Characterization of chitinase activities, and cloning, analysis, and expression of genes encoding pathogenesis-related proteins in strawberry

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CHARACTERIZATION OF CHITINASE ACTIVITIES, AND CLONING, ANALYSIS, AND EXPRESSION OF GENES ENCODING PATHOGENESIS-RELATED PROTEINS IN STRAWBERRY

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College
In partial fulfillment of the Requirements for the degree of Doctor of Philosophy

In

The Department of Biological Sciences

By
Anwar A. Khan
B.Sc. University of Karachi, 1987
B.S. Winona State University, 1994
December, 2002
DEDICATION

This dissertation is dedicated to the memory of my dear brother Ayaz Ahmed Khan (late). He was one of the most brilliant persons I have known in my early age. His encouragement and help is one reason I am able to reach this point in my life. I was fortunate to have him as a brother and it would have made him proud to see me complete a doctorate degree.
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ABSTRACT

The goal of this dissertation research is to investigate the defense systems of strawberry plant by characterizing the genes and their products involved in plant disease-resistance. Pathogenesis-related proteins, including hydrolytic enzymes chitinases and β-1,3-glucanases, have been known to be induced in plants upon infection with various pathogens. Highest total chitinase activity was found in strawberry crown, whereas root, petiole, fruit, leaf, and runner showed successively lower activities. Chitinase isoform analysis showed that up to six acidic and two basic isoforms were present in various organs. The total chitinase activity was stable at 50°C. The pH optimum for chitinase activity was 5. Total chitinase activity was inducible in leaves when plants were treated with fungal spores, salicylic acid, ethephon, or injury.

Genes encoding a class III chitinase and two class II chitinases (designated as FaChi2-1 and FaChi2-2) were cloned and their complete nucleotide sequences were obtained. Of the two class II chitinase genes, FaChi2-1 contains one intron whereas FaChi2-2 contains two introns. cDNA clones, containing the complete protein coding regions, for the two class II chitinase genes were obtained to establish the exact intron splice junctions. All cloned genes were found to be expressed constitutively in the strawberry leaves. Southern blot analyses for the two class II chitinase genes showed that these genes belong to small multi-gene families with no more than two members per haploid genome. Transcription start site for FaChi2-1 was mapped to –87 and –102 positions from the putative translation start site by primer extension analysis. FaChi2-2’s transcription start site was mapped to –52 position upstream of the putative translation start site.
Induction of total chitinase activity, pattern of acidic and basic isoforms, and expression of two class II chitinase genes were analyzed at 2, 6, 12, 24, and 48 h after fungal inoculation of plants with *Colletotrichum fragariae* or *C. acutatum*. The chitinase activity was inducible up to five fold. The expression of FaChi2-1 and FaChi2-2 was quantified by real-time PCR. FaChi2-1 was induced early within 2 h whereas FaChi2-2 was induced only at 24-48 h post-infection.
CHAPTER 1
LITERATURE REVIEW

Higher plants respond to various physical, chemical and biological stresses in a variety of ways. These stress factors include wounding, exposure to heavy metals such as mercury, salinity, drought, cold, and invasion by fungi, bacteria and viruses. Plants defend themselves against such insults by physical strengthening of the cell wall through lignification, suberization, and callose deposition; by synthesizing low molecular weight compounds known as phytoalexins which are toxic to the invading organisms; by producing various pathogenesis-related (PR) proteins such as chitinases, β-1,3-glucanases, and thaumatin-like proteins (Bowles, 1990). PR-proteins were first discovered, through advances in electrophoretic techniques, in tobacco plants hypersensitively reacting to tobacco mosaic virus (TMV) infection (Van Loon, 1970). Later, these proteins were found to be induced by bacterial and fungal infections in a number of plant species (Redolfi, 1984). Observations such as induction of PR-genes and occurrence of systemic acquired resistance (SAR) in plants to subsequent infections after an initial necrotic infection (Kassanis et al., 1974; Van Loon, 1975; Ward et al., 1991), absence of the substrate for one of the PR proteins (chitinase) in plants but its presence in the cell wall of certain fungi, and the in vitro antifungal activity of chitinases (Mauch et al. 1988) suggested a defensive role for PR-proteins.

PR-proteins are currently grouped into eleven families (PR-1 through 11) based on their primary structures and immunological properties (van Loon et al., 1994). The PR-1 family comprises small proteins of unknown function that are induced upon pathogen invasion. The PR-2 family comprises β-1,3-glucanases, acting on β-1,3-glucan,
a biopolymer found in plant and fungal cell walls. PR-3, 4, 8, and 11 families comprises plant chitinases belonging to various classes (I-VII). PR-5, 6, 7, 9, and 10 families comprises thaumatin-like proteins, protease-inhibitors, endoproteinases, peroxidases, and ribonuclease-like proteins, respectively (van Loon, 1999). Proteins such as thionins and ribosome-inactivating proteins are also considered as PR-proteins. While the mode of action of many PR-proteins in plant-defense remains unclear, chitinase, a hydrolytic PR-protein, appears to exert its effect through degradation of the chitin-containing fungal cell wall and release of elicitor molecules. There is a growing body of evidence supporting the notion that higher chitinase activity helps plants resist fungal infection (Grison et al., 1996; Terakawa et al., 1997).

**PLANT CHITINASES**

Chitinases (E.C. 3.2.1.14) are poly (1,4-(N-acetyl-β-D-glucosaminide))-glycanohydrolases. They are widely distributed in nature, occurring in bacteria, fungi, animals, and plants. Plant chitinases are usually endo-chitinases capable of hydrolyzing chitin, a major constituent of certain fungal cell walls as well as arthropodal and nematodal exoskeletons and insect gut linings. Figure 1.1 shows the enzymatic reaction catalyzed by endochitinases.

**General Characteristics**

Multiple chitinase isoforms and gene clusters have been detected in many plants analyzed to date. Plant chitinases are generally small proteins of molecular weight 25-40 kDa, with wide range of isoelectric points (3-10), and post-translational modifications such as glycosylation and prolyl-hydroxylation (Sticher et al., 1992; Colinge et al., 1993; Nielsen et al., 1994). Chitinases generally show wide pH optima (4-9) for activity.
Figure 1.1 Enzymatic reaction catalyzed by endo-chitinases. Chitin chain of length n is reduced to N,N’-diacetylchitobiose and higher oligomers of reduced length n-x where x ≥ 2.

Some chitinases, such as a yam class III chitinase, show two pH optima depending on the substrate used (Tsukamoto et al., 1984). This particular chitinase is also stable at 80°C, whereas other plant chitinases show moderate temperature tolerance of up to 60°C.

Chitinases contain several disulfide linkages through conserved cysteine residues in their tertiary structure. Crystal structures of heveamine, a class III chitinase/lysozyme from rubber tree (Hevea brasiliensis) (Terwisscha van Scheltinga et al., 1994 and 1995), and class II chitinases from barley (Hordeum vulgare) (Hart et al., 1995) and jack bean (Canavalia ensiformis) (Hahn et al., 2000) have been determined.

Based on their primary structures, plant chitinases have been classified into seven classes, class I through VII. Different chitinase classes have no apparent correlation to
being present in a particular plant species and plant organ or tissue. However, certain chitinase isoforms are sometimes induced by a particular elicitor. For example, in potato, a class I basic chitinase was strongly induced by ethylene and wounding whereas a class II acidic chitinase was induced by salicylic acid (Buchter et al., 1997). Also, only particular isoforms show antifungal activities and certain isoforms have additional novel functions such as antifreeze activity (Sela Buurlage et al., 1993; Yeh et al., 2000).

Class I and II Chitinases

Class I and II chitinases belong to the PR-3 family of pathogenesis-related proteins with the tobacco chitinases as the prototypical members. All members of the PR-3 family belong to family 19 of glycosyl-hydrolases, which catalyzes sugar hydrolysis with the inversion of configuration at the anomeric carbon.

Class I chitinases have an N-terminal cysteine-rich chitin-binding domain (CBD) that is homologous to hevein, a chitin-binding lectin from the rubber tree. CBD is separated from the catalytic domain by a proline- and glycine-rich hinge or spacer region, variable both in size and composition. For a tobacco class I chitinase, the deletion of CBD and the spacer region singly or in combination reduces the hydrolytic activity by 50%, whereas antifungal activity is reduced by 80% (Suarez et al., 2001). Class I chitinases are synthesized as propeptides, directed to the secretory pathway and eventually directed to the vacuole by a short C-terminal signal sequence. Deletion of this C-terminal signal peptide redirects a class I chitinase to the apoplast while retaining the enzymatic activity (Grover et al., 2001). Addition of this six amino acid signal (GLLVDTM) to an unrelated, usually secreted class III chitinase redirects this protein to the vacuole in tobacco (Neuhaus et al., 1991). Class II chitinases are similar to class I but
they lack the N-terminal CBD and the hinge region. In the catalytic domain they
sometimes have a deletion as compared to class I chitinases. Class II chitinases are
usually secreted to the apoplast as they lack the C-terminal vacuolar-targeting signal.

**Protein Structure of Class I and II Chitinases**

A barley class II monomeric 26 kDa chitinase, and a jack bean class II chitinase
are the only members of the PR-3 family whose crystal structures are known (Hart et al.,
1995; Hahn et al., 2000, respectively). The structure of barley chitinase is mostly α-
helical and forms a globular structure of approximately 42 Å³. The barley enzyme is
composed of one short antiparallel β-strand and ten α-helices occupying 47% of the
primary structure. The jack bean chitinase also shows similar structure with ten helices
of 7-8 amino acids. These structures have similarity to the lysozyme fold of hen egg-
white lysozyme (HEWL) and other family 19 glycosyl-hydrolases without sequence
homology. In the barley enzyme four loops have been identified. Loops 1 and 2
surround the catalytic site and are held by conserved disulfide bonds, whereas loops 3
and 4 are located at the surface and are not likely to participate in the catalysis. In the
barley enzyme three disulfide bonds, between cysteine residues 24-86, 98-104, and 203-
222, are held by conserved cysteines. These disulfide linkages are also found in jack
bean chitinase at similar locations.

Two active site glutamic acid residues have been identified in the crystal
structures. In the barley enzyme these residues are identified at amino acid positions 67
and 89. Mutation of either active site glutamate turned a class I chitinase into a chitin
binding lectin (Iseli-Gamboni et al., 1998). The precursor of stinging nettle (*Urtica
dioica*) lectin sequence is related to chitinases, but it has both the active-site Glu residues
mutated and the protein does not show chitinase activity. Jack bean chitinase also has catalytic site residues located at similar positions. Interestingly, the HEWL Glu 35 is essential for catalysis and superimposes with Glu 67 of barley chitinase upon superimposition of the crystal structures. Other active site residues have been modified or mutated to show their functions in catalysis. Mutation of Tyr 123 of *Zea mays* chitinase and a similar tyrosine of Arabidopsis chitinase in the conserved NYNY catalytic site motif, present in most class I and class II chitinases at similar locations, causes greatly reduced chitinase activities (Verburg et al., 1992 and 1994). Other residues such as Asn 124, 199, Trp 103, and Try 123 of barley chitinase are implicated in substrate binding and are conserved in other chitinases.

Chitin binding to the barley enzyme has been proposed only hypothetically since chitinase crystals dissolve upon substrate binding. The crystal structure shows the active site nicely fitting the substrate in an elongated cleft running though the length of the protein. Six sugar binding sites, labeled A-F, have been identified in this cleft (Honda and Fukamizo, 1998). Catalysis requires that an acid (Glu 67) attack the glycosidic bond at C4 oxygen and a base (Glu 89) activate a water molecule that attacks C1 position of the sugar. The catalysis occurs between sugar binding sites D and E of the enzyme. NMR analysis of the cleavage products has shown that this enzyme acts by inversion of the configuration at the anomeric carbon (Hollis et al., 1997).

**Class I and II Chitinase Gene Structure and Regulation**

A large number of cDNAs, but fewer gene sequences, have been obtained for PR-3 family chitinases. The genomic sequences of class I and II chitinases show none, one, or two introns. The first intron is located after the position corresponding to the
conserved catalytic site motif SHETTG whereas the second intron is located just before the conserved motif NYNY. An exception to these locations is a class II chitinase gene from Bermuda grass which has two introns but the first intron of 94 bases is located upstream of the normal first intron at the catalytic site SHETTG motif in other chitinases (de los Reyes et al., 2001). The introns are usually small, ranging in size from approximately 50-200 bases. An unusual *Beta vulgaris* chitinase gene, however, has two introns of 2.5 and 1.5 kb (Bergland et al., 1995). This particular gene codes for a chitinase with a relatively short CBD but an unusually long spacer region of 131 amino acids, of which 90 are proline residues, as compared to 5-22 amino acid spacers in the majority of class I chitinases. Any significance of such a structure is unknown. Genomic structures of various chitinases show that they exist as single-copy to large multi-gene families. For example, potato class I chitinase genes (Ancillo et al., 1999), strawberry class II genes (this study), and maize class I genes (Wu et al, 1994) exist as one or two copies per haploid genome. Cotton and rice show complex genomic organizations of chitinase genes with four and as many as 8-10 members, respectively (Chlan and Bourgeois, 2001; Takakura et al., 2000, respectively). These genes appear in clusters as shown for Arabidopsis, cucumber, and potato. The gene expression is complex and varies among different plant species. Chitinase genes show differential induction in various plants upon challenge with pathogens or treatment with ethylene, salicylic acid, jasmonic acid, and fungal elicitors such as chitosan.

Currently, the study of signaling events from signal perception to the transcription of PR-genes is an intense area of research. Several elicitor molecules capable of inducing PR-gene expression have been characterized. These elicitors include β-glucans, peptides,
and avirulence (Avr) gene products of the pathogens. Proteins able to interact with these elicitors have been isolated from plants, but no receptor on the plant cell membrane has unequivocally been identified (Zhou, 1999; Nurnberger and Scheel, 2001). Various secondary messengers, including salicylic acid, ethylene, jasmonic acid, and nitric oxide have been shown to be required in the signaling events leading to PR-gene activation (Grant and Loake, 2000; Feys and Parker, 2000; Glazebrook, 2001; Nurnberger and Scheel, 2001; Wendehanne et al., 2001). Salicylic acid appears to be involved in the induction of genes associated with the development of systemic acquired resistance (SAR). In Arabidopsis, NPR1 (non expresser of PR-genes), an important switch controlling a number of PR-genes and responding to salicylic acid and pathogens has been identified. NPR1 is an ankyrin repeat protein presumed to mediate protein-protein interactions (Cao et al., 1997) and has been shown to translocate to the nucleus upon stimulation (Dong, 1998). NPR1 appears to work downstream of salicylic acid and acts to negatively regulate the salicylic acid production. Similar to salicylic acid, NPR1 is required for the establishment of SAR and expression of PR-genes (Cao et al., 1994). Ethylene and jasmonic acid appear to function in a pathway different from salicylic acid leading to the induction of a variety of genes including basic PR-genes, thionins and defensins (Dong, 1998; Feys and Parker, 2000; Glazebrook, 2001). Ethylene and jasmonic acid are also involved in a pathway stimulated by non-pathogenic organisms such as rhizobacteria (Pieterse, 1998). This pathway leads to induced systemic resistance (ISR). ISR is a salicylic acid-independent pathway leading to small alteration of gene expression. Interestingly, NPR1 is also a central control element in this pathway, providing evidence that NPR1 is a common adapter involved in different pathways.
There is considerable cross-talk between different pathways and activation of a particular PR-gene by a particular pathway utilizing certain second messengers seems to be dependent upon individual pathogen/elicitor recognition events (Feys and Parker, 2000).

The signaling pathway mediating elicitor-inducible gene expression appears to be conserved in distantly related plant species. The promoter of a pine class II chitinase gene which is responsive to chitosan, a deacetylated derivative of chitin, in pine suspension culture cells, mediated chitosan induced expression in tobacco plants (Wu et al., 1997). The fact that a chitinase gene promoter from a gymnosperm showed similar regulation in an angiosperm illustrates that at least the major components of signaling events in plants are conserved.

Chitinase genes are regulated at the transcription level. The most direct evidence came from promoter studies of a bean class I chitinase gene. A bean chitinase gene is activated by ethylene (75-100 fold), oligosaccharide elicitors, and fungal pathogens (Broglie et al., 1986). The 1.6 kb promoter region of this gene was able to confer the ethylene regulation on a β-glucuronidase (GUS) reporter gene transformed into tobacco plants (Broglie et al., 1989). The ethylene responsive element was localized to the –422 to-195 region from the transcription start site of the gene. The region contains two DNA sequences imparting quantitative expression and ethylene response for the gene. The GUS gene was also induced when tobacco plants were challenged with fungal pathogens. The GUS activity closely correlated with the induction of endogenous tobacco chitinase activity (Roby et al., 1990). The promoter was only active at the areas of infection and the signal was sharply reduced away from the infection site. Such ethylene responsiveness has also been studied in a class I tobacco chitinase promoter (Shinshi et
al., 1995). The ethylene responsiveness was localized to the −503 to −358 region from the transcription start site. This sequence, in either orientation, was sufficient to confer ethylene responsiveness in a heterologous construct containing a cauliflower mosaic virus (CaMV) 35S RNA promoter, suggesting that this sequence also acts as an enhancer. Within this region a 71 base sequence was further localized (−480 to −410) for ethylene regulation. This sequence contain two GCC boxes, (TAAGAGCCGCC), which are frequently found in other pathogenesis-related gene promoters responding to ethylene. One such gene is a basic class I β-1,3-glucanase gene (Hart et al., 1993; Ohme-Takagi and Shinshi, 1990).

Induction of class I chitinase synthesis has been demonstrated through Northern and/or Western blot analyses in a number of plants and cell cultures in response to various biotic and abiotic stress factors. However, relatively few studies have been done for chitinases belonging to other classes. In cultured tobacco cells, induction of a class I basic chitinase, a class II acidic chitinase, and a class I β-1,3-glucanase have been investigated in response to fungal elicitors from Phytophthora infestans (Suzuki et al., 1995). The class II acidic chitinase gene was induced rapidly within 30 min of treatment and the induction reached maximum level at 4-5 h. The expression decreased to background levels within 24 h. In contrast, the basic class I chitinase and the glucanase genes were induced after a 2 h lag period, reaching a maximum level at 6 h and maintaining that level over a 24 h period. The induction was shown to be dependent on protein synthesis for class I chitinase and glucanase genes but not for class II chitinase gene. Also, inhibition of protein phosphorylation prevented induction of basic class I chitinase and glucanase but had no effect on class II chitinase. These results demonstrate
that regulation of the expression of these genes, at least in response to the elicitors studied, is accomplished through separate signal transduction pathways. In a similar study, Kim et al. (1998) showed that the induction of a rice class II acidic chitinase in response to fungal elicitors was repressed by protein phosphatase 1 and 2A. The investigators suggest that protein dephosphorylation might be a key step in the regulation of class II acidic chitinases, which is in agreement with the results reported by Suzuki et al. (1995). It should be noted that differences in the activation of rice class II chitinase gene were observed in rice cell culture and rice leaves. The gene was induced by ethephon and HgCl$_2$ in leaves but not in suspension cells. It was induced by glycol chitin and fungal elicitors in suspension cells but not in leaves. Salicylic acid and β-1,3-glucan had no effect in either system. This demonstrates that cell culture, although a simple and an effective system, may not reproduce organismal level results.

**Class III Chitinases**

Class III chitinases are unique in having a structure unrelated to any other class of plant chitinases. These chitinases belong to the PR-8 family and family 18 of glycosyl-hydrolases. Members of family 18 glycosyl-hydrolases catalyze sugar hydrolysis with the retention of configuration at the anomeric carbon. Class III chitinases generally have lysozyme activity and appear to be more closely related to the bacterial chitinases. A class III chitinase enzyme was purified from the seeds of *Benincasa hispida* (white gourd or wintermelon), a Chinese medicinal plant (Shih et al., 2001). The enzyme is a 29 kDa protein with 27 amino acid N-terminal signal peptide (as deduced from N-terminal amino acid and genomic DNA sequences). The length of signal peptides and molecular weights are similar in other class III chitinases such as a pumpkin chitinase of 29 kDa with a 27-
amino acid signal peptide (Kim et al., 1999) and a sugar beet chitinase of 29 kDa with a 25-amino acid signal peptide (Nielsen et al., 1993). The pumpkin class III chitinase was purified by chitin affinity chromatography, which showed strong retention of this protein. This is unusual for a class III chitinase since they do not have a chitin-binding domain. Class III chitinases show a wide range of isoelectric points, activity over a wide range of pH, and temperature stability at 60-70°C. The B. hispida chitinase has a pH optimum of 2 and retains approximately 50% activity at pH 8 (Shih et al., 2001). Some class III chitinases, such as a yam enzyme, show two pH optima and heat stability at 80°C (Tsukomoto et al., 1984). There is no post-translational modification reported for class III enzymes analyzed to date.

The three-dimensional structure of heveamine, a chitinase/lysozyme from rubber tree, and its complex with the inhibitor allosamodin has been determined (Terwisscha van Scheltinga et al., 1994, 1995, and 1996). The structure is an (α/β)8 barrel similar to the bacterial family 18 glycosyl-hydrolases without significant sequence identity. These enzymes contain a substrate-binding cleft located at the C-terminal end of the β-strand in the barrel structure. The active site residue Glu127 of heveamine is required for activity whereas Asp125 allows a wider pH range for catalysis. Heveamine requires chitopentose as minimum substrate. The catalysis occurs by retention of configuration at the anomeric carbon, and is substrate assisted. Generally class III chitinases also act as lysozymes. However, Bokma et al. (1997) showed that heveamine hydrolyzes the glycosidic bond of the peptidoglycan between C-1 of N-acetylglucosamine and C-4 of N-acetylmuramic acid as opposed to lysozyme which catalyzes the hydrolysis of peptidoglycan by cleavage of C-1 of N-acetylmuramic acid and C-4 of N-acetylglucosamine. Therefore, heveamine
and possibly other class III plant chitinases, are not strictly lysozymes. Some class III chitinases such as a sugar beet enzyme do not exhibit lysozyme activity (Nielsen et al., 1993). A recent study shows kinetic constants of heveamine by an improved assay method (Bokma et al., 2000). The $k_m$ and $k_{cat}$ for N-acetylglucosamine-pentamer (GlcNac)$_5$ and (GlcNac)$_6$ were measured to be 13.8 $\mu$M, 0.355/s and 3.2 $\mu$M, 1.0/s, respectively. Allosamodin was found to be a competitive inhibitor with a Ki of 3.1 $\mu$M.

Class III chitinase genes in *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 copies. In contrast, heveamine from *H. brasiliensis* is encoded by a small multigene family (Bokma et al., 2001). Also, class III chitinase genes from *B. hispida* and *H. brasiliensis* (Shih et al., 2001 and Bokma et al., 2001, respectively) lack introns.

Various class III chitinase genes showed distinct regulation upon stress treatment. For example, a *Lupinus albus* gene was shown to be induced by infection with *Colletotrichum gloesporioides*, by treatments with UV light, and by wounding (Regalado et al., 2001). No antifungal activity was observed for *Trichosanthes kirilowii* class III chitinase, and it was not induced by salicylic acid (Savary et al., 1997). Both acidic and basic isoforms of tobacco class III chitinases were induced upon infection of plants with TMV (Lawton et al., 1992). The level of induction was about 5-10 fold and was observed in the infected leaves as well as in secondary non-infected leaves. This suggests that class III chitinases act as a generalized systemic-acquired resistance (SAR) response instead of being induced in response to pathogen as has been seen for class I or II chitinases. A grape class III chitinase was also shown to be induced in infected and
non-infected leaves upon fungal infection (Busam et al., 1997). The induction showed two maxima at 2 d and 6 d in the susceptible *Vitis vinifera* whereas the level was steeply induced up to 4 d and declined to the basal level by day 7 in the resistant *V. rupestris*. This gene showed SAR whereas a class I gene analyzed simultaneously did not. A pumpkin class III gene was also responsive to the fungal elicitor and glycol chitin (Kim et al., 1999). This gene showed maximal induction within 1 h of fungal elicitor treatment and the transcript disappeared within 6 h. In contrast, glycol chitin induced this gene at 3 h and the expression gradually decreased to background level at 24 h. The gene was not induced by salicylic acid or by UV irradiation. Protein accumulation took 6-10 h after transcription.

**Class IV, V, VI, and VII Chitinases**

Class IV, V, VI, and VII chitinases belong to the PR-3 family of pathogenesis-related proteins. The structure of class IV chitinases is similar to class I chitinases except that they are shorter due to four deletions. Class V, VI, and VII chitinases have unique structures and are represented by one example each.

**Functions of Plant Chitinases**

Plant chitinases have been known to be induced upon fungal infection and inhibit fungal growth in vitro, criteria initially used to implicate chitinases in plant defense (Schlumbaum et al., 1986; Mauch et al., 1988a and 1988b). The induction of chitinases was initially shown in pea plants infected with *Fusarium solani*, or challenged with other biotic or abiotic stress factors (Mauch et al., 1988a). Protein extracts made from infected pea plants were able to inhibit growth of 15 of the 18 fungal species tested in vitro. Purified chitinase inhibited growth of only one fungal species whereas a combination of
chitinase and another PR-protein, β-1,3-glucanase, inhibited the growth of all fungi tested showing a synergism in activities (Mauch et al., 1988b). Subsequently, a number of studies verified these results in tobacco (Yun et al. 1996), grapes (Derckel et al., 1998), chickpea (Giri et al., 1998), rice (Velazhahan et al., 2000) and other plants. The current view is that only specific isoforms are induced in response to a particular pathogen and only certain isoforms are able to inhibit specific fungi (Ji et al., 2000; Sela-Buurlage et al., 1993). For example, a class I chitinase from tobacco showed antifungal activity against *Fusarium solani*, but class II chitinases showed only a slight growth inhibitory effect when used with high concentrations of a β-1,3-glucanase (Jach et al., 1996).

Constitutive chitinase expression is higher and induction is stronger and quicker in the resistant varieties as compared to the susceptible varieties in some phyto-pathogen systems such as sugar beet (Nielsen et al., 1993), wheat (Anguelova et al., 2001) and tomato (Lawrence et al., 2000). However, contrary data also exist showing no difference in the timing, induction, or intensity of PR-gene expression in susceptible and resistant cultivars, for example in cotton (McFadden et al., 2001). Quick response in the resistant cultivars might affect the cell wall of germinating fungal spores, releasing elicitors leading to the expression of PR-genes and disease resistance. It was shown for *Alternaria solani* that a basic chitinase was only active on the germinating spores and not on the mature fungal cell wall for generation of elicitor molecules able to induce disease resistance (Lawrence et al., 2000). The difference may be in the length of these fragments as it is known that 4-5 N-acetylglucosamine residues are necessary for defense elicitation. In an interesting study in potato, it was shown that chitinase and osmotin-like proteins interact with actin filaments (Takemoto, et al. 1997). It has been known that
actin filaments show cytoplasmic aggregation at the site of fungal penetration. It was hypothesized that PR-proteins are translocated to the site of fungal penetration for effective blocking of the pathogen and/or for the release of elicitors.

Class IV chitinases are similar to class I, but they are smaller in size due to four deletions. A class IV chitinase was found to rescue a somatic embryo mutant of carrot unable to differentiate (de Jonge, 1992). It was found that a sugar beet class IV chitinase was not able to substitute, however a class I chitinase could. A possible substrate for this activity was found to be the arabinogalactan proteins (AGP) functioning in somatic embryogenesis in carrot (van Hengel et al., 2001). AGPs have a complex structure with a β-1,3-galactan backbone and highly heterogeneous side chains. These proteins are produced in somatic embryos and have half-lives of 10-15 min. Thus, it is thought that chitinases might act on these proteins for degradation, either destroying or generating signaling molecules. A specific chitinase isoform in *Pinus caribaea* was shown to interact with AGP present in embryogenic tissue but not with AGP from non-embryogenic calli (Domon et al., 2000). In arabidopsis, a class IV chitinase was shown to be expressed only in seedpods and the promoter region fused with GUS showed expression in Arabidopsis and tobacco embryos (Gerhardt et al., 1997). Albeit the fact that exact role of chitinases in embryogenesis is not clear, there is ample evidence that chitinases are important in this process and the nature of physiological chitinase substrates are beginning to emerge. In *Cichorium*, chitinase as well as β-1,3-glucanase and osmotin-like protein are synthesized at higher levels and secreted in the culture medium of somatic embryogenesis-competent cells (Helleboid et al., 2000). It would be interesting to know if these enzymes exist in oligomeric complexes functioning in
embryogenesis. Chitinases along with other PR-proteins have been implicated in freeze tolerance (see below) where they do form oligomeric complexes.

A class III chitinase gene from *Medicago truncatula* was shown to be specifically expressed in root cortical cells containing developing or mature arbuscules (Bonanomi et al., 2001). This gene was not induced by phytopathogenic fungi and the expression was limited to cells harboring the arbuscules. Investigators hypothesize that this gene functions in suppression of the defense response in these cells by degrading fungal chitin elicitors. The hypothesis was substantiated by the fact that H$_2$O$_2$ production, a hallmark of defense response, was absent from these cells (Salzer et al., 1999) and that the other defense related proteins such as β-1,3-glucanses were present at low levels in these cells (Blee and Anderson, 2000). Another class III chitinase from *Sesbania rostrata* lacking the active site glutamic acid residue and devoid of chitinase activity was induced during nodule development (Goormachtig et al., 2001). The active site glutamate is mutated to a lysine residue in this protein and therefore renders this protein a chitin binding lectin. This gene was induced within 4 h of inoculation with nodulation bacteria, and the protein was localized to the outer cell layer of nodules. Investigators suggest a role for this protein in Nod factor binding which would protect, concentrate, or facilitate its interaction with a receptor protein. Increased chitinase activity and induction of new isoforms have been observed in other plants, such as soybean, in symbiosis with nodulation bacteria (Xie et al., 1999). Evidence for other physiological functions of chitinases in flowering, reproduction, germination, and plant growth are also beginning to emerge.
PLANT β-1,3-GLUCANASES

β-1,3-glucanases (E.C.3.2.1.39) belong to the PR-2 family of pathogenesis-related proteins. These enzymes catalyze the cleavage of β-1,3-glucosidic bonds of β-1,3-glucan, another constituent of the fungal cell wall. Unlike chitinases, the substrate for β-1,3-glucanases is widespread in plants and therefore these proteins are implicated in diverse physiological functions as well as in plant defense.

Plant β-1,3-glucanases range in size from 30-40 kDa with both acidic and basic isoforms. Based on their primary structure, pl, and localization, β-1,3-glucanases are divided into two major classes (I and II), whereas two minor classes (III and IV) are represented by only one example each (Buchel and Linthorst, 1999). Tobacco basic glucanases belong to class I and are targeted to the vacuole, whereas acidic isoforms are secreted and belong to class II. Class I β-1,3-glucanases have an N-terminal signal peptide and a short C-terminal signal sequence that is glycosylated. The C-terminal signal sequence is believed to be the vacuolar targeting signal that is removed to generate the mature protein (Sitcher et al., 1992; Shinshi et al., 1988). Class II, III, and IV β-1,3-glucanases lack this C-terminal signal and therefore are secreted to the apoplast. Some β-1,3-glucanases are solely developmentally regulated and do not show a stress-related induction. Two examples are tobacco styler β-1,3-glucanase (Ori et al., 1990) and a tobacco anther β-1,3-glucanase (Bucciaglia et al., 1994). A barley β-1,3-glucanase has been crystallized and its 3-D structure determined (Varghese et al., 1994).

β-1,3-glucanase genes, generally existing in multigene families, show either no introns or one intron located near the N-terminus of the coding region. This intron usually ranges in size from 400-600 bases. β-1,3-glucanase genes are primarily regulated
at the transcriptional level as shown by reporter gene experiments using DNA constructs containing various β-1,3-glucanase gene promoter segments (Henning et al., 1993; Van de Rhee et al., 1993; Shah and Klessig, 1996). Class I β-1,3-glucanase accumulated only at the site of tobacco mosaic virus (TMV) infection in tobacco plants. In contrast class II and III β-1,3-glucanases accumulated both at the site of infection and systemically (Vogeli-Lange et al., 1994; Livne et al., 1997). Similar to chitinases, β-1,3-glucanases can degrade the fungal cell wall by disrupting hyphal tips, especially in combination with a chitinase (Mauch et al., 1988). Release of oligosaccharide elicitors is another mode by which plant glucanases could elicit other defense responses (Ryan and Farmer, 1991). Transgenic plants expressing β-1,3-glucanase, especially in combination with a chitinase, have shown increased tolerance to pathogens (Zhu et al., 1994; Jach et al., 1995; Jongedijk et al., 1995). On the other hand, in experiments where a class I β-1,3-glucanase was suppressed by the antisense technique, tobacco plants were more resistant to TMV, presumably through increased callose deposition. However, a novel β-1,3-glucanase was induced to compensate for the suppressed β-1,3-glucanase suggesting presence of multiple isoforms (Beffa et al., 1993 and 1996).

**PR-PROTEINS EXISTING IN OLIGOMERIC COMPLEXES FOR ANTIFREEZE FUNCTION**

Cold-acclimated plants are more resistant to disease than untreated controls (Tronsmo, 1984; Tronsmo et al., 1993). Winter rye (*Secale cereale*) can withstand freezing temperature of –20°C and the antifreeze proteins (AFPs) in this plant were shown to be similar to plant PR-proteins existing in oligomeric complexes. Cold-acclimated plants showed an approximately 10-fold higher total apoplastic protein
concentration. Some of the abundant proteins had antifreeze activity in vitro and were shown by N-terminal sequencing to be chitinases, $\beta$-1,3-glucanases, and thaumatin-like proteins. Moreover, one of the chitinases showed enzymatic activity (Hon et al., 1995). Interestingly, PR-proteins induced in response to pathogens did not show antifreeze activity. It has been hypothesized that different PR-protein isoforms are present in cold-acclimated plants providing both antifreeze and disease resistance functions as compared to non-acclimated plants having PR-protein isoforms functioning only in disease resistance (Antikainen et al., 1996). Snow mold was able to induce the PR-proteins in winter rye plants both acclimated and non-acclimated to cold. While these proteins showed enzymatic activities in both plants, only PR-proteins from cold-acclimated plants showed antifreeze activity (Hiilovaara-Teijo et al., 1999). It has been hypothesized that these differential activities are due to the expression of slightly different isoforms since no post-translational modification has been detected in isoforms from cold-acclimated and non-acclimated plants (Yeh et al., 2000). In a recent report the effects of salicylic acid and ethylene, known to induce PR-proteins, were studied in winter rye for the induction of PR-proteins with or without antifreeze activities. Whereas salicylic acid induced PR-proteins which did not have antifreeze activities, ethylene induced PR-proteins with antifreeze activities (Yu and Griffith, 2001). The investigators concluded that ethylene might have a regulatory role in cold and drought tolerance as well as in disease resistance.

Six AFPs were detected in winter rye, two each of chitinase, $\beta$-1,3-glucanase, and thaumatin-like proteins. These proteins were shown to have antifreeze activities in vitro (Hon, 1994). Interestingly, these proteins existed in a complex in various combinations.
of the indicated proteins together with some unidentified proteins as shown by Yu and Griffith (1999). When protein bands extracted from native gels were run under denaturing conditions, multiple bands ranging in size from 14-144 kDa were detected. Immunoblots positively identified these bands as chitinase, β-1,3-glucanase, and thaumatin-like proteins. Also, immunoprecipitation of apoplastic extracts by either anti-glucanase-like protein-, anti-chitinase-like protein-, and anti-thaumatin-like protein-antibodies, precipitated all three proteins showing physical association of these proteins. Moreover, β-1,3-glucanase activity of the complex was inhibited severely by the addition of anti-glucanase-like-protein-antibody. β-1,3-glucanase activity was also inhibited by addition of anti-chitinase-like-protein- and anti-thaumatin-like-protein- antibodies.

Extracellular ice formation in plants might be restricted by factors including AFPs which could adsorb on the ice surface, depressing the freezing point and preventing recrystallization (Hon 1995 and ref. therein). The interaction of AFPs with ice would require surface characteristics compatible for such interaction and the existence of AFPs in oligomers might provide a larger surface area for such interactions. Evolution might have chosen a class of proteins for antifreeze function that also have antipathogen activities. Existence of multiple isoforms of PR-proteins would be consistent with this idea. It would be very interesting to study the promoter region, signal transduction, and regulation of such genes. Recently, a class II chitinase gene from bermudagrass, highly homologous to a strawberry gene FaChi2-1, was found to be expressed at high levels in varieties more tolerant to cold. The gene was activated within 48 h of cold acclimatization (de los Reyes et al., 2001). Also, an osmotin-like protein from bittersweet nightshade (Solanum dulcamara) has been shown to function in cryoprotection (Newton and Duman, 2000).
TRANSGENIC PLANTS WITH ENHANCED DISEASE RESISTANCE

Purified chitinases show varying degrees of antifungal activities in vitro. In general, class I chitinases have the highest antifungal activity, perhaps due to the presence of a chitin-binding domain (Sela-Buurlage et al., 1993). They also have higher specific activities compared to other classes of chitinases. While a CBD is not required for chitinolytic or antifungal activities, it increases both, perhaps by anchoring to the substrate and increasing its effective concentration for hydrolysis (Iseli et al., 1993). Another explanation is that CBD might have antifungal activity of its own, acting on another substrate. Hevein and stinging nettle lectin exemplify chitin-binding lectins which are antifungal proteins without having chitinolytic activities (Broekaert et al. 1989; Van Parijs et al., 1991). All other chitinase classes have lower to no antifungal activity as compared to class I chitinases. Based on these observations, most transgenic work to produce plants with elevated chitinase activity has utilized class I chitinase gene(s). A bean (Phaseolus vulgaris) chitinase gene under the control of CaMV 35S promoter was introduced into tobacco plants through Agrobacterium mediated transformation. A high level of chitinase activity of 20-40 fold as compared to control was observed in the transgenic plants. Transgenic plants showed increased resistance to infection by pathogenic fungi Rhizoctonia solani and delayed development of disease symptoms. The transgenic plants showed no resistance to the non-chitin containing fungus Pythium aphanidermatum (Broglie et al., 1991). In one study, oilseed rape (Brassica napus) transgenic plants transformed with a tomato chitinase gene were grown and challenged with three different fungal pathogens at two field locations (Grison et al., 1996). Over a period of 52 days, the protection level against three fungi was 23% to 79% with both
delayed appearance of symptoms and reduced lesion numbers. The highest protection was against *Cylindrosporium concentricum* which contains chitin as a major component in its cell wall and at the tip of growing hyphae. The protection was lower against two other fungi whose cell walls have different distributions and amounts of chitin.

Transformations of various other plants with chitinase genes showing enhanced disease resistance have been achieved. For example, a rice chitinase gene transformed into rice showed enhanced resistance to sheath blight caused by *Rhizoctonia solani* (Datta et al., 2001); a tobacco chitinase gene transformed into peanut showed enhanced resistance to leaf spot disease caused by *Cercospora arachidicola* (Rohini and Rao, 2001); a rice chitinase gene transformed into grapevine increased resistance of these plants against powdery mildew caused by *Uncinula necator* (Yamamoto et al., 2000).

The fact that pathogenesis-related proteins act synergistically in vitro in fungal growth inhibition assays suggests an intriguing possibility of stacking transgenes for crop protection. Such efforts have been made by various groups by transforming plants with combinations of pathogenesis-related protein genes. Jach et al. (1995) transformed tobacco plants with a barley class II basic chitinase along with either a barley class II basic β-1,3-glucanase gene or a barley ribosome-inactivating protein gene. The tobacco plants were challenged with *Rhizoctonia solani* and plants transformed with both sets of genes showed enhanced levels of protection as compared to the single gene transformants. This study demonstrated the usefulness of class II chitinase genes for transgenic crop protection despite its low in vitro antifungal activity. A barley class II chitinase gene under the control of ubiquitin promoter was transformed into wheat (Oldach et al., 2001). The transgenic wheat showed resistance to powdery mildew and leaf rust diseases.
Non-plant chitinases have also been transferred to model plants in order to evaluate their usefulness in disease resistance. A chitinase gene from the mycoparasitic fungus *Trichoderma harzianum* was transformed into tobacco and potato plants. The transgenic plants showed enhanced resistance to various fungal pathogens including *Alternaria alternata*, *A. solani*, *Botrytis cinerea* and *Rhizoctonia solani* (Lorito et al., 1998). Another fungal chitinase gene from *Rhizopus oligosporus* showed suppression of disease symptoms in transgenic tobacco plants when challenged with pathogenic fungi (Terakawa et al., 1997). A chitinase from autograph californica multiple nucleopolyhedro virus (AcMNPV) transformed into tobacco showed enhanced disease resistance in 59% of plants (Shi et al., 2000).

Not all chitinases or other PR-proteins show antifungal activity in vitro, or when their genes are transformed into plants. Class III chitinase of sugar beet induced in sugar beet in response to fungal challenge did not provide any disease resistance when transformed into tobacco (Nielsen et al., 1993). Tobacco plants transformed with barley chitinase, glucanase, and ribosome inactivating protein alone or in combinations were resistant to a AG4 group *Rhizoctonia solani* (Jach et al., 1995), but only chitinase transformants showed resistance to AG8 group fungi (O’Brien et al., 2001). These results suggest that different isolates of the same pathogen might have different sensitivities toward a particular PR-protein.

**STUDY OF STRAWBERRY’S PATHOGENESIS-RELATED GENES / PROTEINS**

Strawberry is an important horticultural crop in southern United States. California and Florida are two largest producers of strawberry. Florida accounts for 12% of the annual domestic production but 100% of the winter crop. During the 1994-95
season 6,000 acres were planted producing 168 million pounds of berries worth approximately $118.6 million. During the same season the cost of strawberry production per acre was over $15,000, which makes strawberries one of the most expensive crops to grow. In the 1997-98 season, however, 27% of the crop in Florida was infected with anthracnose disease leading to high capital loss (http://edis.ifas.ufl.edu; Dr. Barbara Smith, personal communication). In Louisiana, strawberry has been an important horticultural crop, but production has consistently been declining in recent years. This reduction is also mainly due to strawberry diseases, of which anthracnose disease causing crown, fruit, and root rot and damaging petiole and runners, is most important. 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, therefore, is an attractive alternative to generate plants with enhanced resistance to fungal pathogens. Such goals for other crop species have been achieved. While transfer of foreign genes such as chitinase or $\beta$-1,3-glucanase under control of a strong promoter, such as CaMV 35S RNA, have been achieved in strawberry, it is desirable to obtain knowledge of strawberry’s endogenous defense mechanisms. The foremost benefit of such a study is advancement of understanding in plant defense. Also, with long-term goals in mind, strawberry’s own defense genes or their regulatory elements could be used in enhancing disease resistance in strawberry. Therefore, it is the goal of this dissertation to characterize the chitinase activities of strawberry plants, clone genes encoding various chitinases and other PR-proteins, characterize these genes, and evaluate expression of these genes in strawberry plants under diseased conditions.
CHAPTER 2

CHITINASE ACTIVITIES IN STRAWBERRY PLANT

INTRODUCTION

Plants respond to pathogen attack by the activation of a variety of defense mechanisms. These include structural reinforcement of the cell wall (Vance et al., 1980; Bowles, 1990; Brisson et al., 1994), localized cell death (Levine et al., 1996), accumulation of protease inhibitors (Green and Ryan, 1972), synthesis of phytoalexins (Darville and Albersheim, 1984), and synthesis of pathogenesis-related (PR) proteins (Van Loon, 1983). Chitinases, along with β-1,3-glucanases, are among the most extensively studied PR proteins.

Chitinases (EC 3. 2. 1. 14) catalyze the hydrolysis of chitin, a linear homopolymer of β-1,4-linked N-acetylglucosamine. They are found in a wide range of organisms, including bacteria, fungi, higher plants, insects, crustaceans, and some vertebrates. Most plant chitinases are endochitinases, which randomly hydrolyze internal β-1,4- linkages of chitin to N, N’-diacetylchitobiose and higher oligomers. Many plants contain multiple chitinase isoforms. These hydrolytic enzymes are generally expressed constitutively at low levels. However, their synthesis increases upon viral, bacterial, or fungal infection (Neuhaus, 1999). Plant chitinases are currently divided into seven classes based on their primary structures (Neuhaus, 1999).

Some plant chitinases have been shown to be able to inhibit fungal growth in vitro by causing lysis of the hyphal tips (Young and Pegg, 1982; Boller et al., 1983). This lysis is presumably the result of hydrolysis of chitin in the fungal cell wall. The fungal growth inhibitory effect is synergistically increased if β-1,3-glucanase is also present.
Transgenic plants over-expressing chitinase, alone or in combination with another PR protein, have been shown to exhibit higher levels of resistance to fungal infection or delayed development of disease symptoms (Broglie et al., 1991; Jach et al., 1995; Grison et al., 1996; Datta et al., 2001).

Strawberry is a member of the Rosaceae, which is a large plant family with approximately 3,000 members grouped into approximately 100 genera (Baumgardt, 1982). The Rosaceae also includes, for example, roses, apples, peaches, pears, blueberries, and raspberries. Thus far, very few studies have been reported on the PR proteins of the members of this large plant family. Shih’s laboratory has reported the nucleotide sequence of a strawberry class III chitinase gene (Khan and Shih, 1999), and the sequence and partial characterization of a strawberry gene encoding an osmotin-like protein (Wu et al., 2001). No study on any strawberry PR proteins has thus far been reported. In the present study, I examined the total chitinase activity and the isoform distribution pattern of different organs of the strawberry plant.

MATERIALS AND METHODS

Plant Growth Conditions

Dormant strawberry plantlets were purchased from Nourse farms (South Deerfield, MA, USA). 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)] mixed with dolomitic lime (4.7 kg/m³). 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°C/ 18°C (day/night) and an 11-hour photoperiod. General Electric
(T32T8SP41) lamps were used for illumination delivering irradiance of 8 W m\(^{-2}\). The relative humidity was kept at 60% to 70%. The plants were watered with distilled water approximately every other day, and they were used in experiments in about 10-14 days after planting.

**Preparation of Strawberry Protein Extract**

Strawberry leaves or other organs were ground to fine powders in liquid nitrogen with a mortar and pestle. The ground samples were added to the extraction buffer [25 mM Tris-HCl, pH 8.5, 5% polyvinyl polypyrrolidone (PVPP), 14 mM \(\beta\)-mercaptoethanol, and 30 \(\mu\)L/g fresh tissue weight of plant protease inhibitor cocktail (Sigma Chemical Co. St. Louis, MO, USA)]. The tissue to buffer ratio was 1:4. The mixture was ground in an Omni-mixer (DuPont Co. Newtown, CT, USA) homogenizer on ice three to four times with 1 min homogenization followed by 1 min pause each time. The homogenate was centrifuged at 20,000 g for 10 min and the supernatant was passed though one layer of Miracloth (BD Biosciences Clontech, Palo Alto, CA, USA). The crude extract was stored at –20°C.

**Protein Concentration Determination**

Protein concentrations were determined by using the Bio-Rad Coomassie dye reagent (Bio-Rad Laboratories, Hercules, CA, USA) based on the Bradford method (1976).

**Preparation of \(^{3}\)H-Labeled Chitin and Radiometric Chitinase Activity Assay**

The method described by Boller (1992) for the preparation of \(^{3}\)H-labeled chitin was used with some modifications. To 2.5 g of chitosan, 50 mL of 10% acetic acid was slowly added over a period of 30-40 min. After stirring for one hour, this mixture
became viscous. The mixture was stirred for another hour after addition of 225 mL of methanol. This solution was filtered through 53 µm nylon mesh (Spectrum, Laguna Hills, CA) over a buchner funnel. In a separate tube, 10 mCi (15 µL) of [3H]-labeled acetic anhydride (ICN Chemicals) was mixed with a 10 mL mixture of methanol and 10% acetic acid solution in a 9:2 ratio. The radioactive solution was mixed with the chitosan slurry and stirred vigorously for 1 min and then kept at room temperature for 2 h. While stirring vigorously, 3.5 mL of non-radioactive acetic anhydride was added and in less than 2 min the solution gelled and this gel was kept for 1 h at room temperature. The radioactive liquid on top of the gel was removed and the gel was cut into pieces with a spatula. The gel was ground in a blender with 100 mL of water at the lowest speed for 2 min. The amorphous suspension was poured over a nylon net in a buchner funnel. The regenerated chitin was washed with 500 mL of water twice and with 500 mL of acetone twice. Briefly dried regenerated chitin was mixed with 250 mL of ice cold HCl and stirred for 20 min. While mixing vigorously, the HCl-chitin suspension was filtered through two layers of cheesecloth and collected into 2 L of 50% ethanol. This suspension was stirred for 20 min and filtered through a nylon mesh on a buchner funnel. The regenerated chitin was washed three times with 800 mL of water, twice with 100 mM NaAcetate (pH 5.5), and five times with water again. Chitin was collected into a measuring cylinder and the volume was brought to 247.5 mL with water and 2.5 mL of 2% Na-azide was added. The chitin suspension was homogenized in a blender at low speed for 30 s, divided into 25 mL aliquots and kept at 4°C.

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 µL of 0.1 M sodium acetate buffer, pH 5.0, 20 µL of [3H]-chitin (approximately 3x10^5 CPM), various amounts of the enzyme solution, and de-ionized distilled water (ddH2O) in a final volume of 200 µL. The reaction mixtures were incubated in 1.5 mL microcentrifuge tubes for 2 h at 37°C. The tubes were incubated with shaking at 200 rpm in an incubator-shaker. After incubation, the reactions were stopped by addition of 200 µL of 10% trichloroacetic acid (TCA). The undigested substrate was pelleted by centrifugation at 15,000 g for 10 min. Avoiding any visible pieces of the substrate, 220 µL of supernatant was withdrawn from each assay mixture and filtered through a 0.45 µm Millipore Ultrafree-MC filter (Millipore Crop., Bedford, MA, U.S.A.) by centrifuging the filter assembly at 15,000 g for 15 s. A 200 µL aliquot from each filtrate was withdrawn and added to 2 mL of the Liquiscient™ scintillation cocktail (National Diagnostics, Atlanta, GA, U.S.A.) and counted in a Beckman LS 60001 C Scintillation counter (Beckman Instruments, Inc., Fullerton, CA, U.S.A.).

**Detection of Acidic Chitinase Isoforms**

Chitinase isoforms were detected according to the method of Trudel and Asselin (1989) with slight modifications. Gel electrophoresis was carried out using Hoeffer SE250 Mighty Small minigel system (Amersham Pharmacia Biotech, San Francisco, CA, U.S.A.). Separating (12.5%) and stacking (5%) gels of 1.5 mm thickness were prepared according to the Laemmli system (Laemmli, 1970) with the exclusion of SDS. The protein samples were prepared in a 3X sample buffer that did not contain SDS or any reducing agent. Also, no heat treatment of the samples was performed. 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. When the tracking dye reached the bottom of the gel, the
gel was removed and incubated in 100 mL of 0.1 M sodium acetate buffer (pH 5.0) for 5 min. This gel was then overlaid on a 7.5% acrylamide, 0.75 mm thick, substrate gel containing glycol chitin (0.04%) as substrate in 0.1 M sodium acetate buffer (pH 5.0). The gels were sandwiched between two glass plates and incubated at 37°C under moist conditions. A weight of about 200 g was placed on top of this assembly. The incubation time varied between 4-16 h. After incubation, the substrate gel was stained with 0.01% (w/v) Fluorescent Brightener 28 (C.I. 40622; calcofluor white M2R) in 0.5 M Tris-HCl buffer (pH 8.8) for 5 min in the dark (Trudel and Asselin, 1989). Following extensive destaining with water in the dark, the acidic chitinase isoform bands appeared as cleared (dark) zones against bluish fluorescent background when viewed over an UV transilluminator.

**Detection of Basic Chitinase Isoforms**

Gel electrophoresis was carried out using Hoeffer SE250 Mighty Small minigel system. Separating (12.5%) and stacking (5%) gels were prepared according to the method described by Reisfeld et al. (1962). The protein samples were prepared in a 3X sample buffer that did not contain SDS or a reducing agent. Methyl green (1%) was used as a tracking dye in the sample buffer. Also, no heat treatment of the samples was performed. In order to separate the basic proteins, the polarity of electrodes was reversed as compared to the Laemmli system. The gels were loaded and run at 4°C at a constant voltage of 25 V. Once the protein samples compacted, voltage was increased to 100 V. When the tracking dye reached the bottom of the gel, the gel was removed and treated further as in the detection of acidic chitinase isoforms.
Detection of Chitinase Isoforms by SDS-PAGE

Gel electrophoresis was carried out using Hoeffer SE250 Mighty Small minigel system. Separating (12.5%) and stacking (5%) gels were prepared according to the Laemmli system. Glycol chitin (0.01%) as the chitinase substrate was incorporated in the separating gel solution prior to polymerization. The protein samples were prepared in a 3X sample buffer that did not contain any reducing agent. Also, no heat treatment of the samples was performed. The gels were run at 16°C at a constant voltage of 150 V. Once the protein samples compacted, voltage was increased to 200 V. When the tracking dye reached the bottom of the gel, the gel was removed and incubated in 200 mL of 0.1 M sodium acetate buffer (pH 5.0) containing 1% (v/v) Triton X-100 (Research Products International Corp., Elk Grove Village, IL, U.S.A.) at 37°C with mild agitation at 50 rpm in an incubator-shaker (Model G-25, New Burnswick Scientific Co., Edison, NJ, U.S.A.) for 4-16 h. This incubation slowly released SDS from the gel promoting protein renaturation. The gel was then stained with Fluorescent Brightener 28, destained, and viewed as described above.

Effect of pH on Enzyme Activity

The optimal pH for strawberry chitinases was measured by incubating the protein extract (5 µg) in assay solutions of different pH. For assaying the activity at pH 1, 0.1 N HCl was used. For other pH, the following buffers were used: pH 2, Na-phosphate; pH 3, Na-citrate; pHs 4 and 5, Na-acetate; pH 6 MES (2-(N-morpholino) ethanesulfonic acid); pH 7, Na-phosphate; pH 8, Tris (tris(hydroxymethyl) aminoethane); pH 9 and 10, CHES (cyclohexylaminoethanesulfonic acid). The final concentration of all buffers in the assay solutions was 25 mM.
Effect of Temperature on Enzyme Stability

For measuring the effect of temperature on chitinase stability, the protein extract (5 µg) was incubated in 50 mM Na-acetate buffer, pH 5.0, at various temperatures for 1 h. Following this incubation, substrate was added to start the regular assay.

RESULTS

The Linear Range of Chitinase Activity

The linear range of the radioactive chitinase assay was evaluated. Figure 2.1A shows strawberry leaf chitinase activity when total protein concentration was increased from 0-20 µg. Figure 2.1B shows fit of the line for chitinase activity when protein concentration varied from 0-10 µg. The plot shows linearity at least up to 10 µg of total protein. This assay provides confidence in comparative measurements across samples when total protein less than 10 µg was used.

The pH and Temperature Effects

To characterize the strawberry chitinases, the effect of pH on total chitinase activity and the effect of temperature on the stability of the enzyme were examined. Leaf extracts were used as the source of the enzyme.

For determining the pH effect, chitinase activity assays were carried out in buffers with pH values ranging from 1 to 10. The highest activity was observed at pH 5 (Figure 2.2). At pH 4 and 6, the activity was approximately 90% of that of pH 5. Much lower activities were seen at pH lower than 4 or higher than 6. The enzyme activities were slightly above background at pH 1, 2, 9, and 10, and were 12%, 31% and 12% of that of pH 5 at pH 3, 7, and 8, respectively.
Figure 2.1  Linear range of chitinase activity.  A. Chitinase activity measured by radioactive assay in counts per minute as a function of total protein from 0-20 µg.  
B. Fit of the line from A.
Figure 2.2  Chitinase activity as a function of pH. Radioactive chitinase assays were performed using 5 µg total protein with buffers at indicated pH. The highest value at pH 5 was set to 100% and all other values were compared to that. The chitinase activity of each sample was read in triplicate and each bar represents the average of two independent experiments.

The stability of total chitinase activity to heat treatment was measured by incubating protein extracts at 20, 30, 40, 50, 60, 70, and 80°C for 1 h in 50 mM sodium acetate buffer (pH 5.0). The strength of the buffer was increased from regular radioactive assay condition of 25 mM to 50 mM in order to reduce any pH fluctuations due to temperature change. The chitinase activity after exposure of protein sample to 20°C was set at 100% and all other readings were compared to that. Figure 2.3 shows that temperature up to 40°C has no effect on total chitinase activity. At 50°C, 85% activity remains, whereas it drops to 65%, 15%, and 3% at 60, 70, and 80°C, respectively.
Figure 2.3  Total chitinase activity of protein extracts after incubation at indicated temperature for 1 h. The chitinase activity of sample incubated at 20°C was adjusted to 100% and all other values were compared to that. The chitinase activity of each sample was read in triplicate and each bar represents the average of two independent experiments.

Comparison of Chitinase Activity and Isoform Pattern of Different Strawberry Organs

The basal level of total chitinase activity was measured for various strawberry plant organs. For leaves, petioles, crowns, and roots, plants grown in growth chambers were used. The fruit and runner samples were obtained from plants grown in the local fields. Chitinase activities in all samples were compared based on equal total protein. The crown showed the highest level of chitinase activity in plants grown in growth chamber (Figure 2.4). Roots, petioles, and leaves showed successively lower levels of chitinase activities of 70%, 45%, and 38%, respectively, as compared to the crown. Fruit showed approximately equal level of activity as leaf and petiole, whereas runners showed the lowest level of chitinase activity of about 25% of fruit.
Figure 2.4  Total chitinase activities in different strawberry organs. Chitinase activities were measured using chitin as substrate by radioactive assay in 5 µg of total protein extract from the indicated organ. Each chitinase assay was performed in triplicate. Each bar represents an average of four trials with pooled material from two plants.

Presence of acidic and basic isoforms was detected by native gel electrophoresis followed by incubating the gel in contact with glycol chitin containing substrate gel. Figure 2.5A shows acidic chitinase isoforms in strawberry. The acidic isoforms were identified as ChiA1 through ChiA5 with decreasing mobility as seen in the leaf sample of field grown plants. ChiA3 was the predominant isoform in samples from all organs except fruit. In leaf samples of plants grown in a growth chamber, only the major isoform ChiA3 was visible. In all other organs analyzed, additional minor isoforms were also visible. In the crown, which showed the highest chitinase activity, the major acidic isoform was not as prominent as other organs, but the presence of three additional isoforms could explain the high chitinase activity. It is interesting to note that the major acidic isoform (ChiA3) is not as prominent in fruit. When this major acidic isoform was extracted from the native gel, which was loaded with leaf protein extract, and run on
Figure 2.5  Acidic (A) and basic (B) isoforms in different strawberry organs from plants grown in growth chambers. Five µg total protein extract was loaded per lane. Lanes 1 and 8, strawberry leaf extract from field-grown plants; lane 2, leaf; lane 3, petiole; lane 4, crown; lane 5, root; lane 6, runner; lane 7, fruit.
sodium dodecylsulfate-polyacrylamide (SDS-PAGE) activity gel, it dissociated into several chitinase activity bands (see below).

Figure 2.5B shows the basic isoforms in the same sample setup as in Figure 2.5A. In growth chamber plants no basic isoform was detected in any organ, whereas one basic isoform, designated ChiB1, was present in leaf samples in plants from fields, lanes 1 and 8.

Figures 2.6A and B show acidic and basic isoforms, respectively, in plants grown in the field. Lanes 1-4 show chitinase isoforms from leaves, roots, crowns, and fruits, respectively. In these samples, leaves clearly show the presence of five different acidic and one basic isoform. Isoform ChiA1 appears to be expressed exclusively in leaves. Both roots and crowns show three different acidic and two basic isoforms. Fruits show four acidic and one basic isoform. In the fruit sample again, the major acidic isoform (ChiA3) was not so prominent.

**Analysis of ChiA3 on SDS-Polyacrylamide Gels**

Analysis of the leaf extract from growth chamber grown plants on native gels showed that it contained only one acidic isoform (ChiA3) and one basic isoform (ChiB1) (Figure 2.5). However, when the same extract was fractionated on glycol chitin-containing SDS-polyacrylamide gels, at least five major activity bands were observed after the proteins on the gels were renatured by removing of the SDS following electrophoresis (See Figure 2.8 below). A possible explanation of this discrepancy could be that the isoform detected on the native gel is a protein complex consisting of multiple chitinase isoforms. In support of this possibility, it has been shown that fractionation of the apoplastic fluid of cold-acclimated winter rye leaves on native polyacrylamide gels
Figure 2.6 Acidic (A) and basic (B) isoforms in different organs of strawberry plants from field grown plants. Five µg total protein was loaded per lane. Lane 1, leaves; lane 2, root; lane 3, crown; and lane 4, fruit.
resulted in the separation of nine acidic proteins (Yu and Griffith, 1999). Six of these nine proteins were found to be oligomeric protein complexes that have antifreeze activity. These antifreeze protein complexes, two of which contain as many as seven or eight polypeptides, are composed of various combinations of a 35-kDa chitinase-like protein (CLP), one or two glucanase-like proteins, and a thaumatin-like protein as revealed by first isolating complex proteins individually from the native gel followed by fractionating them on SDS gels and probing with specific antisera. The gene encoding the 35-kD CLP was subsequently cloned and sequenced, and the result showed that this protein is indeed a chitinase (Yeh et al., 2000).

To test whether similar chitinase-containing protein complexes exist in the strawberry plant, we chose ChiA3 for further analyses. First, the leaf extract was incubated under different conditions or 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, and the diluted extract was heated at 40°C or 55°C for 30 min in the presence of 0, 10, or 20 mM of additional mercaptoethanol. The result in Figure 2.7A clearly shows that ChiA3 remained as a single band under these treatment conditions, although enzyme inactivation occurred when the extract was incubated at 55°C in the presence of high concentrations of mercaptoethanol. Similarly, ChiA3 remained as a single band when the same set of samples were fractionated on a 20% gel (Figure 2.7B). These results indicated that ChiA3 was not a non-specific aggregate or a fortuitous result of several chitinases migrated together in the 12.5% gel.
Figure 2.7 The stability of ChiA3 after treatments at different conditions. (A) Electrophoresis of a diluted leaf extract incubated at 40° or 55°C in the presence or absence of mercaptoethanol. Lanes 1, untreated control; lanes 2 through 4, diluted extract incubated at 40°C in the presence of 0, 10, and 20 mM mercaptoethanol, respectively; lanes 5 through 7, diluted extract incubated at 55°C in the presence of 0, 10, and 20 mM mercaptoethanol, respectively. (B) Same as A on a 20% native polyacrylamide gel.

Next, Chi3A was extracted from the native polyacrylamide gel and then run on a substrate-containing SDS gel. After renaturing and staining the gel, four or five chitinase activity bands could be seen (Figure 2.8, lane1). These activity bands represent a subset of the bands obtained from total leaf protein extract (lanes 2 and 3). This result suggested that ChiA3 very likely is a complex protein consisted of three or four chitinase isoforms.

**DISCUSSION**

Although various colorimetric methods for the measurement of chitinase activity have been developed, the radiometric chitinase assay remains the most sensitive and easy
to perform. However, the quality and characteristics of tritium labeled chitin substrate are unique for every batch, introducing some variation in the results. In the literature it has been argued that the radiometric assay is not linear in time or with enzyme concentration. For example, chitinases from wheat germ (Molano et al., 1979), bean pea pods (Mauch et al., 1988), and bean leaves (Boller et al., 1983) showed non-linear product formation with varying concentrations of protein. The reason for this effect is not clear, but it might be due to structural differences in various chitinases. The bean and pea chitinases are class I enzymes whereas the wheat germ chitinase classification is unknown. In this report, I show that at low protein concentrations of up to 10 µg this assay performs linearly. In agreement with my findings the total protein extract of wintermelon seeds also showed linear enzyme assay from 1-9 µg of total protein.
Purified wintermelon chitinase showed linear chitinase activity from 0.06-1 µg (Shih et al., 2001). Therefore, for comparative analysis, strawberry protein concentration of 5 µg was chosen for all assay reactions.

Chitinases are generally active over a wide pH range of 3-10. A bean chitinase retained 50% activity at pH 3 and 9 with an optimum at 6.5 (Boller et al., 1983). The strawberry chitinases, however, are active from pH 4 to 6, with chitinase activity dropping sharply on either side of this pH range. This relatively narrow pH range is in agreement with the range for purified chitinases from rye seeds (Yamagami et al., 1993) and carrot (Zhang et al., 1996). A purified chitinase from Japanese radish seeds showed stability over a pH range of 2.2-10.5, and optimal activity at pH 6 (Kondo et al., 1997). However, the purified class III wintermelon chitinase (Shih et al., 2001) showed its optimal activity at pH 2 and retained approximately 50% activity at pH 8. Some chitinases, such as a class III yam chitinase (Tsukamoto et al., 1984) showed two pH optima that are dependent on the substrate used for the assay.

Chitinases are generally stable at moderately high temperatures. This is perhaps because they are relatively small in size (25-40 kDa) with several disulfide bridges in their tertiary structures. The strawberry chitinases retained approximately 85% and 65% activity when exposed to 60°C and 70°C, respectively. This again is in agreement with other chitinases such as that from carrot which retains 65% activity after treatment at 60°C (Zhang et al., 1996). Wintermelon chitinase retained approximately 75% activity after 70°C exposure (Shih et al., 2001). A class III yam chitinase was shown to be resistant to temperatures of 80°C (Tsukamoto et al., 1984). A Japanese radish chitinase showed its optimal activity between 55-65°C. Interestingly, this enzyme retained stability up to 60°C and denaturing completely at 70°C (Kondo et al., 1997).
The highest level of chitinase activity in strawberry was found in the crown followed by root, petiole, fruit, leaf, and runner. It appears that the highest level of chitinase activity in crown is consistent with the idea that storage, regenerative, and embryogenic organs have maximal levels of protective mechanisms. The high chitinase activities of crown and root that are directly in contact with soil might reflect constant challenge by pathogens and could be a defense mechanism. The lower level of activity in berries is somewhat surprising since fruit is more prone to pathogen invasion. It has been shown that grape chitinase activity was about 10 times higher than leaves and the chitinase activity of berries could be correlated with the accumulation of sugar (Derckel et al., 1996 and 1998). Accumulation of PR-proteins during ripening has been reported for other fruits. However, in tomato, a β-1,3-glucanase was reported to be downregulated in fruit (Gonzalez-Bosh et al., 1996).

Chitinase activities correlated well with the number of acidic isoforms in the organs tested. Crown contained four isoforms (Figure 2.5A) and had the highest activity whereas root and petiole had two isoforms followed by leaves which had only one acidic isoform. The molecular weights of chitinase isoforms were 25-65 kDa as determined by SDS-PAGE.

A number of acidic and basic isoforms have been detected in various plants. For example in wheat, 7 distinct isoforms were detected by isoelectric focusing (Botha et al., 1998); 4 isoforms were present in cucumber (Zhang and Punja, 1994) and celery (Krebs and Grumet, 1991); 7 isoforms were present in tobacco (Pan et al., 1992); 13 isoforms were present in grapevine (Derckel et al., 1996); up to 8-10 chitinase isoforms were detected in carrot (Zhang et al., 1996). Our studies showed that in strawberry, up to five acidic and two basic chitinase isoforms were detected in different plant organs. Even
though each chitinase activity band is presumed to be a distinct isoform, it cannot be completely ruled out that some faster migrating bands are proteolytic cleavage products of the larger isoforms. The proteolytic cleavage and generation of isoform diversity, however, could occur in vivo and does not necessarily represent protein extraction artifact. In bean plants a class IV chitinase was shown to be proteolytically cleaved in vivo into three different isoforms (Lange et al., 1996). The processing occurred in plant roots colonized by a compatible fungus, *Fusarium solani*, but not in plants colonized with incompatible or symbiotic fungi. The processing occurred in the hinge region, retaining the catalytic domain intact. The function of this diversification is not known. However, it could be a plant strategy to produce isoforms with altered substrate specificities or a pathogen strategy to reduce the effectiveness of the nascent protein.

The isolated ChiA3 dissociated into three or four chitinase activity bands when it was subjected to electrophoresis on a SDS gel. This observation indicated that, like the winter rye antifreeze proteins, ChiA3 may also be a protein complex. It is intriguing to note here that strawberry plants are generally planted in the field before the winter months, while fruit production occurs after the winter season. The isoform distribution pattern results showed that ChiA3 was the predominant isoform in the leaf, petiole, crown, and root, but not the fruit. It would be interesting to know if ChiA3 is indeed a protein complex containing other PR-proteins. In the future, a western blot could be performed with this complex to address the possibility. However, if it is in fact a complex, it is surprising that it enters the 12.5% and the 20% acrylamide gel. The winter rye complex had multiple PR-proteins with a combined molecular weight of approximately 127 kDa. It is possible that the complex exists as a compact structure in its native form.
CHAPTER 3

CLONING AND CHARACTERIZATION OF PATHOGENESIS-RELATED GENES

INTRODUCTION

Synthesis of hydrolytic enzymes, such as chitinases and β-1,3-glucanases, is among the diverse plant responses to pathogen attack or physical insults such as injury. Chitinases (E.C. 3.2.1.14) hydrolyze the β-1,4-linkages in chitin, a homopolymer of N-acetyl-D-glucosamine. Plant endochitinases cleave chitin into N,N′-diacetylchitobiose and higher oligomers. Chitinases have been shown to lyse fungal hyphae and release chitin oligomers from fungal cell walls in vitro (Boller et al., 1983; Mauch et al., 1988). Based on these and other observations, plant chitinases have been implicated in protection against pathogens (Bowles, 1990; Broglie, 1993). It has been hypothesized that increased chitinase expression in plants restrains fungal infection directly by degrading the chitin containing fungal cell walls and indirectly by releasing elicitors of other plant defense responses. Transgenic plants with constitutive chitinase expression have been shown to exhibit enhanced levels of resistance to fungal infection and delayed disease symptoms when challenged with fungal pathogens (Broglie et al., 1991; Jach et al., 1995; Grison et al., 1996). Transgenic plants co-expressing two PR-genes such as chitinase and β-1,3-glucanase show a synergistic effect on disease resistance.

In an effort to understand the defense systems of strawberry plants on a molecular level, Shih laboratory has been studying the genes and proteins implicated in such systems. Strawberry is a member of the Rosaceae (rose) family. The rose family is a large plant family consisting of more than 3,000 species with approximately 100 genera
(Baumgardt, 1982). Only a few sequences of PR-genes are known from this large family. The only known sequences of any chitinases in this family include an apple class III chitinase (only open reading frame (ORF) sequence shown, it is uncertain if it was a cDNA or genomic DNA, accession # AF309514), a partial cDNA sequence of a class III chitinase from sand pear (accession # AB021786), and a partial genomic sequence of a class I chitinase from peach (accession # AF206635). In comparison, several β-1,3-glucanase sequences are available from rose family members.

In this chapter I report cloning of two class II chitinase genes (designated as FaChi2-1 and FaChi2-2, for *Fragaria ananassa* chitinase class II gene 1 and 2, respectively), which are the only examples of class II chitinases from the rose family members. Genomic Southern blotting and primer extension analyses were performed for FaChi2-1 and FaChi2-2 to analyze the genomic organizations and transcription start sites for these genes. The exact locations of introns in these genes were confirmed by cloning of their corresponding cDNA. These cDNAs likewise represent the only examples of class II chitinase cDNAs from rose family members. A class III chitinase gene was also cloned (designated as FaChi3-1 for *Fragaria ananassa* chitinase class III gene 1). This genomic sequence was the first reported chitinase gene from any rose family member (Khan and Shih, 1999). Sequence information on a class II β-1,3-glucanase (designated as FaGlu2-1) gene and its cDNA was also obtained, establishing the complete coding region, the presence of an intron, and the transcription start site. In addition to the PR-protein genes, partial genomic and cDNA sequences of two distinct strawberry glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes (FaGAPDH1 and FaGAPDH2) were also obtained.
MATERIALS AND METHODS

Preparation of Genomic DNA

DNA was extracted and purified by using the MasterPure Leaf DNA extraction kit (Epicenter Technologies) or by using the method described by Manning (1991). For large-scale DNA preparations by Manning’s method, 3 g of leaves (central vein removed) were ground in liquid nitrogen into very fine powders in chilled mortar and pestle (approximately 50 strokes in 30-40 sec). The ground leaves were transferred into 30 mL Oakridge centrifuge tube containing 10 mL of extraction buffer (0.2 M boric acid, 10 mM Na₂EDTA, pH 7.6). Sodium dodecyl sulfate (SDS) and β-mercaptoethanol were added to the final concentration of 0.5% and 280 mM, respectively, before use. The powdered ground leaves were gently stirred into the buffer. Ten mL of phenol:chloroform:isoamylalcohol (IAA) (equilibrated with extraction buffer without the SDS and β-mercaptoethanol) was added and stirred vigorously for 15 min. This mixture was centrifuged for 10 min at 18,000 g. Eight mL of the upper aqueous layer was divided into 4 mL aliquots into two 30 mL centrifuge tubes. Each 4 mL extract was diluted three fold by addition of 8 mL of water. The Na⁺ concentration was raised by addition of 1 mL of 1 M sodium acetate, pH 4.6. To this mixture, 0.4 volumes (5.2 mL) of 2-butoxyethanol (2-BE) was added and mixed by inversion 4-6 times. This mixture was kept on ice for 30 min to precipitate sugars. The sugars were removed as a gelled pellet by centrifugation at 20,000 g for 15 min. To the supernatant, 2-BE was added to one volume (7.8 mL), mixed, and kept on ice for 30 min to precipitate total nucleic acids. After centrifugation at 20,000 g for 20 min, the pellet was washed sequentially once with 1 mL of 1:1 of extraction buffer and 2-BE, once with 1 mL of 70% ethanol with 0.1 M K-
acetate (pH 7.6), and once with absolute ethanol. The pellet was briefly dried and
dissolved in 0.2 mL of sterile water.

RNA was removed from genomic DNA preparations by RNase A treatment.
RNase A (DNase free) was used at a final concentration of 0.1 mg/mL and the reaction
was carried out at 37°C for 2 h. The DNA was extracted once with
phenol:chloroform:IAA and once with chloroform:IAA. The DNA was precipitated and
dissolved in distilled-deionized water (ddH2O) and analyzed on a 1% agarose gel. If the
isolated DNA was resistant to restriction digest, it was precipitated and dissolved in 0.2
mL of the cleanup solution provided in the Plant Leaf DNA Extraction Kit (Epicentre
Technologies). The DNA was precipitated with isopropanol, washed with 70% ethanol,
and dissolved in 0.2 mL of ddH2O again.

**Cloning of Genes and cDNA Encoding Pathogenesis-Related Proteins**

Genes encoding pathogenesis-related proteins were cloned using PCR based
protocols. Degenerate primers directed towards conserved regions of the defense-related
genones were designed by alignment of protein sequences from other plant sources. Based
on sequence information of the gene fragments obtained by degenerate primers, genome
walking was performed to obtain complete gene sequences. The primer pairs and PCR
conditions for each gene are listed below and as an example, cloning of FaChi2-1 gene is
described in detail. Both DNA strands of the full-length genomic and cDNA clones were
sequenced before sequence submission to the GenBank database.

**Cloning of a Class II Chitinase Gene (FaChi2-1)**

Two degenerate primers were designed to anneal to the conserved regions in the
catalytic domain of the class I or class II chitinase genes. The forward primer was 5’-
CAR CAN WSIn CAY GAR CAN CAN GG-3’ and the reverse primer was 5’-CCR CAY 
TCN ARN CCN CCR TTD A-3’. These primers were designed to anneal to conserved 
regions QTSHETTG and INGGLECG of the protein sequence, respectively. PCR was 
performed in a 50 µL reaction mixture containing 50 ng of strawberry genomic DNA, 
400 µM each of dNTP, 3 mM MgCl₂, 1 X PCR buffer without Mg⁺⁺, and 5 unit (U) Taq 
DNA polymerase (Promega). The degenerate primer set was used at concentrations of 
0.5, 1, and 2 µM of each primer in the PCR reactions. A touch-down PCR program was 
used with one step for 3 min at 94°C, followed by 10 cycles of 1 min at 94°C, 1 min at 
58°C (decreasing 1°C / cycle), 1 min at 72°C, 30 cycles of 1 min at 94°C, 1 min at 48°C, 1 
min at 72°C, and a final step of 10 min at 72°C.

The putative gene fragments were purified from agarose gels using the Concert 
Nucleic Acid Purification System (Gibco, BRL Life Technologies), cloned into pGEM T-
Easy vector (Promega) and sequenced at either Iowa State University’s DNA Synthesis 
and Sequencing Facility or on campus at the Gene Lab of Louisiana State University 
School of Veterinary Medicine. The nucleotide sequences were conceptually translated 
and the identities of the translated products were analyzed using the National Center for 
Biotechnology Information’s (NCBI) BLASTp search program.

Genome walking was performed to obtain the sequence upstream and downstream 
of the PCR products obtained by the degenerate primers. Five genome walker libraries 
(DraI, EcoR V, Pvu II, Sca I, Stu I) were constructed using the Universal Genome 
Walker kit according to the manufacturer’s directions (Clontech). Briefly, 2.5 µg of 
strawberry genomic DNA samples were each completely digested with Dra I, EcoR V, 
Pvu II, Sca I, or Stu I. The digested DNA was extracted with phenol:chloroform:IAA,
precipitated, and synthetic GenomeWalker adaptors were ligated to the genomic DNA fragments using T4 DNA ligase. Two nested primers, specific for the adaptor sequences, were provided in the GenomeWalker kit. Based on the sequence information of initial gene fragments, two nested gene-specific primers were designed for each upstream or downstream genome walk. With these primers and the adapter primers provided by the GenomeWalker kit, PCR reactions were performed according to the Genome Walker kit recommendations. The amplification products of first reactions were used as templates for the second round PCR with nested primers to obtain gene fragments. The fragments from a library producing the cleanest and largest product was purified and sequenced directly or cloned into pGEM T-Easy vector and sequenced to obtain additional sequence information.

After one round of upstream and one round of downstream genome walking, the complete gene sequence of FaChi2-1 was obtained. The following primers were used to obtain a full-length clone of this gene, forward primer, 5’-ACG GAG TCA ACA ATC AAG GTA CCG ACA CGA-3’, reverse primer, 5’-CCA GAC CAA AGG ATC CAT ATA ACT CGC TAG-3’. PCR was performed in a 25 µL reaction mixture containing 20 ng of strawberry genomic DNA, 400 µM each of dNTP, 400 nM of each primer, 1 X PCR buffer with 2 mM MgSO₄, and 4 U Vent DNA polymerase (New England Biolabs). A touch-down PCR program was used with one step for 3 min at 94°C, followed by 5 cycles of 1 min at 94°C, 1 min at 70°C (decreasing 1°C / cycle), 2 min at 70°C, 30 cycles of 1 min at 94°C, 1 min at 65°C, 2 min at 70°C, and a final step of 10 min at 70°C. Since VentR DNA polymerase produces blunt end products, terminal A’s were attached for cloning into pGEM T-Easy vector as follows. The PCR products were extracted with
phenol:chloroform:IAA and precipitated. The pellet was dissolved in 20 µL 1X Taq buffer (Promega) with 200 µM dATP and 3 mM MgCl₂. The reaction was performed at 72°C for 20 min with 2 U of Taq DNA polymerase.

**Cloning of Second Class II Chitinase Gene (FaChi2-2)**

Degenerate primers, described for FaChi2-1, amplified a fragment of another distinct class II chitinase gene. This gene was designated as FaChi2-2 and the complete coding sequence was determined after two rounds of upstream and one round of downstream genome walking. A full-length clone of this gene was obtained using the following primers in a PCR reaction; forward primer, 5’-GCT GGT CCT TTA GGT CTG CCC AAC A-3’, reverse primer 5’-TGT AAC TGG GAA GTA CAT TCG TAT CC-3’. The full-length clone was obtained by using the FailSafe PCR system (Epicenter Technologies). PCR was performed in a 25 µL reaction mixture containing 20 ng of strawberry genomic DNA, 300 nM of each primer, 1 X PCR buffer B, and 1 U DNA polymerase mix. A touch-down PCR program was used with one step for 3 min at 94°C, followed by 6 cycles of 1 min at 94°C, 1 min at 72°C (decreasing 1°C / cycle), 2 min at 72°C, 28 cycles of 1 min at 94°C, 1 min at 65°C, 1 min 35 s at 72°C, and a final step of 10 min at 72°C. The PCR product was cloned into pGEM T-Easy vector and both DNA strands were sequenced before sequence submission to the GenBank database.

**Cloning of FaChi2-1 and FaChi2-2 cDNAs**

The cDNAs containing complete coding sequences of FaChi2-1 and FaChi2-2 were obtained by reverse transcribing strawberry leaf total RNA using the Retroscript First Strand Synthesis Kit (Ambion). Reverse transcription was carried out in a 20 µL reaction mixture containing 2 µg of total leaf RNA, 5 µM oligo (dT) primers, 500 µM
each dNTP, 1 U of placental RNase inhibitor, 10 U of moloney murine leukemia virus (MMLV) reverse transcriptase, and 1X reverse transcription buffer. Reverse transcription was performed at 45°C for 1 h. The reaction was terminated by heating at 92°C for 10 min. The following two primers were used to obtain a fragment of FaChi2-1 cDNA containing the complete open reading frame, forward primer, 5’-GGC CGG CAC ATA CTT ATG ACA A-3’, reverse primer, 5’-AGA GTG TTA AGT GTA GCT GCT CGC-3’. Two µL of the reverse transcription reaction containing cDNA was used as template in a 25 µL PCR reaction mixture containing 400 µM each of dNTP, 300 nM of each primer, 3 mM MgCl₂, 1 X PCR buffer, and 5 U Taq DNA polymerase (Promega). A touch-down PCR program was used with one step for 3 min at 94°C, followed by 8 cycles of 1 min at 94°C, 1 min at 60°C (decreasing 1°C / cycle), 1 min 30 s at 72°C, 30 cycles of 1 min at 94°C, 1 min at 52°C, 1 min 30 s at 72°C, and a final step of 10 min at 72°C. The PCR product was purified and cloned into pGEM T-Easy vector (Promega). Both DNA strands were sequenced and the sequence was deposited in the GenBank database.

The following two primers were used to obtain a cDNA clone of the FaChi2-2 gene, forward primer, 5’-CCA AGC TCT GGC TAT TAC CA-3’, reverse primer, 5’-GGT CTA TGT AAG AAT AAG GGA-3’. PCR conditions as described above for FaChi2-1 were used.

**Cloning of a Class III Chitinase Gene (FaChi3-1)**

The following degenerate primers were initially used to obtain a fragment of class III chitinase gene through PCR, forward primer, 5’-GGN ATH GCN ATH TAY TGG-3’, reverse primer, 5’-CCA NAR CAT NAC NCC NCC RTA-3’. These primers were
designed to anneal to the conserved regions, GIAIYW and YGGVMLW respectively, of the protein sequence. PCR was performed in a 50 µL reaction mixture containing 50 ng of strawberry genomic DNA, 400 µM each of dNTP, 3 mM MgCl₂, 500 nM of each primer, 1 X PCR buffer without Mg ++, and 5 U *Taq* DNA polymerase (Promega). A touch-down PCR program was used with one step for 3 min at 94°C, followed by 10 cycles of 1 min at 94°C, 1 min at 60°C (decreasing 1°C / cycle), 1 min at 72°C, 30 cycles of 1 min at 94°C, 1 min at 50°C, 1 min at 72°C, and a final step of 10 min at 72°C. The complete coding region of this gene was obtained after one round of upstream and downstream genome walks. The primers for the final amplification of FaChi3-1 were 5'-GCC TAC GTG ACT GTA ATG CCA CGG ATC A-3' (forward primer) and 5'-CRC CCA CCR TTC ATC TTA RGC TAC ATG TAG-3' (reverse primer) (note that after sequencing, it was discovered that the forward primer did not contain GCC at the 5’ end). PCR was performed in a 25 µL reaction mixture containing 20 ng of strawberry genomic DNA, 400 µM each of dNTP, 400 nM of each primer, 1 X PCR buffer with 2 mM MgSO₄, and 4 U *Vent* DNA polymerase (New England Biolabs). A touch-down PCR program was used with one step for 3 min at 94°C, followed by 8 cycles of 1 min at 94°C, 1 min at 75°C (decreasing 1°C / cycle), 1 min 30 s at 70°C, 28 cycles of 1 min at 94°C, 1 min at 68°C, 1 min 15 s at 70°C, and a final step of 10 min at 70°C. The amplification product was purified and cloned into pGEM T-Easy (Promega). Both DNA strands were sequenced at the Iowa State University’s DNA Synthesis and Sequencing Facility and the final sequence was submitted to the GenBank database.

**Cloning of a β-1,3-Glucanase Gene (FaGlu2-1)**

The following two degenerate primers (Egea et al., 1999) were used to amplify a fragment of the β-1,3-glucanase gene, forward primer 5’-TAY ATA GCY GTT GGW
PCR was performed in a 25 µL reaction mixture containing 20 ng of strawberry genomic DNA, 400 µM each of dNTP, 3 mM MgCl₂, 1 X PCR buffer without Mg²⁺, and 2.5 U Taq DNA polymerase (Promega). Varying concentrations of primers, 0.12, 0.25, 0.5, 1, and 2 µM, were used in PCR reactions. A touch-down PCR program was used with one step for 3 min at 94°C, followed by 8 cycles of 1 min at 94°C, 1 min at 58°C (decreasing 1°C / cycle), 1 min at 72°C, 32 cycles of 1 min at 94°C, 1 min at 50°C, 1 min at 72°C, and a final step of 10 min at 72°C.

After one downstream walk and two upstream walks, the sequence of the complete coding region was determined. The primers used to obtain a putative full-length clone were 5’-CGG ATA TAG TGT AAC ATA TGG CAT AGG-3’ (forward primer) and 5’-TAG AGC CTC TGA GAT CTC AAT TCT GTG-3’ (reverse primer). PCR was performed with a 25 µL reaction mixture containing 20 ng of strawberry genomic DNA, 400 nM of each primer, 1 X PCR buffer B, and 1 U DNA polymerase mix of the FailSafe PCR system (Epicenter Technologies). A touch-down PCR program was used with one step for 3 min at 94°C, followed by 10 cycles of 1 min at 94°C, 1 min at 70°C (decreasing 1°C / cycle), 2 min 30 s at 72°C, 30 cycles of 1 min at 94°C, 1 min at 58°C, 2 min 15 s at 72°C, and a final step of 10 min at 72°C. The gene was cloned into pGEM T-Easy vector and complete sequences of both strands were determined.

**Sequencing of Glyceraldehyde-3-Phosphate Dehydrogenase Gene and cDNA Fragments**

Two degenerate primers were designed by alignment of GAPDH genes from various plant sources. The primers were 5’-GAR GGN YTN ATG CAN CAN GTN CA-3’ (forward primer), and 5’-ACC ATR CTR TTR CTY ACC CCN ATR-3’ (reverse
primer). These primers were designed to anneal to the conserved regions of GAPDH protein sequences EGLMTTVH and WYDNEWGY, respectively. Two µL of reverse transcribed cDNA or 20 ng of genomic DNA was used as template in a 25 µL PCR reaction mixture containing 400 µM each of dNTP, 400 nM of each primer, 3 mM MgCl₂, 1 X PCR buffer, and 2.5 U Taq DNA polymerase (Promega). A touch-down PCR program was used with one step for 3 min at 94°C, followed by 10 cycles of 1 min at 94°C, 1 min at 60°C (decreasing 1°C / cycle), 1 min at 72°C, 30 cycles of 1 min at 94°C, 1 min at 50°C, 1 min at 72°C, and a final step of 10 min at 72°C. The amplified putative gene fragments were purified and cloned into pGEM T-Easy vector. Two clones from each cDNA and genomic DNA amplification reactions were used for DNA sequencing.

**Southern Blotting**

Strawberry leaf genomic DNA was digested with EcoRI, EcoRV, or doubly digested with both enzymes. The digested DNA (approximately 20 µg) along with markers and controls was electrophoresed on a 0.6% agarose gel 0.6 cm thick in 0.5X Tris-Borate-EDTA (TBE) buffer. A one kb DNA mass ladder and λ/HindIII fragments (Gibco BRL Life Technologies) were used as markers. For controls, 100 pg of purified FaChi2-1 and FaChi2-2 gene fragments (excised by EcoRI digestion of plasmid clones) were electrophoresed. The gel was run at 50 V (approximately 10 V/cm between the electrodes) until the bromophenol blue dye reached the bottom of the gel. The gel was stained with ethidium bromide (5 µg/mL) for 20 min, destained for 5 min in distilled water and photographed with a fluorescent ruler on an UV-transilluminator. The DNA was then nicked for efficient transfer of large DNA fragments from the gel by incubation
in 0.25 N HCl for 10 min with gentle shaking. The gel was washed briefly in distilled water and soaked in transfer buffer (1 M NaCl, 0.4 M NaOH) twice for 15 min each. The DNA was transferred to Zeta-probe-GT (genomic tested) nylon membrane (BioRad) by downward capillary alkaline transfer. The transfer was carried out for 2 h. The DNA was crosslinked to the damp membrane by UV-irradiation in a Stratalinker crosslinker (Stratagene) on the auto setting that delivers $1.2 \times 10^5 \mu$ joules of energy. The membrane was washed briefly in ddH$_2$O, dried and pre-hybridized with 0.1 mL/cm$^2$ of UltraHyb Hybridization Solution (Ambion) containing 20 µL of salmon sperm DNA (10 mg/mL). Pre-hybridization was carried out at 43°C for 1 h in a roller bottle in micro hybridization oven (Bellco Glass Inc.). The $[^{32}\text{P}]$-labeled DNA probe (see below) (1.5 x 10$^6$ CPM/mL) was boiled with 80 µL of salmon sperm DNA for 10 min. An aliquot (0.8 mL) of pre-hybridization solution was heated to 68°C, mixed with the probe, and returned to the roller bottle for hybridization at 43°C overnight. After hybridization, the blot was washed once with low stringency wash buffer (2X SSC, 0.1% SDS) for 10 min at room temperature. The blot was then washed twice with a higher stringency buffer (0.1X SSC, 0.1% SDS) for 30 min each at 43°C. The wash volumes were approximately 20 mL. The blot was then dried or kept moist with wet filter papers, wrapped with plastic wrap and exposed to a phosphoimager screen for detection with Phosphoimager 860 (Molecular Dynamics).

**Preparation of Radioactively Labeled Probe**

PCR was used for synthesizing probes. Using cloned gene plasmids as templates and $[^{32}\text{P}]$-dCTP as label, 50 µL reaction mixes were prepared as follows: 27.5 µL H$_2$O, 5 µL 10X PCR buffer (without MgCl$_2$), 6 µL MgCl$_2$ (25 mM stock to obtain 3 mM final), 1
µL DNA (0.1 ng plasmid DNA), 1.5 µL each primer (300 µM final or 15 pmoles each), 1.5 µL dNTP minus dCTP (1.5 µL of 1.66 mM dATP, dGTP, dTTP each, 50 µM each final concentration), 1 µL dCTP (1 µL of 0.4 mM dCTP, 8 µM final), 5 µL 32P-dCTP (3000 Ci/mmol, from Amersham Pharmacia), and 1 µL (5 U) Taq DNA polymerase (Promega). A touch-down PCR program was used for all probes with one step for 2 min at 94°C, followed by 8 cycles of 1 min at 94°C, 1 min at 60°C (decreasing 1°C / cycle), 1 min at 72°C, 32 cycles of 35 s at 94°C, 35 s at 52°C, 1 min at 72°C, and a final step of 10 min at 72°C.

After PCR, the probes were purified by NucAway spin columns (Ambion) according to the manufacturer’s recommendations. The specific activities of the probes were calculated by counting 2 µL of probe in 10 mL of scintillation fluid (Liquiscent, National Diagnostic) in a Beckman LS 60001 C scintillation counter. Probes with specific activity of approximately 10^8 CPM/µg DNA were obtained. Probes were used immediately or stored at –20°C.

**Primer Extension Analysis**

Primers were designed to anneal to the mRNA sequences around the putative translation start sites. Primer sequences for FaChi2-1 and FaChi2-2 were 5’-GCG AGA GTA CTG CAG CCA TG-3’ and 5’-TAA GGG TGA GTG TCT TCA TTG-3’, respectively. The underlined sequences corresponded to the complementary AUG start codons. The primers were end labeled in a 20 µL reaction containing 20 pmol of each primer, 20 pmol of [32P]-ATP (3000 Ci/mL from Amersham Pharmacia), 1 X polynucleotide kinase buffer, and 15 U T4 polynucleotide kinase (Promega). The kinase reaction was performed at 37°C for 30 min. The reaction was terminated by heating at
90°C for 2 min. Labeled primers were stored at –20°C. Labeled primers were used to synthesize the first strand of the cDNA by using Ambion’s Retroscript cDNA Synthesis Kit. Primers were extended in 20 µL reactions containing 2 µg of RNA, 500 µM of each dNTP, 10 pmol of labeled primer, 1X reverse transcription buffer, 1 U of placental RNase inhibitor, and 10 U of molony murine leukemia virus (MMLV) reverse transcriptase. The reaction was carried out at 50°C for 1 h. The reaction was terminated by heating at 90°C for 10 min.

The same labeled primers were used to sequence the corresponding cloned gene plasmids by using the fmol DNA Cycle Sequencing System (Promega) according to the manufacturer’s recommendations. The sequencing reactions and primer extension reactions were run on a 6% polyacrylamide sequencing gel. The gel was transferred to a filter paper, dried, and exposed a phosphoimager screen to collect data through phosphoimager 860 (Molecular Dynamics).

**Rapid Amplification of cDNA Ends (RACE)**

In order to confirm the presence of an intron and map the transcription start site of the β-1,3-glucanase gene, 5’ RNA-ligase-mediated RACE was performed using FirstChoice RLM-RACE kit (Ambion) according to the manufacturer’s recommendations. Briefly, 10 µg of total RNA was first treated with calf-intestinal phosphatase (CIP) to remove the 5’-phosphate of degraded mRNAs and ribosomal RNAs. After removal of CIP by phenol:chloroform extraction, the RNA was treated with tobacco acid pyrophosphatase to remove the CAP structure of the intact mRNA. Using RNA ligase, a synthetic RNA adapter was then ligated to the mRNAs bearing 5’-phosphate. The RNA was reverse transcribed in a 20 µL reaction containing 2 µL of
ligated RNA as template, 500 µM of each dNTP, 5 µM of random decamers, 1X reverse transcription buffer, 1 U of placental RNase inhibitor, and 10 U of MMLV reverse transcriptase. The reaction was carried out at 42°C for 1 h. A β-1,3-glucanase gene-specific primer and a nested primer were designed for PCR amplification of the cDNA. Together with adaptor-specific primers, primary and secondary PCR reactions were performed in 25 µL PCR reaction mixtures containing 400 µM each of dNTP, 300 nM of each primer, 3 mM MgCl₂, 1 X PCR buffer, and 2.5 U Taq DNA polymerase (Promega). A touch-down PCR program was used with one step for 2 min at 94°C, followed by 8 cycles of 1 min at 94°C, 1 min at 68°C (decreasing 1°C / cycle), 1 min at 72°C, 30 cycles of 30 s at 94°C, 30 s at 60°C, 1 min at 72°C, and a final step of 10 min at 72°C. The PCR product was purified, cloned into pGEM T-Easy vector (Promega), and sequenced.

RESULTS

Cloning and Characterization of FaChi2-1 and Its cDNA

Strawberry chitinase genes were cloned by first obtaining a fragment of the genes through PCR amplification using degenerate primers designed to anneal to sequences corresponding to conserved regions of the proteins. Based upon the sequences of gene fragments, genome walking was performed to obtain additional sequence information. Finally, full-length clones of the genes were obtained by PCR using genomic DNA as templates. Degenerate primers directed towards the conserved regions of class I and II chitinases in the catalytic domain amplified three major fragments (Figure 3.1). The smallest fragment was 587 bp long (indicated by the lower arrow in Figure 3.1), and it was verified to be a class I or II chitinase gene fragment through NCBI’s Blastp search of its translation product.
Figure 3.1  Amplification of PCR products with degenerate primers directed towards the catalytic region of class I and class II chitinases. The smallest fragment and the middle fragment correspond to FaChi2-1 and FaChi2-2, respectively (indicated by arrows). Lane 1, 1 kb ladder; lanes 2-4, PCR products with 0.1, 0.2, and 0.4 µM of each set of the degenerate primers, respectively.

Based on the sequence of this gene fragment, a gene specific primer and a nested primer were designed for both upstream and downstream genome walks. Using genome walker library DNA as templates, an approximately 0.9 kb fragment from the DraI library (Figure 3.2, lane 2) and an approximately 0.9 kb fragment from the ScaI library (Figure 3.3, lane 5) were amplified and sequenced for upstream and downstream walks, respectively.

The fragment from the DraI library for the upstream walk provided sequence information through 309 bases upstream of the putative translation start codon of this gene. The fragment from the ScaI library for the downstream walk provided enough sequence to reach the stop codon of this gene and 210 downstream bases. A full-length clone of this gene was obtained and sequenced. Nucleotide sequence of 1,507 bases was
Figure 3.2 First upstream genome walk for FaChi2-1 gene. Lane 1 shows 1 kb ladder, lanes 2-6 show PCR products using Dral, EcoRI, PvuII, Scal, and StuI genome walker libraries.

Figure 3.3 First downstream genome walk for FaChi2-1 gene. Lane 1 shows 1 kb ladder, lanes 2-6 show PCR products using Dral, EcoRI, PvuII, Scal, and StuI genome walker libraries.
deposited in the GenBank database (accession number AF147091). Figure 3.4 shows nucleotide and translated protein sequences of this gene. This gene had two exons of 467 (nucleotide positions 310-776) and 367 (nucleotide positions 931-1297) bases interrupted by a 154 base intron. The intron position was confirmed by cDNA sequence. This intron had the conserved G/GT and AG/G sequences at the splice junction sites (Shapiro, 1987). The coding region of this gene was 834 bases long with the capacity to encode a protein of 277 amino acids. Three putative TATA like sequences were found in the 309 base upstream region of this gene. These signals were located at positions 192-199 (TATATATA), which corresponded to –119 position from the translation start site, 196-201 (TATAAA), which corresponded to –115 position from the translation start site, and 256-263 (TATATATA), which corresponded to –55 position from the translation start site. No consensus poly-A signal was found in the 210 base region downstream of the stop codon. However, two weak poly-A signals, AAUAUA and AAUAAU were located at positions 1343-1348 and 1396-1401, respectively. These poly-A signals are located 43 and 97 bases, respectively, downstream of the stop codon.

The translation product of this gene is predicted to have a molecular weight of 30.63 kDa and an isoelectric point of 7.91. If processing of the N-terminal end occurs by the rule of von Heijne (1983), 33 amino acids are expected to be removed to produce the mature protein which will contain 244 amino acids with a molecular weight of 27.1 kDa and an isoelectric point of 8.33. This conceptual protein was assigned a protein I.D. AAF00131 by GenBank. Since a chitin-binding domain, the hinge region, and the C-terminal vacuolar-targeting signal were not identified, this protein was classified as a class II chitinase. FaChi2-1 has the highest identities and similarities to a parsley class II
Figure 3.4  Nucleotide and deduced amino acid sequence of the FaChi2-1 gene.  The intron is boxed.  Regions D1 and D2 represent locations of degenerate primers.  Primers C1 and C2 were used to obtain the cDNA fragment.  Putative overlapping TATA box like sequences are underlined.  Potential transcription start sites are marked by asterisks (*).
chitinase (X82329) with 73% identity and 83% similarity. Figure 3.5 shows an alignment of FaChi2-1 with FaChi2-2 (described below) and two class II peanut and parsley chitinases along with a class I tobacco chitinase. The alignment shows several motifs highly conserved in both class II and class I chitinases. For example, catalytic site motifs at amino acid positions 141-149 (QISHETTGG), 192-197 (GRGPIQ), 231-234 (NYNY), and 235-238 (WFWM) are conserved. It is interesting to note that several amino acids are conserved in one intron class II genes (FaChi2-1, Ahchi21, and possibly Pcchi21) but different amino acids are conserved in two intron class II genes (FaChi2-2, Ahchi22, and possibly Pcchi22). For example, FaChi2-1 amino acid positions 142 Ile, 162 Leu, 211 Leu, 229 Ala, 240 Glu, 251 Met, 255 Tyr, 265 Asn, 270 Phe, 297 Tyr, and 302 Ala are conserved in one intron genes, but they are conservatively replaced in two intron genes by Thr, Tyr, Ile, Ser, Pro, Ile, Trp, Gly, Tyr, Phe, and Cys, respectively. A potential glycosylation site (NRT) was located at amino acid position 221 of FaChi2-1. The corresponding cDNA was cloned to verify the intron-exon splice junctions. A cDNA clone containing the complete coding region was obtained (accession No. AF420226). Figure 3.6 shows the nucleotide and translated amino acid sequence of this cDNA. This clone showed four nucleotide substitutions in the coding region leading to three amino acid changes as compared to the genomic sequence. cDNA position 84 C was substituted for T, position 187 G was substituted for A, position 279 A was substituted for G, and position 662 G was substituted for A residue. Even though this clone was obtained by cDNA amplification by Taq DNA polymerase, the frequency of errors, if PCR related, seems high. Genomic sequence, however, is of higher confidence since multiple clones were sequenced for genomic walks in addition to both DNA strands of the final clone.
Figure 3.5 Alignment of two class II chitinases with peanut class II chitinases (X82329 and X82330), parsley class II chitinases (AF141373 and AF141372), and a class I tobacco chitinase (S44869).
To determine the organization of the FaChi2-1 gene a Southern blot analysis was performed. Figure 3.7 shows EcoRI (lane 1), EcoRV (lane 2), and EcoRI+EcoRV (lane 3) genomic digests hybridized to a [32P]-labeled probe specific for FaChi2-1. Lane 4 shows a positive control with 100 pg of purified FaChi2-1 gene fragment, excised by EcoRI digest of the cloned FaChi2-1 plasmid. Lane 5 shows a negative control with 100 pg of FaChi2-2 gene fragment. Absence of any band in lane 5 shows specificity of the probe for FaChi2-1. Since strawberry is octaploid, 8-10 in each lane shows that 1-2 copies of this gene are present per haploid strawberry genome.

The transcription start site of this gene was mapped by primer extension analysis. A major extension product was observed which maps the transcription start site to a G at –103 from the translation start site (Figure 3.8). A minor band was also seen at a T at –88 from the translation start site. The FaChi2-1 sequence in this region is shown in lanes marked G, A, T, and C. The text sequence shown is complementary to the lane assignment.

**Cloning and Characterization of FaChi2-2 and Its cDNA**

The 717 bp fragment of initial PCR amplification with degenerate primers also turned out to be a class I or II chitinase gene fragment (indicated by the upper arrow in Figure 3.1). The complete sequence of the coding region for this gene was obtained through genome walks. A full-length clone of FaChi2-2 was obtained and sequenced. The 1,438 base sequence was deposited in the GenBank (accession number AF320111). Figure 3.9 shows the nucleotide and translated protein sequences of this gene. The coding region of this gene is composed of three exons of 265 (positions 271-535), 151 (positions 660-810), and 367 bases (positions 979-1345). The two introns are 125 and
Figure 3.6 cDNA sequence and the deduced amino acid sequence of FaChi2-1. Numbers on both sides represent nucleotide and amino acid positions. Primers E1 and E2 were used to detect the expression of this gene by RT-PCR. Primers R1 and R2, and probe RP, were used in real-time PCR analysis. Location of intron is represented by an arrowhead.
Figure 3.7 Southern blot analysis of FaChi2-1. Lane 1, 2, and 3 show restriction digests of genomic DNA with EcoRI, EcoRV, and EcoRI + EcoRV, respectively. Lanes 4 and 5 show 100 pg of FaChi2-1 and FaChi2-2 gene fragments respectively. Molecular weight markers are shown on the left.

Figure 3.8 Primer extension analysis of FaChi2-1 to map the transcription start site. The outside lanes were loaded with 3 and 6 µL of primer extension products, and sequencing of corresponding FaChi2-1 plasmid is indicated by bases. Potential transcription start sites are indicated by asterisks.
169 bases long. The intron splice junctions follow the GT and AG rules and were confirmed through cDNA analysis. The upstream intron has consensus G/GT and AG/G sequences, whereas downstream intron has A/GT and AG/C sequences. The 270 base upstream region contains one TATA like signal at position 228-233, corresponding to the –43 position from the translation start site. The 93 base downstream region contains one consensus poly-A signal (AAUAAA) at positions 1427-1432, corresponding to 82 bases downstream of the stop codon.

The translation product of this gene is a 260 amino acid protein with molecular weight of 28.32 kD and an isoelectric point of 8.33. The GenBank protein I.D. for this translation product is AAG37276. The N-terminal 19 amino acids are expected to be removed (Nielsen et al., 1997), yielding a mature protein with a molecular weight of 26.23 kD and a pI of 8.33. This chitinase was also classified as a class II protein since no chitin-binding domain, hinge region, or C-terminal vacuolar-targeting signal were detected.

FaChi2-2 shares 54% and 52% sequence identity with FaChi2-1 at the nucleotide and amino acid level, respectively. FaChi2-2 has the highest identities and similarities to chitinases (AF202731, AF335589) from soybean (79% identity and 86% similarity), pea (X63899) (78% identity and 85% similarity), potato (X07130) (73% identity and 85% similarity), and banana (AJ277278) (75% identity and 85% similarity). Figure 3.5 shows an alignment of both FaChi2-1 and FaChi2-2 with class II chitinases from peanut and parsley along with a class I tobacco chitinase.

A cDNA clone of FaChi2-2 (AF420225) was obtained as described for FaChi2-1. Figure 3.10 shows the nucleotide and translated amino acid sequence of this cDNA. The
Figure 3.9 Nucleotide and deduced amino acid sequence of FaChi2-2. Intron sequences are boxed. Primers C1 and C2 were used to obtain the cDNA fragment. The putative TATA box like sequence is underlined. The potential transcription start site is marked by asterisk (*). The potential poly-A signal is underlined.
cDNA sequence shows one base difference at position 18 (G substitution for A) leading to an amino acid change as compared to the genomic sequence.

To determine the copy number of this gene a Southern blot analysis was performed. Figure 3.11 shows EcoRI (lane 1), EcoRV (lane 2), and EcoRI+EcoRV (lane 3) digests of genomic DNA. Lane 4 shows a positive control with 100 pg of a purified fragment of FaChi2-2 excised by EcoRI digestion of the cloned gene. Lane 5 shows another control with 100 pg of the FaChi2-1 gene fragment. The probe was specific for FaChi2-2, as the purified FaChi2-1 fragment was not detected (lane 5) under the stringency conditions employed. The number of bands, 6-9, in lanes 1 and 2 show that 1-2 copies of this gene are present per haploid strawberry genome.

The transcription start site of this gene was mapped by primer extension analysis. Figure 3.12 shows that this site maps to an A at –52 position from the translation start site. The sequence of FaChi2-2 in this region is shown by lanes marked G, A, T, and C. The indicated text sequence is complementary to the plasmid sequence shown.

**Cloning and Analysis of a Class III Chitinase Gene (FaChi3-1)**

By using degenerate primers a 765 bp fragment was amplified which was found to be a class III chitinase gene fragment. Arrows D1 and D2 in Figure 3.13 show the annealing locations of these primers in the gene. Through one round of upstream and one round of downstream genome walks, the complete coding region of this gene, designated as FaChi3-1, was determined. The final set of primers amplified a PCR product of 1,192 bp. This product was cloned and the sequence was deposited in the GenBank database (accession number AF134347). Figure 3.13 shows the nucleotide and translated protein sequence of this gene. An ORF of 900 nucleotides (position 133-1032) was identified as
Figure 3.10  cDNA sequence and deduced amino acid sequence of FaChi2-2. Numbers on the left and right represent nucleotide and amino acid positions. Primers E1 and E2 were used to detect the expression of this gene by RT-PCR. Primers R1 and R2, and probe RP, were used in real-time PCR analysis. Locations of introns are represented by arrowheads.
Figure 3.11 Genomic Southern analysis of FaChi2-2. Lane 1, EcoRI digest, lane 2, EcoRV digest, lane 3, EcoRI + EcoRV digests, lane 4, 100 pg FaChi2-2 EcoRI fragment, lane 5, 100 pg FaChi2-1 EcoRI fragment.

Figure 3.12 Primer extension analysis of FaChi2-2. Outside lanes were loaded with 4 and 8 µL of reverse-transcription reaction. The sequencing of corresponding FaChi2-2 plasmid is indicated by bases on the top. The transcription start site is represented by an asterisk (*).
the coding region for FaChi3-1. One putative TATA signal (TATAAA) was located at position 47-52 (-86 from the translation start site) in the 132 base upstream sequence. No canonical poly-A signal (AAUAAA) was recognized in the 160 base downstream region. However, two weak poly-A signals were identified. AAUAUA is located at position 1100-1105, 67 nucleotides downstream of the stop codon, whereas AAUAUU is located at position 1184-1189, which is 151 nucleotides downstream of the stop codon.

The translation product of this gene was given GenBank protein I.D. AAD22114. This 299 amino acid protein should have a molecular weight of 31.61 kDa and an isoelectric point of 5.85. The mature protein, however, is expected to have the first 24 amino acids removed. This mature protein is expected to be 29.37 kDa with an isoelectric point of 5.85. This protein has highest homology (77% identity and 87% similarity) to an apple (*Mulus domestica*) chitinase. It has 68% identity and 81% similarity to *H. brasiliensis* heveamine A, 67% identity and 81% homology to an *A. thaliana* chitinase, and 65% identity and 76% similarity to an *Oryza sativa* class III chitinase. Figure 3.14 shows an alignment of this protein with other class III chitinases. A hydrophobic signal peptide of 21-29 amino acids is present in all sequences and the mature N-terminal sequence is conserved. The active site glutamic acid, Glu 152 of FaChi3-1, is present in all sequences suggesting that the proteins are active chitinases. The active site aspartic acid, Asp 150 of FaChi3-1, is also conserved except in *Arabidopsis* where it is mutated to an Asn residue. Gln 34, Asn 69, Tyr 209, and Trp 281 are known to be important for maintaining the active site geometry, and they are conserved in all sequences. Contrary to the other sequences, Gly 105 and 107 of FaChi3-1 occupy similar positions as Gly 81 and Ile 82 of *H. brasiliensis* sequence. They are
Figure 3.13 Nucleotide and deduced amino acid sequence of FaChi3-1. Numbers on both sides represent nucleotide and amino acid positions. Locations of degenerate primers are labeled D1 and D2. Primers used to detect the expression of this gene by RT-PCR are labeled E1 and E2. A putative TATA box signal is underlined.
important in maintaining active site geometry, but they are separated by a Trp residue.

Six Cys residues in FaChi3-1 are also conserved in all aligned sequences.

**Cloning and Analysis of β-1,3-Glucanase (FaGlu2-1) Gene**

Figure 3.15 shows the result of PCR amplification of a β-1,3-glucanase gene fragment with degenerate primers using strawberry genomic DNA as template. Two bands (indicated by arrows) of approximately 0.5 and 0.6 kbp were obtained. After sequencing, the identity of the larger fragment could not be established through a search of its translation products by the NCBI’s Blastp program, whereas the smaller fragment corresponded to a part of a β-1,3-glucanase gene.

The putative complete coding region of the glucanase gene was obtained after two rounds of upstream and one round of downstream genome walk. Two primers were designed to amplify the entire coding region with flanking upstream and downstream sequences. After several failed efforts to obtain a full-length clone of this gene with Vent DNA polymerase (New England Biolabs), the FailSafe PCR system (Epicenter Technologies) was used to obtain a putative full-length (1,575 bp) clone of this gene.

Figure 3.16 shows the DNA sequence and the translation of ORF coding for β-1,3-glucanase. Based on its acidic pI and lack of a C-terminal vacuolar-targeting signal, this glucanase was classified as a class II enzyme.

A close inspection of this gene, however, revealed the possible presence of an intron at the 5’ end of the coding region. β-1,3-glucanases from some plant species have an intron located approximately 80-100 bases downstream of the predicted translation start codon. For example, the first exon in glucanases from tobacco (AF141654), soybean (U41323), and rice (U72249), is 81, 93, and 78 bases long, respectively. The
Figure 3.14  Alignment of FaChi3-1 with *M. domestica* (AF309514), *A. thaliana* (AB006068), *O. sativa* (AB003195), *B. hispida* (AF184884), *H. brasiliensis* (P23472), and *N. tabacum* (Z11563) class III chitinases.
Figure 3.15 Amplification of β-1,3-glucanase gene fragment by PCR with degenerate primers. Lane 1 shows 1kb ladder, lanes 2-6 show PCR reactions with 0.6 µM, 1 µM, 2 µM, and 4 µM each primer.

lengths of the introns in these genes are 0.6 kb, 2 kb, and 0.4 kb, respectively. This observation was consistent with the strawberry coding sequence being approximately 30 amino acids shorter than the other glucanases at the N-terminus. The putative start codon was in frame within the intron sequence. To verify the presence of an intron and to obtain the transcription start site, an RNA-ligase mediated rapid-amplification of cDNA ends (RACE) was performed. Through this technique, the sequence of a 468 bp 5’cDNA fragment was obtained (Figure 3.17). Within this sequence 264 bases overlapped with the genomic sequence downstream of the intron. This establishes 294 bases at the 5’ end of this gene as intron sequence (Figure 3.16), and only 144 bases are required to reach the transcription start site of this gene upstream of this intron. Three potential start codons upstream of the intron were identified. Choosing the first start codon places a 90 base coding region upstream of the intron, in agreement with the glucanases mentioned above. The upstream 54 base cDNA sequence maps the transcription start site to a G at –54 from the translation start site. Since this RACE was performed by a method which chooses
Figure 3.16 Nucleotide and deduced amino acid sequence of FaGlu2-1. Numbers on both sides represent either nucleotide or amino acid positions. Location of degenerate primers are labeled D1 and D2, primers used to detect the expression of this gene by RT-PCR are labeled E1 and E2.
only capped mRNA for participation in the RACE amplification, we assign this G residue as the true transcription start site, despite the lack of primer extension analysis. The length of the 54 base leader sequence is in agreement with leader sequences of 40-80 bases in most plant genes (Joshi, 1987).

The strawberry β-1,3-glucanase gene consists of two exons of 90 bp and 951 bp in length interrupted by an intron of approximately 500 bp (as indicated by a PCR designed to predict the size of the intron in this gene) of which 290 bp sequence is known. In a tobacco glucanase the intron location separated the signal peptide from the catalytic region (Leah et al., 1990). The strawberry gene appears to code for 2 amino acids of mature protein in the first exon.

The putative coding sequence, assembled by combining cDNA and genomic sequences, codes for a protein of 346 amino acids with a molecular weight of 37.3 kDa and an isoelectric point of 4.51. The N-terminal 32 amino acids may constitute a signal peptide and that would be cleaved to produce a mature protein of 33.76 kDa with a pI of 4.5. The acidic pI classifies this protein as a class II β-1,3-glucanase and in the absence of any vacuolar-targeting signal it is expected to be secreted to the apoplast. No potential glycosylation signal was detected. A class I β-1,3-glucanase from tobacco was found to be glycosylated at the C-terminus vacuolar-localization signal that is removed from the mature protein (Shinshi et al., 1988).

A GenBank Blastp search revealed that this gene has highest homology (60% identity, 77% homology) to a Cicer aritinum acidic glucanase (AJ012751). It has 58% identity and 73% similarity to a Prunus persica glucanase (AF435089) and 54% identity, and 71% similarity to an N. tabacum acidic glucanase. This protein also shows relatively
Figure 3.17  Sequence of a β-1,3-glucanase cDNA fragment and predicted protein sequence. An arrowhead shows the location of the intron.

high homology to class I basic vacuolar glucanases from *H. brasiliensis* (U22147) at 58% identity and 72% homology, and *N. tabacum* (M60403) at 55% identity and 71% homology. Figure 3.18 shows an alignment of the strawberry β-1,3-glucanase with other glucanases. The *H. Brasiliensis* basic glucanase, and both basic and acidic tobacco glucanases have extended (approximately 20 amino acid) C-terminal ends. These sequences are expected to be the vacuolar-targeting signals.

**Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) Gene Fragments**

To obtain a cDNA sequence of a housekeeping gene for use in the real-time PCR, two degenerate primers were designed by alignment of various plant cytosolic GAPDH’s. PCR amplification was performed using both genomic DNA and cDNA as templates.
Figure 3.18 Alignment of FaGlu2-1 with *C. arietinum* (AJ012751), *P. persica* (AF435089), *H. brasiliensis* (U22147), *N. tabacum* (M60402, M60403) β-1,3-glucanases.
Two products of each genomic and cDNA amplification were two distinct but corresponding clones. The following accession numbers were assigned for the GAPDH gene and cDNA fragments; 902 bp FaGAPDH1 genomic (AF421492), 399 bp FaGAPDH1 cDNA (AF421144), 845 bp FaGAPDH2 genomic (AF421493), and 399 bp FaGAPDH2 cDNA (AF421145). Four introns were present in the genomic sequences at the same locations but they differed in lengths between the two clones. Figure 3.19 shows the cDNA and translated protein sequence of FaGAPDH2. Since the FaGAPDH2 cDNA sequence was closely related to other plant GAPDHs, it was used for the design of a probe and primers to be used in real-time PCR (Chapter 4).

**DISCUSSION**

Strawberry Class II Chitinase Genes (FaChi2-1 and FaChi2-2)

All three PCR products obtained using degenerate primers were sequenced to establish their identity. The translation product of each fragment was a chitinase gene fragment. The sequences were similar, but showed the presence of one intron in the smallest fragment, two introns in the middle fragment, and two introns (a longer intron as compared to the middle fragment) in the largest fragment when their sequences were aligned with other intron containing class I and class II chitinase genes. Genome walk was performed on all fragments and the complete coding sequences were determined. The smallest PCR fragment was part of the one intron FaChi2-1 gene. The middle PCR fragment was a part of the two intron FaChi2-2 gene, and the largest PCR fragment was a part of the third chitinase gene similar to FaChi2-2 containing a longer second intron. The gene corresponding to the largest initial fragment had its coding region interrupted towards the N-terminus with stop codons. This gene was later found to be located
Figure 3.19 Nucleotide and deduced amino acid sequences of FaGAPDH2 cDNA. Numbers on both sides represent nucleotide or amino acid positions. Primers R1 and R2, and probe RP were used for real-time PCR. Locations of introns in the genomic sequence are represented by arrowheads.

approximately 1 kbp downstream of FaChi2-2. This gene, however, was not further investigated or characterized.

FaChi2-1 is composed of two exons of 467 and 367 bases interrupted by an intron of 154 bases. FaChi 2-2 is composed of three exons of 256, 151, and 367 bases interrupted by two introns of 125 and 169 bp. The size of introns is similar to other chitinase genes. For example, a class II bermudagrass chitinase has 94 and 103 bp introns (de los Reyes, 2001). A cotton class II chitinase gene contains introns of 99 and 154 bases at similar locations as in FaChi2-2 (Hudspeth et al., 1996). However, the peanut class II chitinases show longer introns (Kellmann et al., 1996). The one-intron peanut gene has a 920 bp intron, whereas the two-intron peanut gene has 737 and 197 bp introns located at similar positions as in the strawberry chitinase genes. Therefore, the size of introns in chitinase genes vary considerably. The A+T richness in the intron
sequences is consistent with the idea that this is a signal for splicing. FaChi2-1’s single intron is 71.43\% A+T as compared to the coding region which is 53.12\% A+T. FaChi2-2’s two introns are 68.55 and 66.67\% A+T rich as compared to the coding region which is 52.11\% A+T rich. As a comparison FaChi3-1 is also 52.11\% A+T rich in the coding region. Other plant chitinase gene introns share similar A+T richness, such as a pine gene (Wu et al., 1997). These findings are in agreement with plant introns being 15\% more A+T rich than coding regions as compared to human introns which are about 2\% more A+T rich in the intron region as compared to the exons. It was found that the U richness in maize hnRNAs is a potential signal for processing as U rich introns are more efficiently spliced (Ko et al., 1998).

The predicted translation start codon (underlined) of FaChi2-1 was embedded in the sequence AAATCC\underline{ATG}GCT, whereas for FaChi2-2 the start codon was embedded in the sequence ATAACA\underline{ATG}AAG. FaChi2-1’s sequence around the translation start site is similar to eukaryotic consensus sequence (CCA/GCC\underline{ATG}G) at the +4, -1, and -2, positions. It is similar to plant consensus translation start site sequence (TAAACAA\underline{ATG}GCT) (Joshi, 1987) at +4, +5, +6, -4, -5, and –6 positions. FaChi2-2’s sequence around the putative translation start site bears lower resemblance to the consensus sequences.

In the FaChi2-1 sequence, three potential TATA-like elements were found at –119, -115, and –55 bases relative to the translation start codon. However, the -55 element is not likely to be the true promoter since the corresponding cDNA was obtained by using a primer upstream of this sequence. The upstream TATA-like signals were only 15 and 12 bases away, respectively, from the major transcription start site. The distances
between the TATA signals and the major transcription start site, therefore, are smaller than the consensus 32±7 bases (Joshi, 1987). The second weaker transcription start site was located at 30 and 26 bases downstream of these potential TATA boxes, respectively (Figure 3.4). The distance between two TATA signals and the second weaker transcription start site appears to be in agreement with the consensus (32±7 bases) and with other chitinase genes. The major transcription start site of a tobacco chitinase was found to be 28 bases downstream of the TATA signal and 11 bases upstream of the ATG codon (Shinshi et al., 1990). For two class II chitinase genes from peanut the transcription start sites were mapped to 32±7 bp downstream of the TATA signal (Kellmann et al., 1996). The FaChi2-2’s transcription start site maps to an A at –52 from the translation start site which is in close agreement with the peanut class II, two-intron chitinase gene, whose transcription start site maps to -47 position, and to a two-intron containing cotton chitinase gene whose transcription start site maps to –43 position (Hudspeth et al., 1996). Primer extension results of rice Rcht2 gene, which also has two introns, showed four transcription start sites at 159, 170, 172, and 176 bp upstream of the translation start site (Kim et al., 1998). The only TATA-like sequence in FaChi2-2 is located at position 219, which is actually downstream of the transcription start site and is unlikely to be the true TATA sequence. Absence of a TATA-like element has been detected in plant genes, especially in some housekeeping genes (Joshi, 1987).

Both FaChi2-1 and FaChi2-2 showed presence of multiple bands in Southern blots that represent small multigene families for these genes with no more than two members per haploid genome. Various chitinase genes show a range of hybridizing bands in genomic Southern blots. For example, a pine gene shows 2-4 bands (Wu et al.,
1997), a *Brassica juncea* gene shows 6-9 bands, and an *Oryza sativa* gene shows 1-2 strong bands but up to 8 faint bands. The presence of multiple chitinase homologues in many plant species is consistent with the observation that evolution of disease resistance genes has exceeded other plants genes in general (Richter and Ronald, 2000). The diversification of these genes is an adaptive function to counteract the rapid evolution of pathogens.

FaChi2-1 would encode a mature protein of 27.1 kDa with a pI of 8.33, whereas FaChi2-2 would encode a mature protein of 26.23 kDa with a pI of 8.33. The basic pI of strawberry class II chitinases is somewhat unusual since most reported class II chitinases are acidic. However, the experimental pI must be determined since it is possible that these proteins are post-translationally modified. A potential glycosylation site (NRT) was found at amino acid position 221 of FaChi2-1, whereas FaChi2-2 also showed one potential glycosylation site (NPS) at amino acid position 195. Interestingly, in FaChi2-1 the glycosylation signal is located 10 amino acids upstream of a conserved chitinase sequence (NIINGGLECG). The same glycosylation signal (NRT) was also observed 10 amino acids upstream of this motif in peanut and parsley chitinases as well. A parsley chitinase was indeed shown to be glycosylated (Kirsch et al., 1993) as well a potato class I chitinase (Ancillo et al., 1999). Any significance of this modification in chitinases is not known, but it is thought to function in protein stability and/or localization.

The length of the signal peptide (33 amino acids) of FaChi2-1 is longer than usual (approximately 20 amino acids) but similar to a potato class I chitinase (Gaynor, 1988). The length of the FaChi2-2 signal peptide (19 amino acids) is similar to class I and class II chitinases from potato (Ancillo et al., 1999), and class I chitinases from tobacco (Shinshi et al., 1990) and grape (Busam et al., 1997).
In mature FaChi2-1, the active site glutamates are located at amino acid positions 68 and 90, whereas they are located at positions 67 and 89 in FaChi2-2. The location of FaChi2-1’s catalytic residues are exactly as in the barley chitinase whereas in FaChi2-2 they are positioned exactly as in the jack bean chitinase. Three disulfide bonds identified in the crystal structures of barley and jack bean class II chitinases are expected to be present in strawberry chitinases through cysteines at positions 24-86, 98-107, and 206-218 in FaChi2-1 and at positions 24-85, 96-104, and 203-222 in FaChi2-2. The positions of cysteine residues in the primary structures is very similar to the barley and jack bean chitinases. Other residues important in catalysis or implicated in maintaining the active site geometry are also conserved in strawberry chitinases. These include active site residues Y of the NYNYG motif (Verburg et al., 1992), the first T of the SHETTGGG motif, the second G and the Q of the GRGPIQL motif, and the second N of the NYNY motif.

**Strawberry Class III Chitinase Gene (FaChi3-1)**

The genomic sequence of FaChi3-1 showed a single ORF, similar to other class III chitinase genes. The putative translation start site maps to 87 bases downstream of the putative TATA box. In comparison, two peanut chitinase genes were shown to have translation start sites 69-86 bases downstream of the TATA box (Kellman et al., 1996). The putative ATG initiation codon of FaChi3-1 was embedded in a sequence (CTTTATATGGCT) similar only in three downstream nucleotides to the plant consensus translation start site sequence (TAAACAAATGGCT). It is, however, interesting to note that T residues in the strawberry gene replace all upstream A residues of the consensus sequence at similar locations. Any significance of this is not known.
The translation product of this gene shows high degrees of homology to other class III chitinases. A potential glycosylation site (NVT) was found at amino acid position 220. In general, class III chitinases have not been shown to be glycoproteins. The active site glutamic acid is located at position 128 of the mature protein, and the active site aspartic acid is at position 126. The active site residues of other chitinases are present at similar locations, and therefore the strawberry enzyme is expected to be active. In hevamine the Glu 127 is indispensable for catalysis whereas Asp 125 widens the pH range for catalysis. The *Arabidopsis* enzyme has Asp 125 mutated to Asn, and therefore this enzyme is expected to be active over a narrow pH range (Samac and Shah, 1990). Conconavalin B is sequence-related to class III chitinases but its active site residue (Glu 127) has changed to Gln, turning this protein into a storage protein (Hennig et al., 1995). The predicted signal peptide of 24 amino acids is similar in size and it is expected to be cleaved at location similar to that as experimentally shown for class III chitinases of wintermelon (Shih et al., 2001) and pumpkin (Kim et al., 1999). The enzyme also has six conserved cysteine residues, at locations similar to other class III chitinases.

*FaChi3-1* is expressed constitutively at low levels in strawberry leaves as analyzed by RT-PCR (see chapter 4, Figure 4.3). This is in agreement with findings of Yeboah et al. (1998) that the constitutive expression of a class III gene in *Glycine max* leaves and stems was not detectable by Northern hybridization, although a low level of expression was shown by RT-PCR analysis. In soybean the highest level of constitutive expression was seen in seeds. Regalado et al. (2000) showed the presence of class III chitinase in *Lupinus albus* vegetative tissues at the protein level. In *L. albus*, the protein was also present in seeds. Purification of a class III chitinase from seeds of *B. hispida*
was achieved (Shih et al., 2001). It appears that class III chitinases have some role, maybe defensive, in embryonic tissues. In banana, however, a class III chitinase-like gene and protein were shown to be expressed at high levels in unripe pulp, but not in ripe pulp. The investigators suggested that this protein is probably not functioning in plant defense, but rather as a storage protein (Clendennen et al., 1998).

When evaluated for their antifungal activity or plant protection against pathogens, class III chitinases showed mixed results. For example, a *Trichosanthes kirilowii* chitinase showed no in vitro antifungal activity even when used at high concentrations (Savary et al., 1997); a *Medicago truncatula* class III chitinase was not induced by infection with several fungi (Bonanomi et al. 2000); and a *Beta vulgaris* class III chitinase induced in response to fungi showed no protection in transgenic tobacco (Nielsen et al., 1993). In contrast, other reports show induction of this class of chitinase in response to fungal and viral infections (Lawton et al., 1992; Busam et al., 1997).
CHAPTER 4

ANALYSIS OF CHITINASE ACTIVITIES, ISOFORMS, AND EXPRESSION OF TWO CLASS II CHITINASE GENES IN STRAWBERRY PLANTS INOCULATED WITH Colletotrichum fragariae OR C. acutatum

INTRODUCTION

When plants are invaded by pathogens, a number of metabolic changes take place within the plant as a defense response. These changes may include an immediate early response of the invaded cells resulting in necrosis or a hypersensitive reaction limiting the spread of the pathogen. In the vicinity of infection, various genes are activated that result in the production of compounds toxic to the pathogen and reinforcement of the cell wall. Finally, systemic expression of pathogenesis-related (PR) genes, such as chitinase and β-1,3-glucanase, leads to systemic acquired resistance (SAR) in the plant (Ponath et al., 2000).

Plant chitinases have been of particular interest since they are known to be induced upon pathogen invasion. A variety of abiotic stress factors, including UV-irradiation, ozone, salts, heavy metals, and drought, are also able to induce the expression of chitinase genes (Hong et al., 2000; Kim and Hwang, 1996). Chitinases catalyze the hydrolysis of N-acetylglucosamine chain of chitin, a biopolymer found in the cell wall of certain fungi, exoskeleton of arthropods, and the gut lining of insects. No chitin substrate, however, has been found in plants. Chitinases have been shown to inhibit fungal growth in vitro, especially when tested together with a second PR-protein, such as β-1,3-glucanase (Mauch et al., 1988; Sela-Buurlage et al., 1993). Transgenic plants expressing chitinase genes at high levels have shown increased tolerance to diseases or
delayed development of disease symptoms (Zhu et al., 1994; Jach et al., 1995; Yamamoto et al., 2000; Datta et al., 2001; Rohini and Rao, 2001).

Multiple chitinase isoforms have been detected in various plant species analyzed to date. They differ in primary structure, isoelectric point, and cellular localization. Based on their primary structures, chitinases have been divided into seven classes (Neuhaus, 1999). Most chitinase genes or proteins studied, however, belong to the first four classes. Class I chitinases have an N-terminal chitin-binding domain (CBD) separated by a hinge region from the catalytic domain. They also have a C-terminal vacuolar-localization signal. Class II chitinases have a catalytic domain similar to class I chitinases but are devoid of the CBD, the hinge region, and the C-terminal extension. Class IV chitinases are shorter than class I chitinases due to deletions in the catalytic domain. Class III chitinases are unique in having a structure more closely resembling bacterial chitinases than other plant chitinases.

Class I chitinases show antifungal activity in vitro, whereas chitinases belonging to other classes have none to low antifungal activity (Mauch et al., 1988; Sela-Buurlage et al., 1993; Jach et al., 1996). Therefore, most studies have used class I chitinase genes or their promoter regions in delineating the events leading to plant disease or resistance (Broglie et al., 1986; Shinshi et al., 1995; Fukuda and Shinshi, 1994; Fukuda, 1997). Class I chitinases have been extensively used in transgenic studies showing, in many cases, enhanced disease resistance of plants. However, a major role of hydrolytic enzymes in disease resistance appears to be the release of pathogen-derived elicitor molecules that stimulate plant defense response. Therefore, it is logical to expect that plants transformed with class II chitinases, despite having low in vitro antifungal activity,
may also show enhanced disease resistance (Jach et al., 1995). Only a small number of studies have analyzed the expression of class II chitinase genes and the effectiveness of this class of chitinases in transgenic research has not been firmly established. Regulation of class II chitinase genes has been studied in parsley (Ponath et al., 2000), pepper (Hong et al., 2000), bermuda grass (de los Reyes et al., 2001), potato (Buchter et al., 1997), rice (Kim et al., 1998), peanut (Kellmann, 1996) and tobacco (Suzuki et al., 1995). Without consensus, various class II chitinase genes show induction in response to ethylene, salicylic acid, wounding, and fungal elicitors. It is important to note that in some cases class I chitinases have been shown to be plant allergens. The CBD has been implicated in the allergenic reaction whereas class II chitinases lacking this domain were shown not to elicit an allergenic reaction (Sanchez-Monge et al., 2000; Blanco et al., 1999; Posch et al., 1999; Sanchez-Monge et al., 1999; Diaz-Perales et al., 1998). Therefore, in certain crop plants the incorporation of a class II chitinase might be a better strategy for the production of disease-resistant transgenic plants.

Strawberry is an important horticultural crop in southern U.S.A. However, fungal diseases, such as anthracnose crown rot caused by Colletotrichum sp., are major limiting factors in strawberry production. Virtually no information is available on the molecular events in strawberry plants subsequent to the fungal infection. With the cloning of two class II chitinase genes, it would be interesting to learn the pattern of regulation of these genes when plants are infected with fungi. This information would be useful in understanding plant responses to fungal infection. Also, based upon this information, new strategies could be developed to produce transgenic strawberry plants with enhanced disease resistance. In this chapter, analyses of uninoculated and infected
strawberry plants are presented. *Colletotrichum fragariae* and *C. acutatum*, the fungal species in Louisiana affecting strawberries, were used for infection. These two fungi are the most important fungal pathogens that cause the severe strawberry disease anthracnose crown rot in the Gulf state region of the United States and in other parts of the world, respectively (Smith and Black 1986; Smith, 1990). Total chitinase activity, the acidic and basic chitinase isoform distribution patterns, and the expression of two class II chitinase genes in these plants were analyzed.

**MATERIALS AND METHODS**

**Growth and Preparation of Fungal Inoculum and Plant Inoculation**

In this study, *Colletotrichum fragariae* isolate CF-75 and *C. acutatum* isolate Goff were used to infect the strawberry plants (Bonde et al., 1991). Fungal cultures were obtained from Dr. Barbara Smith (USDA Small Fruit Research Station, Poplarville, MS) and maintained on half strength fungal growth media. The media was prepared by mixing 2.44 g of potato dextrose agar, 4.55 g oatmeal agar (Sigma) and 3 g of Bacto agar in 250 mL deionized-distilled water (ddH₂O) (Smith et al., 1990). Media was autoclaved, stirred vigorously and poured into petri dishes. Fungal cultures were grown at room temperature under constant fluorescent light. To prepare the fungal spore inoculum for spray, actively growing cultures in petri dishes were flooded with approximately 15 mL of 0.01% Tween-20. Spores were dislodged into the liquid by a round end glass rod and filtered through two layers of cheesecloth. A drop of this spore suspension was placed on each counting chamber of a hemocytometer. Number of spores were counted in five large squares in each counting chamber and averaged. This average was used to calculate the spore concentration. The spore suspension was diluted with 0.01% Tween-20 to
obtain $1.5 \times 10^6$ spores/mL concentration. Plants were sprayed with fungal spore suspension until the liquid ran off from the leaves. Control plants were sprayed with 0.01% Tween-20. Plants were immediately transferred to a dew chamber at 28°C. Four plants of each treatment at 2, 6, 12, 24, and 48 h were harvested. RNA and protein samples were extracted immediately after harvest.

**Isolation of Total RNA from Strawberry Leaves**

RNase free plasticware and reagents were from Ambion. Total cellular RNA from strawberry leaves was extracted using the method described by Manning (1991). Leaves were ground in liquid nitrogen in a pre-chilled mortar and pestle to fine powders (approximately 40-50 hard strokes in 30 sec.). Approximately 100 mg of the ground leaf 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$_2$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 maximum speed (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$^+$ 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. The pellet was dried for approximately 5 min. Total nucleic acids were dissolved in 20 µL of RNA-Secure (Ambion), which had been pre-heated to 60°C, and the dissolved nucleic acids were incubated at 60°C for 10 min for inactivation of any contaminating RNases. The nucleic acid samples were either frozen at –80°C or immediately treated with DNase I. DNase treatment of RNA samples was done by using the DNA-free kit (Ambion). One µL (10 units) of DNase I was added to 20 µL sample in 1X DNase I buffer. The DNase treatment was carried out at 37°C for 30 min. Five µL of DNase I/divalent ions removal reagent was added and the solution was kept at room temperature for 2 min. After centrifugation for 1 min, the supernatant was transferred to a new tube. The quantity and quality of RNA samples were determined by measuring UV absorbencies at 230, 260, 270, 280, and 320 nm and by electrophoresis on 1% agarose gel, respectively.

**Isolation of Total Protein, Chitinase Activity, and Isoform Pattern Analysis**

Protein extracts from leaf samples for assaying chitinase activity and isoform patterns were prepared by homogenization with a polytron (PRO Scientific Inc. Monroe, CT, USA, Model PRO 200), using the same homogenization conditions as with the Omni-mixer except omitting the liquid nitrogen grinding step (Chapter 2). Protein concentration determination, chitinase activity analysis, and chitinase isoform analysis were performed as described in Chapter 2.
Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

cDNA was synthesized by using Ambion’s Retroscript cDNA synthesis kit. The RNA was reverse transcribed in a 20 µL reaction containing 2 µg of total RNA as template, 500 µM of each dNTP, 5 µM of oligo-dT primers, 1X reverse transcription buffer, 1 U of placental RNase inhibitor, and 10 U molony murine leukemia virus (MMLV) reverse transcriptase. In the control reactions, reverse transcriptase was omitted. Reverse transcription was carried out at 48°C for 1 h. The reaction was stopped by heating at 90°C for 10 min. Two µL of this reverse transcription reaction was used in the PCR to detect the presence or absence of a transcript. Possibility of DNA contamination was eliminated if the minus reverse transcriptase control did not show the corresponding product.

Real-Time PCR (RTi-PCR)

The expression levels of the two class II chitinase genes and the glyceradehyde-3-phosphate dehydrogenase gene (FaGAPDH) at various time points after fungal inoculation were determined by real-time PCR using a Perkin-Elmer 7700 thermal cycler. The primers and TaqMan probes were designed using the Sequence Detection System software and following the guidelines not incorporated in the software. These guidelines entail visual examination of the primers for the following two requirements: 1. There should be no more than two G and/or C residues in the five nucleotides at the 3’ end. 2. C content of the primers should be equal to or greater than the G content (Perkin Elmer, User Bulletin 2). The primers and 6-FAM labeled probes with quencher TAMRA for FaGAPDH2, FaChi2-1, and FaChi2-2 genes are shown below.
FaGAPDH2:
Forward primer: 5’-CTG CCT GCT CTC AAT GAC AAG T-3’
Reverse primer: 5’-GAG GTC AAC AAC TGA AAC ATC AAC A-3’
TaqMan Probe: 6-FAM-CTG GAA TGG CCT TCC GTG TAC CCA-TAMRA

FaChi2-1:
Forward primer: 5’-TCG GCA CCA CCG GAA GT-3’
Reverse primer: 5’-TGG GAG ATC TGA GCA AGA AAT G-3’
TaqMan probe: 6-FAM-AGC CAC CCG AAA GCG TGA GAT TGC-TAMRA

FaChi2-2:
Forward primer: 5’-GGT CAA ACC TCT CAC GAA ACC A-3’
Reverse primer: 5’-ATC CCC AAG CAT AAG GAC CAT-3’
TaqMan probe: 6-FAM-TGG TGC ACT TGG CCA TCC TCC A-TAMRA

The primers and probes were obtained from Applied Biosystems (Foster City, CA). Primers were dissolved in RNase free water at a concentration of 10 µM. The TaqMan probe was supplied at a concentration of 100 µM. Using one-step RT-PCR master mix reagents (Applied Biosystems), 40 µL reactions were setup in a 96-well plate where for each gene TaqMan probe was used at 250 nM final concentration, whereas each primer was used at 500 nM final concentration. For each sample, 5 ng of total RNA was analyzed and all three genes (FaGAPDH2, FaChi2-1, and FaChi2-2) were always analyzed simultaneously. Each sample was run in triplicate. Universal thermal cycler conditions 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.
RTi-PCR Validation Experiment

RNA samples from a strawberry plant infected with *C. fragariae* at 48 h time point were used for the validation experiment. A serial dilution of total RNA, 10, 5, 1, 0.5, 0.1, 0.05, and 0.01 ng, was used in RTi-PCR. The threshold cycle (C_T) for each gene (FaGAPDH2, FaChi2-1, and FaChi2-2) was determined with the indicated RNA dilution series. Threshold cycle was defined as a particular PCR cycle when a specific amount of PCR product accumulated. The plots of log ng total RNA versus difference in C_T for each RNA dilution between FaGAPDH2 and FaChi2-1 and between FaGAPDH2 and FaChi2-2 were plotted. FaGAPDH2 represented a housekeeping gene whose expression was invariant in different samples and therefore used to normalize the expression of FaChi2-1 and FaChi2-2.

In another preliminary experiment, efficiency of RTi-PCR with cloned cDNA templates (for genes under study) was determined. The template for FaChi2-1 was also amplified with FaChi2-2 primers and probe, and vice versa to check for product formation from closely related sequences during PCR.

Salicylic Acid, Ethephon, and Injury Treatments

Salicylic acid (5 mM) and ethephon (10 mM) solutions were prepared in 0.01% Triton X-100 (Hong et al., 2000). Plants were sprayed twice at 30 min interval each time until liquid started to drip from the leaves. For injury, the leaves were rubbed approximately 0.5 cm² on both sides of the central vein with 3M sandpaper. Plants were kept in growth chamber as indicated above. Samples were harvested 24 h after the treatments to extract total protein and RNA.
RESULTS

Examination of the Constitutive Expression of the Cloned PR-Genes in Uninoculated Strawberry Plants

Constitutive expressions of FaChi2-1, FaChi2-2, FaChi3-1, and FaGlu2-1 genes were determined in uninoculated plant leaves by RT-PCR. Since FaChi2-1 and FaChi2-2 have intron(s) in their genomic sequences, the PCR products from genomic or cDNA templates were distinguishable based on size by using primers encompassing the intron(s). Figures 4.1 (FaChi2-1) and 4.2 (FaChi2-2) show, respectively, PCR products with genomic DNA (lane 2) and cDNA (lane 3) as templates. Expression of these genes in plant leaves is evident in lanes 3 and different size products from genomic and cDNA templates confirm no contamination of genomic DNA in the RNA preparations. Since FaChi3-1 does not have any intron and primers for the FaGlu2-1 expression were in the second exon, their RT-PCR products were confirmed to be from cDNA by running negative reverse transcriptase controls during the reverse transcription step. Two uninoculated strawberry plants were analyzed for both genes and they were found to express both genes constitutively in leaves. Figures 4.3 and 4.4, for FaChi3-1 and FaGlu2-1, respectively, show the presence of the corresponding transcripts in two plants (lanes 2 and 4). Controls in lanes 3 and 5 for the two plants show absence of any DNA contamination.

Total Chitinase Activity and Isoform Pattern of Fungal Infected, Injured, and Salicylic Acid or Ethephon Treated Plants

The level of total chitinase activity and the pattern of isoform distribution in infected plants were analyzed. Also, total chitinase activity and the pattern of isoform distribution were analyzed after 24 h in plants either injured or treated with salicylic acid
Figure 4.1  Constitutive expression of FaChi2-1 in strawberry plant leaves as detected by RT-PCR. Lane 1, 1 kb ladder; lane 2, PCR with genomic DNA as template; lane 3, RT-PCR with cDNA made from leaf total RNA. Primers used are shown in Figure 3.6 as E1 and E2.

Figure 4.2  Constitutive expression of FaChi2-2 in strawberry plant leaves as detected by RT-PCR. Lane 1, 1 kb ladder; lane 2, PCR product with genomic DNA as template; lane 3, RT-PCR product using cDNA synthesized from leaf total RNA. The primers used are shown in Figure 3.10 as E1 and E2.
Figure 4.3  Constitutive expression of FaChi3-1 gene in strawberry leaves. Lane 1, 1 kb ladder; lane 2, plant 1 RT-PCR; lane 3, plant 1 control RT-PCR; lane 4, plant 2 RT-PCR; lane 5, plant 2 control RT-PCR. Primers used are shown in Figure 3.13 as E1 and E2.

Figure 4.4  Constitutive expression of FaGlu2-1 gene in strawberry plant leaves. Lane 1, 1 kb ladder; lane 2, plant 1 RT-PCR; lane 3, plant 1 control RT-PCR; lane 4, plant 2 RT-PCR; lane 5, plant 2 control RT-PCR. Primers used are shown in Figure 3.16 as E1 and E2.
or ethephon. The total chitinase activities of infected plants were normalized to those of the control plants. Two separate infection experiments were performed. Infection of plants by 48 h was evident by dark lesions on the leaves and the petioles. In the first experiment, induction of the total chitinase activity was observed in the infected plants after two hours of fungal inoculation (Figure 4.5). The level of total chitinase activity in plants inoculated with *C. acutatum* was 2.1-, 3.43-, 3.3-, 1.66-, 1.67-, and 1.24-fold as compared to the control plants at 2, 6, 12, 24, and 48 h post-inoculation, respectively. In comparison, the induction of total chitinase activity in response to *C. fragariae* was 3.73-, 3.36-, 1.66-, 3.06-, and 4.1-fold as compared to the controls at 2, 6, 12, 24, and 48 h post-inoculation, respectively. The replication of this experiment (Figure 4.6) showed gradual induction of total chitinase activity in response to both fungi up to 24 h. *C. acutatum* elicited induction of 1.5-, 2-, 2.5-, 5.1-, and 3-fold as compared to the controls at 2, 6, 12, 24, and 48 h post-inoculation, respectively. At 2 h the chitinase activity of *C. fragariae* infected plants was somewhat lower than control plants, but the induction was 2-, 2.2-, 4.5-, and 2.2-fold at 6, 12, 24, and 48 h post-inoculation, respectively. The level of chitinase induction in plants treated with either salicylic acid or ethephon, or injured was 1.74-, 2.02-, and 2.8-fold, respectively, after 24 h as compared to the control plants (Figure 4.7).

The chitinase isoforms were analyzed for two control plants and two plants infected with each fungus at 2, 6, 12, 24, and 48 h post-inoculation. Individual plant samples showing highest total chitinase activities were chosen for the isoform analysis (Figure 4.8). The major acidic isoform (ChiA3) and the two basic isoforms (ChiB1 and ChiB2) were always present in all of the control and the infected plants. No
Figure 4.5  Total chitinase activities of plants infected with *C. acutatum* (C.acut.) or *C. fragariae* (C.frag.) at various time points (Experiment 1). Induction levels were normalized to controls set at 1. Each bar represents an average reading from three plants. Each assay was performed in triplicate.

Figure 4.6  Total chitinase activities of plants infected with *C. acutatum* (C.acut.) or *C. fragariae* (C.frag.) at various time points (Experiment 2). Induction levels were normalized to controls set at 1. Each bar represents an average reading from three plants. Each assay was performed in triplicate.
Figure 4.7 Induction of total chitinase activity in plants treated with salicylic acid (SA), ethephon, and injury after 24 h of treatment. Induction levels were normalized to controls. Each bar represents an average reading from three plants. Each assay was performed in triplicate.

Drastic changes of the isoform pattern were observed, and no distinctive new isoforms were detected in the infected samples. Acidic isoform ChiA3 was induced at 24 h in *C. acutatum* infected plants (Figure 4.8, a24h, lanes 3 and 4) and in one *C. fragariae* infected plant (Figure 4.8, a24h, lane 5). At 48 h, an induction of ChiA1, ChiA3, ChiA5, and ChiA6 was observed in plants infected with *C. fragariae* (Figure 4.8, a48h, lanes 5 and 6). Induction of both basic isoforms was observed in plants infected with *C. fragariae* at 12 h (Figure 4.8, b12h, lanes 5 and 6) and 24 h (Figure 4.8, b24h, lanes 5 and 6) post-inoculation. At 24 h, appearance of a possible minor basic isoform in one of the *C. acutatum*-infected plant (marked by an arrow in lane 4 of Figure 4.8, b24h) was also observed.
Figure 4.8 Acidic and basic isoforms in control and infected plants. 2, 6, 12, 24, 48 h post-infection chitinase isoform distribution are shown by (a) acidic and (b) basic gels. Lanes 1, 2, control plants; lanes 3, 4, plants infected with \textit{C. acutatum}; lanes 5, 6, plants infected with \textit{C. fragariae}. (Figure 4.8 cont.)
In plants treated with salicylic acid, ethephon, or injury, ChiA2, ChiA4, ChiA5, and ChiA6 were induced (Figure 4.9, a24h, lanes 3-8). In control plants only ChiA3 was present which was induced in ethephon and injury treatments. Basic isoforms were only induced in plants treated with injury and a new minor isoform, ChiB1a (indicated by an arrow in Figure 4.9, b24h, lane 7) was induced in injury treatment and in one plant from the salicylic acid treatment.

**Quantitation of FaChi2-1 and FaChi2-2 Expression by RTi-PCR**

In this study, expression levels of the two class II chitinase genes, FaChi2-1 and FaChi2-2, in uninoculated and in infected plants were compared. In preliminary experiments, it was established that each primer and probe combination was specific for the intended gene. A relative quantitation was performed instead of absolute quantitation. Absolute quantitation produces results in copy numbers but necessitates running standards in every run occupying ¼ of the plate. A relative quantitation is performed by calibrating the expression of each gene under study with a normalizer gene, known to be expressed at a constant level (a housekeeping gene, such as glyceraldehyde-3-phosphate dehydrogenase, GAPDH). This corrects against slight variations in RNA loading in different samples. The expression levels in infected plants were then compared with control plants to obtain the induction level (if any) of a particular gene. The threshold cycle (C\textunderscore T) values, defined as a particular PCR cycle when a specific amount of PCR product accumulated, were transformed mathematically by the ΔΔC\textunderscore T method into relative expression levels (Perkin Elmer User Bulletin 2).

**RTi-PCR Validation Experiment**

In order for quantitation to be accurate, the relative efficiencies of reverse-transcription of normalizer gene transcript and gene transcripts under study must be
Figure 4.9 Acidic (a) and basic (b) isoforms in plants challenged with salicylic acid (lanes 3, 4), ethephon (lanes 5, 6), injury (lanes 7, 8), and control plants in lanes 1, 2 after 24 h post-treatment.

equal. A sensitive measure of this is a plot of the difference in C\textsubscript{T} values of normalizer and experimental genes when amplified with a dilution series of RNA. The slope of the line with a value less than the absolute value of 0.1 suggests relative reverse transcription efficiencies to be approximately equal for quantitation purposes. Figure 4.10 shows the validation experiment for the strawberry genes under study. Figure 4.10A shows fit of the lines for the threshold cycles as a function of RNA concentration for FaGAPDH2, FaChi2-1, and FaChi2-2 genes. The relationship is linear in the total RNA range of 0.05-10 ng under the assay conditions employed. This graph establishes that C\textsubscript{T} is 30 and 22
when total RNA was 0.05 ng and 10 ng, respectively, for FaGAPDH2 gene. Figure 4.10A also shows similar plots of FaChi2-1 and FaChi2-2 genes. The difference of \( C_T \) values (\( \Delta C_T \)) between FaGAPDH2 and the two chitinase genes for each RNA concentration was plotted (Figure 4.10B). The slope of the line for FaChi2-1 was –0.06 and for FaChi2-2 it was 0.05, which passes the test for the validity of the relative quantitation.

The threshold cycles of each gene were determined in uninoculated plant leaves. The \( C_T \) values for FaGAPDH2, FaChi2-1, and FaChi2-2 were 24.2, 35.1, and 31.44, respectively (Figure 4.11). These values are inversely proportional to the relative abundance of the gene transcripts. Therefore, in uninoculated plant leaves FaChi2-2 gene transcript is present at higher levels as compared to the FaChi2-1 gene transcript.

Figure 4.12 shows expression of FaChi2-1 in response to \( C. acutatum \) and \( C. fragariae \) at various time points as compared to the controls. The induction levels of infected plants were normalized to those of the control plants at each time point. \( C. acutatum \) induced the expression of FaChi2-1 by 1.31-fold at 2 h, 2.66-fold at 6 h, and 3.55-fold at 12 h post-inoculation. However, the expression of this gene decreased to the background level at 24 h (1.14-fold) and remained at that level at 48 h(1.19-fold). \( C. fragariae \) caused a rapid induction of FaChi2-1 to 3.18-fold at 2 h, 4.47-fold at 6 h, 4.76-fold at 12 h, 3.86-fold at 24 h, and 6.68-fold at 48 h. Figure 4.13 shows the expression pattern of FaChi2-2 in the same experiment. The expression of this gene was similar in the control and infected plants up to 12 h. Only slight induction of 1.52-fold and 2.19-fold was evident at 24 h in response to \( C. acutatum \) and \( C. fragariae \), respectively. Interestingly, at 48 h post-inoculation, while this gene was induced only 1.68-fold in plants infected with \( C. acutatum \), it was induced 8.28-fold in plants infected with \( C. fragariae \).
Figure 4.10  Real Time PCR validation experiment.  
A. Plot of $C_T$ values versus various concentrations of total RNA for FaGAPDH2 (♦), FaChi2-1 (■), and FaChi2-2 (▲) genes. 
B. $\Delta C_T$ for FaChi2-1 (■) and FaChi2-2 (▲) versus various RNA concentrations.
Figure 4.11 Threshold cycles of FaGAPDH2, FaChi2-1, and FaChi2-2 gene transcripts in uninoculated plant leaves. Each bar represents an average reading from three plants. Each plant was individually assayed and each gene was assayed in triplicate.

Second RTi-PCR infection experiment showed a quicker response of FaChi2-1 in plants infected with *C. acutatum*. The induction was 3.39-fold at 2 h and 4.89-fold at 6 h post-inoculation. The induction of this gene, 1.68-fold, was slightly over the background level at 12 h and remained approximately at background level through 24 h (0.85-fold). At 48 h the gene was induced again at 3.81-fold. Plants infected with *C. fragariae* showed induction of 3.43-, 3.03-, 4.41-, 3.27-, and 8.4-fold at 2, 6, 12, 24, and 48 h post-infection (Figure 4.14). The expression level of FaChi2-2 was similar to controls up to 12 h. The gene was slightly induced to 1.8-fold in response to both fungi at 24 h. The expression level was 3.73- and 6.11-fold in plants infected with *C. acutatum* and *C. fragariae*, respectively, at 48 h (Figure 4.15).
Figure 4.12  Relative quantities of FaChi2-1 gene transcript at various time points post-inoculation with *C. acutatum* (C.acut.) and *C. fragariae* (C.frag.). Control (Cont.) levels were set to one arbitrarily. Each bar represents an average reading from three plants. Each plant was individually assayed and each gene was assayed in triplicate.

Figure 4.13  Relative quantities of FaChi2-2 gene transcript at various time points post-inoculation with *C. acutatum* (C.acut.) and *C. fragariae* (C.frag.). Control (Cont.) levels were set to one arbitrarily. Each bar represents an average reading from three plants. Each plant was individually assayed and each gene was assayed in triplicate.
Figure 4.14 Relative quantities of FaChi2-1 gene transcript at various time points post-inoculation with *C. acutatum* (C.acut.) and *C. fragariae* (C.frag.). Control (Cont.) levels were set to one arbitrarily. Each bar represents an average reading from three plants. Each plant was individually assayed and each gene was assayed in triplicate.

Figure 4.15 Relative quantities of FaChi2-2 gene transcript at various time points post-inoculation with *C. acutatum* (C.acut.) and *C. fragariae* (C.frag.). Control (Cont.) levels were set to one arbitrarily. Each bar represents an average reading from three plants. Each plant was individually assayed and each gene was assayed in triplicate.
DISCUSSION

Total chitinase activities, isoform patterns, and expression of FaChi2-1 and FaChi2-2 at the level of transcription were analyzed in plants infected with \textit{C. fragariae} or \textit{C. acutatum} at various time points. Individual plants showed variable responses to fungal infections when analyzed for total chitinase activity. The first infection experiment showed induction of total chitinase activity after 2 h of fungal inoculation. The induction elicited by \textit{C. acutatum} and \textit{C. fragariae} reached maximum level at 6 h and 48 h, respectively. The rapid induction is likely to be due to the release of elicitors from germinating fungal spores. Strawberry’s constitutive chitinase activity might have a role in the release of these elicitors. Alternatively, these molecules could also be released as a result of fungal cell wall growth and re-organization during germination. The induction of chitinase activity was slower in the 2$^{nd}$ infection experiment. This may be due to the use of plants or fungal cultures from different batches. In this experiment the chitinase induction gradually increased to maximum level within 24 h, decreasing slightly at 48 h. It appears that the maximum level of chitinase induction was only maintained for a short period of at least 6 h and no more than 24 h. These experiments established that total chitinase activity in strawberry leaves could only be inducible up to 5-fold maximum under the experimental conditions employed. Also, various abiotic treatments induced chitinase activity only 2-3 fold.

The induction level of total chitinase activity is similar to other reports. For example in bean, total chitinase activity was induced five fold upon infection with \textit{Fusarium solani} but the induction was two-fold with an incompatible fungus (Lange et al., 1996). Soybean varieties resistant to the \textit{Meloidogyne incognita} infection showed 3-4
fold chitinase induction (Qiu et al., 1997). In grapevine, wounding and salicylic acid
induced total chitinase activity by a factor of 4.9 and 5.5, respectively (Deckel et al.,
1996). A 5-fold increase in chitinase activity was detected after 24 h in rice cells treated
with fungal elicitors (Velazhahan et al., 2000). Therefore, it appears that the total
chitinase activity is inducible approximately 5-fold in various plants during pathogen
ingress or abiotic challenge. The level of induction is sufficient in some systems to
overcome the pathogen, perhaps in coordination with other defense mechanisms.
However, as opposed to the total chitinase activity, the induction of specific isoforms that
are effective against the invading organism would be of crucial importance.

Analysis of the isoform pattern of control and fungal infected plants showed that
the major acidic isoform (ChiA3) and the two basic isoforms (ChiB1 and ChiB2) were
present in all the control and the infected plants. However, other than the possible
presence of a new minor basic isoform (ChiB1a) (indicated by an arrow in Figure 4.8,
b24h, lane 4), no induction of the synthesis of any new major acidic or basic isoform was
detected. This suggested that the observed increase in the total chitinase activity after
Colletotrichum infection was not caused by the synthesis of new isoforms, but was a
result of an increased synthesis of the isoforms that were already present in the plant
tissues before infection. On the other hand, plants treated with salicylic acid, ethephon, or
injury clearly showed induction of four acidic isoforms (ChiA2, ChiA4, ChiA5, and
ChiA6) at 24 h as compared to the controls that did not show presence of these isoforms.
ChiA3 showed slight induction in samples treated with ethephon and injury as compared
to the control plants. Only injury induced the basic isoforms ChiB1 and ChiB2. A new
minor isoform ChiB1a was detected in this treatment as well (indicated by an arrow in
In terms of the synthesis of new chitinase isoforms after fungal infection, different plants respond differently. In the rose plant, infection by *Diplocarpon rosae* causes the synthesis of two new acidic isoforms in the intercellular fluid (Suo and Leung, 2001). Sorghum leaves showed two constitutive chitinase isoforms. Four new isoforms appeared in response to injury but only minor changes were seen in the isoform pattern in leaves challenged with the fungus *Fusarium moniliforme* (Krishnaveni, et al., 1999). In wheat, 7 distinct isoforms were detected in healthy plants with induction of one and two new isoforms in injury and ethylene treated plants, respectively (Botha et al., 1998). In grapevine, 4 basic and 2 acidic isoforms were detected in healthy plant leaves, but wounding induced 4 new acidic isoforms whereas salicylic acid induced these acidic isoforms slightly but induced three constitutive basic isoforms significantly (Derckel et al., 1996). Interestingly, in bean, new isoforms were shown to be the in vivo-proteolytic cleavage products of a class IV chitinase. This processing occurred only in plants infected with the compatible fungus generating three new isoforms processed at the hinge region (Lange et al., 1996). In light of this important finding, it cannot be completely ruled out that new isoforms induced in response to stress might have been generated by proteolytic processing.

Despite the fact that total chitinase activity was induced and multiple chitinase isoforms were present in strawberry leaves, the fungal species tested were able to infect these plants. It is possible that speed and intensity of the induction are determining factors for plant disease or resistance (Ryals et al., 1996). Also, some studies have shown that only certain isoforms are able to defend plants against particular pathogens (Sela-Buurlage et al., 1993; Sticher et al., 1997). In order to identify the most effective
chitinases against a particular pathogen, chitinase genes and their products must be 
evaluated individually for their induction kinetics and antifungal activities against the 
pathogen.

In this study, two class II chitinase genes, FaChi2-1 and FaChi2-2, were examined 
for their expression levels in the uninoculated control plants and in fungal infected plants. 
Only a few class II chitinase genes have been cloned and a limited number of studies 
have been done to detect their expression pattern in disease or stress conditions. Most 
available sequences are derived from cDNA and therefore do not show the complete 
structure of the genes. Studies have been performed on class II chitinases from peanut 
and parsley. It is interesting to note that the peanut chitinases, A.h.Chi2;1 and 
A.h.Chi2;2, are also one and two-intron containing genes, respectively. Only cDNA 
sequences are known for the parsley chitinases (PcCHI1-1 and PcCHI2). FaChi2-1 has 
high degree of amino acid identity to A.h.Chi2;1 (72.9%) and PcCHI1-1 (75.8%), 
whereas FaChi2-2 shares a higher identity with the two-intron A.h.Chi2;2 chitinase 
(61.9%) and PcCHI2 (66.5%) chitinase. It appears that FaChi2-1, A.h.Chi2;1, and 
PcCHI1-1 could be placed in a sub-group within the class, whereas FaChi2-2, 
A.h.Chi2;2, and PcCHI2 could constitute another sub-group based on sequence similarity 
and genomic structure. This might have implications in similar functions for these genes 
as their expression pattern appears to be similar. FaChi2-1 is a quick response gene 
induced within 2 h of fungal inoculation. In strawberry, the two fungi tested induced 
FaChi2-1 with different kinetics. In response to *C. fragariae*, FaChi2-1 induced 
approximately 3-4 fold and remained at that level for 24 h period with more pronounced 
induction of approximately 7.5 fold at 48 h. However, in one experiment (Figure 4.14)
FaChi2-1 showed biphasic behavior in response to *C. acutatum* with two peaks around 6 h and 48 h. The biphasic induction of this gene might have been true in Figure 4.12 since expression past 48 h was not analyzed. The biphasic induction of genes has been reported previously. For example, two chitinase genes from sugar beet (Nielsen et al., 1993) and grape (Busam et al., 1997) showed biphasic induction kinetics. Any significance of such induction pattern is not known. The early induction of FaChi2-1 is in close agreement with the related PcCHI1-1 and A.h.Chi2;1 genes. Parsley cell culture when treated with Pep25, a defined oligopeptide fungal elicitor (Nurnberger, 1994), showed a rapid induction of PcCHI1-1 mRNA at 0.75 h. The induction reached its peak at 2 h and went to low levels at 8-10 h, and remained at that level throughout the length of the experiment, 32 h. Parsley leaves challenged with *Phytophthora sojae* also showed PcCHI1-1’s rapid and transient induction that peaked at 10-12 h and returned to background levels at 20-25 h (Ponath et al., 2000). The peanut A.h.Chi2;1 gene showed induction within 1 h, reaching maximum level at 4 h in suspension culture cells in response to conidia from *Botrytis cinerea* (Kellmann et al., 1996). Transgenic tobacco plants carrying the GUS reporter gene fused to the 1.2 kb upstream region of A.h.Chi2;1 showed transient induction of this gene that peaked within 3 h of challenge with *B. cinerea*. The level of induction with both fungal elicitor and *B. cinerea* was only 2-3 fold as monitored by GUS activity. In comparison, the level of induction was approximately 3.5 fold thorough 24 h for the FaChi2-1 gene in response to *C. fragariae*.

FaChi2-2 showed induction within 48 h of fungal inoculation. *C. fragariae* caused sharp induction (approximately 7 fold) of this gene whereas *C. acutatum* showed moderate induction of this gene (only in one experiment, Figure 4.15). It should be noted
that the constitutive level of FaChi2-2 was higher as compared to FaChi2-1 in the uninoculated plant leaves. This might be a reason a slow induction of this gene was observed. The late induction of this gene is in agreement with kinetics of parsley PcCHI2 gene (Ponath et al., 2000). PcCHI2 mRNA was detectable only at 16 h and reached its peak at 32 h (last time point in this study) in response to a fungal elicitor. In parsley leaves challenged with *Phytophthora sojae* the expression of PcCHI2 was not detectable up to 25 h. The peanut gene A.h.Chi2;2 was induced in response to conidia from *Botrytis cinerea*, ethylene, and salicylate (Kellmann et al., 1996). The maximum expression was reached with these treatments between 1-4 h. Another example of a related gene is from rice, Rcht2, that shows similar structure as FaChi2-2 with two introns (Kim et al., 1998). The expression of Rcht2 was inducible in leaves by ethephon and mercuric chloride after 48 h post-treatment. The timing of induction is in close agreement with FaChi2-2.

At least at organ or organismic levels, one intron containing class II chitinase genes appear to be induced faster than two intron containing genes. It is tempting to speculate that their regulation is geared towards immediate, and slow but perhaps longer lasting protections, respectively. Molecular analysis of the promoter regions to identify the elements important in the timing of induction, and determination of the signaling events leading to the expression of these genes will provide further insight into the regulation of class II chitinase genes.

The presence of large number of chitinase isoforms and their differential expression patterns in response to various stimuli shows complex gene regulation. Closely related, but distinct, isoforms are induced in response to specific stimuli. Their induction kinetics could depend on the plant species, invading organisms, growth
conditions, and the environment. Also, only specific isoforms show antifungal activity against a given pathogen. Therefore, for transgenic application of a gene, the pathogen targeted must be defined, and the candidate gene products must be evaluated for their antifungal activity towards that pathogen. Strawberry’s two class II chitinase genes are good candidates for such studies against *C. fragariae* and *C. acutatum*. In the absence of CBD, the gene products are expected not to be allergenic, a problem with class I chitinases. Alternatively, the promoter regions of these genes may be used with other antifungal agents desiring the kinetics of expression observed for these genes.
CHAPTER 5

SUMMARY, CONCLUSIONS, AND FUTURE DIRECTIONS

Pathogenesis-related (PR) proteins play an important role in plant defense against invading organisms. PR-proteins were initially described approximately 30 years ago 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. However, only plant chitinases and β-1,3-glucanases have been extensively studied. This in part is due to the immediate realization that these hydrolytic enzymes could degrade cell walls of certain fungi. These enzymes were shown to be induced in plants upon infection and some purified proteins were observed to have antifungal activity in vitro (Mauch et al., 1988; Sela Buurlage et al., 1993; Regalado et al., 2001). Chitinases and glucanases have been purified and characterized from a number of plant sources. Genes encoding these enzymes have also been cloned from a variety of plant sources. Currently, there is an immense interest in delineating the molecular events from pathogen recognition to the expression of these genes. In an effort to enhance the disease resistance, PR-genes have been used to transform a variety of plant sources. There is considerable evidence showing positive correlation between the expression of PR-genes and disease resistance (Broglie et al., 1991; Jach et al., 1995; Grison et al., 1996; Terakawa et al., 1997; Yamamoto et al., 2000; Datta et al., 2001; Oldach et al., 2001).

Strawberry is an important horticultural crop in the southern United States. However, in recent years fungal infections have become serious problems for sustained strawberry production. While efforts are under way for the production of transgenic
strawberries with enhanced disease resistance, no information is available on strawberry’s endogenous defense systems. This dissertation is an effort to delineate these defense systems. Characterization of the chitinase activities and chitinase isoforms in strawberry plants were performed. PR-genes were cloned and two class II chitinase genes were partially characterized. Finally, the induction of chitinase activities and the expression of two class II chitinase genes were analyzed in plants infected with either *Colletotrichum fragariae* or *C. acutatum*.

Chitinase activities and isoforms were analyzed in various strawberry organs. Crown showed the highest activity in uninoculated plants, whereas root, petiole, fruit, leaf, and runner showed successively lower activities. High constitutive chitinase activity in crown might be an adoptive feature since *Colletotrichum* sp. are capable of causing anthracnose crown rot leading to plant death. While up to six acidic and two basic chitinase isoforms were detected in various plant organs, a major acidic isoform (ChiA3) was predominant in every sample tested. An exception was the fruit where this particular isoform was not as prominent. Unexpectedly, this particular isoform, when isolated from the native gel and run under semi-denaturing conditions, dissociated into 3-4 chitinase activities. These observations indicated that this particular isoform might be a complex of proteins. It has been shown in winter rye that such a complex in fact exists consisting of chitinases, β-1,3-glucanases, and osmotin-like proteins (OLP) (Yu and Griffith, 1999). Interestingly this complex functions in cold tolerance. It would be interesting to study strawberry’s ChiA3 isoform further by identification of its constituents. Possibility of the presence of β-1,3-glucanase and OLP in this complex could be checked by performing a Western blot. If this is indeed a complex, in analogy to winter rye complex, its antifreeze
activity could be determined. If this complex shows antifreeze function, the identity of
proteins in this complex and the protein domains required for the complex formation
would be interesting to know.

Two class II chitinase genes were cloned and their corresponding cDNAs were
obtained. FaChi2-1 and FaChi2-2 contain one and two introns in their genomic
sequences, respectively. The intron splice junctions were verified by the cDNA analysis.
These genes belong to a small multigene family as shown by multiple bands on genomic
Southern blots. The transcription start site for these genes were determined by primer
extension analyses. The transcription start sites of FaChi2-1 maps to –103 G or –88 T
from the translation start site. The transcription start site of FaChi2-2 maps to –52 A
from the translation start site. These genes were expressed constitutively in the
strawberry leaves. An interesting pattern of regulation was observed for these genes in
plant leaves infected with either *C. fragariae* or *C. acutatum*. FaChi2-1 is a quick
response gene induced within 2 h of fungal inoculation. FaChi2-2 expressed later in the
infection process within 24-48 h. This type of induction pattern has been seen for other
chitinase genes (Kellmann et al., 1996; Kim et al., 1998; Ponath et al., 2000). It would be
interesting to obtain the promoter sequences of these genes. Genome walking could be
utilized to obtain approximately 2 kbp of upstream sequence for both genes. These
promoter regions could be fused to *uidA* gene, coding for β-glucuronidase (GUS), and
introduced into tobacco or arabidopsis plants by transformation. A quantitative measure
of the mRNA in response to various stimuli, such as salicylic acid, ethylene, jasmonic
acid, and injury, would help in delineating the signaling pathways utilized by these genes.
Also, it would be interesting to find the regulatory regions of these genes important in
timing of the induction for these genes. Regulatory elements important in salicylic acid, ethylene, jasmonic acid, and injury response would also be interesting to find. A promoter deletion series linked to a reporter gene could be constructed and transformed into a model plant to identify such elements.

A class III chitinase gene, FaChi3-1 was cloned. Partial genomic and cDNA sequences of a β-1,3-glucanase gene, FaGlu2-1, were also obtained. In the future, complete genomic and cDNA sequences of FaGlu2-1 should be obtained. Both of these genes were found to be expressed constitutively in the strawberry leaves. However, no studies on their induction in the infected plants were performed. For future experiments, it would be interesting to know if these genes are also induced during fungal infection. The kinetics of their induction could be determined by similar experiments as presented in this dissertation using real-time PCR or by Northern blot analyses.

It would be interesting to study the induction of individual proteins coded by the cloned genes. Antibodies should be produced against the recombinant proteins. The recombinant proteins can easily be produced in vitro in a cell-free translation system. Availability of antibodies would also open the avenues for detailed microscopic examination of where and when exactly in the plant a particular protein is accumulating in response to the fungal infection (Avant, 2001). Antifungal activities of the recombinant proteins could be analyzed. If a synergistic effect between one of the class II chitinases and β-1,3-glucanase is observed, they would be ideal candidates for the strawberry transformation with a strong promoter.

Plant PR-genes have been studied extensively in the last 20 years. However, regulation of these genes are still poorly understood. With the cloning of strawberry PR-
genes, we can dissect the molecular events leading to the induction of these genes during plant disease. In future experiments, these genes can be used to transform strawberry plants for enhanced disease resistance. Further experiments will provide basic biochemical knowledge of these genes in various plant functions.
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