2 and 3, chitinase isoforms (indicated by arrows) in crude protein extract.

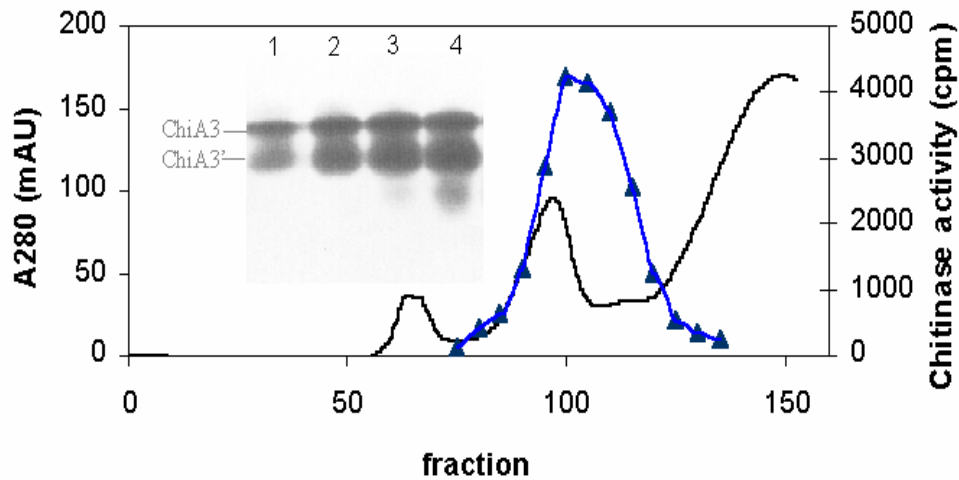


Figure 2.4 Elution profile from the Sephacryl S-200 HR FPLC. Column size, 2.6 cm x 60 cm; flow rate, 1 ml/min; elution buffer, 25 mM Tris-HCl (pH 8.5)/150 mM NaCl containing 14 mM  $\beta$ -mercaptoethanol; fraction size, 1.5 ml. Absorbance at 280 nm, continuous line; chitinase activity, solid triangle. Inset: Fractions from Sephacryl S-200 HR FPLC were analyzed on native activity gel. Equal volumes of each fraction were loaded on each lane. Lanes 1 through 4 are fraction 97, 99, 101, 103, respectively.

fractions contained ChiA3 and an additional distinctive band, which migrated ahead of ChiA3 in the gel. Since the protein sample obtained from the previous batch-wise elution step contained predominately ChiA3 (see lane 1, Fig. 2.6A), the additional band must be a related complex derived from ChiA3. This new complex was designated as ChiA3'.

Fractions from the peak area (fractions 97 to 102) of the Sephacryl S200 column were pooled, and the pooled proteins were further fractionated on a Mono Q column with a linear gradient of 0-0.5 M NaCl. Fig. 2.5 shows the elution profile. Again, the isoform patterns of chitinase-containing fractions were analyzed on native activity gels. As shown by gel patterns in the inset of Fig. 2.5, the amount of ChiA3 decreased as the salt concentration increased until a salt concentration was reached where while only ChiA3' was detected (lanes 1 through 4, Fig. 2.5 Inset picture).

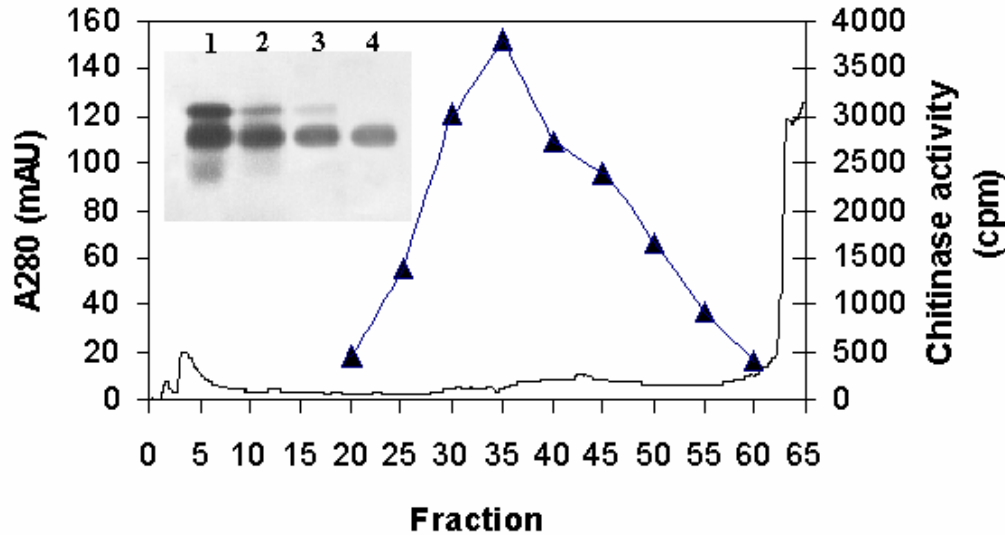


Fig. 2.5 Elution profile from the Mono Q FPLC. Column size, 5 mm x 50 mm; flow rate, 1 ml/min; elution with 0-0.5 M linear gradient of NaCl in total 60 ml of 25 mM Tris-HCl (pH 8.5) containing 14 mM  $\beta$ -mercaptoethanol; fraction size, 1 ml. Continuous line represent the A280 reading. Solid line with solid triangle represents chitinase activity. Inset: Fractions from Mono Q FPLC was analyzed on acidic native gel. Equal volumes of each fraction were loaded on each lane. Lane 1, fraction 35; lane 2, fraction 40; lane 3, fraction 45; lane 4, fraction 50.

Fractions containing only ChiA3' were pooled, and the proteins were examined both on a native activity gel and a SDS activity gel. The native gel result showed that ChiA3' present in the pooled fraction from the Mono Q column appeared as a somewhat diffused band (Fig. 2.6A, lane 3). In comparison, the same diffused band was also observed with the pooled fraction from the Sephacryl S200 column, which, in addition, contained a ChiA3 band (lane 2). On the other hand, as already pointed out, the sample from the batch-wise elution step contained predominately ChiA3 (lane 1).

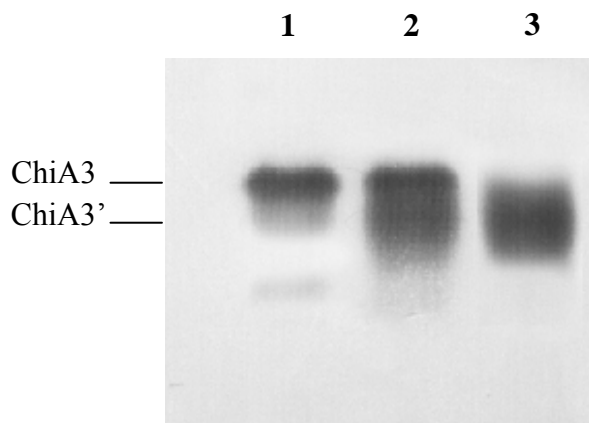
Fig. 2.6B shows the SDS activity gel pattern of the pooled fraction from the Mono Q column (lane 3), together with the patterns from the pooled fraction from the Sephacryl S200 column (lane 2) and from the batch-wise elution step (lane 1). These patterns were almost completely identical. The samples from the batch-wise elution and Sephacryl

S200 each contained five chitinase bands (bands 1 through 5 with increasing mobility), whereas the sample from the Mono Q column contained the same five bands plus an additional, relatively minor band, which had a higher electrophoretic mobility than the other five bands on the SDS gel (band 6). This result further established the multi-component nature of ChiA3/ChiA3'. The estimated molecular masses of chitinases represented by bands 1 through 6 are approximately 49 kDa, 46 kDa, 41 kDa, 35 kDa, 22 kDa and 18 kDa, respectively.

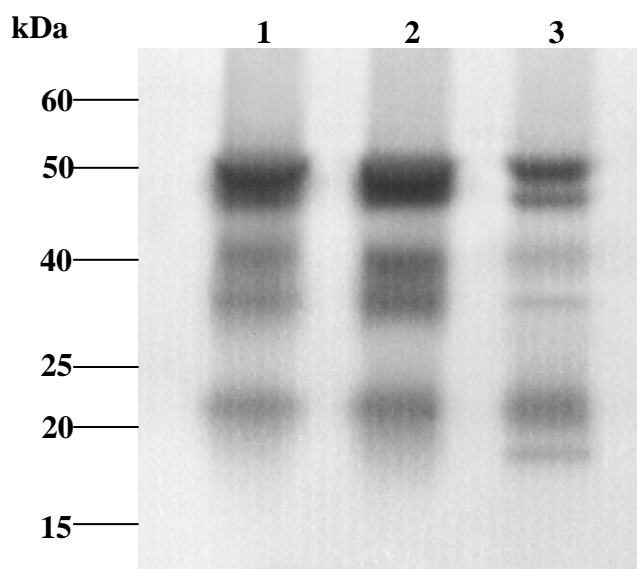
The pooled fraction from Mono Q column was also fractionated on a native gel and stained with silver staining to examine the degree of purity of the isolated protein complex. As shown on Fig. 2.7 (panel A and B), the silver-stained Mono Q fraction contained a diffused band similar to that in the activity gel. In addition, the  $R_f$  values of the diffuse band in the silver staining gel and in the activity gel were almost identical, 0.714 and 0.711, respectively, suggesting that the two bands are corresponding to each other. In addition, besides the major ChiA3' band, only one minor band with slower electrophoretic mobility than that of ChiA3' was detected on the stained gel, indicating that ChiA3' was purified to near homogeneity.

### **2.3.3 Western Blot Analysis**

The winter rye antifreeze protein complexes contain GLPs and TLPs in addition to the presence of a chitinase. To determine whether ChiA3/ChiA3' contains these PR proteins, western blot analyses were carried out using antisera against a barley  $\beta$ -1,3-glucanase and a rice thaumatin. The results are shown in Fig. 2.8. With the anti-glucanase antiserum, two hybridization bands were observed with a sample from the batch-wise elution step (Fig. 2.8A, lane 1) and one band was observed with a sample



A



B

Figure. 2.6 Chitinase isoform profile on acidic native gel (A) or SDS activity gel (B) after each chromatography step. Same amount of chitinase, in terms of total chitinase activity, was loaded in each lane. Lane 1, DEAE-cellulose batch-wise elution; lane 2, pooled ChiA3-containing fractions after HiPrep Sephacryl S-200 FPLC; lane 3, pooled ChiA4-containing fractions (F50-54) after Mono Q FPLC.

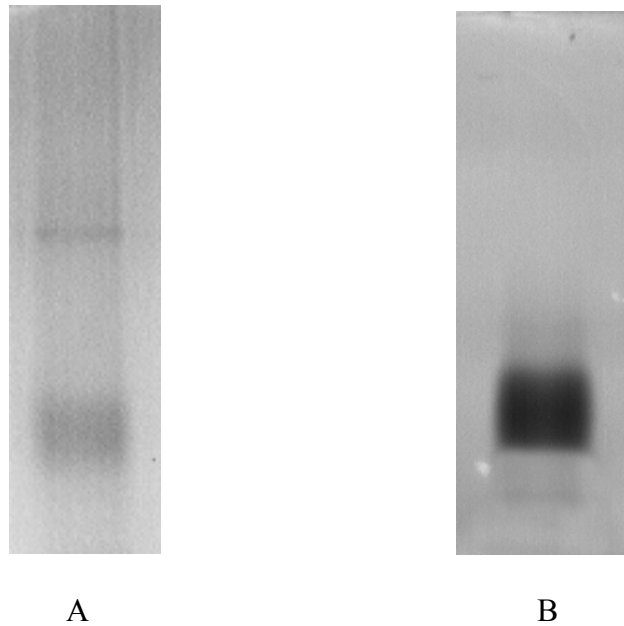


Figure 2.7 Examination of pooled fraction (F50-54) after Mono Q FPLC on native gel stained by silver staining (A) or on native activity gel (B). Equal volume of pooled fraction was loaded onto two separate lanes in the same gel. The gel was run under native conditions. After electrophoresis, one lane was stained by silver staining; one lane was used for detection of chitinase activity band.

from the pooled fraction from the Sephacryl S200 column (lane 2). However, no band was observed with a sample from the pooled fraction from the Mono Q column. With the anti-thaumatococcus antiserum, multiple bands were observed with the batch-wise elution sample (Fig. 2.8B, lane 1), one band with the Sephacryl S200 column sample (lane 2), and, again, no band was observed with the sample from the Mono Q column (lane 3). These results indicated that ChiA3/ChiA3' may not contain a GLP or a TLP.

## 2.4 Discussion

The purification procedure for the strawberry chitinase complex consisted of three consecutive chromatographic steps: a batch-wise elution step using a DEAE-Sepharose column, fractionation on a Sephacryl S-200 HR column, and fractionation on a Mono Q

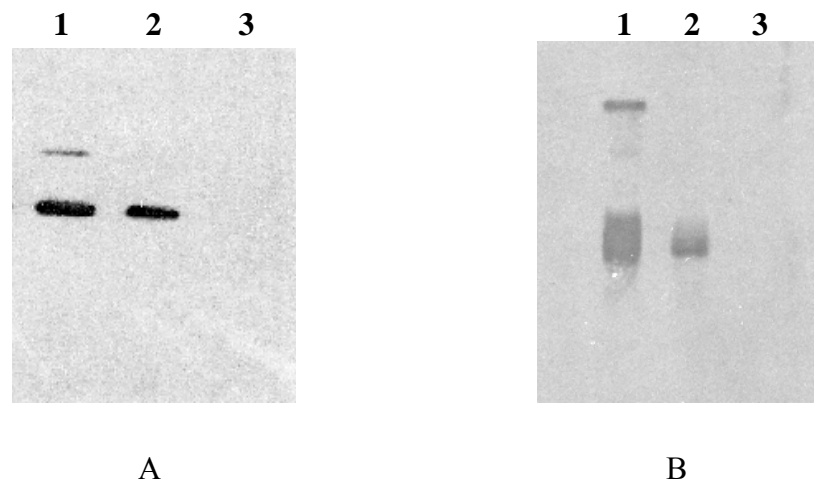


Figure 2.8 Western blot analyses of protein samples after each chromatography step. Equal amounts of protein (5  $\mu$ g) were loaded on each lane. Lane 1, protein sample after DEAE-Sepharose batch-wise elution; lane 2, pooled ChiA3/ChiA4 containing fractions after Sepharacryl S200 FPLC; lane 3, pooled ChiA4 containing fraction after Mono Q FPLC. After SDS-PAGE, the gels were blotted and probed with anti-GLP antiserum (A), and anti-TLP antiserum (B).

column. Analysis of samples from these three steps revealed that there was a change in the acidic chitinase isoform pattern during the purification process. A new chitinase activity band, ChiA3', which had a higher electrophoretic mobility than ChiA3 on the native gel, appeared after gel filtration chromatography (Fig. 2.6A). Since the protein sample obtained from the previous step (batch-wise elution) contained predominately ChiA3 (Fig. 2.6A, lane 1), ChiA3', then, must be derived from ChiA3. An explanation for the appearance of ChiA3' could be that ChiA3 lost one or more loosely bound protein component(s) during the gel filtration step, which resulted in a change in the composition of the protein complex. Another explanation could be that ChiA3 underwent structural changes caused by repeated cycles of freeze-thaw of the protein samples, which occurred when protein samples were stored at  $-20^{\circ}\text{C}$  and thawed subsequently for the next fractionation step. This explanation is in agreement with the finding of Stressmann et al.

(2004) who observed that freeze-thaw cycles led to alterations of the secondary and tertiary structures of the winter rye antifreeze protein complexes.

In addition, it should be pointed out that in the gel filtration step, ChiA3/ChiA3' was eluted in fractions appearing between where the molecular weight markers bovine serum albumin (molecular weight 66,000) and carbonic anhydrase (molecular weight 29,000) were eluted. This elution profile was in contradiction to the expected high molecular weight of the protein complex. However, a similar observation was described by Yu and Griffith (1999) on the winter rye antifreeze protein complexes. They found that when these complexes were fractionated on either a Sephacryl 200 column or a Bio-Gel P-100 column, all the complexes were eluted in fractions after where the 12-kDa molecular weight marker was eluted. The reason for observed retardation in the migration of the winter rye protein complexes as well as the strawberry complex in the gel media is not known. It is possible that these complexes contain high amounts of carbohydrates which could cause the retardation.

Analysis of the pooled fraction from Mono Q column on native gel revealed only one chitinase activity band (ChiA3'). Yet when the same pooled fraction was resolved on a SDS activity gel, at least five chitinase activity bands were detected (Fig 2.6). These results indicated that ChiA3/ChiA3' is indeed a protein complex that contains multiple chitinase molecules. In addition, the ChiA3/ChiA3' complex appears not to have any TLPs or GLPs, based on western blot analysis results (Fig 2.8). These observed characteristics of ChiA3/ChiA3' demonstrated that this complex is structurally different from the winter rye complexes which are composed of various combinations of a chitinase, a TLP, two GLPs, and other unidentified proteins (Yu and Griffith, 1999).

Hence, the strawberry complex may represent a new structural form of a complex-carbohydrate degrading enzyme that has not been previously observed in higher eukaryotes. At the least, the strawberry complex represents the second case of a chitinase-containing protein complex in plant cells.

In terms of protein complex structures, it may be relevant to point out that certain cellulolytic bacteria and fungi produce large multi-subunit protein complexes called cellulosomes, which are involved in degrading cellulose (Lamed et al., 1983; Shoham et al., 1999). Cellulosomes contain all the proteins and enzymes necessary for degrading cellulose to glucose monomers. For example, the cellulosome from *Clostridium thermocellum* strain YS contains 14 different polypeptide chains. A model for the structure of this particular cellulosome has been proposed (Béguin and Lemaire, 1996). According to this model, catalytic components of the cellulosome, which include endoglucanases, cellulases, and  $\beta$ -glucosidases, are organized around a large noncatalytic glycoprotein termed CipA. CipA acts both as a scaffolding and cellulose-binding factor. Each catalytic component of the cellulosome contains a conserved, noncatalytic region called the dockerin domain, which binds to a receptor domain on CipA, termed the cohesion domain. It is possible that the ChiA3/ChiA3' complex might have a structure like that of a cellulosome, or a smaller and less complex version of it.

Interestingly, in addition to the cellulose degrading enzymes, a chitinase named Chi18A was also identified in the *C. thermocellum* cellulosome (Bayer et al. 1998; Zverlov et al., 2002). Chi18A was shown to consist of a catalytic domain and a duplicated 24-amino acid dockerin domain at the C-terminus, with a molecular weight of 51,000. It is interesting to note that three of the chitinases contained in the ChiA3/ChiA3'

complex had a molecular weight between 40,000 and 50,000 (Fig. 2.6B). These values are higher than the molecular weight of the majority of plant chitinases which fall in the range of 24,000-35,000. It is intriguing to think that the high molecular weight of ChiA3/ChiA3' chitinases might be due to the presence of additional domains such as a dockerin domain.

The biological functions of the multi-chitinase protein complex in strawberry remain unknown at the present time. However, since chitinases are generally believed to take part in plant defense against pathogen infection via their hydrolytic activity, it is logical to assume that this complex also has such a role. In fact, when strawberry plants were infected with *Colletotrichum fragariae* or *Colletotrichum acutatum*, two important strawberry fungal pathogens, the level of ChiA3 was indeed increased by several fold (Khan, 2002). A study by Brunner et al. (1998) demonstrated that chitinases of different classes had differential hydrolytic activity toward different substrates, including chitin, chitosan (partially deacetylated chitin), and chitin oligomers of different length. Therefore, the assembly of several chitinases into one complex could have a synergistic effect on the hydrolysis of chitin, and as a consequence, repress the spread of pathogens more effectively. In the case of the cellulosome, synergistic effects on insoluble cellulose degradation were observed between three cellulosomal glucanases from *Clostridium cellulovorans* (Murashima et al., 2002).

In future studies, it would be intriguing to identify and characterize the chitinase constituents as well as other possible components in the strawberry chitinase complex. This could bring some insight into the structural organization and the possible biological

functions of this complex. Also, it would be tempting to speculate on the existence of such a complex in other plant species.

## CHAPTER 3

### ISOLATION OF AN OSMOTIN-LIKE PROTEIN GENE FROM STRAWBERRY AND ANALYSIS OF THE RESPONSE OF THIS GENE TO ABIOTIC STRESSES\*

#### 3.1 Introduction

Plants respond to pathogen invasions or severe environmental stresses by the activation of several different mechanisms. One of the most important mechanisms is the accumulation of pathogenesis-related (PR) proteins. Osmotin and osmotin-like proteins (OLPs) belong to the thaumatin-like protein (TLP) or PR-5 family (van Loon, 1999). Osmotin was originally identified as the predominant protein accumulated in NaCl-adapted tobacco cell cultures (Singh et al., 1985). Many studies have demonstrated that the expression of OLPs can be activated by microbial infections and by a variety of abiotic stress factors (reviewed in Velazhahan et al., 1999).

Although the biological functions of OLPs have not yet been fully established, some have been shown to act as antifungal proteins *in vitro*. For example, tobacco osmotin was reported to cause spore lysis and growth inhibition of *Phytophthora infestans* (Abad et al., 1996). Grape osmotin exhibited inhibition of the hyphal growth of *Botrytis cinerea* (Salzman et al., 1998). Moreover, studies have demonstrated that over-expression of PR-5 proteins in transgenic plants conferred enhanced resistance to pathogens (reviewed in Velazhahan et al., 1999; Velazhahan and Muthukrishnan 2003). However, the molecular mechanism that accounts for the antifungal activity of PR-5 proteins is still not clear. A mechanism involving membrane permeabilization was

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proposed by Abad et al. (1996). Another study conducted by Yun et al. (1998) demonstrated that osmotin could subvert target cell signal transduction pathway to increase its cytotoxic efficacy. A more recent study showed that tobacco osmotin induced apoptosis in *Saccharomyces cerevisiae*, which was correlated with intracellular accumulation of reactive oxygen species and was mediated via the RAS2/cAMP pathway (Narasimhan et al., 2001).

In addition to their antifungal activities, OLPs have also been indicated in other developmental and physiological functions, including roles in flower formation and fruit ripening (Neale et al., 1990; Salzman et al., 1998), protections against osmotic stress (Zhu et al., 1995a) and antifreeze activities (Hon et al. 1995).

Strawberry is a member of the *Rosaceae* family, which is a large plant family with approximately 3,000 species grouped into approximately 100 genera (Baumgardt, 1982). This plant family also includes, for example, roses, peaches, blueberries, pears and raspberries. Thus far, relatively few studies have been reported on PR protein genes or PR proteins of the *Rosaceae* family members. No studies have been reported on the expression of any PR genes in response to any abiotic stresses in strawberry plants. In fact, to our knowledge, only two reports have been published thus far on the effect of abiotic stresses on PR genes in the *Rosaceae* family -- one on a TLP gene in apple plants and the other on two TLPs gene and one chitinase gene in peach plants (Ruperti et al., 2002; Kim et al., 2003). In a previous study conducted by Dr. Shih's laboratory, the first OLP gene was isolated from strawberry (Wu et al. 2001). Here, I report the cloning and characterization of a second strawberry OLP gene. Moreover, I report the response of this OLP gene to abscisic acid (ABA) and salicylic acid (SA), which are the signal molecules

implicated in plant response to osmotic-related stresses and pathogenic invasion, respectively (Skriver and Mundy, 1990; Gaffney et al., 1993). The expression of this OLP upon mechanical wounding was also investigated, since wounding is a common damage occurring to plants as a result of abiotic or biotic stresses.

## **3.2 Materials and Methods**

### **3.2.1 Plant Materials**

Dormant strawberry (*Fragaria ananassa* Duchesne) plantlets were purchased from Nourse farms (Deerfield, MA). The plantlets were planted into 9 cm<sup>2</sup> containers (Kord, Ontario, Canada) that contained a soil mix [bark, peat moss, and perlite (7:2:1, v/v/v)] mixed with dolomitic lime (4.7 kg m<sup>-3</sup>). Approximately 5 g of Osmocote-plus fertilizer (15-9-12; Scotts-Sierra, Marysville, OH) was spread on top of each container. The plants were grown in Percival growth chambers (Percival Scientific, Boone, IO, USA, Model AR-60L) at 26/18°C (day/night) and an 11-h photoperiod. General Electric (T32T8SP41) lamps were used delivering irradiance of 8 W m<sup>-2</sup>. The relative humidity was kept at 60% to 70%. The plants were watered with distilled water approximately every other day. For abiotic stress study, the plants were treated with abiotic stimuli about 10-14 days after planting. For analysis of gene expression in different organs, samples were collected from leaves, roots and crowns of strawberry plants one month after planting. In addition, samples were collected from green fruits of plants 5 month after planting and from ripe red fruits about two week later.

### **3.2.2 Isolation of Genomic DNA and Total RNA**

Total nucleic acids were extracted from various strawberry organs according to the method described by Manning (1991). Organs were ground in liquid nitrogen into

very fine powders using a chilled mortar and pestle. Approximately 100 mg of the ground organs was transferred with a chilled spatula to a 2 mL microcentrifuge tube containing 0.7 mL of extraction buffer (0.2 M boric acid, 10 mM Na<sub>2</sub>EDTA, pH 7.6, 0.5% SDS, and 280 mM  $\beta$ -mercaptoethanol [SDS and  $\beta$ -mercaptoethanol were added to the extraction buffer immediately before use]). To this mixture, 0.7 mL of phenol:chloroform:isoamyl alcohol (24:4:1) equilibrated with extraction buffer without SDS and  $\beta$ -mercaptoethanol was added. The mixture was shaken vigorously for 5 min and centrifuged in a microcentrifuge at maximum speed (15,000 g) for 5 min. The aqueous phase was withdrawn and distributed into two 2 mL tubes, 275  $\mu$ L each. Two volumes, 550  $\mu$ L, of RNase-free water was added to each tube and the Na<sup>+</sup> concentration was raised to 80 mM by adding 72  $\mu$ L of 1 M Na-acetate, pH 4.5. This mixture was mixed briefly and 0.4 volume, 359  $\mu$ L, of 2-butoxyethanol (2-BE) was added and mixed thoroughly by inversion (4-6 times). Carbohydrates were removed by incubating the solution on ice for 30 min followed by centrifugation at 15,000 g for 5 min. The supernatant was transferred to a new tube and 538  $\mu$ L of 2-BE was added. The mixtures were mixed thoroughly by inversion (4-6 times) and incubated on ice for 30 min to precipitate total nucleic acids. Nucleic acids were pelleted by centrifugation at 15,000 g in a microcentrifuge for 10 min. The pellet was washed successively once with 1:1 of cold extraction buffer (without SDS and  $\beta$ -mercaptoethanol):2-BE, once with 75% ethanol containing 0.1 M KCl, and once with absolute ethanol. The total nucleic acid pellet was dried for approximately 5 min.

For genomic DNA preparation, the pellet was dissolved in 20  $\mu$ L of sterile water. RNA was removed from the total nucleic acid sample by treating with RNase A at a final concentration of 0.1 mg/mL, at 37°C for 2 h. The DNA was subsequently extracted once

with phenol:chloroform:IAA and once with chloroform:IAA. The DNA was precipitated and dissolved in distilled-deionized water (ddH<sub>2</sub>O) and analyzed on a 1% agarose gel.

For total RNA isolation, the pellet was dissolved in RNA-Secure (Ambion, Austin, TX) and incubated at 60°C for 10 min to inactivate any contaminating RNases. The RNA samples were then purified with RNeasy kit (QIAGEN, Valencia, CA), and treated with DNase I by using the DNA-free kit (Ambion) following the manufacturer's instructions.

### **3.2.3 Cloning of the Osmotin-Like Protein Gene**

According to the sequences of two conserved regions of OLPs, a set of degenerate primers was designed and synthesized (primer set 1, Table 1). Polymerase chain reaction (PCR) was performed in a 25 µL reaction mixture containing 20 ng of strawberry leaf DNA, 400 µM of each dNTP, 3 mM of MgCl<sub>2</sub>, 400 nM of each primer, 1 x PCR buffer, and 5 units of Taq DNA polymerase (Promega, Madison, WI). A touchdown PCR program as following was used: 3 min at 94°C, 10 cycles with 1 min at 94°C, 1 min at 60°C (decreasing 1 °C/cycle), 1 min at 72°C, followed by 30 cycles with similar parameters except that the annealing temperature was kept constant at 50°C, followed by a final step of 10 min at 72°C. A 380-bp DNA fragment was obtained and cloned into pGEM T-Easy vector (Promega) and sequenced. Nucleotide sequence analysis was performed by the Gene and Probe Laboratory of the School of Veterinary Medicine, Louisiana State University.

Based on the sequence of the 380-bp fragment, nucleotide sequences from both the upstream and downstream regions of the 380-bp fragment were obtained by applying a genomic walking procedure using the Universal Genome Walker kit (Clontech, Palo Alto, CA). These additional sequences allowed the design of a final set of primers

(primer set 2, Table 1), which allowed the amplification of a DNA fragment containing the complete coding region of an OLP gene. The amplification conditions were the same as described above for obtaining the 380-bp fragment. The amplification product, designated FaOLP2, was then cloned into pGEM T-Easy vector. The recombinant plasmid was designated as pFaOLP2. Both DNA strands of the insert were sequenced.

#### **3.2.4 Sequence Analysis**

Potential signal peptide cleavage site was identified using SignalP 3.0 (Bendtsen et al., 2004). Various transcription regulatory elements were identified using the PLACE database (Higo et al. 1999).

#### **3.2.5 Genomic DNA Hybridization Analysis**

Strawberry leaf DNA (approximately 15 µg) was digested with *EcoRI*, *EcoRV*, or *NcoI*, and the digested samples were subjected to electrophoresis on a 0.8% agarose gel. The separated DNA fragments were transferred to a Zeta-probe-GT nylon membrane (BioRad, Hercules, CA) by downward capillary alkaline transfer. The DNA fragments were cross-linked to the membrane by UV-irradiation and pre-hybridized with DIG Easy Hyb solution (0.1 mL/cm<sup>2</sup> of membrane; Roche Applied Science, Indianapolis, IN) containing 200 µg of fragmented salmon sperm DNA for 1 h at 45°C. Hybridization with a DIG-labeled probe was carried out at 45°C overnight. The blot was washed twice with low stringency wash buffer (2 x SSC, 0.1% SDS) for 5 min each at room temperature, and twice with high stringency buffer (0.1 x SSC, 0.1% SDS) for 30 min each at 65°C. The *FaOLP2* was detected by using the DIG High Prime DNA Labeling and Detection Starter Kit I (Roche Applied Science) following the manufacturer's protocol.

The DNA probe was labeled with digoxigenin-11-dUTP by random priming, using the DIG High Prime DNA Labeling and Detection Starter Kit I according to the manufacturer's recommendations. The DNA probe for the random priming reaction was synthesized using PCR with primer set 3 (Table 1) and pFaOLP2 as the template. The resulting PCR product spans the first 196 bp of *FaOLP2*.

### **3.2.6 Hormone Treatments and Mechanical Wounding**

For ABA and SA treatments, plants were sprayed to run-off, respectively, with 100  $\mu$ M ABA and 5 mM SA (Sigma, St Louis, MO), both of which were dissolved in 0.01% (v/v) Tween 20. Control plants were sprayed with 0.01% (v/v) Tween 20. For the mechanical wounding treatment, all the fully extended leaves were punctured 6 times along the midvein using a sterile needle (22 G x 1 in, PrecisionGlide needle, Becton Dickinson, Rutherford, NJ). Three plants of each treatment were collected at 2, 6, 12, 24, 48 and 72 h post-treatment. RNA samples were immediately extracted and stored at -80°C. The experiment was repeated once.

### **3.2.7 Relative Quantification by Real-Time PCR**

The expression levels of *FaOLP2* in different strawberry organs as well as in leaf samples collected at different time points post-abiotic treatments were determined by real-time PCR using a Perkin-Elmer 7000 thermal cycler. Strawberry glyceraldehyde-3-phosphate dehydrogenase gene (*FaGAPDH2*) (Khan and Shih 2004), a housekeeping gene, was used as the reference gene. The primers and TaqMan MGB probes for *FaGAPDH2* and *FaOLP2* were designed using the Sequence Detection System software, and are shown as primer sets 4 and 5 in Table 1, respectively. The primers were

synthesized by the Gene and Probe Laboratory. The probes were obtained from Applied Biosystems (Foster City, CA).

Using one-step RT-PCR master mix reagents (Applied Biosystems), 50  $\mu$ L reaction mixtures were set up in a 96-well plate. For *FaGAPDH2*, the primers were used at a final concentration of 500 nM, and the TaqMan MGB probe was used at a final concentration of 250 nM. For *FaOLP2*, the primers were used at a final concentration of 900 nM, and the TaqMan MGB probe was used at a final concentration of 50 nM. For each plant sample, 100 ng of total RNA was analyzed for each gene, and the two genes (*FaGAPDH2* and *FaOLP2*) were always analyzed simultaneously. Each sample was run in triplicate. The thermal cycler conditions recommended by the manufacturer were used with first stage at 48°C for 30 min for reverse transcription, second stage at 95°C for 10 min, and third stage (40 cycles) at 95°C for 15 s followed by 60°C for 1 min.

The comparative  $C_T$  method was used in the quantification analysis, which mathematically transforms the threshold cycle ( $C_T$ ) into the relative expression level of genes (Perkin-Elmer User Bulletin 2). When comparing the expression of *FaOLP2* in different strawberry organs, the relative quantification of the expression of *FaOLP2* was achieved by calibrating its expression level to that of the reference gene, *FaGAPDH2*, which was arbitrarily set as 1. When analyzing the expression of *FaOLP2* at different time points post-abiotic treatments, the expression levels of *FaOLP2* in treated plants were compared with those of the control plants to obtain the induction level of *FaOLP2*.

Table 3.1 List of primers and TaqMan probes used in different experiments

1	Forward	TGY CAR ACN GGN GAY TGY GG
	Reverse	AAN GTN SWN GTN GGR TCR TC
2	Forward	CAC AAA CTC ACT CAT AAT CTT AGG
	Reverse	GCT CCC AAG AAC TCA AAC TGC AAT TT
3	Forward	ATG AGC ATC CAC AAA AGC TTC T
	Reverse	TGC CCG CAT TCA CGT CTA G
4	Forward	CAG ACT TGA GAA GAA GGC CAC CTA
	Reverse	GAT ACC CTT CAT CTT TCC CTC AGA
	Probe	ATC AAG GCT GCT ATC AAG
5	Forward	CGG CGG CAG ACA GCT TAA
	Reverse	TGC CCG CAT TCA CGT CTA G
	Probe	CAG GGA GAA TCT TGG

### 3.3 Results

#### 3.3.1 Cloning and Sequence Analysis of *FaOLP2*

A strawberry gene encoding an OLP, designated *FaOLP2*, was isolated using a PCR-based, genome walking procedure. The sequence data of *FaOLP2* have been deposited in GenBank under accession number DQ325524. As shown in Fig 3.1, *FaOLP2* is 1442 bp in length, and it has an open reading frame (ORF) of 690 nucleotides with a 658-nucleotide upstream sequence and a 94-nucleotide downstream sequence. Sequence comparison showed that *FaOLP2* and *FaOLP1* share no significant homology in the 5' and 3' flanking region.

```

1  CACAAACTCACTCATAATCTTAGGCCAACATGCAATTGTCATATGATCGATGGTCCAAAT
61  ATATATGGTTGTCGTATATATAAACTCTTATAGCTATGTTGGCTTTTACTGCTAACCTCA
121 AATACTTGAGCTAATAACATTTACCGTGCAGCCTTGGTGGAGAAAATATTAAGAGTTCA
181 ACCTAACGTGAAGAGGCTCTATCTTCTTTTGAGAGCTCCTGATGCCAACTATATGGTAGA
241 ATCGCCAACAAGCCAGTAGAATGATAAACATATATAAAAAAGAATATCCTAGCTATTTTC
301 CTCCAGATGCCATGGACATGAACTGCAATTTACGTTTATACTGACAATTTTCATTGAGACTA
361 TTCTTCAACGTGAACCGGACGACATTCACCTCCCCACAACCTTCTTCCACTAATTACGCAC
421 TAATTATTTCTGGAAACCGTCCAATCTTATTGTCTTTGTGGAAAATGAACATATGGAAAT
481 TTCCAGGAGTTAAGAAAGAGATGTCATAGCGATCGGGAAGACGAAAATGTCGAATTTTTT
541 TGCATGCTTGTGTCTTGTATTGTTATGCCAATAGCTTATTTTGTACCCTATATAAACT
601 CATCAAATTCCTCAGAATTCAGACACAATCACACATCGAAACCAATATTTTACAAAAT
                                     M 1
661 GAGCATCCACAAAAGCTTCTCAGTTTTGTCCATCCTATGGATCACCGCCCTTTGCTTTGC
   S I H K S F S V L S I L W I T A L C F A 21
721 TCCATCAACCATCAGTGCAGCAGGATTTCGATATCCGAAACAACCTGCCCTTCCCTGTCTG
   P S T I S A A R F D I R N N C P F P V W 41
781 GGCCGCCGAGTCCAGGCGGCGGACAGCTTAACCAGGGAGAATCTTGGCCCTTAGA
   A A A V P G G G R Q L N Q G E S W P L D 61
841 CGTGAATGCGGGCACTACAGGTGGTGCAGTGTGGGCACGAACCGGATGCAACTTTGATGC
   V N A G T T G G R V W A R T G C N F D A 81
901 TTCCGGACATGGCAGCTGTCAGACCGGTGACTGCGGCGGCCTCCTTGAGTGCCAAGCCTA
   S G H G S C Q T G D C G G L L E C Q A Y 101
961 CGGTCAACCACCAAACACCCTAGCCGAATACGGGCTTAACCAGTTCAACAATTTGGATTT
   G Q P P N T L A E Y G L N Q F N N L D F 121
1021 CATCGACATCTCTCTCGTTGACGGGTTCAATGTGGCTATGGACTTTAGTCCTACTTCTCC
   I D I S L V D G F N V A M D F S P T S P 141
1081 TTGCGACCGTGGGATTTCAGTGCACGGCGGATATCAACGGGCAGTGCCCTAATGAGCTGAA
   C D R G G I Q C T A D I N G Q C P N E L K 161
1141 AGCCCCGGGTGGCTGTAACAATCCCTGTACCGTGTACAAGACTGATGAGTACTGCTGCAA
   A P G G C N N P C T V Y K T D E Y C C N 181
1201 TTCTGGGAGCTGTCAACCCACGGATTTGTCTAGGTTTTTCAAGCAGCTGTGCCCGGATGC
   S G S C Q P T D L S R F F K Q L C P D A 201
1261 TTACAGTTACCCTAAGGATGATAACAACAGCACTGTGGTTTTTACTTGCCCCGGCGGGAC
   Y S Y P K D D T T S T V V F T C P G G T 221
1321 TAACTATAGGGTTGTGTTCTGCCCATAGATCTTCGCTATATATAGGTAGTAAAATTACAA
   N Y R V V F C P * 229
1381 CAATAACTAAGAACAAGATGTTTGGGATCGAATAATAAATTGCAGTTTGTAGTTCTTGGGA
1441 GC

```

Fig. 3.1. Nucleotide sequence and deduced amino acid sequence of *FaOLP2*. Numbers on the left represent nucleotide positions. Numbers on the right represent amino acid positions. Transcriptional regulatory signals are underlined. Stress-related *cis*-elements are double underlined. The predicted N-terminal signal sequence is shown by bold letters.

The ORF of *FaOLP2* starts at nucleotide position 659 and ends at position 1348.

Computational analysis revealed three potential TATA box sequences within the upstream region of *FaOLP2*, at positions 77, 271, and 591. Another conserved eukaryotic promoter element, the CAAT box, was also observed at positions 33, 326, 344, 442, 570,

627 and 644. A canonical plant polyadenylation signal, AATAAT, was present at position 1411.

Several stress-related *cis*-elements were also identified in the 5' upstream region of *FaOLP2*. One MYB-like recognition site (WAACCA, position 640) and four MYC-like recognition sites (CANNTG, positions 33, 40, 304 and 470) were identified. Protein members of both MYB and MYC families have been shown to play important roles in the plant's response to pathogens, low temperatures and dehydration. In *Arabidopsis*, both the AtMYB2 and AtMYC2 proteins function as transcription activators in ABA signaling (Abe et al., 2003). A T/G-box motif (AACGTG) was observed at positions 185 and 367, which is involved in induction of genes by jasmonates (Boter et al., 2004). Jasmonates are well-known regulators of plant defense responses to wounding. A gibberellin-responsive element (TAACGTA) was identified at position 331 in reverse direction; gibberellin regulates plant growth and development (Sutoh and Yamauchi 2003).

The coding region of *FaOLP2* contains no intron. It encodes a precursor protein of 229 amino acid residues with a predicted signal peptide of 27 amino acid residues at the N-terminus. The calculated molecular mass of the mature protein is approximately 21.6 kDa with a predicted isoelectric point of 4.24.

A GenBank Blastp search revealed that *FaOLP2* shares 71% identity and 81% similarity with *FaOLP1* (AAF13707). However, *FaOLP2* has the highest sequence homology to the soybean PR-5 protein p21 (P25096) with 78% identity and 84% similarity. It also shares a high degree of homology with tobacco osmotin (P14170) (62% identity and 77% similarity), tobacco PR-5d protein (2981950) (62% identity and 75% similarity) and maize zeamatin (P33679) (65% identity and 74% similarity). Fig. 3.2

shows a sequence alignment of FaOLP2, FaOLP1, zeamatin, PR-5d and osmotin. The five amino acid residues reported to be crucial to the antifungal activity of the latter three proteins are also conserved in both FaOLP2 and FaOLP1, as indicated by arrows in Fig. 3.2. In contrast, FaOLP2 shares lower degrees of homology to known PR-5 proteins of other rose family members. For example, the homology scores are 39% identity and 51% similarity for a peach TLP (AAM00216; Ruperti et al., 2002), and 38% identity and 49% similarity for a sweet cherry TLP (AAB38064; Fils-Lycaon et al., 1996).

### **3.3.2 DNA Hybridization Analysis of *FaOLP2* Gene**

The copy number of *FaOLP2* gene was examined using genomic DNA hybridization analysis. Fig. 3.3 shows the hybridization patterns of strawberry DNA digested with three different restriction enzymes. Multiple hybridization bands were observed with each digest, indicating that a multi-gene family encodes FaOLP2 or related proteins. The complete absence of any hybridization band in the negative control (lane 5) demonstrated the specificity of the probe toward the intended gene

### **3.3.3 Differential Expressions of *FaOLP2* in Different Strawberry Organs**

The expression patterns of *FaOLP2* in different strawberry organs were examined using real-time PCR analysis. From the results shown in Fig. 3.4, it is clear that *FaOLP2* is constitutively expressed in leaves, roots, crowns, green fruits and ripe red fruits. Among these organs, relatively higher levels of *FaOLP2* mRNA were observed in crowns and leaves. A moderate level of *FaOLP2* mRNA was observed in roots. In contrast, the expression of *FaOLP2* was barely detectable in green fruits; however, its expression increased approximately 2 fold in ripe red fruits.



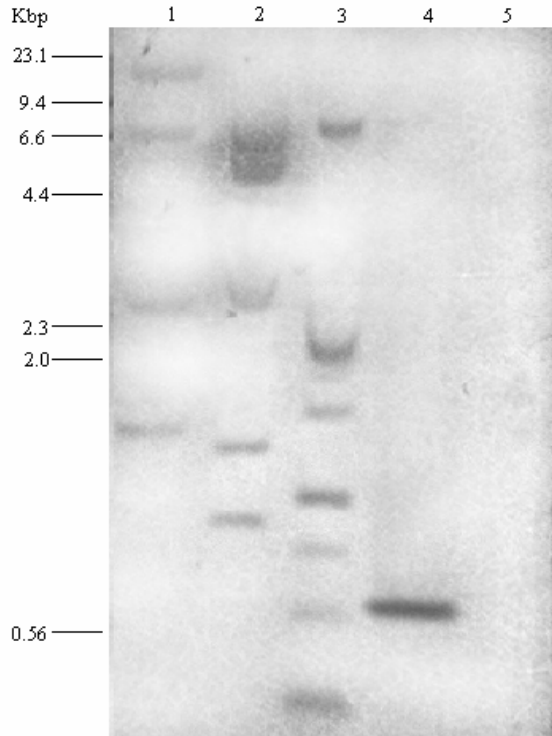


Fig. 3.3. DNA hybridization analysis of *FaOLP2*. Lanes 1-3 show restriction digests of genomic DNA with *Nco*I, *Eco*RI, and *Eco*RV, respectively. Lane 4 shows 100 pg of *FaOLP2* PCR product as the positive control. Lane 5 shows 100 pg of the PCR product from *FaChi2-1* (a strawberry chitinase gene) as the negative control. Molecular weight markers are shown on the left.

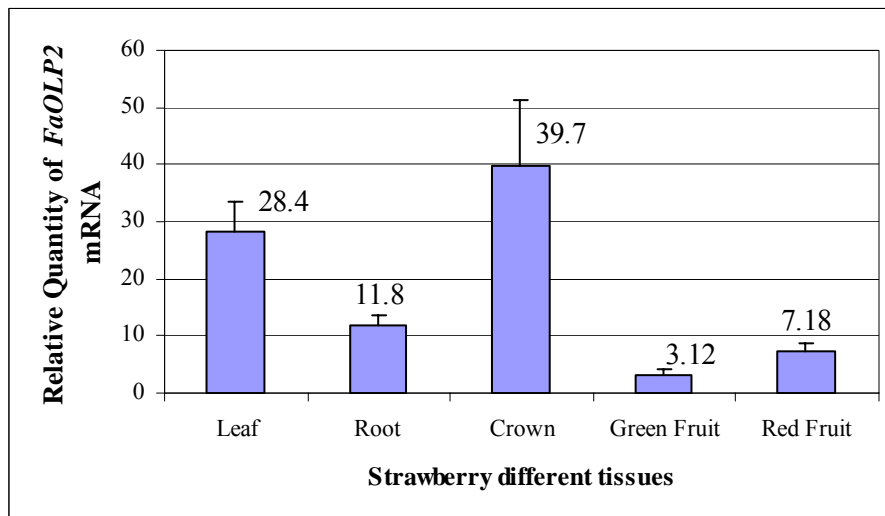
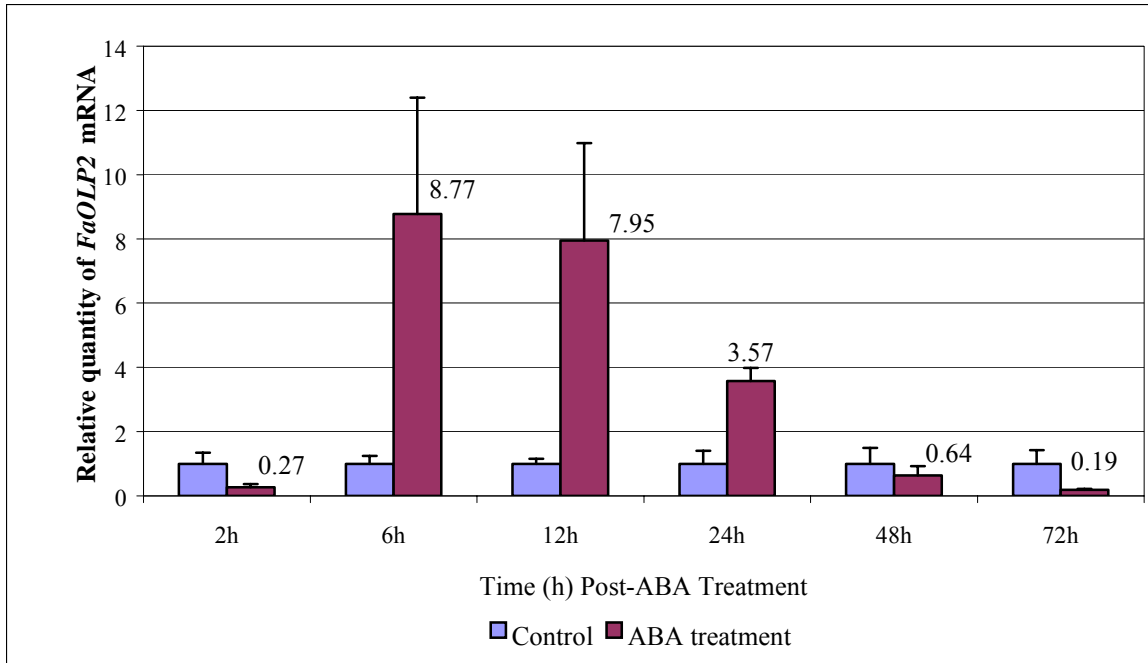


Fig. 3.4. Relative quantities of *FaOLP2* mRNA in different strawberry organs. Each organ sample was individually assayed in triplicate. Values shown represent the mean reading from three plants and the error bars indicate the standard errors of the means.

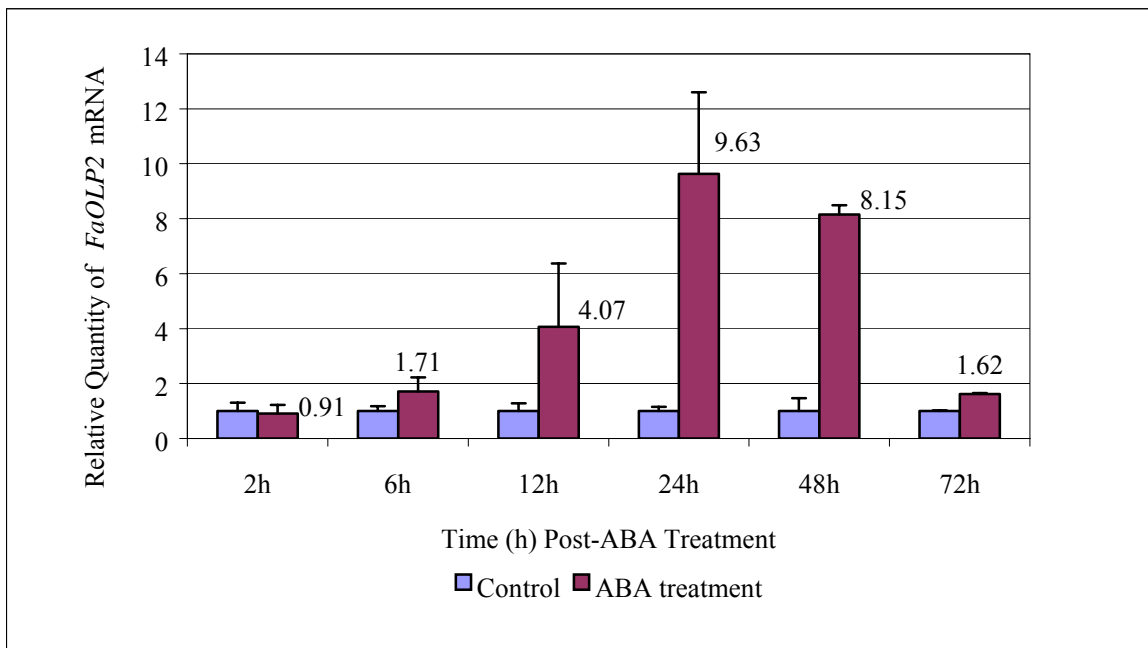
### 3.3.4 Effects of ABA, SA and Mechanical Wounding on Expression of *FaOLP2*

To study the response of *FaOLP2* to abiotic stresses, strawberry plants were treated with three different abiotic stimuli. The expression patterns of *FaOLP2* at different time points after treatments were analyzed using real-time PCR. Fig. 3.5A shows the induction pattern of *FaOLP2* by ABA treatment at various time points as compared to control plants in the first induction experiment. It appeared that *FaOLP2* was down-regulated by ABA at the early stage after treatment. At 2 h post-treatment, the expression of the gene was only 0.27-fold of the background level. The expression level quickly increased to 8.77-fold at 6 h, and maintained at approximately the same level (7.95-fold) at 12 h. However, *FaOLP2* expression decreased rapidly after the 12 h time point, 3.57-fold at 24 h and lower than the background level at 48 and 72 h (0.64-fold and 0.19-fold, respectively). In the second trial, *FaOLP2* exhibited a slower response to ABA (Fig 3.5B). The gene was slightly induced at 6 h, only 1.7-fold higher than the control plants. The expression level then steadily increased, 4.07-fold at 12 h, and peaked at 24 h with 9.63-fold induction. The expression of *FaOLP2* was kept at a similar level at 48 h (8.15-fold), and decreased to 1.62-fold at 72 h post-ABA treatment.

Fig. 3.6A shows the accumulation of *FaOLP2* mRNA in response to SA. Exogenous application of SA appeared to cause a rapid induction of *FaOLP2* gene to 5.61-fold at 2 h post-treatment. The expression level then decreased to 2.47-fold at 6 h, and remained at that level until 12 h (2.19-fold). The expression of *FaOLP2* increased again to 11.14-fold at 24 h and peaked at 48 h (30.53-fold). After that, the expression level decreased, yet still 15.12-fold higher than the background level at 72 h after ABA



A



B

Figure 3.5 Relative quantities of *FaOLP2* mRNA at various time points post-ABA treatment. Each plant was individually assayed in triplicate. Values shown represent the mean reading from three treated plants and the error bars indicate the standard errors of the means. Panel A, data from the first trial; panel B, data from the second trial.

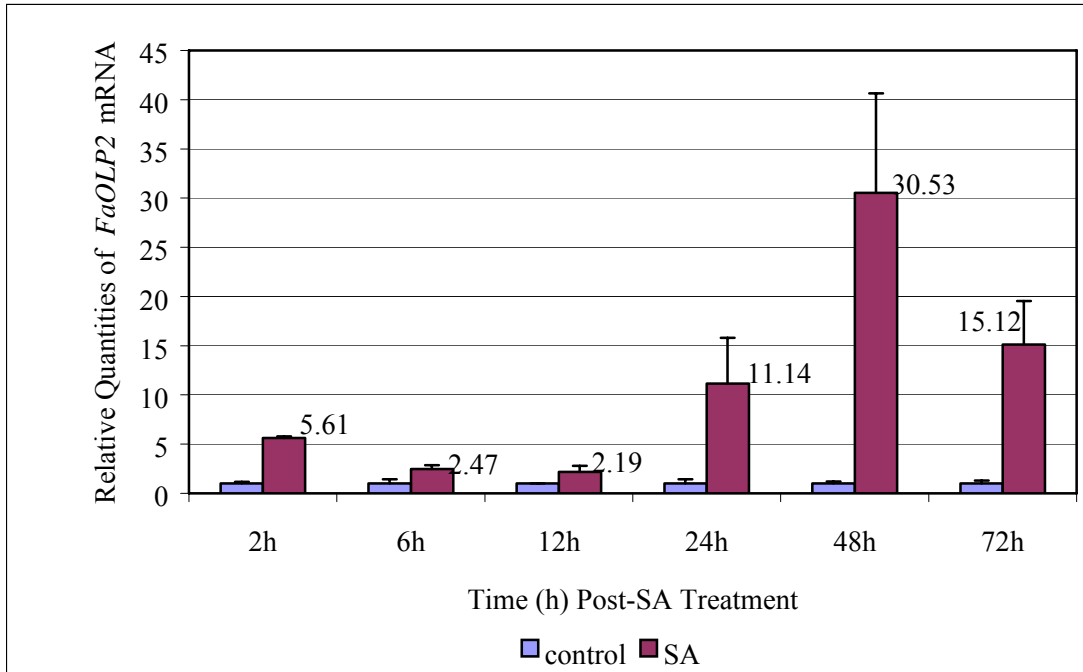
treatment. In the second trial (Fig 3.6B), *FaOLP2* was still induced within 2 h, although to a lower level (2.58-fold) than that in the first trial. The expression of this gene then decreased to background level at 6 h, but increased again to 9.25-fold at 12 h and remained at that level through 24 h. The expression level continued to increase and peaked at 48 h, 32.34-fold higher than the background level. At 72 h, *FaOLP2* was still induced by 20.1-fold.

The expression pattern of *FaOLP2* upon mechanical wounding in the first trial is shown in Fig. 3.7A. It appeared that wounding triggered the response of *FaOLP2* at a very early stage. A significant induction (5.25-fold) of *FaOLP2* was observed at 2 h post-treatment. The expression level continued to increase till the 12 h time point, 6.08-fold at 6 h and 8.83-fold at 12 h. The expression of the gene then decreased steadily, 4.36-fold at 24 h, 3.77-fold at 48 h, and 2.13-fold at 72 h. In the second trial (Fig 3.7B), the induction of *FaOLP2* by wounding within the first 2 h was even more significant, which was 8.26-fold. However, the expression quickly reduced to background level at 6 h and remained at the same level through 12 h. The expression of *FaOLP2* was then increased again, 3.17-fold at 24 h, 4.21-fold at 48 h, and back to the baseline level at 72 h.

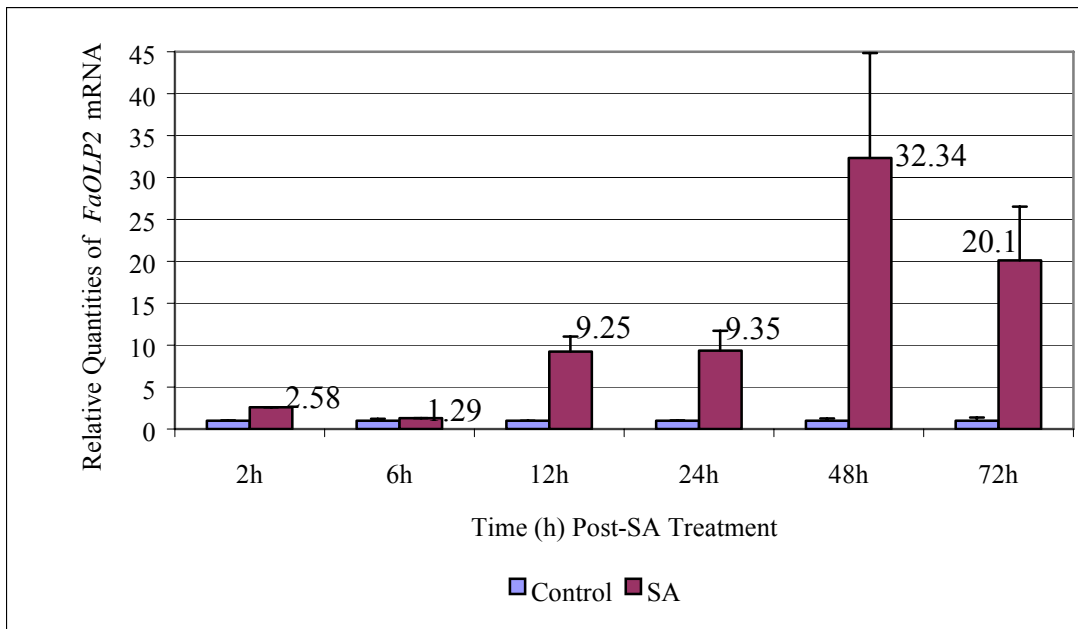
Overall, all of the three tested abiotic stresses appeared to be able to trigger a significant accumulation of *FaOLP2* mRNA within 6 h post-treatment. Moreover, *FaOLP2* was more prominently induced by SA (approximately 30-32 fold) than by ABA or mechanical wounding (approximately 8-9 fold).

### **3.4 Discussion**

In the present chapter, a PCR-based genome walking procedure was used to isolate a strawberry OLP gene, designated *FaOLP2*. Like other PR-5 protein genes,

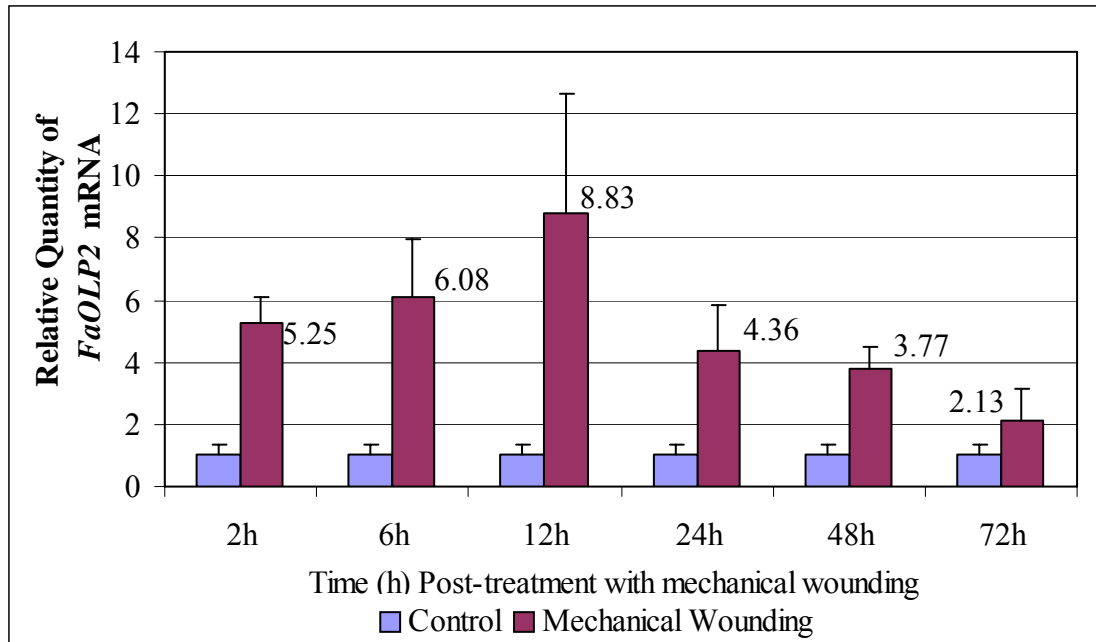


A

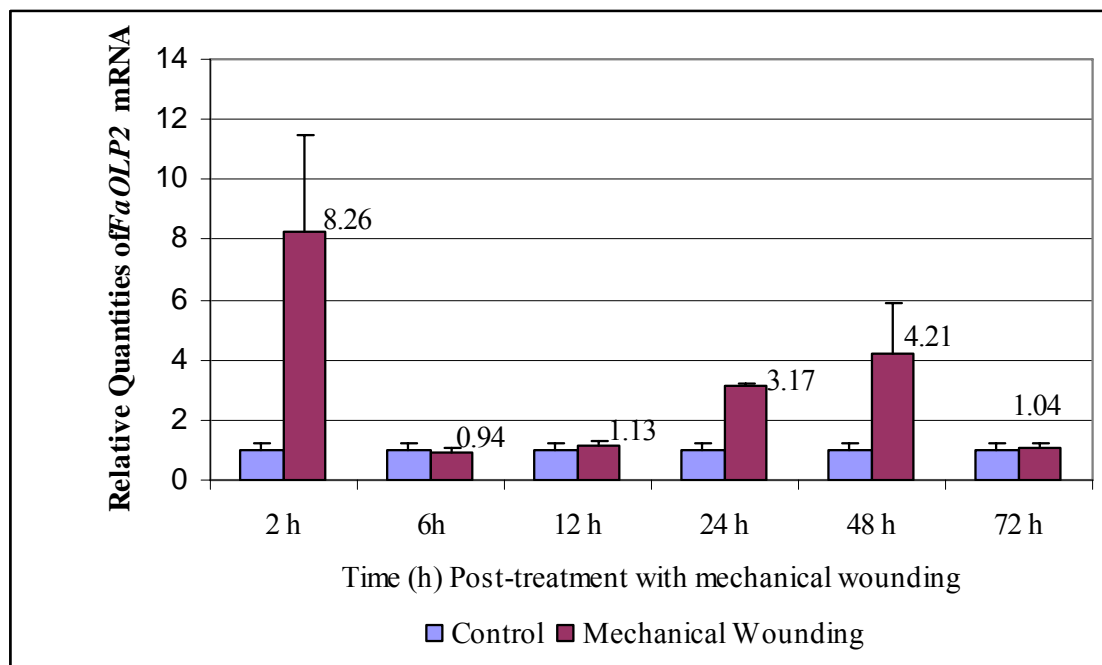


B

Figure 3.6 Relative quantities of *FaOLP2* mRNA at various time points post-SA treatment. Each plant was individually assayed in triplicate. Values shown represent the mean reading from three treated plants and the error bars indicate the standard errors of the means. Panel A, data from the first trial; panel B, data from the second trial.



A



B

Figure 3.7 Relative quantities of *FaOLP2* mRNA at various time points post-wounding treatment. Each plant was individually assayed in triplicate. Values shown represent the mean reading from three treated plants and the error bars indicate the standard errors of the means. Panel A, data from the first trial; panel B, data from the second trial.

*FaOLP2* contains no intron. The only exception so far is a pistil-specific TLP gene from Japanese pear whose coding region is interrupted by an intron of 351 bp (Sassa et al., 2002). *FaOLP2* would encode a mature protein of 21.6 kDa with a pI of 4.24, and it contains a signal sequence at the amino-terminus. Therefore, like most acidic TLPs, *FaOLP2* would be secreted to the extracellular space.

The deduced amino acid sequence of *FaOLP2* shares high degree of homology with many other PR-5 proteins, including zeamatin, tobacco PR-5d and osmotin. The crystal structures of the latter three proteins have been determined, all revealing the presence of a similar acidic cleft that is crucial to their antifungal activities (Batalia et al., 1996; Koiwa et al., 1999; Min et al., 2004). In comparison to the sequences of these three PR-5 proteins, the four amino acid residues forming the acidic cleft -- a Glu residue and three Asp residues -- are well conserved in *FaOLP2* (Fig. 3.2). Moreover, *FaOLP2* also has the sixteen cysteine residues, which form eight disulfide bonds, at locations similar to those of other TLPs. These observations suggest that *FaOLP2* may also possess antifungal activity. Indeed, synthesis of another strawberry OLP, *FaOLP1*, was found to be induced when the strawberry plants were inoculated with either of the two fungal pathogens, *Colletotrichum fragariae* or *Colletotrichum acutatum* (discussed in the following chapter).

Multiple hybridization bands were observed in the genomic DNA blot of *FaOLP2* (Fig 3.3), indicating that *FaOLP2* is present as a multi-gene family consisting of similar or related genes in the strawberry genome. This observation is in agreement with that of *FaOLP1* as well as PR-5 protein genes from several other plant species such as tomato and *Solanum nigrum* (King et al., 1988; Wu et al., 2001; Campos et al., 2002). In contrast,

some PR-5 genes have been shown to be single-copy genes. For example, two PR-5 genes from *Arabidopsis* and the OLP gene from *Benincasa hispida* belong to this category (Capelli et al., 1997; Hu and Reddy 1997; Shih et al., 2001).

The level of constitutive expression of *FaOLP2* appeared to be different among different strawberry organs (Fig 3.4). Higher levels of *FaOLP2* transcripts were detected in crowns and leaves, while relatively lower level was detected in roots. This is in contrast to the usually high levels of expression of PR-5 protein genes in the roots of other plant species presumably due to constant exposure to soil-associated microbes (Dudler et al., 1994). However, since the strawberry plants were grown in the growth chamber, it is possible that the lower expression of *FaOLP2* in the roots is due to fewer microbes present in the growth-chamber soil than in the open field soil. On the other hand, from the stages of green fruits to ripe red fruits, the level of *FaOLP2* transcripts increased approximately 2-fold. Although the expression levels in green and ripe red fruits were relatively low, this increase still suggests that the expression of *FaOLP2* could be developmentally regulated in strawberry fruits. In fact, the accumulation of OLPs has been observed during the fruit ripening of bananas (Clendennen and May, 1997), cherries (Fils-Lycaon et al., 1996), and grapes (Salzman et al., 1998).

Plant OLPs are implicated in plant response to pathogen attacks and adverse environmental stresses. In the present study, I demonstrated that *FaOLP2* was regulated by the signal molecules abscisic acid and salicylic acid at the transcriptional level. It is well known that ABA modulates gene expression under osmotic-related stresses such as freezing, drought, and high salt (Skriver and Mundy, 1990). The exogenous application of ABA triggered an early and significant induction of *FaOLP2* in both experiments. The

induction was observed within 6 h and reached as high as 8-9 fold (Fig. 3.5). This significant enhancement of *FaOLP2* expression in strawberry leaves upon ABA treatment, together with the identification of homologous MYB/MYC recognition sites in the promoter region of *FaOLP2*, suggests that *FaOLP2* could be involved in strawberry's response to osmotic stresses. In fact, in the study conducted by LaRosa et al. (1992), accumulation of tobacco osmotin mRNA was induced by ABA as well as NaCl and water deficit. Moreover, transgenic potato plants carrying the GUS reporter gene fused to the promoter regions of potato OLPs showed enhanced GUS expression in response to ABA and NaCl (Zhu et al., 1995b).

Salicylic acid has been demonstrated to serve as an important signal molecule in plants' local response to pathogens and also in the development of systemic acquired resistance (SAR) (Gaffney et al., 1993). A number of PR protein families involved in SAR have been identified in *Arabidopsis* and tobacco, which include PR-1, PR-2 and PR-5 family members (Uknes et al., 1992; Ward et al., 1991). In our study, exogenous SA greatly induced the expression of *FaOLP2* (Fig. 3.6). In both experiments, the induction level reached to 9-11 fold within 24 h, and continued to increase until 48 h to approximately 30-fold. Although the expression of the gene decreased afterward, at 72 h after treatment, it was still at a considerably higher level than the background level. In apple plants, a TLP gene, *MdTL1a*, was also shown to be induced by SA (Kim et al., 2003). The high induction of *FaOLP2* by SA is comparable to the study conducted by Uknes et al (1992) in which the induction of an *Arabidopsis* PR-5 gene was greater than 20-fold within one day after SA treatment. These observations indicate that *FaOLP2* could be an active member in SAR as well as in plant defense against local pathogen

attack. In future studies, it will be interesting to investigate if *FaOLP2* is induced both locally and systemically upon pathogen invasion.

The induction of *FaOLP2* by mechanical wounding should not be very surprising, since a homologue of a jasmonate-responsive element was identified in the 5' upstream region of *FaOLP2* (Fig. 3.1). Wounding not only physically damages plant tissues, but also provides potential infection sites for pathogens. Therefore, in order to build a barrier against opportunistic microorganisms, defense related genes are expected to be expressed at the wounding site. The induction of PR genes by wounding has been demonstrated in many studies (e.g. Wu and Bradford, 2003; Hong et al., 2004). The induction of *FaOLP2* by wounding was very early and significant; at only 2 h after wounding, the *FaOLP2* expression increased by 5.25-fold in the first trial and by 8.26-fold in the second trial (Fig. 3.7). The early induction of *FaOLP2* by wounding is different from the TLP genes reported in the other two members of the *Rosaceae* family, peaches and apples (Rupert et al., 2002; Kim et al., 2003). In peach plants, a TLP gene, *PpAz8*, was shown first to be down regulated and then underwent a continuous accumulation from 12-48 h after wounding, while a second TLP gene, *PpAz44*, was not significantly induced until 48 h after wounding. On the other hand, the TLP gene from apple plants, *MdTLL1a*, was not induced by wounding within the time period tested (i.e., 3 h after wounding). The response of *FaOLP2* to wounding is consistent with the proposed role of OLP genes in plant defense.

Taken together, these observations suggest that *FaOLP2* may be actively involved in protecting strawberry plants against pathogen invasion and adverse environmental conditions including osmotic stress. Further studies on the evaluation of the regulatory

elements of *FaOLP2* on conferring the observed induction by different abiotic stress, the antifungal activities of this gene and its application in transgenic plants will expand our knowledge of plant defense mechanisms.

## CHAPTER 4

### EXPRESSION OF A STRAWBERRY OSMOTIN-LIKE PROTEIN GENE, *FAOLP1*, IN RESPONSE TO FUNGAL INFECTION

#### 4.1 Introduction

When plants are invaded by pathogens, a number of metabolic changes occur within the plant as a defense response. Initially, a localized resistance reaction, known as the hypersensitive response, takes place to confine the spread of the pathogen. At the same time, in the vicinity of infection, various genes are activated, which results in the production of antimicrobial compounds, the phytoalexins, and various antimicrobial proteins, including pathogenesis-related (PR) proteins. Finally, systemic expression of PR protein genes is also activated, leading to the establishment of systemic acquired resistance (SAR) in the plant.

Osmotin and osmotin-like proteins (OLPs) belong to the thaumatin-like protein (TLP) or PR-5 family (van Loon and van Strien, 1999). The PR-5 proteins from a variety of plant sources have been shown to inhibit fungal growth *in vitro* (e.g., Vigers et al., 1992; Salzman et al., 1998). They caused lysis of fungal spores, inhibition of hyphal growth and/or reduction of spore germination (Woloshuk et al., 1991; Abad, et al., 1996; Koiwa et al., 1997). In addition, transgenic plants constitutively expressing PR-5 proteins have been shown to exhibit enhanced disease resistance (reviewed in Velazhahan et al., 1999; Velazhahan and Muthukrishnan 2003). In this respect, PR-5 proteins must have an important role in plant defense against pathogens.

However, the molecular mechanism that accounts for the antifungal activity of PR-5 proteins is still not clear. It has been proposed to involve interaction with specific

plasma membrane component(s) of the fungal target and/or destabilizing the fungal plasma membrane. Abad et al. (1996) reported that tobacco osmotin caused membrane leakage and dissipated the pH gradient across the cell wall/membrane of sensitive fungal species. Using *Saccharomyces cerevisiae* as a model system, several studies have found that the antifungal activity of PR-5 protein was mediated by the composition of fungal cell wall (Coca et al., 2000; Ibeas et al., 2001). In particular, fungal cell wall phosphomannans were shown to facilitate the toxic activity of PR-5 proteins (Ibeas et al., 2000; Salzman et al., 2004). Surprisingly, in a more recent study, six TLPs from barley, tomato, cherry and tobacco were shown to have  $\beta$ -1,3-glucanase activity (Grenier et al., 2001), which suggest that some TLPs might inhibit fungi growth via direct action on fungal  $\beta$ -1,3-glucans.

In addition to their antifungal activity, OLPs have been implicated in normal developmental processes such as flower formation and fruit ripening (Neale et al., 1990; Salzman et al., 1998). Some OLPs also displayed protections against osmotic stress (Zhu et al., 1995a) or against freezing (Hon et al. 1995).

Strawberry belongs to the *Rosaceae* family, which consists of more than 3000 species (Baumgardt, 1982). This large plant family includes important fruit crops such as apples, pears and raspberries. Thus far, relatively few studies have been reported on PR proteins or PR protein genes of the *Rosaceae* family members. Dr. Shih's laboratory has previously isolated a strawberry osmotin-like protein gene, designated as *FaOLP1* (Wu et al., 2001). Amino acid sequence alignment analysis revealed that the 5 amino acid residues crucial to the antifungal activity of maize zeamatin, tobacco PR-5d protein and tobacco osmotin are all conserved in *FaOLP1*, indicating that *FaOLP1* may posses

antifungal activity. To investigate this possibility, we examined the response of *FaOLPI* to *Colletotrichum fragariae* and *Colletotrichum acutatum*, which can cause the severe strawberry disease, anthracnose crown rot, in the Gulf states region of the United States and in other parts of the world (Smith and Black, 1986).

## **4.2 Materials and Methods**

### **4.2.1 Plant Growth Conditions**

Dormant strawberry plantlets (*Fragaria ananassa* Duchesne) were purchased from Nourse Farms (Deerfield, MA). The plantlets were planted into 9 cm square containers (Kord, Ontario, Canada) that contained a soil mix (bark, peat moss, and perlite (7:2:1, v/v/v)) with dolomitic lime (4.7 kg/m<sup>3</sup>). Approximately 5 g of Osmocote-plus fertilizer (15-9-12; Scotts-Sierra, Marysville, OH) was spread on top of each container. The plants were grown in Percival growth chambers (model AR-60L; Percival Scientific, Boone, IO) at 26/18 °C (day/night) and an 11 h photoperiod. General Electric (T32T8SP41) lamps were used for illumination, delivering irradiance of 8 W/m<sup>2</sup>. The relative humidity was kept at 60–70%. The plants were watered with distilled water approximately every other day, and they were ready for infection experiments 10 days after planting.

### **4.2.2 Isolation of Total RNA from Leaf Tissues**

Total RNAs were extracted from strawberry leaves according to the method described by Manning (1991). Leaves were ground in liquid nitrogen into very fine powders using a chilled mortar and pestle. Approximately 100 mg of the ground leaves was transferred with a chilled spatula to a 2 mL microcentrifuge tube containing 0.7 mL

of extraction buffer (0.2 M boric acid, 10 mM Na<sub>2</sub>EDTA, pH 7.6, 0.5% SDS, and 280 mM β-mercaptoethanol). SDS and β-mercaptoethanol were added to the extraction buffer immediately before use. To this mixture, 0.7 mL of phenol:chloroform:isoamyl alcohol (24:4:1) equilibrated with extraction buffer without SDS and β-mercaptoethanol was added. The mixture was shaken vigorously for 5 min and centrifuged in a microcentrifuge at 15,000 g for 5 min. The aqueous phase was withdrawn and distributed into two 2 mL tubes, 275 μL each. Two volumes, 550 μL, of RNase-free water was added to each tube and the Na<sup>+</sup> concentration was raised to 80 mM by adding 72 μL of 1 M Na-acetate, pH 4.5. This mixture was mixed briefly and 0.4 volume, 359 μL, of 2-butoxyethanol (2-BE) was added and mixed thoroughly by inversion (4-6 times). Carbohydrates were removed by incubating the solution on ice for 30 min followed by centrifugation at 15,000 g for 5 min. The supernatant was transferred to a new tube and 538 μL of 2-BE was added. The mixtures were mixed thoroughly by inversion (4-6 times) and incubated on ice for 30 min to precipitate total nucleic acids. Nucleic acids were pelleted by centrifugation at 15,000 g in a microcentrifuge for 10 min. The pellet was washed successively once with 1:1 of cold extraction buffer (without SDS and β-mercaptoethanol):2-BE, once with 75% ethanol containing 0.1 M KCl, and once with absolute ethanol. After being dried approximately 5 min, the pellet was dissolved in RNA-Secure (Ambion, Austin, TX) solution and incubated at 60°C for 10 min to inactivate any contaminating RNases. The RNA samples were further purified with RNeasy kit (QIAGEN, Valencia, CA), and treated with DNase I by using the DNA-free kit (Ambion) following the manufacturer's instructions.

### **4.2.3 Growth and Preparation of Fungal Inoculums and Plant Inoculation**

In this study, *C. fragariae* isolate CF-75 and *C. acutatum* isolate Goff were used to inoculate the strawberry plants. Fungal cultures were obtained from Dr. Barbara Smith (USDA, Small Fruit Research Station, Poplarville, MS) and maintained on half strength fungal growth media (Smith et al., 1990). To prepare the fungal conidia inoculums, actively growing cultures in Petri dishes were covered with approximately 15 ml of 0.01% Tween 20 per dish. Conidia were dislodged into the liquid by using a round end glass rod, and the suspension was filtered through two layers of cheesecloth. The conidia concentration was determined by a hemocytometer and adjusted to  $1.5 \times 10^6$  conidia/ml. Plants were sprayed to run-off with the conidial suspension. Control plants were sprayed with 0.01% Tween 20. After spraying, plants were immediately transferred to a dew chamber maintained at 28 °C. Three plants of each treatment were harvested at 2, 6, 12, 24, and 48 h post-inoculation. RNA samples were extracted immediately and stored at –80 °C. The infection experiment was repeated once.

### **4.2.4 Relative Quantification by Real-time PCR**

The expression levels of *FaOLPI* (GenBank accession number AF199508) in leaf samples collected at various time points post-inoculation were determined by real-time PCR using a Perkin-Elmer 7700 thermal cycler. Strawberry glyceraldehyde-3-phosphate dehydrogenase gene (*FaGAPDH2*) (Khan and Shih 2004), a housekeeping gene, was used as the reference gene. The primers and TaqMan MGB probes for *FaGAPDH2* and *FaOLPI* were designed using the Sequence Detection System software, and are shown below.

*FaGAPDH2*

Forward primer: 5'-CAG ACT TGA GAA GAA GGC CAC CTA-3'

Reverse primer: 5'-GAT ACC CTT CAT CTT TCC CTC AGA-3'

Probe: ATC AAG GCT GCT ATC AAG

*FaOLP1*

Forward primer: 5'-CCA GGC GGT GGC AAA C-3'

Reverse primer: 5'-GCT GCC ACA TTT ATC GTC CAT-3'

Probe: CTC GGC ACC GGC CA

The primers were synthesized by the Gene and Probe Laboratory of the School of Veterinary medicine, Louisiana State University. The probes were obtained from Applied Biosystems (Foster City, CA).

Using one-step RT-PCR master mix reagents (Applied Biosystems, Foster City, CA), 50  $\mu$ l reaction mixtures were setup in a 96-well plate. For both genes, the primers were used at a final concentration of 500 nM, and the TaqMan MGB probe was used at a final concentration of 250 nM. For each plant sample, 200 ng of total RNA was analyzed for each gene (*FaGAPDH2* and *FaOLP1*) and the two genes were always analyzed simultaneously. Each sample was run in triplicate. The thermal cycler conditions recommended by the manufacturer were used with first stage at 48 °C for 30 min for reverse transcription, second stage at 95 °C for 10 min, and third stage (40 cycles) at 95 °C for 15 s followed by 60 °C for 1 min.

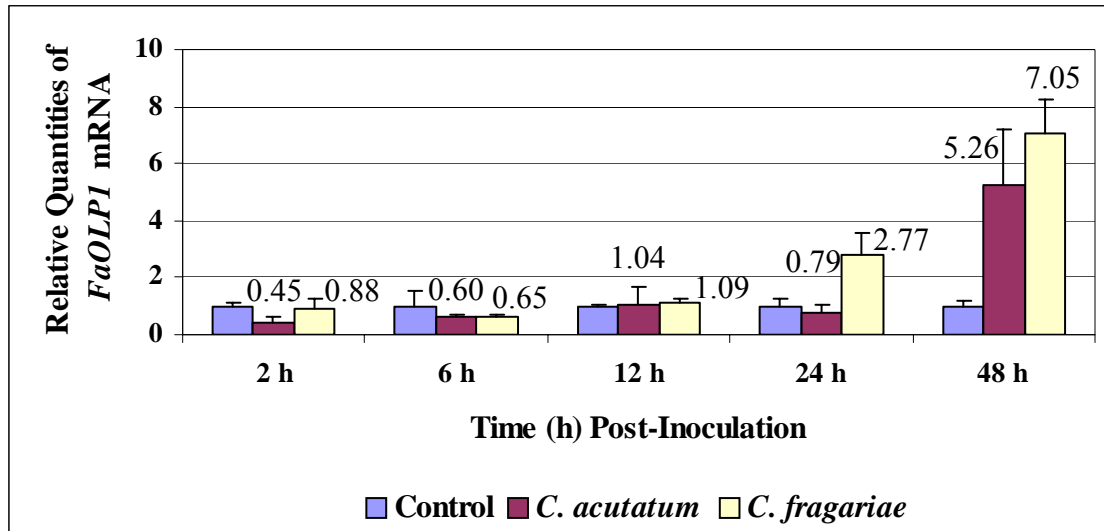
The comparative  $C_T$  method was used in the quantification analysis, which mathematically transforms the threshold cycle ( $C_T$ ) into the relative expression level of genes (Perkin-Elmer User Bulletin 2). A relative quantification of the expression of

*FaOLP1* was achieved by calibration with the expression of the reference gene, *FaGAPDH2*. The calibrated *FaOLP1* expression levels in infected plants at different time points post-inoculation were then compared with that of the respective control plant which was arbitrarily set as 1 to obtain the induction level of *FaOLP1*.

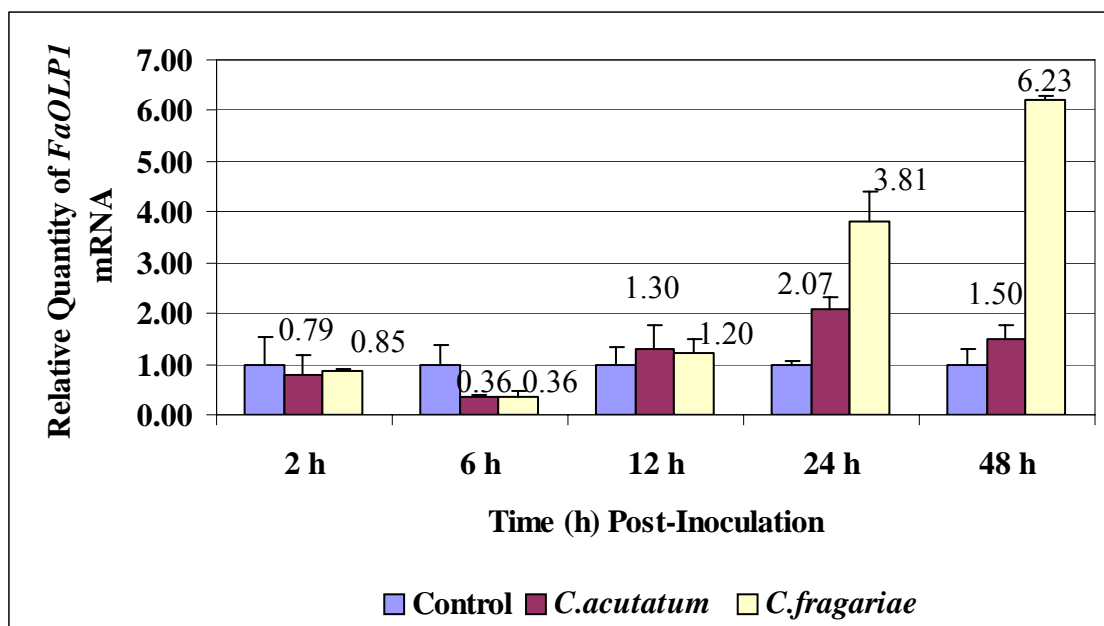
### 4.3 Results

The expression patterns of *FaOLP1* in response to *C. acutatum* and *C. fragariae* in the first infection experiment are shown in Fig. 4.1A. For strawberry plants inoculated with *C. acutatum*, no increase in the expression level of *FaOLP1* was observed during the first 24 h after infection. The expression level then quickly increased to 5.26-fold at 48 h (Fig. 4.1A). Similarly, it appeared that *FaOLP1* did not respond to *C. fragariae* at the early stage of infection either. The expression of *FaOLP1* remained approximately at the background level up to 12 h. The expression then increased to 2.77 fold at 24 h and 7.05-fold at 48 h.

In the second infection study, *C. acutatum* caused a quicker response of *FaOLP1*; however the induction level was lower than that in the first trial (Fig. 4.1B). Slight induction of 1.30-fold was evident at 12 h post-inoculation. The induction increased to 2.07-fold at 24 h, but it decreased to 1.5-fold at 48 h. On the other hand, the induction pattern of *FaOLP1* by *C. fragariae* was essentially the same as that in the first trial. The expression level was not increased up to 12 h. The expression of *FaOLP1* was induced to 3.81-fold at 24 h and 6.23-fold at 48 h.



A



B

Figure 4.1 Relative quantities of *FaOLP2* mRNA at various time points after inoculation with *C. acutatum* or *C. fragariae*. Results from two separate experiments are shown in panels A and B. Each plant was individually assayed in triplicate. Values shown represent the mean reading from three treated plants and the error bars indicate the standard errors of the means.

## 4.4 Discussion

Many studies have demonstrated that the expression of OLPs can be induced by microbial infection (reviewed in Velazhahan et al., 1999), but relatively few studies have been reported on the temporal expression pattern of OLPs upon pathogen infection. In the present study, I examined the expression pattern of *FaOLP1* within the time frame of 48 h after inoculation. Samples were not collected beyond the 48 h time point, since some plants were severely diseased after 48 h, especially plants inoculated with *C. fragariae*. The results showed that both of the tested pathogenic fungi, *C. acutatum* and *C. fragariae*, were able to trigger the induction of *FaOLP1*, and that noticeable induction did not occur until 24-48 h post-inoculation (Fig. 4.1). In addition, it appeared that the induction of *FaOLP1* caused by *C. fragariae* was higher than that induced by *C. acutatum*. The positive response of *FaOLP1* to the two *Colletotrichum* species indicated that *FaOLP1* could be involved in strawberry defense reactions.

Similar timing of induction of OLPs by pathogen attacks has been reported in a number of studies. In oat seedlings infected with stem rust fungus (*Puccinia graminis*), four distinct OLP genes were induced: two were induced between 24-30 h post-inoculation, and the other two between 42-48 h post-inoculation (Lin et al., 1996 and 1998). Similarly, infection of rice plants with the sheath blight fungus resulted in the accumulation of two TLP mRNAs within 1 to 2 days and peaked around 4 days (Velazhahan et al., 1998). In addition, in potato plants infected with *Phytophthora infestans*, no appreciable increase in the levels of three OLP mRNAs occurred up to 12 h after infection (Zhu et al., 1995a). Significant accumulation of these three OLP mRNAs was observed 4 days after infection. In contrast, a pepper OLP gene was induced within

3-5 h after inoculation with three different pathogenic or non-pathogenic bacteria (Hong et al., 2004).

The expression of two strawberry chitinase genes (*FaChi2-1* and *FaChi2-2*) and two strawberry  $\beta$ -1,3-glucanase genes (*FaBG2-2* and *FaBG2-3*) in response to the same *Colletotrichum* species were investigated in previous studies (Khan and Shih, 2004; Shi et al., 2005). When comparing all these infection results, some similarities in the induction patterns of these five PR genes were noticed. Firstly, higher levels of induction of the five genes were always observed in plants inoculated with *C. fragariae* than those inoculated with *C. acutatum*. Secondly, except for the *FaChi2-1* gene, none of the PR protein genes responded to the *Colletotrichum* fungi until 24-48 h post-inoculation. This latter observation indicates that the induction of the four PR genes (*FaOLP1*, *FaChi2-2*, *FaBG2-2* and *FaBG2-3*) by these two particular fungi might involve the same signal molecule or signaling pathway. In future studies, quantitative determination of the mRNAs produced in response to various signal molecules, such as salicylic acid, ethylene, and jasmonic acid, would help in delineating the signaling pathways utilized by these genes. Moreover, studies on the antifungal activities of the proteins encoded by these genes and their usefulness in transgenic plants to enhance the disease resistance should further broaden our knowledge in plant defense mechanisms.

## CHAPTER 5

### SUMMARY AND CONCLUSIONS

Pathogenesis-related (PR) proteins play an important role in protecting plants from invading microorganisms as well as various environmental stresses. PR proteins were originally identified in tobacco plants hypersensitively reacting to the tobacco mosaic virus (TMV) infection (van Loon and van Kammen, 1970). A number of different PR proteins have been identified since then. Plant chitinases are one of the most extensively studied PR proteins, partly due to their ability to degrade cell walls of certain fungi. These enzymes were shown to be induced in plants upon infection, and some were observed to have antifungal activity *in vitro* (e.g., Kragh et al., 1993; Chang et al., 1995; Sela-Buurlage et al., 1993). Chitinases have been purified and characterized from a number of plant species. All chitinases examined so far are present as single-polypeptide molecules, except one chitinase identified in winter rye (Yu and Griffith, 1999). This chitinase, together with glucanase-like proteins, thaumatin-like proteins and other unidentified proteins, were found to form protein complexes in the apoplastic space of cold-adapted winter rye leaves.

Osmotin-like proteins (OLPs) are members of thaumatin-like, PR-5 protein family. OLPs from different plant sources have been shown to inhibit fungal growth *in vitro* (e.g., Abad et al., 1996; Salzman et al., 1998). Expression of OLPs can be induced by pathogen infection, by abiotic stresses such as drought and cold, and by exogenous application of signal molecules such as salicylic acid and abscisic acid (reviewed in Velazhahan et al., 1999). Considerable evidence has demonstrated that constitutive expression of PR proteins in transgenic plants delayed the development of disease symptoms caused by

pathogen attacks (Tabei et al., 1998; Chen et al., 1999; Datta K et al., 2001; Velazhahan and Muthukrishnan 2003). Currently, there is a great interest in dissecting the molecular events from pathogen recognition to the induction of these PR protein genes.

Strawberry is an economically important fruit crop in the southern United States. However, in recent years fungal infections have become serious problems for sustained strawberry production. While some transgenic strawberries with enhanced disease resistance have been generated, no information is available on strawberry's endogenous defense systems. This dissertation is an effort to broaden our knowledge about PR proteins in general and also their reactions in a plant defense response. A chitinase-containing protein complex was partially purified from strawberry leaves. A gene encoding an OLP was cloned and partially characterized. The induction patterns of this OLP gene upon treatment with salicylic acid, abscisic acid or mechanical wounding were studied. In addition, the expression of a previously isolated OLP gene (Wu et al., 1999) in response to fungal infection was analyzed.

A chitinase-containing protein complex was purified to near homogeneity from strawberry leaf extracts after three consecutive chromatographic steps: batch-wise elution on a DEAE-Sepharose column, fractionation on a Sephacryl S-200 HR column, and fractionation on a Mono Q column. Analysis of the pooled fraction from Mono Q column on native gel revealed only one chitinase activity band (ChiA3'). Yet when the same pooled fraction was resolved on a SDS activity gel, at least five chitinase activity bands were detected. These results indicated that ChiA3' is indeed a protein complex that contains multiple chitinase molecules. In addition, western blot analysis of the protein complex sample from the mono Q column showed no detectable band when antiserum

prepared against a rice thaumatin and a barley  $\beta$ -1,3-glucanase were used. This result suggested that the strawberry chitinase complex probably does not contain any thaumatin-like or glucanase-like proteins. The strawberry complex, then, would be different from the structures of the winter rye apoplastic antifreeze protein complexes, which consist of different combinations of a chitinase, two thaumatin-like proteins, two glucanase-like proteins, and other proteins. By forming a protein complex, these chitinases might act synergistically to inhibit fungal growth. In future studies, it would be interesting to identify these chitinases as well as other possible constituents in this protein complex. An *in vitro* antifungal activity assay of these chitinases would then show if there is a synergetic effect against fungal growth. In addition, characterization of the chitinase genes and their regulation would bring some insight into the biological functions of this chitinase-containing complex.

A strawberry genomic clone containing an osmotin-like protein (OLP) gene, designated *FaOLP2*, was isolated and sequenced. The coding region of *FaOLP2* contains no intron, and is predicted to encode a precursor protein of 229 amino acid residues. There is a predicted signal peptide of 27 amino acid residues at the N-terminus; therefore, *FaOLP2* likely is secreted to the apoplastic space. The predicted amino acid sequence of *FaOLP2* shares high degrees of homology with a number of other OLPs. Genomic DNA hybridization analysis indicated that *FaOLP2* represents a multi-gene family. *FaOLP2* was constitutively expressed in strawberry leaves, roots, crowns, green fruits and ripe red fruits, although at different levels. High levels of *FaOLP2* mRNA were found in crowns and leaves. In addition, from green fruit stage to ripe red fruit stage, the expression of *FaOLP2* was increased by 2 fold, suggesting a developmental regulation of *FaOLP2* in

strawberry fruits. In addition, the regulation pattern of *FaOLP2* under different abiotic stresses was analyzed at different time points. All of the three tested abiotic stimuli, abscisic acid, salicylic acid and mechanical wounding, triggered a significant accumulation of the *FaOLP2* transcript within 2-6 h post-treatment. Moreover, *FaOLP2* was more prominently induced by salicylic acid than by abscisic acid or mechanical wounding. The positive responses of *FaOLP2* to the three abiotic stimuli suggested that strawberry *FaOLP2* may help to protect plants against osmotic-related environmental stresses and that it may also be involved in plant defense against pathogens. In future studies, it would be intriguing to identify the regulatory elements that are important for the response of *FaOLP2* to these signal molecules. A promoter deletion series linked to a reporter gene could be constructed and transformed into a model plant to identify such elements. Additionally, since salicylic acid is a key signal in systemic acquired resistance, it would be interesting to know if *FaOLP2* can be induced locally and/or systemically upon pathogen attack.

The expression of a previously isolated osmotin-like protein gene (*FaOLP1*) (Wu et al., 1999) in response to two strawberry fungal pathogens, *Colletotrichum acutatum* and *Colletotrichum fragariae*, was examined at different time points after inoculation with each fungus separately. Both of the two *Colletotrichum* fungi caused an increase in *FaOLP1* transcription at 24-48 h after infection. Moreover, accumulation of the *FaOLP1* transcript caused by *C. fragariae* was higher than that caused by *C. acutatum*. Similar induction patterns have been observed for one strawberry class II chitinase and two strawberry  $\beta$ -1,3-glucanases (Khan and Shih, 2004; Shi et al., 2005), suggesting that the regulation of these four PR protein genes by these two fungi could be involved in the

same signaling pathway. Further studies on the expression pattern of these genes in response to the known signal molecules, salicylic acid, jasmonic acid, and ethylene, could help to evaluate this possibility.

Plant PR proteins have been studied extensively in the last 20 years. However, our understanding about their roles in plants and the regulation of their expression are far from complete. In this dissertation, a protein complex was identified in the strawberry plant, and it was shown to consist of multiple chitinase isoforms. It would be intriguing to know if such a chitinase-containing complex also exists in other plant species. On the other hand, with the cloning of strawberry OLP genes, we can dissect the *cis*-elements and *trans*-factors involved in the induction of these genes, and the molecular events leading to the induction of these genes under biotic or abiotic stress. Such knowledge could bring new insight into the regulation of other PR protein genes.

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## **VITA**

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