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Isolation, characterization, and expression analysis of $\beta$-1, 3-glucanase genes from strawberry plants

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ISOLATION, CHARACTERIZATION, AND EXPRESSION ANALYSIS OF \( \beta-1, 3 \)-GLUCANASE GENES FROM STRAWBERRY PLANTS

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

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ABSTRACT

Plant β-1, 3-glucanases are pathogenesis-related proteins, which are implicated in plant defense responses against pathogen infection. As an initial step in understanding the roles of β-1, 3-glucanases in the strawberry plant defense system, genome walking, and 3′ and 5′ RACE were performed to isolate β-1, 3-glucanase genomic and cDNA clones. In addition, real time PCR was performed to determine the expression levels of two of the isolated β-1, 3-glucanase genes in healthy and fungal infected plants.

Two genomic clones, FaBG2-1 and FaBG2-2, and a cDNA clone, FaBG2-3, encoding three different β-1, 3-glucanases, were isolated. FaBG2-1 was comprised of two exons and one intron. The first exon of FaBG2-1 encodes the major part of a signal peptide. Results of Southern blotting analysis indicated that the strawberry genome contains several copies of FaBG2-1 or related genes. FaBG2-2 appears to be an intronless gene and does not encode a signal peptide. FaBG2-3, like FaBG2-1, also encodes a signal peptide, but is different from FaBG2-1 in 3′ and 5′ non-coding regions. The proteins encoded by these three genes share a high degree of sequence homology to plant class II β-1, 3-glucanases.

The expression of FaBG2-1 and FaBG2-3 in strawberry plants infected with Colletotrichum fragariae and Colletotrichum acutatum, two important strawberry fungal pathogens, were examined. High levels of induction of both genes were observed in plants infected with C. fragariae, whereas lower levels of induction were observed in plants infected with C. acutatum. Moreover, the expression of FaBG2-3 was much greater than FaBG2-1 in both the uninfected and the infected plants.
The expressions of *FaBG2-1* and *FaBG2-3* in leaves, crowns, and roots were examined at different time points during a 7 month growth period. Different organs showed different expression patterns for the two genes. Furthermore, the total β-1, 3-glucanase activity and isozyme pattern were analyzed. The isozyme patterns were different between the uninfected and the infected plants. Also, the differences were observed between young plants and older plants. This research shows that β-1, 3-glucanase in strawberry plant may play roles in plant defense and plant development.
CHAPTER 1

LITERATURE REVIEW

1.1 Pathogenesis-Related (PR) Proteins

Higher plants have developed different mechanisms to protect themselves from various biotic and abiotic stresses, including pathogen attacks, wounding, exposures to heavy metals, salinity, drought, cold, air pollutants and ultraviolet rays (Agrios, 1997). These stresses can provoke structural and biochemical changes in plants, such as formation of cellular defense structures and production of phenolic compounds and phytoalexins (Bowles, 1990). In addition, a group of novel proteins are induced, which are collectively referred to as pathogenesis-related (PR) proteins. Unlike phytoalexins, which are mainly produced by healthy cells adjacent to localized damaged and necrotic cells, PR proteins accumulate not only locally in the infected and surrounding tissues, but also in remote uninfected tissues. This production of PR proteins in the remote uninfected parts of plants can lead to the occurrence of systemic acquired resistance, protecting the affected plants from further infection (Ryals et al., 1996; Delaney, 1997).

PR proteins were originally identified in tobacco plants infected by tobacco mosaic virus (Van Loon and Van Kammen, 1970). These proteins were subsequently detected in a wide range of plant species. Most PR proteins are acid-soluble, low molecular weight, and protease-resistant proteins (Leubner-Metzger and Meins, 1999; Neuhaus, 1999). PR proteins can be acidic or basic proteins depending on their isoelectric points (pI). Acidic PR proteins are predominantly located in the intercellular spaces. Basic PR proteins, while having similar functions to acidic PR proteins, are mainly located...
intracellularly in the vacuole (Legrand et al., 1987; Niki et al., 1998; Van Loon and Van Strien, 1999). Based on amino acid sequence similarities, serologic relationships, and/or enzymatic or biological activities, PR proteins can be categorized into 17 families (Table 1), including β-1, 3-glucanases, chitinases, thaumatin-like proteins, peroxidases, ribosome-inactivating proteins, defensins, thionins, nonspecific lipid transfer proteins, oxalate oxidase, and oxalate-oxidase-like proteins (Van Loon et al., 1994; Van Loon, 1997; Van Loon and Van Strien, 1999; Görlach et al., 1996; Okushima et al., 2000; Christensen et al., 2002).

Table 1. Recognized families of pathogenesis-related proteins*

<table>
<thead>
<tr>
<th>Families</th>
<th>Type member</th>
<th>Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR-1</td>
<td>Tobacco PR-1a</td>
<td>antifungal</td>
</tr>
<tr>
<td>PR-2</td>
<td>Tobacco PR-2</td>
<td>β-1,3-glucanase</td>
</tr>
<tr>
<td>PR-3</td>
<td>Tobacco P, Q</td>
<td>chitinase type I,II, IV,VI,VII</td>
</tr>
<tr>
<td>PR-4</td>
<td>Tobacco ‘R’</td>
<td>chitinase type I,II</td>
</tr>
<tr>
<td>PR-5</td>
<td>Tobacco S</td>
<td>thaumatin-like</td>
</tr>
<tr>
<td>PR-6</td>
<td>Tomato Inhibitor I</td>
<td>proteinase-inhibitor</td>
</tr>
<tr>
<td>PR-7</td>
<td>Tomato P69</td>
<td>endoproteinase</td>
</tr>
<tr>
<td>PR-8</td>
<td>Cucumber chitinase</td>
<td>chitinase type III</td>
</tr>
<tr>
<td>PR-9</td>
<td>Tobacco ‘lignin-forming peroxidase’</td>
<td>peroxidase</td>
</tr>
<tr>
<td>PR-10</td>
<td>Parsley ‘PRI’</td>
<td>‘ribonuclease-like’</td>
</tr>
<tr>
<td>PR-11</td>
<td>Tobacco ‘class V’ chitinase</td>
<td>chitinase, type I</td>
</tr>
<tr>
<td>PR-12</td>
<td>Radish Rs-AFP3</td>
<td>Defensin</td>
</tr>
<tr>
<td>PR-13</td>
<td>Arabidopsis THI2.1</td>
<td>Thionin</td>
</tr>
<tr>
<td>PR-14</td>
<td>Barley LTP4</td>
<td>lipid-transfer protein</td>
</tr>
<tr>
<td>PR-15</td>
<td>Barley OxOa (germin)</td>
<td>oxalate oxidase</td>
</tr>
<tr>
<td>PR-16</td>
<td>Barley OxOLP</td>
<td>‘oxalate oxidase-like’</td>
</tr>
<tr>
<td>PR-17</td>
<td>Tobacco PRp27</td>
<td>unknown</td>
</tr>
</tbody>
</table>

*This table is extracted from web site: [http://www.bio.uu.nl/~fytopath/PR-families.htm](http://www.bio.uu.nl/~fytopath/PR-families.htm)

The modes of action of PR proteins in plant-defense remain unclear (Blume et al., 2000; Zhang and Klessig, 2001). Studies on *Arabidopsis thaliana* suggested that signaling events initiated by diverse pathogens are converged into a conserved mitogen-
activated protein kinase (MAPK) cascade. The MAPK cascade leads to the expression of WRKY proteins, which are transcription factors (Du and Chen, 2000). WRKY proteins contain a WRKY domain, defined by the conserved amino acid sequence WRKYGQK at their N-termini, together with a novel zinc-finger-like motif. They can bind to W-box DNA elements (containing a TGAC core sequence) that are found in the promoters of many defense-related genes. This binding may activate the promoters and induce the expression of the defense-related genes (Eulgem et al., 1999, 2000; Du and Chen, 2000). Defense responses activated by the MAPK cascade are effective against both fungal and bacterial pathogens (Asai et al., 2002).

MAPKs are important enzymes in MAPK cascades. These enzymes are regulated by jasmonic acid (JA), salicylic acid (SA), ethylene, abscisic acid (ABA), hydrogen peroxide, protein phosphatase inhibitors, chitosan, high salt/sugar, and heavy metals (Agrawal et al., 2003). After pathogen infection, many secondary signal molecules can be produced by plants, including JA, SA, and ethylene.

JA can be produced by the octadecanoid-signaling cascade. In this cascade, the linolenic acid in plant membrane is released into the cytosol and then is converted to hydroperoxylinolenic acid. This hydroperoxide is converted to an unstable allene oxide and then into 12-oxo-phytodienoic acid (PDA). PDA is then reduced to 10, 11-dihydro-PDA and degraded by three cycles of consecutive β-oxidation cycles, to epi-JA. Epi-JA is then changed to JA. Intermediate products of this pathway, including acyclic octadecanoids, cyclopentanoid C18 fatty acids, and epi-JA, may also play roles in regulating the expression of PR protein genes (Koch et al., 1999).
In the case of ethylene biosynthesis, the two key steps are the conversion of S-adenosyl-methionine to 1-aminocyclopropane-1-carboxylic acid (ACC) and the oxidative cleavage of ACC to form ethylene. The enzymes that catalyze these two reactions are ACC synthase (ACS) and ACC oxidase (ACO), respectively. Upon stimulation, ACS activity is induced rapidly. However, the signal transduction pathways that modulate ethylene biosynthesis in plants under stress are largely unknown (Kim et al., 2003).

1.2 Plant β-1, 3-Glucanases

β-1, 3-glucanases (glucan endo-1, 3-β-glucosidases, E.C. 3.2.1.39) catalyze the hydrolysis of β-1, 3-glucan, which is a polymer of β-1, 3-linked glucose residues (Simmons, 1994). β-1, 3-glucanases have been found in plants, yeasts, actinomycetes, bacteria, fungi, insects, and fish (Boller, 1985; Pan et al., 1989).

β-1, 3-glucan is a major structural component of the cell walls of many pathogenic fungi (Wessels and Sietsma, 1981, Adams, 2004). For example, *Phytophthora infestans* is an oomycete pathogen that causes late blight of potato and tomato. Oomycetes have a cell wall that is comprised of 80-90% β-1, 3-glucan. β-1, 3-glucan (called callose in plants) is also a cell-wall component of certain cell types during specific developmental stages of plants (Abeles and Forrence, 1969; Kauss, 1987, 1992; Kotake et al., 1997). For example, callose acts as a permeability barrier in pollen mother cell walls (Heslop-Harrison, 1964) and muskmelon endosperm envelopes (Yim and Bradford, 1998).

Plant β-1, 3-glucanases are pathogenesis-related proteins and they are classified as members of the PR-2 family. Syntheses of these enzymes can be induced by pathogens or other stimuli. Also, because the substrate for β-1, 3-glucanases exists in plants, these
enzymes may be involved in various physiological and developmental processes, such as cell elongation (Masuda and Wada, 1967; Heyn, 1969), cell division (Fulcher et al., 1976), fruit ripening (cell wall degradation leading to fruit softening) (Hinton and Pressey, 1980), fertilization (Ori et al., 1990), pollen germination and tube growth (Roggen and Stanley, 1969; Meikle et al., 1991), bud dormancy (removal of phloem callose) (Krabel et al., 1993), microsporogenesis (dissolution of pollen tetrads into free microspores) (Bucciaglia and Smith, 1994), somatic embryogenesis (Helleboid et al., 2000), seed germination (Morohashi and Matsushima, 2000; Buchner et al., 2002), and flower formation (Neale et al., 1990; Akiyama et al., 2004).

1.2.1 The Structures and Coding Properties of Plant β-1, 3-Glucanase Genes

All β-1, 3-glucanase genes examined thus far, perhaps with two exceptions, contain a single intron. The position of the intron is usually the same in all these genes whether they code an acidic or a basic enzyme. The first exon of these genes encodes the major portion of a signal peptide (Von Heijne, 1983). The lengths of the intron in different β-1, 3-glucanase genes can vary. The introns usually range in size from 400-600 base pairs, but some β-1, 3-glucanase genes have shorter or longer introns. For example, introns in three peach β-1, 3-glucanase genes are 160, 256 and 677bp (Thimmapuram et al., 2001; Ko et al., 2003). In comparison, the curled-leaved tobacco β-1, 3-glucanase gene has a 975-bp intron (Gheysen et al., 1990).

A β-1, 3-glucanase gene (gn1) isolated from Nicotiana plumbaginifolia contains two introns (Castresana et al., 1990). This gene encodes 370 amino acids. Like β-1, 3-glucanase genes with one intron, the first exon of gn1 encodes part (25 amino acid residues) of a signal peptide (30 amino acid residues). The first intron is 669 bp in length.
The second intron, which is 199 bp in length, divided the rest of the gene into two more exons of 273 bp and 952 bp.

The gene encoding the barley β-1, 3-glucanase isozyme GI has no intron and this gene does not encode a signal peptide (Burton et al., 1998). GI has been shown to localize intracellular in barley leaves because it has no vacuolar or endoplasmic reticulum targeting signals or any other obvious sequence motifs that are needed for intracellular transport. In addition to GI, the barley β-1, 3-glucanase isozyme GV (Xu et al., 1992) and a rice β-1, 3-glucanase (OsGLN1) (Akiyama and Pillai, 2001) are believed not to have a signal peptide based on cDNA sequences of these two isozymes. Hence, these two β-1, 3-glucanase isozymes, like the barley GI isozyme, may also be derived from intronless genes.

A number of various cis-acting regulatory elements are found to be involved in the expression of β-1, 3-glucanase genes. For example, a TCA-like motif (TCATCTTCTT), known to bind a nuclear protein induced by SA, has been found in the upstream regions of tobacco (Goldsbrough et al., 1993) and peach β-1, 3-glucanase genes (Ko et al., 2003). A highly conserved AGC element (AGCGCC) has been found in the promoter regions of many β-1, 3-glucanase and chitinase genes, and is known to be an ethylene-responsive element (Hart et al., 1993; Ohme-Takagi and Shinshi, 1995, Ko et al., 2003). Studies of a tobacco β-1, 3-glucanase gene showed that enhancer activity and ethylene-responsiveness also depend on the presence of a GCC box (GCGGCC) (Grimmig et al., 2003; Brown et al., 2003). A W-box sequence (TTGAC) also present in the promoters of some β-1, 3-glucanase genes, is recognized specifically by SA-induced WRKY DNA binding proteins (Yu et al., 2001).
Other cis-acting regulatory elements necessary for response to different stimuli have been found in the 5’ upstream region of some plant PR protein genes. For example, an ASF-1 binding site (TGACG) has been found to be present in the promoters of some PR protein genes and this site is involved in transcriptional activation of these genes by SA (Despres et al., 2003).

1.2.2 Plant β-1, 3-Glucanases and Their Structures

The majority of known β-1, 3-glucanase genes encodes precursor proteins that are 340-400 amino acids in length. Some precursor proteins contain a signal peptide and are secreted into extracellular space. Many of these precursor proteins also contain a sequence at the C-termini called the C-terminal extension (about 20 amino acids in length). This C-terminal extension has a single N-glycosylated site and is glycosylated. The C-terminal sequence contains a signal for targeting the enzyme to the vacuole and is responsible for the transport of these proteins from the endoplasmic reticulum via the Golgi compartment to the vacuole where the sequence is removed (Shinshi, 1988; Sticher et al., 1992). The mature proteins of β-1, 3-glucanases are variable in length with molecular masses ranging from 33 to 41 kDa. PI of β-1, 3-glucanases are also variable. Some are acidic and some are basic or neutral. Amino acid sequences of all β-1, 3-glucanases examined thus far are different from 32% to more than 90% identity.

The three-dimensional structure of a barley β-1, 3-glucanase, isozyme GII, has been determined by x-ray crystallography. The enzyme has an alpha/β-barrel structure. A deep groove runs along the full length of the upper surface of the molecules, perpendicular to the barrel axis, and is suggested to be the polysaccharide substrate binding site. There is a deep cleft parallel to the major axis of the molecule. The length of
the cleft is sufficient to accommodate 8 residues of extended $\beta$-1, 3-glucan chain. The two catalytic glutamic acid residues (E231 and E288) lie about one-third of the way down the cleft. It is assumed that the glycosidic oxygen is positioned between these two residues. Protonation of the glycosidic oxygen by the catalytic residue is followed by stabilization of the intermediate oxycarbonium ion by the catalytic nucleophile. E231 functions as a catalytic nucleophile and E288 functions as a proton donor. Adjacent to the E288 residue are the strictly conserved residues K282 and E279. These residues appear to be important in influencing the protonation state of the catalytic acid E288 (Varghese et al., 1994) (Fig. 1).

Figure 1. Structure of barley $\beta$-1, 3-glucanase (GeneBank accession number 1GHSA). The active site and essential catalytic residues are indicated in the figure. This figure is constructed by Cn3D 4.1 program.
The specific enzymatic activities and substrate specificities of different β-1, 3-glucanases are different. For example, class I and class II PR-2c enzymes were 50 to 250 times more active in degrading laminarin than the class II PR-2a, PR-2b and the class III (PR-2d) enzymes (Kauffmann et al., 1987; Linthorst, 1991) (See classification of the enzymes below).

1.2.3 Classification of Plant β-1, 3-Glucanases

β-1, 3-glucanase genes have been identified in a variety of plants, including tobacco (De Loose et al., 1988), soybean (Takeuchi et al., 1990), rubber tree (Chye et al., 1995), banana (Peumans et al., 2000), and rice (Yamaguchi et al., 2002). Not only different plant species have different β-1, 3-glucanase genes, a single plant species may also have several different copies of β-1, 3-glucanase genes. For example, more than 14 β-1, 3-glucanase genes have been found in tobacco plants (Leubner-Metzger and Meins, 1999).

The proteins encoded by different β-1, 3-glucanase genes show differences in size, pI, primary structure, cellular localization, and pattern of regulation (Meins et al., 1992). Based on these differences, especially primary amino acid sequence differences, β-1, 3-glucanases in the genus Nicotiana were divided into four classes (Payne et al., 1990; Ward et al., 1991; Leubner-Metzger and Meins, 1999). These classifications have also been applied to β-1, 3-glucanases of other plant species which have similar structural isoforms (Van Kan et al., 1992; Beerhues and Kombrink, 1994; Domingo et al., 1994; Oh et al., 1995).

Most class I β-1, 3-glucanases are basic proteins which are localized in the cell vacuole, while most class II, III, and IV β-1, 3-glucanases are acidic proteins which are
secreted into the extracellular space. Premature class I β-1, 3-glucanases contain a signal peptide. Most premature class I β-1, 3-glucanases also contain a C-terminal extension. However, some class I enzymes do not contain C-terminal extension. For example, two Arabidopsis β-1, 3-glucanases, BG1 and BG3, showed structural homology to tobacco class I proteins, which are basic vacuolar enzyme, except for the absence of a C-terminal extension (Uknes et al., 1992).

In addition to the differences in pI and cellular localization, classes II, III, and IV β-1, 3-glucanases are also different from class I β-1, 3-glucanase in molecular mass and amino acid sequence. For example, the molecular mass of Nicotiana tabacum class I β-1, 3-glucanase is 33 kDa whereas the tobacco class II (PR-2a, PR-2b, PR-2c), class III (PR-Q), and class IV (tag1) enzymes range in size from 34 to 36 kDa on denaturing gels (Beffa et al., 1993; Bucciaglia and Smith, 1994). Furthermore, almost all classes II, III, IV enzymes do not contain a C-terminal extension (Linthorst et al., 1990; Ward et al., 1991; Beffa et al., 1993). Most class II β-1, 3-glucanases, like the class I β-1, 3-glucanases, also have signal peptides (Von Heijne, 1983). Based on amino acid sequences of the mature proteins, class II, III and IV β-1, 3-glucanases can be distinguished from each other. Different classes differ in sequence homology by at least 40% to 50% (Meins et al., 1992; Simmons, 1994). For example, class IV β-1, 3-glucanase tag1 shows 37-38% identity in sequence to the mature forms of tobacco class I (Gla), class II (PR-2) and class III (PR-Q) β-glucanases (Payne et al., 1990; Bucciaglia and Smith, 1994; Leubner-Metzger and Meins, 1999).

For tobacco class II β-1, 3-glucanases at least six different isoforms have been identified (PR2a, 2b, 2c, 2d, SP41a, and SP41b) based on amino acid sequences (Ward et
al., 1991; Leubner-Metzger and Meins, 1999). These tobacco class II isoforms are at least 82% identical in amino acid sequence to each other and differ from tobacco class I enzymes at a minimum of 48.8% of amino acid residues. Although most class II β-1, 3-glucanases are acidic extracellular proteins without a C-terminal extension, there are a few exceptions. For example, tobacco β-1, 3-glucanases GL153 and GL161, which showed high sequence homology to class II enzymes (90% identity), are either neutral or basic vacuolar proteins with a C-terminal extension (Ward et al., 1991).

It should be pointed out that some recently discovered enzymes do not fit in the established classification system. For example, two new *Arabidopsis* isoforms, BG4 and BG5, showed relatively low homology (32% - 41% identity) to all established classes of β-1, 3-glucanases (Delp and Palva, 1999).

### 1.3 Possible Role of β-1, 3-Glucanases in Plant Defense

Plant β-1, 3-glucanases are pathogenesis-related proteins and they are classified as members of the PR-2 family. Based on the hydrolytic activities of β-1, 3-glucanases and their relationships to pathogen infections, β-1, 3-glucanases have been suggested as an important component of plant defense mechanisms against pathogens (Kauffmann et al., 1987; Mauch and Staehelin 1989; Linthorst, 1991; Cordero et al., 1994). It has been suspected that β-1, 3-glucanases play a direct role in defending against fungi by hydrolyzing fungal cell walls, which consequently causes the lysis of fungal cells. In addition, these enzymes may play an indirect role in plant defense by causing the formation of oligosaccharide elicitors, which elicit the production of other PR proteins or low molecular weight antifungal compounds, such as phytoalexins (Keen and Yoshikawa, 1983; Ham et al., 1991; Klarzynski et al., 2000).
1.3.1 Induction of β-1, 3-Glucanases by Pathogens and Pathogen-Derived Elicitors

Many studies have shown that β-1, 3-glucanases are constitutively expressed at low levels in plants, but are dramatically increased when plants are infected by fungal, bacterial, or viral pathogens. For example, upon infection by the fungal pathogen *Rhizoctonia solani*, two rice β-1, 3-glucanases were shown to be induced in all rice cultivars tested (Anuratha et al., 1996). The mRNA for a tomato acidic β-1, 3-glucanase accumulated to a higher level in leaves infected by the fungal pathogen *Cladosporium fulvum* (Van Kan et al., 1992). Tobacco β-1, 3-glucanase was transcriptionally induced up to 21-fold by infection with the bacterium *Pseudomonas syringae pv syringae* (Castresana et al., 1990; Alonso et al., 1995) and up to 10-fold by TMV infection (Livne et al., 1997).

Different β-1, 3-glucanases may be induced in different ways. For example, studies with tobacco plants showed that class I β-1, 3-glucanases were induced as a local response, while classes II and III β-1, 3-glucanases were induced both locally and systemically (Tuzun et al., 1989; Ward et al., 1991; Lusso and Kuc, 1995; Bol et al., 1996).

Induction of β-1, 3-glucanases by pathogens also depends on the plant clones. For example, when the production of a β-1, 3-glucanase upon infection with *Corynespora cassiicola* was compared in different clones of *Hevea brasiliensis*, considerable variability of the enzyme’s activity was observed among different clones during pathogenesis. Increased enzyme activity was found in the tolerant clone, while a decrease was observed in the susceptible clone (Philip et al., 2001).
There are many other examples which showed that the expression levels of these enzymes increased after infected with pathogens, such as barley infected by powdery mildew (Ignatius and Chopra, 1994), maize infected with *Aspergillus flavus* (Lozovaya et al., 1998), soybean infected with *Pseudomonas syringae* (Cheong et al., 2000), pepper infected with *Xanthomonas campestris* pv. *vesicatoria* and *Phytophthora capsici* (Jung and Hwang, 2000), wheat infected with *Fusarium graminearum* (Li et al., 2001), chickpea infected with *Ascochyta rabiei* (Pass.) Labr. (Hanselle and Barz, 2001), and peach infected with *Monilinia fructicola* (Zemanek et al., 2002).

Some components of pathogens or degraded components of pathogens can serve as elicitors of β-1, 3-glucanases and other PR proteins. These elicitors may be components of the cell surface of the pathogen that are released by host enzymes, including fungal β-glucan, chitin, chitosan, glycoproteins and N-acetylchitooligosaccharides (Chang et al., 1992; Kaku et al., 1997; Muench-Garthoff et al., 1997). They may also be synthesized and released by the pathogen after it enters the host in response to host signals, including harpin proteins of bacteria, certain hydroxylipids, peptides, and carbohydrates.

Many studies showed that plant β-1, 3-glucanases can be induced by pathogen elicitors alone without the presence of a pathogen. For example, when cultured parsley cells (*Petroselinum crispum*) were treated with heat-released soluble cell-wall fragments (elicitors) from fungi, β-1, 3-glucanase activity increased strongly in elicitor-treated cells (Kombrink and Hahlbrock, 1986). Another study showed that a *Phytophthora infestans*-derived elicitor can induce β-1, 3-glucanase activities in potato leaves (Kombrink et al., 1988). Similar results were also found with pea β-1, 3-glucanases which can be induced
by the fungal pathogen, *Fusarium solani f. sp. phaseoli* and by autoclaved spores of the pathogen (Mauch et al., 1984).

**1.3.2 Induction of β-1, 3-Glucanases by Other Stimulatory Factors**

Plant β-1, 3-glucanases are induced not only by pathogen infection, but also by other factors. For example, SA strongly induced accumulation of mRNAs of classe II and III β-1, 3-glucanases in wild-type tobacco plants (Ward et al., 1991; Niki et al., 1998). Many other chemical compounds have been reported to regulate β-1, 3-glucanase synthesis. Examples include abscisic acid (ABA) in tobacco (Rezzonico et al., 1998; Akiyama and Pillai, 2001; Wu et al., 2001) and methyl jasmonate, ethylene and gibberellin A₃ in tomato seeds and leaves (Wu and Bradford, 2003). Most studies showed ABA enhances the expression of β-1, 3-glucanases. However, a study showed that ABA strongly suppressed the expression of a β-1, 3-glucanase which had been enhanced by gibberellin A₃ in rice (Akiyama and Pillai, 2001). Other studies showed that accumulation of a pepper basic β-1, 3-glucanase gene transcript was strongly induced in pepper leaves by both ethephon (a precursor of ethylene) and methyl jasmonate (Jung and Hwang, 2000). Systemic acquired resistance in *Carica papaya* L. is induced by benzothiadiazole (BTH). Plants treated with BTH showed increased levels of β-1, 3-glucanase and chitinase activities and these plants also showed increased tolerance to infection by the virulent pathogen *Phytophthora palmivora*.

In addition to the stimulatory factors described above, synthesis of β-1, 3-glucanases can also be stimulated by a number of other stress factors, such as wounding, drought, exposure to heavy metals, air pollutant ozone, and ultraviolet radiation (Zemanek et al., 2002; Akiyama and Pillai, 2001; Fecht-Christoffers et al., 2003;
Sandermann et al., 1998; Thalmair et al., 1996). These various factors often appear to interact, resulting in a dynamic response to biotic, as well as abiotic stimuli. Furthermore, plant β-1, 3-glucanases can be induced by its substrate, β-1, 3-glucan. This fact indicates that β-1, 3-glucan can be used to increase defense responses in plants. For example, laminarin (a β-1, 3-glucan derived from the brown algae *Laminaria digitata*) has been shown to be an efficient elicitor of defense responses in grapevine plants and it can effectively reduce *B. cinerea* and *P. viticola* growth on the infected plants. Additionally, laminarin not only induces the expression of the β-1, 3-glucanase enzyme activity in grapevine, but also elicits other defense reactions in this plant, including calcium influx, alkalinization of the extracellular medium, oxidative burst, activation of two mitogen-activated protein kinases, expression of 10 defense-related genes with different kinetics and intensities, and the production of two phytoalexins (resveratrol and epsilon-viniferin) (Aziz et al., 2003).

### 1.3.3 Inhibition of Fungal Growth and Production of Elicitors

Several class I β-1, 3-glucanases from tobacco have been shown to inhibit the growth of *Fusarium solani* *in vitro* (Sela-Buurlage et al., 1993). Class I β-1, 3-glucanases from tomato also inhibited the growth of certain pathogenic fungi (*Alternaria solani, Trichoderma viride* and *Phytophthora infestans*) *in vitro* (Lawrence et al., 1996; Anfoka and Buchenauer, 1997).

This *in vitro* inhibitory effect can be enhanced in combination with class I chitinases. Besides β-1, 3-glucan, chitin is also a major structural component of the cell walls of many pathogenic fungi. β-1, 3-glucanases and chitinases are two hydrolytic enzymes that are abundant in plant species. β-1, 3-glucanases appear to be coordinately
expressed along with chitinases after fungal infection. This co-induction of the two hydrolytic enzymes has been described in many plant species, including pea, bean, tomato, tobacco, maize, soybean, potato, and wheat (Mauch et al., 1988a, b; Vogelsang and Barz 1993; Jach et al., 1995; Bettini et al., 1998; Lambais and Mehdy, 1998; Petruzzelli et al., 1999, Cheong et al., 2000, Li et al., 2001).

In vitro, class II β-1, 3-glucanases from tobacco and tomato have no inhibitory activity on fungal growth (Joosten et al., 1995; Anfoka and Buchenauer, 1997). However, it should be pointed out that class II β-1, 3-glucanases may still play a role in plant defense because of their ability to generate pathogen-derived elicitor molecules, which, in turn, stimulate the production of various PR proteins and other defense-related molecules (Keen and Yoshikawa, 1983; Ryan and Farmer, 1991; Côté and Hahn, 1994). One of the examples of defense response induced by an elicitor is the generation of the oligoglucoside, β-1, 3-β-1, 6-heptaglucoside, from the pathogenic oomycete Phytophthora megasperma f. sp. Glycinea by soybean class III β-1, 3-glucanases. This elicitor then induces accumulation of the phytoalexin glyceollin (Sharp et al., 1984). The protein that binds this elicitor is called β-glucan elicitor binding protein (GEBP) and is located in the plasma membranes of soybean root cells. This protein may be a receptor of the elicitor, because the binding affinities of the oligoglucoside for the binding protein correlate well with its abilities to induce phytoalexin accumulation in the soybean cotyledon tissue (Cheong et al., 1993).

1.3.4 Age-Related Pathogen Resistance

A variety of studies revealed a direct relationship between plant age and pathogen resistance or susceptibility (Allen at al, 1983; Miller, 1983; Reuveni et al., 1986;
Pretorius et al., 1988; Memelink et al., 1990; Koch and Mew, 1991; Wyatt et al., 1991; Rupe et al., 1995; Reuveni, 1998; Kus et al., 2002; Panter and Jones, 2002; Gaudet et al., 2003). A few cases indicated that plants are more susceptible to pathogens as they develop, while in most cases, plants show higher levels of resistance as they develop. This difference in resistance to pathogens depending on plant age is called age-related pathogen resistance. For example, age-related pathogen resistance was found during the development of the leaves of some plant species. It has been shown that the older leaves from bottoms of the shoots of grapevines exhibited a higher resistance to *Plasmopara viticola* than younger leaves from upper shoots (Reuveni, 1998). In addition, both young and mature leaves in older tobacco and wheat plants showed higher resistance than the leaves in younger plants (Reuveni et al., 1986; Pretorius et al., 1988).

PR proteins are among many factors which play roles in age-related pathogen resistance. As tobacco ages, it becomes naturally resistant to blue mold caused by *Peronospora tabacina*. A study showed that β-1, 3-glucanase, chitinase and peroxidase activities increased in tobacco with age (Wyatt et al., 1991). The studies also showed that these enzyme activities were higher in leaf tissue from the main stalk (resistant to blue mold) as compared to leaf tissue from suckering stems (susceptible to blue mold) on the same plant (Wyatt et al., 1991). β-1, 3-glucanases were also found to increase in grapevine leaves that correspond to its age-related resistance (Reuveni, 1998). Other studies also showed that in healthy plants, higher concentrations of β-1, 3-glucanases were found in roots and lower leaves, relative to younger leaves (Felix and Meins, 1986; Keefe et al., 1990; Memelink et al., 1990, Neale et al., 1990; Van de Rhee et al., 1993; Vogeli-lange et al., 1994).
1.3.5 β-1, 3-Glucanase Genes in Transgenic Plants

β-1, 3-glucanase genes, alone or together with chitinase genes, have been transferred to a number of plant species and expressed constitutively in these plants. In most cases, the resulting transgenic plants exhibit enhanced levels of fungal disease resistance or delayed symptom development as compared to the control plants (Zhu et al., 1994; Jach et al., 1995; Jongedijk et al., 1995). It has been shown that in some cases, expression of β-1, 3-glucanase transgenes alone can reduce the susceptibility of plants to infection by certain fungi. For example, transgenic tobacco plants that expressed a soybean β-1, 3-glucanase showed reduced symptoms when infected with *Alternaria alternata* (Yoshikawa et al., 1993). Transgenic tobacco plants that expressed a tobacco class II β-1, 3-glucanase showed reduced symptoms when infected with *Phytophthora parasitica* var *nicotinae* or with *Peronospora tabacina* (Lusso et al., 1996). Transgenic rice plants that expressed a β-1, 3; 1,4-glucanase gene *Gns1* showed less severe symptoms comparing to the control plants when infected with virulent blast fungus *Magnaporthe grisea* (Nishizawa et al., 2003).

Other studies showed that plants transformed with β-1, 3-glucanase alone did not exhibit resistance to certain pathogens or showed less resistance compared to plants which were transformed with both the β-1, 3-glucanase and chitinase genes. Similarly, plants transformed with chitinase gene alone also did not show an adequate level of resistance, because plant chitinases alone usually affect only the hyphal tip and are unable to effectively degrade harder chitin structures of fungi. In addition, like β-1, 3-glucanase, chitinases inhibit only a limited number of fungal species. However, when the two enzymes are combined, a synergic effect can usually be observed. For example, tobacco
plants transformed with a barley class II basic chitinase along with a barley class II basic β-1, 3-glucanase gene showed enhanced levels of protection against *Rhizoctonia solani* as compared to plants transformed with a single gene (Jach et al., 1995). Tomato plants expressing tobacco class I β-1, 3-glucanase and chitinase transgenes showed reduced susceptibility to infection by *Fusarium oxysporum f.sp. lycopersici* (Jongedijk et al., 1995). Other examples of transgenic plants which expressed both types of genes and exhibited strong defense effects are tobacco plants against *Rhizoctonia solani* (Broglie et al., 1991), *Fusarium solani* (Melchers et al., 1993; Sela-Buurlage et al., 1993), and *Cercospora nicotianae* (Zhu et al., 1994), and Alfalfa plants against *Phytophthora megasperma f. sp medicaginis* (Masoud et al., 1996).

**1.4 Expression Analysis of β-1, 3-Glucanase Gene**

**1.4.1 Northern Blot Analysis**

Northern blot analysis and reverse-transcriptase (RT) PCR have been used to detect β-1, 3-glucanase gene transcripts. Northern blot has also been used to compare the levels of gene transcript. For example, Northern blot analysis of a tobacco β-1, 3-glucanase gene (NbPR2) in an infected plant showed increasing levels of the transcript throughout the infection by *C. destructivum* and a faster rate of increase appeared to occur 48 h post infection, while the level of a control gene (NbEF-1a) transcript showed no significant change (Dean, 2002). A Northern blot analysis of a wheat β-1, 3-glucanase gene transcript showed that the expression patterns of this β-1, 3-glucanase gene in a normal wheat line (Sumai 3) and a mutant line were different. The level of transcript reached the maximum point at or before 24 h after infection in a normal wheat line, but in the mutant line, the peak values were not reached until 48 h or later, indicating a slower
defense response to infection by *F. graminearum* in the mutant (Li et al., 2001). Northern blot analysis of a pepper β-1, 3-glucanase gene (CABGLU) transcript showed that the expression pattern of the infected plants depended on plant growth stage. At the 4-leaf stage, accumulation of the pepper β-1, 3-glucanase gene transcript was strongly induced at 48 h after infection with *Colletotrichum coccodes*. In contrast, strong induction of the β-1, 3-glucanase gene transcript occurred at 24 h after fungal infection at the 8-leaf stage (Hong and Hwang, 2002).

**1.4.2 Real-Time PCR**

Real-time PCR is a relatively new method to quantify expression levels of mRNAs. Compared to other PCR methods, that determine the amount of final products at the end-point, real-time PCR is more specific, sensitive and reproducible (Freeman et al., 1999; Raeymaekers, 2000). The real-time PCR system is based on the detection and quantitation of a fluorescent reporter which is part of a molecular beacon (Livak et al., 1995). Molecular beacons are short segments of single-stranded DNA with a fluorescent reporter dye and a quencher at opposite ends. When the molecular beacon is in the hairpin conformation, any fluorescence emitted by the reporter is absorbed by the quencher and no fluorescence is detected. However, during PCR, molecular beacons are denatured and annealed to the target DNA sequence. Therefore, when molecular beacons anneal to PCR products, they are no longer in the hairpin form and thus are able to fluoresce. Consequently, as PCR product accumulates, the fluorescence increases. The signal level of the fluorescent reporter depends on the quantity of the PCR products. The higher the starting copy number of the mRNA, the sooner a significant increase in signal level of fluorescence is observed. A fluorescence threshold is set at a signal level which is
significantly above the baseline. A threshold cycle ($C_T$) can then be obtained for each sample. $C_T$ is the cycle number at which the fluorescence emission exceeds the threshold. From the $C_T$ value, the relative amount of the target mRNA can be calculated.

Relative gene expression levels can be obtained by choosing an endogenous /internal control gene which is abundant and remains constant, in proportion to the total RNA, among the samples. By using endogenous control gene as a reference gene, the quantity of an mRNA target can be normalized. The most commonly used reference genes are housekeeping genes, including genes for 18S RNA, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and $\beta$-actin (Suzuki et al., 2000; Bustin, 2002). GAPDH is an enzyme involved in glycolysis (catalyzing the inter-conversion between glyceraldehyde-3-phosphate and 1, 3-bisphosphoglycerate). GAPDH is constitutively expressed in almost all tissues at high levels. A strawberry GAPDH gene, FaGAPDH2, was used successfully as the reference gene in a previously study by Dr. Ding S. Shih’s laboratory (Department of Biological Sciences, Louisiana State University, Baton Rouge, LA) in quantifying the expression of strawberry chitinase genes (Khan and Shih, 2004).

### 1.4.3 Quantification of PR Protein Gene Expression by Real-Time PCR

Real-time PCR has been used in quantifying the expression levels of PR protein genes in a number of plants. For example, the expression of three chitinase genes, that belong to chitinase classes I, II, and IV, were monitored with real-time PCR after infection by Heterobasidion annosum in Norway spruce (Picea abies). Transcript levels of classes II and IV chitinases increased after infection, but the transcript level of a class I chitinase declined after infection (Hietala et al., 2004). The expression of two chitinase genes, FaChi2-1 and FaChi2-2 in strawberry plants inoculated with Colletotrichum
fragariae or Colletotrichum acutatum was examined in Shih’ laboratory (Khan and Shih, 2004). The results showed a rapid induction of FaChi2-1 transcript (3 to 5 fold), which occurred within 2-6 h post-inoculation. Induction of FaChi2-2 transcript occurred slower, at 24-48 h post-infection (4 to 8 fold).

1.4.4 β-1, 3-Glucanase Isozymes

All plants species examined thus far show the presence of multiple β-1, 3-glucanase isozymes. Furthermore, the β-1, 3-glucanase isozyme patterns in infected plants are usually different from uninfected plants. Most studies showed that the quantity of isozymes (based on band intensity on native polyacrylamide gels) increased after infection with pathogens (Pan et al., 1991; Shimoni et al., 1992; Churngchow et al., 1995; Roulin and Buchala, 1995; Lawrence et al., 1996; Roulin et al., 1997; Burketova et al., 1999; Egea et al., 1999; Urquhart and Punja, 2002; Shinya et al., 2004). In some cases, new isozymes appear in infected plants. For example, a study of tobacco plants showed that normal tobacco leaves contained one major β-1, 3-glucanase isozyme, but an additional isozyme was detected in the tobacco mosaic virus-infected leaves. Furthermore, the intensities of both isozyme bands were considerably greater than those of the uninfected tobacco leaves (Pan et al., 1991). A study on groundnut showed that three isozymes of β-1, 3-glucanase existed in the plant and that the activities of these isozymes increased after infection by Cercospora arachidicola (Roulin and Buchala, 1995). In the case of barley (Hordeum vulgare L.), the predominant isoforms in the normal leaves were the isozyme GI and, to a lesser extent, the isozyme GIII. After infection with Rhynchosporium secalis, GI remained approximately constant, while the
level of GIII increased. However, the most noticeable response to infection was the
induction of a high level of the isozyme GII (Roulin et al., 1997).

Changes in isozyme patterns were also observed when plants were treated with
other stimuli. For example, a study of sugar beet plants showed that BTH induced the
synthesis of at least three acidic $\beta$-1, 3-glucanase isozymes and a basic isozyme
(Burketova et al., 1999).

1.5 The Strawberry Plant

Strawberry plants are members of the family Rosaceae, which is a large plant
family with approximately 100 genera and more than 3000 species (Baumgardt, 1982).
Other Rosaceae family members include roses, apples, peaches, pears, blueberries, and
raspberries. All varieties of the strawberry plant belong to the genus Fragaria in the
family Rosaceae. The strawberry plant is a perennial plant and grows both as a wild plant
and as a cultivated plant. There are about 12 native species of Fragaria in the north
temperate regions. These include Fragaria vesca (common strawberry or wild
strawberry), Fragaria virginiana (prairie strawberry, or scarlet strawberry), Fragaria
ananassa (commercial strawberry, cultivated strawberry, or garden strawberry). The
number of sets of chromosomes varies from species to species, ranging from 2 sets in the
wild strawberry Fragaria vesca to 8 sets in the cultivated strawberry Fragaria ananassa,
which is a hybrid between the domesticated Fragaria virginiana and Fragaria chiloensis
(Brown, 2002). Strawberry is a non-climacteric fruit which derives from the enlarged
flower receptacle (Perkins-Veazie, 1995).

Like other plants, strawberry plants are susceptible to attack by different
pathogens. For example, some species in the genus Colletotrichum, such as C. fragariae,
*C. acutatum*, and *C. gloeosporioides*, can cause anthracnose diseases in strawberry crowns, fruits, and roots. The genus *Colletotrichum* causes anthracnose by a series of events that begins with the adhesion of fungal spores (conidia) to host surface tissue, followed by spore germination to form appressoria on plant surfaces, from which penetration hyphae develop into the first subcuticular cell (Bailey et al., 1992). In suitable conditions, for example, relatively humid microclimate, the fungus may grow rapidly inside the plant and cause severe symptoms quickly. When the fungus is allowed to grow for enough time, dark fruiting-bodies can form, and typical anthracnose symptoms will appear. Spores or conidia are then produced and they are dispersed by water splash (Yang et al., 1992; Pielaat and van den Bosch, 1998). These spores could be in a dormant condition in soil or dead plant material for a long time until they infect new plants (Eastburn & Gubler, 1992 Feil et al., 2003).

1.6 Studies on PR Proteins and PR Protein Genes in Strawberry

A main research interest of Shih’s laboratory is to use chitinase and β-1, 3-glucanase genes to construct transgenic plants resistant to fungal pathogen, including transgenic strawberry plants. Another main research interest of his laboratory is to understand the defense system of the strawberry plant, particularly the involvement of PR proteins in the defense process. To this end, Shih’s laboratory has cloned and determined the nucleotide sequences of two strawberry class II chitinase genes, a class III chitinase gene, and an osmotin-like protein gene (Khan et al., 1999; Wu et al., 2001; Khan and Shih 2004). Also, his laboratory has studied the expression of the two class II chitinase genes in strawberry plants infected with either *C. fragariae* or *C. acutatum* (Khan and Shih, 2004). To gain further understanding of the roles of PR proteins in the plant defense
system, this research was directed toward work on β-1, 3-glucanases. The major goals of this dissertation research were to characterize the β-1, 3-glucanase activities of strawberry plants, clone the genes encoding these enzymes, characterize these genes, and evaluate expression of these genes in fungal infected and uninfected strawberry plants. During the course of isolating the cDNA of β-1, 3-glucanase genes, a cDNA was accidentally obtained that shares sequence homology with zinc transporter genes in other plants. The complete cDNA clone and a related genomic clone were sequenced and partially characterized (see appendix A).
CHAPTER 2

CLONING AND CHARACTERIZATION OF \( \beta-1, 3 \)-GLUCANASE GENES

2.1 Introduction

Plant \( \beta-1, 3 \)-glucanases are pathogenesis-related (PR) proteins, which are believed to play important roles in plant defense responses to pathogen infection. The synthesis of PR proteins is induced upon pathogen infection and upon treatment of plants with a variety of abiotic stress factors. \( \beta-1, 3 \)-glucanases catalyze the cleavage of \( \beta-1, 3 \)-glucosidic linkages in \( \beta-1, 3 \)-glucan (Simmons, 1994), which is a major structural component of the cell walls of many pathogenic fungi (Wessels and Sietsma, 1981).

Plant \( \beta-1, 3 \)-glucanases are divided into four classes on the basis of their amino acid sequence differences, structural properties and cellular localizations (Leubner-Metzger and Meins, 1999). The majority of these hydrolytic enzymes identified thus far is class I and class II enzymes. Class I \( \beta-1, 3 \)-glucanases are usually basic proteins localized in the cell vacuole. Class I enzymes contain a C-terminal sequence, commonly referred to as the C-terminal extension, which is believed to be the signal sequence responsible for vacuolar localization of these enzymes (Shinshi et al., 1988). Classes II, III and IV proteins are usually acidic and are secreted into extracellular spaces. Enzymes in classes II, III and IV do not have the C-terminal extension.

Class I enzymes from tobacco and tomato can inhibit the \textit{in vitro} growth of certain pathogenic fungi. This \textit{in vitro} inhibitory effect can be enhanced by using combinations of class I \( \beta-1, 3 \)-glucanases and class I chitinases. In contrast, class II \( \beta-1, 3 \)-glucanases...
3-glucanases from tobacco and tomato do not have the \textit{in vitro} fungal growth inhibitory activity (Sela-Buurlage et al., 1993; Joosten et al., 1995; Anfoka and Buchenauer, 1997). However, the role of the class II enzymes in plant defense may reside in their ability to generate pathogen-derived elicitor molecules, which stimulate the production of PR proteins and other defense-related molecules (Ryan and Farmer, 1991).

In some cases, transgenic plants constitutively expressing $\beta$-1, 3-glucanase, either alone or in combination with a chitinase, exhibit enhanced levels of fungal disease resistance or delayed symptom development as compared to the control plants (Zhu et al., 1994; Jach et al., 1995; Jongedijk et al., 1995).

In addition to their possible roles in plant defense, $\beta$-1, 3-glucanases may also have other physiological functions, including roles in the processes of pollen development, seed germination, and fruit ripening (Leubner-Metzger and Meins, 1999).

Strawberry is a member of the family \textit{Rosaceae}, which is a large plant family comprised of approximately 3000 members (Baumgardt, 1982). This plant family includes other important fruit crops such as apples, peaches, pears, raspberries and blackberries. Only few studies have been reported on PR proteins or PR protein genes of the \textit{Rosaceae} family members.

This chapter describes the isolation of two $\beta$-1, 3-glucanase genomic clones, designated as \textit{FaBG2-1} and \textit{FaBG2-2}, and a cDNA clone, designated as \textit{FaBG2-3}. The complete sequences of these clones were determined, and the translated amino acid sequences were compared with each other and with other known plant $\beta$-1, 3-glucanases.
2.2 Materials and Methods

2.2.1 Isolation of DNA and RNA

Total nucleic acids were extracted from strawberry tissues according to the method described by Manning (1991). For large-scale DNA or RNA preparations, 3g leaves (central vein removed) were ground in liquid nitrogen into a fine powder in a chilled mortar and pestle (approximately 50 strokes in 30-40 sec.). The ground leaves were transferred into a 30 ml centrifuge tube containing 10 ml of extraction buffer (0.2 M boric acid, 10 mM Na₂EDTA, pH7.6). Sodium dodecyl sulfate (SDS) and β-mercaptoethanol were added to a 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) (25:24:1) 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 30 ml centrifuge tubes. Each 4 ml extract was diluted three fold by addition of RNase-free 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 sugar. The sugar was 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, pH7.6, and once with absolute ethanol. The pellet was briefly dried and dissolved in 0.2 ml of sterile water.
For DNA preparation, RNA was removed from total nucleic acid samples by treating them with RNase A (final concentration 0.1 mg/ml) at 37 °C for 2 h. The DNA was extracted once with phenol: chloroform: IAA (25:24:1) and once with chloroform: IAA (24:1). The DNA was precipitated and dissolved in distilled-deionized water (ddH₂O) and analyzed on a 1% agarose gel.

For RNA preparation, total nucleic acids were extracted immediately after tissue samples were collected. RNase-free plasticware and reagents (Ambion, Austin, TX) were used in all procedures. The total nucleic acids (described above) were dissolved in water to give a concentration not less than 1mg/ml and adjusted to 3 M LiCl by adding 1/3 volumes of 12 M LiCl. After 1 h on ice, RNA was precipitated by centrifugation at 11,600 g, 10 min, and the pellet washed twice with 1 ml of 3 M LiCl. The RNA was dissolved in an RNA-Secure solution (Ambion), which had been pre-heated to 60 °C, and the dissolved nucleic acids were incubated at 60 °C for 10 min to inactivate any contaminating RNase. These RNA samples were frozen at −80 °C.

2.2.2 Cloning of Gene FaBG2-1

Based on the conserved sequences present in plant β-1, 3-glucanase genes (Egea et al., 1999), two degenerate primers (primer set 1, Table 2) were synthesized. Polymerase chain reaction (PCR) (program 1, Table 3) was performed in a 25 µl reaction mixture containing 20 ng of strawberry genomic DNA, 400 µM of each dNTP, 3 mM MgCl₂, 2 µM of each primer, 1 × PCR buffer, and 2.5 units Taq DNA polymerase (Promega, Madison, WI). From this amplification reaction, a 466-bp DNA fragment was obtained. This fragment was cloned into pGEM T-Easy plasmid (Promega, Madison,
Table 2. List of primers (and TaqMan probes) used in different experiments.

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<tr>
<th></th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Probe Primer</th>
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<tbody>
<tr>
<td>1</td>
<td>TAY ATA GCY GTT GGW MYG AA</td>
<td>CWG AMG GCC AWC CAC WYT C</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>AAG AGT ATA CAC ATA CAG ATC AGT TTG</td>
<td>CAT CAG TAA GCG CTT GCA GGT TGT TGT T</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>ATG TAC TGC TGC TAG ACC TTG TGG AAA AC</td>
<td>TAG AGC CTC TGA GAT CTC AAT TCT GTG</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>AAC CTC GAA GCC CTT ACT GAT G</td>
<td>CGG TCT TCA CAT GAT TGA TCA A</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>GTA ATA CGA CTC ACT ATA GGG C</td>
<td>TGG CAT CGC GTT AGA AAG AGA CAA AG</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>ACT ATA CCC CAC GCG TGG T</td>
<td>TAG CGG AAC ATA CAT ACT TAA CAA AC</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>GCT GAT GCC GAT GAA TGA ACA CTG</td>
<td>AAC TTC TGC ACC GTC CTT GA</td>
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<tr>
<td>8</td>
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<td>9</td>
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<td>AAA TTG TTG CCA TTT CGT CCA T</td>
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</tr>
<tr>
<td>12</td>
<td>TTG TTC TGT TTA TGC CTG CTC TAC A</td>
<td>AAA TTG TCA CCG TTT CGT CCA T</td>
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</tr>
</tbody>
</table>

Table 3. List of PCR conditions used in different experiments.

<table>
<thead>
<tr>
<th>Initial denaturation</th>
<th>Touch down cycles</th>
<th>Main program</th>
<th>Final extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycles</td>
<td>Denaturation</td>
<td>Anneal (-1°C/ per cycle)</td>
<td>Extension</td>
</tr>
<tr>
<td>1</td>
<td>94°C, 3 min</td>
<td>8</td>
<td>94°C, 1 min</td>
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<tr>
<td>2</td>
<td>94°C, 2 min</td>
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<td>3</td>
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<td>94°C, 1 min</td>
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<td>4</td>
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<td>8</td>
<td>94°C, 2 min</td>
<td>10</td>
<td>94°C, 1 min</td>
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</table>
Based on the sequence of the 466-bp fragment, additional genomic sequence information and a short cDNA (500-bp) fragment were obtained (Note: Above experiments in section 2.2.2 were done by a former graduate student, Anwar A. Khan in Shih’s laboratory).

To find complete the sequence of this gene, two gene specific primers (primer set 2, Table 2) were designed based on cDNA sequence. A PCR (program 2, Table 3) was performed in 25 µl PCR reaction mixtures containing 20 ng of strawberry genomic DNA, 400 µM of each dNTP, 400 nM of each primer, 3 mM MgCl2, 1 × PCR buffer, and 2.5 units Taq DNA polymerase (Promega, Madison, WI). A DNA fragment of 740-bp was obtained. The fragment was cloned into the pGEM T-easy vector and sequenced. This DNA fragment contains an intron and a part of the first exon sequences.

Based on this DNA sequence, new primers were designed and used in a genome walking procedure to find additional sequence information at both ends of the gene. This additional sequence information allowed the design of a final set of primers (primer set 3, Table 2) to obtain a clone encompassing the complete coding region of the gene. A PCR (program 3, Table 3) was performed in a 25 µl reaction mixture containing 20 ng of strawberry genomic DNA, 400 nM of each primer, 1 × PCR buffer A, and 1 unit DNA polymerase mix of the FailSafe PCR system (Epicenter Technologies, Madison, WI). The PCR product was cloned into pGEM T-Easy plasmid, and the resulting recombinant plasmid was designated as pFaBG2-1. Both strands of the insert of pFaBG2-1 were sequenced.
2.2.3 Southern Blot Analysis of *FaBG2-1*

Strawberry leaf genomic DNA (10 mg) was digested with *Eco* RI, *Eco* RV or *Hind* III. The digested DNA samples were subjected to electrophoresis on a 0.8% agarose gel, and the separated DNA fragments were transferred to a Zeta-probe-GT nylon membrane (BioRad, Hercules, CA) by downward capillary alkaline transfer. The DNA fragments were crosslinked to the membrane by UV-irradiation in the Stratalinker Crosslinker (Stratagene, La Jolla, CA) on auto setting delivering $1.2 \times 10^5 \mu J$ of energy.

The membrane was pre-hybridized with 0.1 ml/cm$^2$ of Ultrahyb Hybridization Solution (Ambion, Austin, TX) containing 20 µl of fragmented salmon sperm DNA (10 mg/ml) for 1 h at 43 °C. A [$^{32}$P]-labeled probe (see below, $1.5 \times 10^6$ CPM/ml of hybridization solution) was boiled with 80 µl of salmon sperm DNA for 10 min. An aliquot (0.8 ml) of Ultrahyb Hybridization Solution was heated to 68 °C, mixed with the heated probe solution, and added to the prehybridization solution containing the membrane blot. Hybridization was carried out at 43 °C over-night. The blot was washed once with low stringency wash buffer (2 × SSC, 0.1% SDS) for 10 min at room temperature and twice with high stringency buffer (0.1 × SSC, 0.1% SDS) for 30 min each at 43 °C. The blot was then exposed to a phosphoimager screen for signal-detection with the Storm Phosphoimager 860 (Molecular Dynamics, Piscataway, NJ).

A random priming method was used for preparing the probe. The template DNA for the random priming reaction was synthesized by using PCR (primer set 4, Table 2). The resulting 625-bp PCR product, that spans nucleotide positions 1069–1693 in the second exon of *FaBG2-1*, was used for labeling using the Random Primers DNA
Labeling System according to the manufacturer’s recommended conditions (Invitrogen Corporation, Carlsbad, CA).

2.2.4 Cloning of Gene *FaBG2-2*

During the course of isolating *FaBG2-1*, a DNA fragment was obtained that showed a high degree of homology to *FaBG2-1* in the coding region, but was different in the 5′ flanking region. To obtain the complete DNA sequence of this new strawberry β-1, 3-glucanase gene, a PCR-based genome walking experiment was performed using the Universal GenomeWalker kit (Clontech, Palo Alto, CA).

The primers used for PCR amplification are given in Table 2 and the PCR conditions are given in Table 3. All the primers used in this investigation were synthesized by the Gene and Probe Laboratory of the School of Veterinary Medicine, Louisiana State University. Primary PCR (program 4, Table 3) was performed using 25 µl reaction mixtures that each contained 1 µl of one of the 5 strawberry genomic DNA libraries (derived from 5 different restriction enzyme digestions: *Dra I*, *EcoRV*, *PvuII*, *ScaI*, and *StuI*), 400 µM of each dNTP, 3 mM MgCl₂, 1 × PCR buffer without Mg²⁺, 2 µM of each primer (primer set 5, Table 2), and 2.5 units *Taq* DNA polymerase (Promega, Madison, WI).

Secondary PCR (program 5, Table 3) was performed using 25 µl reaction mixtures that each contained 1 µl of diluted (1:49) primary PCR reaction, 400 µM of each dNTP, 3 mM MgCl₂, 1 × PCR buffer without Mg²⁺, 2 µM of each primer (primer set 6, Table 2), and 2.5 units *Taq* DNA polymerase. The PCR product obtained was purified using the Concert nucleic acid purification system (Invitrogen, Carlsbad, CA), cloned into the pGEM T-Easy vector (Promega) and sequenced. Based on the sequence of the
PCR product, another round of genome walking was performed. The additional sequence information allowed the design and synthesis of a set of primers (primer set 7, Table 2) to generate a full-length clone containing the entire gene. PCR (program 6, Table 3) was performed using a 25 µl reaction mixture that contained 20 ng of strawberry genomic DNA, 400 µM each of dNTP, 400 nM of each primer (primer set 7, Table 2), 1× PCR buffer with 2 mM MgSO₄, and 1 unit DNA polymerase mix of the FailSafe PCR system (Epicenter Technologies, Madison, WI). The amplification product, designated as FaBG2-2, was 2415 bp in length. This DNA fragment was cloned into the pGEM T-Easy vector and the resulting recombinant plasmid was designated as pFaBG2-2. Both strands of the insert of pFaBG2-2 were sequenced.

### 2.2.5 Cloning of cDNA FaBG2-3

To obtain the cDNA corresponding to the FaBG2-2 gene, a 5’RNA ligase mediated-rapid amplification of cDNA ends (RLM-RACE) procedure was performed using the FirstChoice RLM-RACE kit (Ambion) according to the manufacturer’s recommendations. Briefly, 10 µg of total leaf RNA was treated with calf-intestinal phosphatase to remove the 5’-phosphate of any degraded RNAs. After removal of the phosphatase by phenol: chloroform extraction, the RNA sample was treated with tobacco acid pyrophosphatase to remove the cap structure of intact mRNA molecules. Using RNA ligase, a synthetic RNA adapter was then ligated to each RNA chain bearing a 5’-phosphate. The RNA was reverse transcribed in a 20 µl reaction mixture containing 2 µl of ligated RNA as template, 500 µM of each dNTP, 5 µM of random decamers, 1× reverse transcription buffer, 1 unit of placental RNase inhibitor, and 10 units of molony murine leukemia virus (MMLV) reverse transcriptase. The reaction was carried out at
42 °C for 1h. The cDNA was amplified using PCR. In the primary amplification, program 7 (Table 3), primer set 7 (Table 2) was used, while in the secondary amplification, program 8 (Table 3), primer 8 (Table 2) was used. Each amplification was performed in a 25 µl PCR reaction mixture containing 1 µl reverse transcription reaction mixture for primary PCR or 1 µl of primary PCR reaction mixture for secondary PCR, 400 µM of each dNTP, 400 nM of each primer, 3 mM MgCl₂, 1 × PCR buffer, and 2.5 units Taq DNA polymerase. A 574-bp DNA fragment product was obtained and purified. The fragment was cloned into the pGEM T-easy vector and sequenced.

To obtain the complete cDNA sequence, a 3'RLM-RACE procedure was performed, using again the FirstChoice RLM-RACE kit. Leaf RNA was reverse transcribed in a 20 µl reaction mixture containing 1 µg of RNA as template, 500 µM of each dNTP, 2 µl of 3' RACE Adapter (5'-GCGAGCACAAGATTTACGACTCAGCTTAGGT VN-3'), 1 × reverse transcription buffer, 1 unit of placental RNase inhibitor, and 10 units of MMLV reverse transcriptase. The reaction was carried out at 42 °C for 1 h. PCR (program 8, Table 3) was performed in a 25 µl PCR reaction mixture containing 1 µl reverse transcription reaction mixture, 400 µM of each dNTP, 400 nM of each primer (primer set 9, Table 2), 3 mM MgCl₂, 1 × PCR buffer, and 1 unit DNA polymerase mix of the FailSafe PCR system. A product of 1262-bp DNA fragment was obtained. The fragment was purified and cloned into the pGEM T-easy vector and the insert was sequenced. By combining with cDNA sequence obtained from the 5' RLM-RACE experiment, a composite sequence of 1271 bp was obtained. The cDNA represented by this 1271-bp composite sequence was designated as FaBG2-3.
2.2.6 Sequence Analyses

Nucleotide sequences were determined with an Applied Biosystems model 373A Automated Sequencer (Applied Biosystems, Foster city, CA), using the Ready-reaction Dye-Terminator kit with AmpliTaq FS (Applied Biosystems). Sequence determination was performed by the Gene and Probe laboratory. Standard protein-protein BLAST (blastp) was used for finding homologous sequences in protein databases (Altschul et al., 1990). Potential N-glycosylation sites were identified by using NetNGlyc. Multiple sequence alignment was performed by using Clustal X (Higgins and Sharp, 1989; Thompson et al., 1997) and modified by using GeneDoc (Nicholas et al., 1997). Molecular mass and pI were calculated by using the Compute pI/Mw tool (Bjellqvist et al., 1993, 1994). Various transcription regulatory elements were identified by using the PLACE database of plant enhancer elements (Higo et al., 1999).

2.3 Results and Discussion

2.3.1 Structure and Coding Properties of FaBG2-1

A PCR-based genome walking procedure was used to isolate a 2167-bp DNA fragment containing a strawberry β-1, 3-glucanase gene, designated FaBG2-1. Figure 2 shows the nucleotide sequence (GenBank accession number AY170375) and a deduced amino acid sequence of the encoded protein. FaBG2-1 comprises two exons and an intron of 397 bp. The intron had the conserved G/gt and ag/G sequences at the splice junction sites (Shapiro and Senapathy, 1987). The intron sequence has 66.25% A + T content as compared to the coding sequence, which has 56.7% A + T content. Other straw-berry intron-containing genes have similar A + T ratio in the intron sequences, such as two class II chitinase genes (GenBank accession numbers AF147091 and AF320111). It has
Figure 2. Nucleotide sequence and deduced amino acid sequence of FaBG2-1. Intron sequence is shown in lower case letters. The transcription start site is indicated by a rightward arrow. The signal peptide cleavage site is indicated by an arrowhead. Numbers on the left and right represent nucleotide and amino acid positions, respectively.
been proposed that introns with high A + T ratio are more efficiently spliced (Ko et al., 1998).

The positions of the splice junction sites were confirmed by the sequence of a 468-bp cDNA (not shown, GenBank accession number AY170376) obtained by using an RLM-RACE procedure. Within this sequence, 323 bases overlapped with the genomic sequence downstream of the intron. Upstream of the intron, three in-frame ATG codons were present in the \textit{FaBG2-1} sequence. Choosing the first ATG as the start codon placed a 91-base coding region upstream of the intron. This start codon assignment is in agreement with the predicated positions of the start codons of several plant \(\beta\)-1, 3-glucanase genes. For example, the predicated start codons in the \(\beta\)-1, 3-glucanase genes from tobacco (AF141654), soybean (U41323) and rice (U72249), are 81, 93 and 78 bp, respectively, from the introns.

The remaining upstream 54-bases from the first ATG in the cDNA sequence represent the 5'-untranslated region of the gene. This length of a 54-bp 5'-untranslated region is in agreement with the length of 40–80 bp in most plant genes (Joshi, 1987). Furthermore, since the RLM-RACE procedure used in the experiment amplifies only the RNA chains that were capped, the 5' end of the isolated cDNA, a G residue at -54 position, should represent the transcription start site.

Four CAAT box-like signals were present in the upstream region of the gene. A TATA box sequence (TATATAA) was present at position 312. Two weak polyadenylation signals, AATACA and AATAAG, were present at positions 1905 and 1915, respectively. Various cis-acting regulatory elements, necessary for response to different stimuli, have been identified in plant PR genes. These include, for example, the ethylene
responsive element called GCC or AGC box (AGCCGCCC), the salicylic acid responsive
element (SARE) (TTCGACCTCC), and the W-box (TTGACC or TGAC-[N]x-GTCA)
(Zhou, 1999). A W-box sequence was identified at position 27 in reverse orientation in
the FaBG2-1 5′ upstream region along with a W-box like sequence (TGAC-GG-GTTCA)
at nucleotide position 228. The W-box has been implicated in elicitor-induced expression
of PR and other pathogen inducible genes such as those encoding phytoalexins. The W-
box is required and sufficient to drive the elicitor-inducible expression of PR genes in
parsley and maize (Raventos et al., 1995; Rushton et al., 1996). Proteins capable of
binding to W-box and transactivating the genes have been identified in parsley and
Arabidopsis (De Parter et al., 1996; Rushton et al., 1996).

*FaBG2-1* has a potential to code for a protein of 347 amino acids with a
molecular weight of 37.58 kDa and an isoelectric point of 4.80. The N-terminal 32 amino
acids may constitute a signal peptide (Von Heijne, 1983) that would be cleaved to
produce a mature protein of 34.02 kDa with a pI of 4.79. The gene product lacks the C-
terminal extension contained in class I β-1, 3-glucanases.

A GenBank blastp search revealed that FaBG2-1 has highest homology, 59%
identity and 76% homology, to a *Cicer arietinum* acidic β-1, 3-glucanase (CAA10167). It
has 56% identity and 72% similarity to a *Prunus persica* β-1, 3-glucanase (AAL30426),
and 52% identity and 70% similarity to a *Nicotiana tabacum* acidic β-1, 3-glucanase
(AAD33881). FaBG2-1 also shows relatively high homology to class I basic vacuolar β-
1, 3-glucanases from *Hevea brasiliensis* (AAA87456) with 58% identity and 72%
homology, and *N. tabacum* (AAA63540) with 53% identity and 68% homology. Figure 3
shows an alignment of FaBG2-1 with these class I and class II β-1,3-glucanases.
Figure 3. Alignment of the deduced amino acid sequences of FaBG2-1 with the sequences of class II β-1, 3-glucanases, *Cicer arietinum* (CAA10167), and *Prunus persica* (AAL30426), and class I β-1, 3-glucanases, *Hevea brasiliensis* (AA87456) and *Nicotiana tabacum* (AA63539, AA63540). Fully conserved residues are shaded in black while semi-conservative substitutions are shaded in gray. The signal peptide of FaBG2-1 and C-terminal extension of class I enzymes are indicated with a bar. Numbers on the right represent amino acid positions.
The *H. brasiliensis* class I basic β-1, 3-glucanase, and both basic and acidic tobacco β-1, 3-glucanase have the C-terminal extensions (approximately 20 amino acids in length). Based on its high sequence homology to other class II enzymes, its predicted acidic pI, and the lack of the C-terminal extension, FaGB2-1 was assigned as a class II β-1, 3-glucanase.

To determine the copy number of *FaBG2-1*, a Southern blot hybridization analysis was performed. As shown in Fig. 4, several major bands were visible in lanes 1, 2, and 3, suggesting that *FaBG2-1* is present in several copies in the strawberry genome. This is similar to the tobacco acidic β-1, 3-glucanases which are encoded by

![Southern blot analysis of FaBG2-1](image)

**Figure 4.** Southern blot analysis of *FaBG2-1*. Lane 1, Eco RI digest; lane 2, Eco RV digest; lane 3, Hind III digest; lane 4, positive control (100 pg of pFaBG2-1 fragment); lane 5, negative control (100 pg of a recombinant plasmid containing a strawberry class II chitinase gene).
approximately eight genes in the tobacco genome (Linthorst et al., 1990). In addition, peach β-1, 3-glucanases are encoded by a small gene family consisting of at least three members (Thimmapuram et al., 2001). This part of research work has been published in DNA sequence (Khan et al., 2003) (http://www.tandf.co.uk).

2.3.2 Structure and Coding Properties of FaGB2-2

During the course of cloning FaBG2-1, a DNA fragment was found that showed high homology with FaBG2-1 in the coding region, but was different in the upstream region. By using a PCR-based genome walking procedure, a 2415 bp DNA fragment, designated as FaGB2-2, was cloned (GenBank accession number AY989818). The nucleotide sequence and the deduced amino acid sequence of the translation product are shown in Fig.5.

FaBG2-2 shows a high sequence homology in the coding region to FaBG2-1. However, other than a short segment immediately preceding the coding regions, FaBG2-2 and FaBG2-1 show no significant homology in the 5’ and 3’ flanking regions. Furthermore, FaBG2-2 does not contain a DNA sequence corresponding to the first exon of FaBG2-1, which encodes 31 of the 32 amino acids of the putative signal peptide of the translation product of FaBG2-1. The coding region of FaBG2-1 contains two exons interrupted by a 397-bp intron. The presence of a signal peptide-coding exon corresponding to the first exon of FaBG2-1 was not detected in FaBG2-2 even when sequence analysis was extended up to 3000 bp upstream (data not shown). This observation strongly suggested that FaBG2-2 is an intron-less gene and that it has only one open reading frame that encodes the entire sequence of the enzyme.
Figure 5. Nucleotide sequence and deduced amino acid sequence of FaBG2-2. Numbers on the left and right represent nucleotide and amino acid positions, respectively. Transcriptional signals are underlined.
The translation product of \textit{FaBG2-2} is predicted to be a protein of 320 amino acids, which is six amino acids longer than the mature protein encoded by \textit{FaBG2-1}. \textit{FaBG2-2} is predicted to have a molecular mass of 34.28 kDa and a pI of 4.56. No potential N-linked glycosylation site was found. Furthermore, the protein does not contain a C-terminal extension sequence for vacuolar targeting.

Computer analysis of the 5′ upstream region of \textit{FaBG2-2} revealed no obvious TATA box sequence present in this gene. However, a number of other potential cis-acting regulatory elements were identified. These include the following: four CAAT box-like signals (positions 54, 125, 146, and 182) (Shirsat et al., 1989), an ASF-1 binding site motif (TGACG, position 867) (Despres et al., 2003), four W-box-like sequences (TTGAC, position 70 and positions 144, 691, and 765 in reverse orientation) (Yu et al., 2001), three RY repeat sequences (CATGCA, position 684 and positions 686 and 940 in reverse orientation) (Ezcurra et al., 1999), three GCC-box core element sequences (GCCGCC, position 26 and positions 32 and 431 in reverse orientation) (Brown et al., 2003), a gibberellin-responsive element (TAACAGA, position 1170 in reverse orientation) (Sutoh and Yamauchi, 2004), and a GAGA element (GAGAGAGAGAGAGAGAGA, position 521) (Sangwan and O'Brian, 2002). In the 3′ flanking region, two weak polyadenylation signal sequences, AATAAG and AATATA are present at 31 and 69 nucleotides, respectively, from the TAG termination codon.

2.3.3 Structure and Coding Properties of \textit{FaGB2-3}

For isolating the cDNA corresponding to \textit{FaBG2-2}, 3′ and 5′ RLM-RACE were performed. A 574-bp cDNA fragment was isolated using the 5′ RLM-RACE. Based on the sequence of this cDNA, a 1262-bp cDNA fragment was subsequently obtained by
using the 3’ RLM-RACE. The two cDNA fragments have a 565-bp overlap. The sequences of the overlap of the two fragments are completely identical, indicating that the two cDNA fragments indeed originated from identical mRNA molecules. A composite sequence was then constructed from the two cDNA sequences. This derived sequence is 1271 bp in length (GenBank accession number AY989819), which is 9-bp longer than the sequence of 3’ RLM-RACE cDNA fragment. As described below, the cDNA molecule (FaBG2-3) represented by this composite sequence is actually not a cDNA derived from FaBG2-2, but rather, a cDNA derived from a different β-1, 3-glucanase gene.

FaBG2-3 is composed of a 50-bp 5’-untranslated region, a 1038 bp coding region, and a 169-bp 3’-untranslated region preceding a stretch of (A) residues (Fig. 6). The 5’- and the 3’-untranslated regions of FaBG2-3 showed significant difference from the corresponding regions of either FaBG2-2 or FaBG2-1. The coding region of FaBG2-3, like that of FaBG2-1, encodes a putative signal peptide of 32 amino acids. The mature protein contains 314 amino acids with a predicted molecular mass of 33.68 kDa and a pI of 4.69. No potential N-linked glycosylation signal was found in the predicted sequence. Furthermore, like both FaBG2-1 and FaBG2-2, FaBG2-3 does not have a C-terminal extension sequence.

2.3.4 Sequence Comparison of FaBG2-1, FaBG2-2, FaBG2-3, and Other β-1,3-Glucanases

The predicted sequence of FaBG2-2 (AAX81589) contains 320 amino acids. The main differences between FaBG2-2 and mature proteins of FaBG2-1 and FaBG2-3 are that FaBG2-2 has a stretch of six amino acids at the N-terminus that does not appear in the mature proteins of FaBG2-1 and FaBG2-3 (Fig. 7). The sequence of FaBG2-2 shows 98 % identity and 99 % similarity to the sequence of mature protein of FaBG2-3, and
Figure 6. Nucleotide sequence and deduced amino acid sequence of FabG2-3. Numbers on the left and right represent nucleotide and amino acid positions, respectively. The predicted splice site is indicated by an arrowhead. The amino acid residues of predicted signal peptide are in bold. The nucleotide sequences of primers for real-time PCR are underlined. The nucleotide sequence of the probe for real-time PCR is underlined and in bold (see Section 3.2.4).
92 % identity and 95 % similarity to the sequence of mature protein of FaBG2-1. The sequence of FaBG2-3 (GenBank accession number AAX81590) is very similar to FaBG2-1 (AAO16642). Both proteins are predicted to contain a signal peptide of 32 amino acids. The predicted mature protein of FaBG2-3 is 314 amino acids long whereas that of FaBG2-1 is one amino acid longer (Fig. 7). The sequences of the two proteins share 93 % identity and 95 % similarity. When comparing the sequence of FaBG2-3 to the class II β-1, 3-glucanases from other plant species, the protein also shows high levels of homology with the enzymes from *Glycine max* (AAR26001) (64 % identity and 80 % similarity), *Cicer arietinum* (CAA10167) (60 % identity and 77 % similarity), and *Vitis riparia* (AAR06588) (63 % identity and 79 % similarity) (Fig. 7).

Based on the sequence homology of FaBG2-2 and FaBG2-3 to other class II β-1, 3-glucanases, their acidic pI values, and their lack of a C-terminal extension sequence, it can be concluded that, like FaBG2-1, FaBG2-2 and FaBG2-3 are also class II β-1, 3-glucanases.

The crystal structure of a barley β-1, 3-glucanase has been determined (Varghese et al., 1994). In comparison with the barley β-1, 3-glucanase, the two essential glutamate residues at the active site are present in FaBG2-1 (270E and 328E), FaBG2-2 (243E and301E), and FaBG2-3 (269E and 327E). Furthermore, two other amino acid residues important for catalysis are also present in FaBG2-1 (319E and 322K), FaBG2-2 (292E and 295K), and FaBG2-3 (318E and 321K) (Fig.7). Therefore, FaBG2-1, FaBG2-2, and FaBG2-3 are expected to be active enzymes.
Figure 7. Comparison of the deduced amino acid sequences of FaBG2-1, FaBG2-2 and FaBG2-3 with the sequences of closely related proteins: Glycine max (AAR26001), Cicer arietinum (CAA10167), and Vitis riparia (AAR06588) β-1, 3-glucanases. Fully conserved residues are shaded in black while semi-conservative substitutions are shaded in gray. The signal peptide of FaBG2-1 is indicated with a bar. The conserved active site and essential catalytic residues (glutamate or lysine residues) are indicated by asterisks (*). Numbers on the right represent amino acid positions.
2.3.5 Discussion

A PCR-based genome walking procedure was used to isolate two strawberry β-1, 3-glucanase genes, FaBG2-1 and FaBG2-2. Also, 3′ and 5′ RLM-RACE PCR methods were used to isolate a β-1, 3-glucanase cDNA, FaBG2-3. The sequences of FaBG2-2 and FaBG2-3 are homologous to each other in the coding regions, but are different in the non-coding regions. FaBG2-2 is predicted to encode a protein of 320 amino acids without a signal peptide at the N-terminus. On the other hand, FaBG2-3 is predicted to encode a protein of 346 amino acids with a signal peptide. The predicated signal peptides of FaBG2-3 and FaBG2-1 are both 32 amino acids long and the sequences of the two peptides differ in only one amino acid. Also, the sequences of the first 11 amino acids following the predicated cleavage sites of these two proteins are identical (Fig. 7). Therefore, it is reasonable to assume that the gene encoding the FaBG2-3 mRNA, like FaBG2-1 and many other plant β-1, 3-glucanase genes, contains two exons with its first exon encoding at least a part of the signal peptide.

In the case of FaBG2-2, the presence of a signal peptide encoding-exon was not detected in a genomic segment of approximately 3000 nucleotides upstream of the coding region of the gene. This finding strongly suggested that FaBG2-2 is an intronless gene and that it does not encode a signal peptide. It has been shown that the gene encoding the barley β-1, 3-glucanase isozyme GI has no introns and this gene does not encode a signal peptide. This GI isozyme has been shown to localize intracellularly in barley leaves. Furthermore, another barley β-1, 3-glucanase isozyme, isozyme GV, as well as a rice β-1, 3-glucanase OsGLN1, have been shown not to have a signal peptide. This suggests that FaBG2-2, like the barley isozyme GI (and also possibly GV), has an intracellular
location. The role of this type of β-1, 3-glucanases in plant defense or in other cellular processes needs to be further investigated.
CHAPTER 3
EXPRESSION ANALYSIS

3.1 Introduction

β-1, 3-glucanases catalyze the hydrolysis of β-1, 3-glucan, which is a polymer of β-1, 3-linked glucose residues. β-1, 3-glucan is a major structural component of the cell walls of many pathogenic fungi, and also a cell-wall component of certain cell types during specific developmental stages of plants (Wessels et al., 1981; Kauss, 1987). β-1, 3-glucanases are members of a large family of proteins collectively referred to as the pathogenesis-related (PR) proteins. These hydrolytic enzymes are implicated in plant defense mechanisms against pathogen infection. Synthesis of β-1, 3-glucanases in plants is induced upon pathogen attack (Kombrink et al., 1986, 1988; Mauch et al., 1984; Chang et al., 1992; Kaku et al., 1997; Muench-Garthoff et al., 1997).

In addition to their proposed role in plant defense, these enzymes may also play a role in cell division, seed germination, bud dormancy, flower formation, and fruit ripening processes (Roggen and Stanley, 1969; Fulcher et al., 1976; Hinton and Pressey, 1980; Neale et al., 1990; Meikle et al., 1991; Krabel et al., 1993; Bucciaglia and Smith, 1994; Morohashi and Matsushima, 2000; Buchner et al., 2002; Akiyama et al., 2004).

A variety of β-1, 3-glucanase genes have been identified in a wide range of plant species. The proteins encoded by these genes show differences in size, isoelectric point (pI), primary structure, cellular localization, and pattern of regulation. Based on these differences, β-1, 3-glucanases are divided into four classes (classes I–IV) (Payne et al., 1990; Ward et al., 1991; Leubner-Metzger and Meins, 1999). Several class I β-1, 3-
glucanases have been shown to inhibit the growth of pathogenic fungi \textit{in vitro}. These class I \( \beta \)-1, 3-glucanases may also directly inhibit fungal growth \textit{in vivo}. Other \( \beta \)-1, 3-glucanases can also play roles in plant defense by generating pathogen-derived elicitors. These enzymes digest fungal cell walls, leading to the release of oligosaccharide elicitors, which, in turn, promote the production of various PR proteins and other defense-related molecules.

Many studies have shown that the synthesis of \( \beta \)-1, 3-glucanases is stimulated by pathogen infections. For example, one tobacco \( \beta \)-1, 3-glucanase gene transcript was induced up to 21-fold by the bacterium \textit{Pseudomonas syringae pv syringae} (Castresana et al., 1990; Alonso et al., 1995). The induction of the \( \beta \)-1, 3-glucanase gene transcript is usually observed within 24 h to 48 h, along with higher enzyme activity and/or different isozyme patterns. New isozymes were observed in some plant infection studies and some isozymes were expressed at higher levels than other isozymes. For example, two barley \( \beta \)-1, 3-glucanase isozymes, GI and GIII, were observed in healthy barley leaves, while three isozymes, GI, GII, and GIII, were observed in infected leaves at different expression levels (Roulin et al., 1997). Moreover, the \( \beta \)-1, 3-glucanase activity can change during plant development. This change also corresponds to the age-related pathogen resistance in plants (Reuveni, 1998).

Real-time PCR is one of the best methods to quantify gene expression differences. However, relatively few studies have been reported on quantifying the expression level of PR protein genes in infected plants using this PCR method. Shih’s laboratory has previously studied the expression of the two classes II chitinase genes, \textit{FaChi2-1} and \textit{FaChi2-2}, in strawberry plants infected with \textit{C. fragariae} or \textit{C. acutatum}, which are two
important fungal pathogens that cause the severe strawberry disease anthracnose crown rot (Smith and Black, 1986). The results showed a rapid induction (2-6 h) of FaChi2-1 transcript and a slower induction (24-48 h) of FaChi2-2 transcript (Khan and Shih, 2004).

This chapter reports the expression patterns of two strawberry β-1, 3-glucanase genes, FaBG2-1 and FaBG2-3, after inoculation with C. fragariae or C. acutatum by using real-time PCR. The total β-1, 3-glucanase activity and the isozyme pattern were also determined in both inoculated and control plants. In addition, the expression levels of FaBG2-1 and FaBG2-3 were examined in different strawberry organs and in strawberry leaves at different ages.

3.2 Materials and Methods

3.2.1 Plant Growth Conditions

Dormant strawberry plantlets were purchased from Nourse Farms (Deerfield, MA). The strawberry variety is Fragaria ananassa var. Chandler. The plantlets were planted in 9 cm square containers (Kord, Ontario, Canada) that contained a soil mix [bark, peat moss, and perlite (7:2:1, v/v/v)] with dolomitic lime (4.7 kg/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 (model AR-60L; Percival Scientific, Boone, IO) at 26/18 °C (day/night) and an 11 h photoperiod. General Electric (T32T8SP41) lamps were used for illumination delivering irradiance of 8 W/m². The relative humidity was kept at 60–70%. The plants were watered with distilled water approximately every other day, and they were used in experiments 10 days after planting.
3.2.2 Growth and Preparation of Fungal Inoculum and Plant Infection

*C. fragariae* isolate CF-75 and *C. acutatum* isolate Goff were used in this study. Fungal cultures were obtained from Dr. Barbara Smith (USDA Small Fruit Research Station, Poplarville, MS) and maintained on half strength fungal growth media (Smith et al., 1990).

To prepare the conidial inoculum, actively growing cultures in Petri dishes were covered with approximately 15 ml of 0.01% Tween 20 per dish. Conidia were dislodged into the liquid by an autoclave sterilized round end glass rod and filtered through two layers of cheesecloth. The spore concentration was determined by using a hemocytometer and adjusted to $1.5 \times 10^6$ conidia/ml.

Plants were sprayed with the conidial suspension until imminent liquid runoff from the leaves. Control plants were sprayed with 0.01% Tween 20. After spraying, plants were immediately transferred to a dew chamber and maintained at 28 °C. Three plants of each treatment were harvested at 2, 6, 12, 24, and 48 h post-infection. RNA samples were extracted immediately from the infected or control plant leaves and stored at −80 °C. The infection experiment was repeated once.

3.2.3 Isolation of RNA for Real-Time PCR

RNase-free plasticware and reagents (Ambion) were used in all procedures. Total nucleic acids were extracted immediately after tissue samples were collected. 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 Na₂EDTA, pH 7.6, 0.5% SDS, and 280 mM β-mercaptoethanol (SDS and β-mercaptoethanol were added to the extraction buffer before use)]. To this mixture, 0.7 ml
of phenol: chloroform: isoamylalcohol (IAA) (25:24:1) was added and stirred vigorously for 5 min. This mixture was centrifuged for 5 min at 15,000 g. 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 of 72 µl of 1 M sodium acetate, pH 4.5. To this mixture, 0.4 volumes (359 µl) 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 sugar. The sugar was removed as a gelled pellet 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, mixed, and kept on ice for 30 min to precipitate total nucleic acids. After centrifugation at 15,000 g for 10 min, the pellet was washed once with absolute ethanol. The pellet was briefly dried and dissolved in 20 µl of RNA-Secure solution (Ambion), which had been pre-heated to 60 °C, and the dissolved nucleic acids were incubated at 60 °C for 10 min to inactivate any contaminating RNase. These treated total nucleic acid samples were either frozen at −80 °C and processed later or processed immediately as follows.

The samples were first treated with the RNeasy Mini Kit (QIAGEN, Valencia, CA) to remove the bulk of the DNA present in the samples, followed by DNase I treatment, using the DNA-free™ kit (Ambion) to completely remove any residual contaminating DNA.

To make certain the complete absence of DNA in the RNA samples, each sample was subjected to real-time PCR without including reverse transcriptase in the reaction mixture. The probe and primers (primer and probe set 10, Table 2) that target the strawberry housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (FaGAPDH2)
was used in the reaction (see section 3.2.4 below for the experimental conditions for real-time PCR). If the result showed the presence of DNA, the sample was further treated with DNase I. Real-time PCR was performed again with the sample to verify the complete removal of DNA.

### 3.2.4 Analysis of the Expression Patterns of *FaBG2-1* and *FaBG2-3* in the Leaves of Infected Plants

The expression levels of *FaBG2-1* and *FaBG2-3* at various time points post-infection were determined by using real-time PCR. The housekeeping gene *FaGAPDH2* was used as the reference gene. The GenBank accession numbers for the genomic and cDNA sequences of *FaGAPDH2* are AF421493 and AF421145, respectively. Real-time PCR experiments were carried out using a Perkin-Elmer 7700 thermal cycler and TaqMan MGB probes. For *FaBG2-1* and *FaBG2-3*, the probes were designed to span the exon-exon junction of each gene (Fig. 6). Use of such probes, which would anneal only to the cDNAs, should eliminate false-positive results originated from amplification of contaminating genomic DNA in the cDNA preparation. Since all RNA samples had been checked for absence of DNA contamination (Section 3.2.3), use of these probes provided an additional level of safeguard for false-positive results.

The primers and 6-FAM-labeled probes for *FaGAPDH2, FaBG2-1, FaBG2-3* are shown as primer and probe sets 10–12, respectively, in Table 2. The probes were obtained from Applied Biosystems. Using the one-step RT-PCR master mix reagents (Applied Biosystems), 50 µl reaction mixtures were set up in a 96-well plate. The universal thermal cycler conditions (TaqMan One-Step RT-PCR Master Mix reagents Kit Protocol, Applied Biosystems) were adapted, as the follows: first stage at 48 °C for 30 min for reverse transcription, second stage at 95 °C for 10 min, and third stage (40
cycles) at 95 °C for 15 s followed by 60 °C for 1 min. The optimal probe and primer concentrations were determined by following the manufacturer’s recommendations.

Real-time PCR assays were performed in 50 µl reaction mixtures that each contained 25 µl TaqMan 2 × Universal PCR Master Mix, 1.25 µl 40 × Mutiscribe™ and RNase inhibitor Mix, 250 nM of TaqMan MGB probe (probe set 10, 11, or 12, Table 2), 500 nM of primer set (primer set 10, 11, or 12, Table 2), and 200 ng of RNA. For the control (without reverse transcription), the 40 × Mutiscribe™ and RNase inhibitor Mix was omitted. For each RNA sample, the three genes (FaGAPDH2, FaBG2-1, and FaBG2-3) were always analyzed simultaneously. Each RNA sample was run in triplicate.

Relative quantification of the expression of FaBG2-1 and FaBG2-3 was obtained by calibrating the mRNA level of each gene with that of the reference gene, FaGAPDH2. The comparative C_T method (ΔΔC_T) was used in this quantification analysis (ABI Prism 7700 Sequence detection System User Bulletin #2). The mRNA level of each gene in the infected plants at each time point was normalized to the mRNA level of the same gene in the 2 h control plants, which was arbitrarily set as one. To compare expression levels of FaBG2-1 and FaBG2-3, the mRNA level of each gene in the plants at 2h and 48h was also normalized to the mRNA level of the FaGAPDH2 in the same plant, which was arbitrarily set as $1 \times 10^6$ units.

### 3.2.5 Time-Course Analysis of the Expression of FaBG2-1 and FaBG2-3 in Different Organs

Strawberry plantlets were planted and grown under the same conditions as described in Section 3.2.1 for the infection experiment. Leaves, roots, and crowns from three plants were harvested at 1, 3, 5, and 7 months after planting. In addition, at the 5 month time point, fruits were collected from three sample plants. When collecting the
leaf sample from each sample plant, only the three newest, fully-expanded leaves were harvested. This sample collecting practice was adapted to avoid any possible positional variation effect. After tissue samples were collected, RNA was immediately isolated from the samples. Each RNA sample was run in triplicate in the real-time PCR assays. The expression levels of \textit{FaBG2-1} and \textit{FaBG2-3} were determined by using the same primers, probes and real-time PCR conditions as described in Section 3.2.4 above. The mRNA level of each gene in the plants at each time point was normalized to the mRNA level of the \textit{FaGAPDH2} in the same plant, which was arbitrarily set as $1 \times 10^6$ units.

3.2.6 Protein Preparation

The same strawberry plants used in the second infection experiment for preparing total nucleic acid samples for real-time PCR analysis were used to isolate proteins. Leaves, fruits, crowns or roots (approximately 5 g) were ground to a fine powder in liquid nitrogen with a mortar and pestle. Each sample was added to the extraction buffer (25 mM Tris-HCl, pH 8.5, 5% polyvinyl polypyrrolidone, 14 mM $\beta$-mercaptoethanol). The tissue to buffer ratio was 1:4. The mixture was ground in the mortar and pestle for approximately 1 min. The homogenate was centrifuged at 15,000 g for 30 min at 4 °C and the supernatant was passed through a layer of miracloth (BD Biosciences Clontech, Palo Alto, CA). To the filtrate, ammonium sulfate was added to 60% saturation. The mixture was stirred at 4 °C overnight and centrifuged at 15,000 g for 30 min at 4 °C. The precipitate was dissolved in 800 µl Tri-EDTA buffer (25 mM Tris-HCl, pH 8.5, 1 mM EDTA, 15 mM $\beta$-mercaptoethanol).

The protein solution was dialyzed at 4 °C against 4 L of H$_2$O for 1 h with two changes. This was followed by dialyzing the protein solution against 4 L 0.01M sodium
acetate buffer, pH5.0, overnight at 4 °C with two changes. The dialyzed protein samples were stored at -80 °C.

**3.2.7 Determination of the Total β-1, 3-Glucanase Activity**

The total β-1, 3-glucanase activity in the protein sample was measured by colorimetric determination of the reducing groups formed in the enzyme reaction (Boller, 1992). Laminarin (Sigma, St. Louis, MO) which is extracted from *Laminaria digitata* was used as a substrate for the β-1, 3-glucanase.

**3.2.7.1 Preparation of Reduced Laminarin**

The terminal reducing groups of the laminarin chains were reduced with NaBH₄ to eliminate background due to the relatively short chain lengths of laminarin. Three g laminarin was dissolved in 150 ml water and the solution was stirred at 80 °C for 20 min. One g NaBH₄ was added to the laminarin solution and stirring continued at 80 °C for 1 h. Then, the solution was cooled to 40 °C and adjusted to pH5.5 with glacial acetic acid. A mixed-bed ion exchange resin (Amberlite MB3) was added to the solution and stirred for 30 min to remove salts. The suspension was passed through a filter and the ion exchange beads were washed by resuspension in 100 ml water and the suspension filtered. The filtrates were combined and adjust to a 300 ml to obtain a 1% solution of reduced laminarin. The reduced laminarin was stored at -20 °C.

**3.2.7.2 Colorimetric Assay**

The enzyme assay mixture (E) (250 µl) contains 7.5 µl of 1 M sodium acetate, pH 5.5, 2 µg total proteins, 50 µl of 1% reduced laminarin. Several control mixtures were prepared and assayed at the same time. Substrate blank (SB) contained no protein. Reagent blank (RB) contained no protein and laminarin. Reagent blanks with internal
standard (RI) contained no protein or laminarin, but contained 50 μl of 3 mM glucose. Enzyme blank (EB) contained no laminarin. Enzyme blanks with internal standard (EI) contained no laminarin, but contained 50 μl of 3 mM glucose.

The enzyme assay mixture and control mixtures were incubated in a water bath at 37 °C for 1 h. Then, 37.5 μl of the mixture was removed to a new tube. In the new tube, 300 μl of the basic copper reagent (40 g Na₂CO₃, 16 g glycine, 0.45 g CuSO₄·5H₂O in 1L water) and 300 μl of a fresh neocuproin solution (0.12 g neocuproin-HCl in 100 ml water) were added. The mixture was incubated in a boiling water bath for 12 min and cooled to room temperature. To the assay mixture, 450 μl of water was added and the absorbance was measured at 450 nm with a Beckman DU-64 spectrophotometer.

The amount of reducing sugar formed in the assay was calculated as follows:

\[
\frac{[A_{450}(E) - A_{450}(EB)]}{[A_{450}(EI) - A_{450}(EB)]} - \frac{[A_{450}(SB) - A_{450}(RB)]}{[A_{450}(SB) - A_{450}(RB)]} \times 150 \text{ nmol}
\]

3.2.8 Analysis of Isozyme Pattern

β-1, 3-glucanase isozymes were separated by using a polyacrylamide gel electrophoresis (PAGE) method described by Pan et al. (1989, 1991) with slight modifications. Gel electrophoresis was carried out using a Hoeffer SE250 Mighty Small minigel system (Amersham Pharmacia Biotech, San Francisco, CA). Separating (15%) 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 sample was dissolved in the 3 × sample buffer that did not contain SDS or a reducing agent. Electrophoresis was performed at 4 °C at a constant voltage of 150 V. Once the protein samples compacted in the gel, voltage was increased to 200V.
When the tracking dye reached the bottom of the gel, the gel was removed and briefly rinsed with water, incubated with 0.05 M sodium acetate, pH 5.0, for 5 min, and then incubated at 40 °C for 1 h in a mixture containing 75 ml of 0.05 M sodium acetate, pH 5.0, and 1 g of laminarin dissolved in 75 ml of water by heating in a boiling water bath. The gel was then incubated in a mixture of methanol, water, and acetic acid (5:5:2) for 5 min, washed with water, and the gel was stained with 0.3 g of 2,3,5-triphenyltetrazolium chloride in 200 ml of 1.0 M NaOH in a boiling water bath until red bands appeared (about 10 min). The gel was then placed in 7.5% acetic acid solution for viewing.

3.3 Results and Discussion

3.3.1 Quantification of *FaBG2-1* and *FaBG2-3* Expression in Infected Plants

A relative quantification of the expression of *FaBG2-1* and *FaBG2-3* was achieved by calibrating the expression of each gene with the reference gene, *FaGAPDH2*. Fig. 8A shows the expression of *FaBG2-1* in plants infected with *C. fragariae* or *C. acutatum* at various time points post-infection as compared to the control plants at 2 h. The expression level of this gene in response to infection by *C. fragariae* remained at low levels until 24 h post-infection. At 24 h and 48 h, *C. fragariae* induced the expression of *FaBG2-1* by 80.4-, 116.9-fold respectively. *C. acutatum* infection also caused an induction of the expression of *FaBG2-1*, but at a considerably lower level than that caused by *C. fragariae*. *C. acutatum* induced the expression of *FaBG2-1* by 9.3-fold at 48 h (Fig. 8A). No significant induction by this fungus was observed before 48 h.
Figure 8. Relative quantities of the FaBG2-1 mRNA at various time points after infection with *Colletotrichum fragariae* or *Colletotrichum acutatum*. The average of the levels of the mRNA in the 2 h control plants was set as one. Values represented are the mean of three plants and the bars indicate the standard error of the mean. Each plant was assayed in triplicate with each gene. Results from two separate experiments are shown in panels A and B.
Figure 9A shows the expression patterns of FaBB2-3. The patterns are similar to those of FaBG2-1. In C. fragariae infected plants, the expression of this gene increased by 4.4-, 9.5-, and 57.1-fold at 6 h, 24 h and 48 h time points, respectively. Also, similar to the effect observed with FaBG2-1, C. acutatum caused a considerably lower degree of induction of the expression of FaBG2-3 than that caused by C. fragariae. An increase of 5.4-fold in the expression of FaBG2-3 in response to C. acutatum was observed at 48 h. The infection experiment was repeated. The results from the second infection experiment (Fig. 8B and Fig. 9B) were generally similar to those from the first experiment.

The results in Figure 8 and 9 show the extent of stimulation of the expression of FaBG2-1 and FaBG2-3 caused by Colletotrichum infection. The data were obtained by comparing the levels of expression of each gene in the infected plants to the expression level of the same gene in the uninfected 2 h control plants. These data, however, do not show a comparison of the expression levels of the two genes relative to each other in the uninfected and the infected plants. To obtain this information, the mRNA level of each β-1, 3-glucanase gene was normalized to the mRNA level of FaGAPDH2, which was arbitrarily set as $1 \times 10^6$ units. The results for the control plants and the 48 h infected plants are shown in Fig. 10. It can be seen from the figure that the expression level of FaBG2-1 in the control plants was very low, or 0.5 and 0.6 unit in the first and second experiments, respectively. On the other hand, the expression level of FaBG2-3 in the control plants was one to two thousand-fold higher, or 618.0 and 1281.4 units for the two experiments). In the C. fragariae and C. acutatum infected plants, the levels of FaBG2-3 expression were several hundred to approximately a thousand-fold higher than the
Figure 9. Relative quantities of the FaBG2-3 mRNA at various time points after infection with *Colletotrichum fragariae* or *Colletotrichum acutatum*. The average of the levels of the mRNA in the 2 h control plants was set as one. Values represented are the mean of three plants and the bars indicate the standard error of the mean. Each plant was assayed in triplicate with each gene. Results from two separate experiments are shown in panels A and B.
Figure 10. Comparison of the expression levels of $FaBG2-1$ and $FaBG2-3$ in 2 h control plants and 48 h infected plants. The relative expression levels of $FaBG2-1$ and $FaBG2-3$ are calculated by using gene $FaGAPDH2$ as reference. The levels of $FaGAPDH2$ transcript in the same plants were set as $10^6$. Values represented are the mean of three plants and the bars indicate the standard error of the mean. Each plant was assayed in triplicate for each gene. Results from two separate experiments are shown in panels A and B.
expression levels of \( FaBG2-1 \) in the two infection experiments. Also, as already shown by the results in Figure 8 and 9, the expression levels of the two genes in \( C. fragariae \) infected plants were considerably higher (approximately five to twenty fold) than the expression levels of these genes in \( C. acutatum \) infected plants. With regard to the low expression level of \( FaBG2-1 \), it should be pointed out that the expression level of this gene in the \( C. fragariae \) infected plants (56.1 and 82.9 units) was considerably lower than the constitutive expression level of \( FaBG2-3 \) in the uninfected control plants (618.0 and 1281.4 units).

### 3.3.2 The Expression Patterns of \( FaBG2-1 \) and \( FaBG2-3 \) as a Function of Time in Different Organs

The expression levels of the two \( \beta-1, 3 \)-glucanase genes in different strawberry organs were compared, and the effect of plant age on the expression of the two genes was examined. Figure 11A shows the expression levels of \( FaBG2-1 \) and \( FaBG2-3 \) mRNAs in the leaf, root, crown, and fruit at 5 months after planting. Constitutive expression of both genes was observed in these organs, although the expression of \( FaBG2-1 \) was very low in the root. The expression levels of both genes were highest in the leaf, second highest in the fruit, and about the same in crowns and the roots. In all the organs, the expression of \( FaBG2-3 \) was higher than that of \( FaBG2-1 \).

Figure 11B shows the relative expression levels of \( FaBG2-1 \) in the leaf, crown, and root at 1, 3, 5, and 7 months after planting. The results show that the expression of \( FaBG2-1 \) in the leaf increased greatly during the seven-month test period, changing from 6.7 units at the beginning to 5031.4 units at the end. In contrast, expression of \( FaBG2-1 \) in the root was not detectable at 7 month and was at very low levels (4.5, 3.2, and 87.1
Figure 11. Expression patterns of *FaBG2-1* and *FaBG2-3* as function of time in different organs. (A) Comparison of the expression of *FaBG2-1* and *FaBG2-3* in different organs at five month after planting. (B) Expression of *FaBG2-1* in different organs during a 7-month growth period. (C) Expression of *FaBG2-2* in different organs during a 7-month growth period. The level of *FaGAPDH2* mRNA was set as $1 \times 10^6$ units. Values represented are the mean of three plants and the bars indicate the standard error of the mean. Each plant was assayed in triplicate for each gene.
units) at 1, 3, 5 months. Low levels of expression of the gene in the crown were detected at all four time points (217.3, 55.6, 239.0 and 22.4 units).

The expression patterns of *FaBG2-3* in the different organs are shown in Fig. 11C. In the leaf, the mRNA level was 1447.8 units at 1 month, increased to 14904.9 units at 3 months, further increased to 16787.9 units at 5 month and then decreased to 5899.9 units at 7 months. Comparatively, the mRNA level in the crown was quite high, 22070.3 units, at 1 month. However, it decreased drastically to 1276.6 units at 3 months, and then further decreased to 926.5 units at 7 months. The expression of *FaBG2-3* in the root was fairly constant during the first 5 months (1232.9, 1613.5, and 1931.3 units), but decreased to an undetectable level after 7 months. It can be concluded from the results of Fig. 11B and 10C that the expression patterns of the two β-1, 3-glucanase genes were different in leaves, crowns, and roots.
3.3.3 The Total $\beta$-1, 3-Glucanase Activities and Isozyme Patterns of Infected Plants

To examine $\beta$-1, 3-glucanase gene expression at the protein level, the total $\beta$-1, 3-glucanase activity and the isozyme pattern of the enzyme were examined. Figure 12 shows the total $\beta$-1, 3-glucanase activities in leaf samples from *C. acutatum* and *C. fragariae* infected plants at different time points post-infection.

![Figure 12](image-url)

Figure 12. Relative total $\beta$-1, 3-glucanase activities at various time points after infection with *Colletotrichum acutatum* and *Colletotrichum fragariae*. A colorimetric assay was performed using 2 µg total proteins in each assay reaction. The levels of the activity in the control plants were set as one. Each bar represents an average reading from three individual assays.

A generally gradual increase of enzyme activity from 2 h to 48 h post-infection was observed with both the *Colletotrichum* species. In the 48 h samples, increases of 1.7-fold and 3.5-fold in the total $\beta$-1, 3-glucanase activity over the activity of the control samples were observed with both *C. acutatum* and *C. fragariae* infected plants. It should
be noted that no significant difference in the total β-1, 3-glucanase activities was observed with the control plants through the 48 h experimental period.

The isozyme patterns of β-1, 3-glucanases at different time points after infection with *C. fragariae* were determined using PAGE. Figure 13A shows the isozyme patterns in leaf samples from infected plants. Three enzyme bands, designated as GI-1, GI-2, and GI-3, were detected. The GI-2 isozyme was seen in the control plants (Fig. 13A, lane 1) and in the 2 h infected sample. The band intensity of GI-2 showed an increase in the 6 h infected sample (Fig. 13A, lane 3) and reached a maximum in the 12 h infected sample. This was followed by decreases in band intensity in the 24 h and 48 h infected samples. The GI-1 isozyme band started to appear in the 12 h infected sample and reached a maximum in the 48 h samples (Fig. 13A, lanes 4 to 6). The GI-3 isozyme band started to appear in the 6 h sample, increased in intensity in the 12 h sample and remained relatively unchanged in the 24 h and 48 h samples (lanes 3 to 6).

The three isozyme forms were also detected in *C. acutatum* infected plants (Fig. 13B). Relatively strong bands of GI-2 and GI-3 were seen in all samples (Fig. 13B, lanes 3 to 6), except the 2 h sample. The GI-1 isozyme band was relatively week but was detectable starting from the 12 h sample (Fig. 13B, lanes 4 to 6).

From the results shown in Fig. 13, it can be concluded that only one β-1, 3-glucanase isozyme (GI-2) could be detected in leaves of uninfected plants, whereas fungal infected leaves contain three isozymes (GI-1 through GI-3). The amounts of these three isozymes varied during the course of the 48 h infection period.
Figure 13. The isozyme patterns of β-1, 3-glucanases at various time points after infection with Colletotrichum fragariae (A) and Colletotrichum acutatum (B). Ten µg total proteins were applied per lane. Lane 1 represents the control leaf sample; Lanes 2-6 represent 2 h, 6h, 12 h, 24 h, 48h post-infection leaf samples, respectively.
3.3.4 The Total β-1, 3-Glucanase Activity and Isozyme Pattern in Different Strawberry Organs

The total β-1, 3-glucanase activities in leaf, root and crown samples obtained from plants seven months after planting were determined (Fig. 14). In addition, the enzyme activities of leaf samples collected from plants 10 days, 1 month, 5 months and 7 months after planting were compared (Fig. 15).

![Bar chart showing enzyme activity in different plant organs](image)

Figure 14. The total strawberry β-1, 3-glucanase activities in plant leaves, roots and crowns at 7 months. A colorimetric assay was performed using 2 µg total proteins in each assay reaction. Each bar represents an average reading from three individual assays.

The results showed that leaves had the highest β-1, 3-glucanase activity, which was approximately three fold higher than the activities of roots and crowns (Fig. 14). The time course result indicated that the enzyme activity remained relatively constant during the first five month growth period, but increased greatly at the seven month time point (Fig. 15).
Figure 15. The total strawberry β-1, 3-glucanase activities in strawberry leaves at various time points after planting. A colorimetric assay was performed using 2 µg total proteins in each assay reaction. Each bar represents an average reading from three individual assays.

The isozyme patterns of leaves, roots and crowns from plants 1 month and 7 months after planting are shown in Fig. 16A and B, respectively. In the 1-month-old plants, GI-2 was the only isozyme detected in leaves (Fig. 16A, lane 1), whereas GI-3 was the predominant isozyme in roots (Fig. 16A, lane 2). None of the three isozymes was detected in crowns (Fig. 16A, lane 3).

The isozyme pattern changed as the strawberry plants grew older. At 7 months after planting, the GI-3 isozyme became the major isozyme in leaves and crowns (Fig. 16 B, lanes 1 and 3). This isozyme was also the major isozyme in the roots (Fig. 16B, lane 2), but the band intensity was much lower than that shown in the 1 month sample (Fig. 16A, lane 2).
Figure 16. The isozyme patterns of β-1, 3-glucanases from different strawberry organs 1 month (A) and 7 months (B) after planting. 5 µg total proteins were applied per lane. Lane 1, plant leaves; lane 2, plant roots; lane 3, plant crowns.
Figure 16 shows that the isozyme patterns are different in different organs and the pattern changed after different growth times. To further examine this change of isozyme pattern, leaf samples were collected at 10 days, 3 months, 5 months and 7 months after planting, and the isozyme patterns of the samples were analyzed (Fig. 17). It can be seen from the gel patterns that in the 10 day sample, GI-2 was the major isozyme (Fig. 17, lane 1), which was barely or not detectable in other samples. On the other hand, GI-3 became the predominant isozyme in the late growth stage (Fig. 17, lanes 3 and 4).

Figure 17. The isozyme patterns of β-1, 3-glucanase from strawberry leaves at various time points. 5 µg total proteins were applied per lane. Lane 1, plant leaves at 10 days; lane 2, plant leaves at 3 months; lane 3, plant leaves at 5 months; lane 4, plants leaves at 7 months.

3.3.5 Discussion

To assess the role of strawberry β-1, 3-glucanases in plant defense against fungal infection, the expression levels of FaBG2-1 and FaBG2-3 in strawberry plants infected
with *C. fragariae* or *C. acutatum* were examined. For both genes, no induction or low levels of induction were observed at the 2, 6, and 12 h time points, and greater levels of induction were seen at the 24 and 48 h time points. Comparing the two *Colletotrichum* species, the induction levels produced by *C. fragariae* were substantially higher than those produced by *C. acutatum*. Shih’s laboratory had previously studied the expression of two strawberry chitinase genes, *FaChi2-1* and *FaChi2-2*, in strawberry plants infected with the two *Colletotrichum* species (Khan and Shih, 2004). Higher levels of induction of both chitinase genes were also observed in *C. fragariae* infected plants than in *C. acutatum* infected plants. One aspect of the induction patterns of the chitinase genes that appeared different from the patterns of the β-1, 3-glucanase genes is that *FaChi2-1* showed a rapid induction, which occurred within 2 to 6 h post-infection. On the other hand, induction of *FaChi2-2*, like the induction of *FaBG2-1* and *FaBG2-3*, occurred slower, at 24 to 48 h post-infection.

Analysis of the expression of *FaBG2-1* and *FaBG2-3* in different organs of uninfected strawberry plants showed that the two genes are constitutively expressed, although they were expressed at widely different levels in these organs. In leaves, the synthesis of *FaBG2-1* mRNA increased continuously during the seven-month test period. The synthesis of *FaBG2-3* mRNA increased initially, peaked at 5 months, and then decreased significantly at seven months. Synthesis of *FaBG2-1* mRNA in crowns was low throughout the test period. However, a high level of *FaBG2-3* mRNA was observed in crowns at one month, drastically decreased at three months, and remained at low level during the rest of the test period. One may postulate that this initial high level of expression of *FaBG2-3* mRNA could be related to plant development. Furthermore, it has
been shown in some plant species that the level of resistance or susceptibility to pathogen infection is affected by plant age (Reuveni et al., 1986; Pretorius et al., 1988). It would be interesting to determine whether changes in the expression levels of β-1, 3-glucanase genes at different strawberry growth periods can be related to the plant’s resistance to pathogens.

The real-time PCR results showed that the synthesis of the FaBG2-1 and FaBG2-3 mRNA significantly increased after infection by the two Colletotrichum species compared to control plants. However, these high degrees of stimulation of the transcription of the two β-1, 3-glucanase genes was not reflected in the enzyme assay results of the β-1, 3-glucanase activity. In the latter case, only 2-4 fold enzyme activity increase over the control plants was observed. The reason for this large discrepancy is not clear. A most likely explanation is that there are many β-1, 3-glucanase genes in strawberry plants and the protein products of FaBG2-1 and FaBG2-3 only constitute a small extent of the total β-1, 3-glucanase activity in strawberry leaves.

The results on the β-1, 3-glucanase isozyme patterns were rather interesting. Only one isozyme was in control plant leaves at 10 days. But three isozymes were in infected plant leaves. The data indicated that concentrations (intensities) of the three isozymes increased after infection by either C. acutatum or C. fragariae, and the kinetics of the expression of the three isozymes were also different. GI-2 decreased at late stage in the C. fragariae infected plants and it did not decrease at late stage in C. acutatum infected plants. Furthermore, in uninfected leaves, which exhibited approximately three fold higher total β-1, 3-glucanase activity than those of roots and crowns, the GI-2 isozyme
appeared to the main isozyme form whereas the GI-3 isozyme was the predominant form at the late stage of strawberry growth.
CHAPTER 4

SUMMARY AND CONCLUSIONS

The production of PR proteins is one of a plant’s reactions to pathogen attacks. PR proteins were originally identified in tobacco plants infected by tobacco mosaic virus (Van Loon and Van Kammen, 1970). Different types of PR proteins have been identified since then. Currently, 17 PR families have been defined (Van Loon et al., 1994; Görlach et al., 1996; Van Loon, 1997; Van Loon and Van Strien, 1999; Okushima et al., 2000; Christensen et al., 2002). Plant β-1, 3-glucanases are members of PR proteins and were shown to be induced in plants upon pathogen infection and other stimuli (Van Kan et al., 1992; Ignatius and Chopra, 1994; Kombrink et al., 1988; Mauch et al., 1984; Anuratha et al., 1996; Kaku et al., 1997; Munch-Garthoff et al., 1997; Lozovaya et al., 1998; Cheong et al., 2000; Li et al., 2001; Hanselle et al., 2001; Zemanek et al., 2002). Plant β-1, 3-glucanases can directly inhibit the fungal growth in vitro by catalyzing the hydrolysis of β-1, 3-glucan which is a major structural component of the cell walls of many pathogenic fungi. They can also generate elicitors which induce other plant defense reactions. In an effort to enhance disease resistance, β-1, 3-glucanase gene constructs were introduced into a variety of plant species. There was considerable evidence of positive correlation between the over-expression of β-1, 3-glucanase gene and enhanced disease resistance (Broglie et al., 1991; Melchers et al., 1993; Sela-Buurlage et al., 1993; Yoshikawa et al., 1993; Zhu et al., 1994; Jach et al., 1995; Jongedijk et al., 1995; Masoud et al., 1996; Lusso et al., 1996; Nishizawa et al., 2003).
Strawberry is an important horticultural crop in the southern United States. The two fungal pathogens, *C. acutatum* and *C. fragariae* can cause anthracnose diseases in strawberry crowns, fruits and roots. Few studies have been reported on the PR proteins or PR protein genes of strawberry. This dissertation research is an effort to gain information on the role of β-1, 3-glucanase in strawberry defense systems. Two strawberry β-1, 3-glucanase genes and a complete cDNA of a third β-1, 3-glucanase gene were isolated and characterized. The expression of these genes in fungal infected and uninfected strawberry plants was evaluated. Characterization of the total β-1, 3-glucanase activities and isozyme patterns in strawberry plants was performed.

Two strawberry β-1, 3-glucanase genes, *FaBG2-1, FaBG2-2*, and one cDNA, *FaBG2-3* were obtained by a PCR-based genome walking procedure and 3′ and 5′ RLM-RACE PCR methods. *FaBG2-1* is comprised two exons and one inton and has a potential to encode a protein of 347 amino acids. The first exon of *FaBG2-1* may encode 31 of the 32 amino acids of a putative signal peptide. Southern blot analysis showed there were several copies of *FaBG2-1* and related genes in the strawberry genome. The sequences of *FaBG2-2* and *FaBG2-3* shared a high homology in the coding regions, but are different in the non-coding regions. *FaBG2-2* is predicted to encode a protein of 320 amino acids. No signal peptide encoding-exon was detected in a genomic segment of approximately 3000 nucleotides upstream of the coding region of the gene. This finding strongly suggested that *FaBG2-2* is an intronless gene and that it does not encode a signal peptide. On the other hand, *FaBG2-3* is predicted to encode a protein of 346 amino acids with a signal peptide. *FaBG2-3*, like *FaBG2-1* and many other plant β-1, 3-glucanase genes, may contain two exons with its first exon encoding at least a part of the signal peptide.
The predicted signal peptides of FaBG2-1 and FaBG2-3 are both 32 amino acids long and the sequences of the two peptides differ in only one amino acid. FaBG2-1, FaBG2-2 and FaBG2-3 were classified as class II β-1, 3-glucanase genes based on their high amino acid sequence homology to other class II β-1, 3-glucanases, their predicted acidic pI, and the lack of the C-terminal extension. The deduced amino acid sequences of FaBG2-1, FaBG2-2 and FaBG2-3 are different. FaBG2-2 has a stretch of six amino acids at the N-terminus that does not appear in the mature proteins of FaBG2-1 and FaBG2-3. FaBG2-1 has 92 % identity and 95 % similarity to FaBG2-2, and 93 % identity and 95 % similarity to FaBG2-3. FaBG2-2 has 98 % identity and 99 % similarity to FaBG2-3. FaBG2-2 has 98 % identity and 99 % similarity to FaBG2-3.

The expression levels of FaBG2-1 and FaBG2-3 were examined in strawberry plants infected with *C. fragariae* or *C. acutatum*. Upon fungal infection, no induction or low levels of induction were observed at 2, 6, and 12 h post-inoculation, and greater levels of induction were seen at 24 and 48 h for both genes. Comparing the response of both genes to the two *Colletotrichum* species, the induction by *C. fragariae* was substantially higher than by *C. acutatum*. *C. fragariae* induced the expression of FaBG2-1 by 116.9-fold and FaBG2-3 by 57.1-fold at 48 h, while *C. acutatum* only induced the expression of FaBG2-1 by 9.3-fold and FaBG2-3 by 5.4-fold at 48 h. The repeat infection experiments showed similar results. Comparing infected plants by the two fungi, the symptom caused by *C. fragariae* is severer than *C. acutatum*. *C. fragariae* is more virulent than *C. acutatum* to the strawberry plants. This virulence is positively related the induction of FaBG2-1 or FaBG2-3 transcripts.

The expression levels of FaBG2-3 were considerably higher than those of FaBG2-1 in both the control and the infected plants. In the control plants, the expression
level of FaBG2-3 was one to two thousand-fold higher than that of FaBG2-1. In the C. fragariae and C. acutatum infected plants, the expression levels of FaBG2-3 were several hundred to approximately a thousand-fold higher than the expression levels of FaBG2-1. Furthermore, even though the expression levels of FaBG2-1 in the C. fragariae infected plants was higher than its expression in control plants, it was still considerably lower than the constitutive expression level of FaBG2-3 in the uninfected control plants.

The expression levels of FaBG2-1 and FaBG2-3 were also determined in different organs of healthy strawberry plants. The results showed that the two genes were constitutively expressed in these organs at widely different levels. The synthesis of FaBG2-1 mRNA in leaves increased continuously during the seven-month test period. The synthesis of FaBG2-3 mRNA increased initially, peaked at 5 months, and then decreased significantly at seven months. Synthesis of FaBG2-1 and FaBG2-3 mRNA in crowns was low throughout the test period, except a high level of FaBG2-3 mRNA at one month.

In addition, the total β-1, 3-glucanase activity and the isozyme patterns were examined in both fungi-infected and healthy plants. A generally gradual increase of β-1, 3-glucanase activity was observed from 2 h to 48 h post infection with both the C. acutatum and C. fragariae infected plants. The total β-1, 3-glucanase activity increased 1.7-fold and 3.5-fold in the plants infected with C. acutatum and C. fragariae at 48 h, respectively. The result of isozyme pattern showed that there were three isozymes, designated as GI-1, GI-2, and GI-3, in infected leaves with both fungi, while in uninfected leaves there was one major isozyme, GI-2, with lesser intensity. The total β-1, 3-glucanase activity and the isozyme pattern also changed in different uninfected organs
at different growth periods. The uninfected leaves exhibited approximately three fold higher total β-1, 3-glucanase activity than those of roots and crowns. The GI-2 isozyme appeared to be the main isozyme form in one month leaves whereas the GI-3 isozyme was the predominant form in seven month leaves.

In summary, the expression of FaBG2-1 and FaBG2-3 was greatly enhanced by infection with fungal pathogen in this study. It would be interesting to determine whether these genes can be induced by other stimuli. It would also be interesting to determine whether changes in the expression levels of β-1, 3-glucanase genes at different strawberry growth periods can be related to the plant’s resistance to pathogens.
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APPENDIX A:
IDENTIFICATION OF A ZINC TRANSPORTER GENE IN STRAWBERRY*

YANLIN SHI and DING S. SHIH

ABSTRACT

We have cloned a ZIP [ZRT (Zn-regulated transporter), IRT (Fe-regulated transporter) -like protein] gene from the strawberry plant (*Fragaria x ananassa* Duch). This gene, designated as *FaZIP1*, is composed of three exons and two introns. The locations of the two introns (546 base pairs and 157 base pairs) in the gene were confirmed by sequence analysis of cDNA clones obtained by using 3’ and 5’ RNA ligase mediated-rapid amplification of cDNA ends (RLM-RACE) PCR. Also, based on the cDNA sequence, the transcriptional start site of *FaZIP1* was assigned. *FaZIP1* is predicted to code for a protein of 353 amino acids containing a signal peptide and eight potential transmembrane domains. Southern blot analysis indicated that *FaZIP1* is a member of a multi-gene family. Reverse transcriptase-polymerase chain reaction analysis revealed that the *FaZIP1* gene is expressed constitutively in strawberry leaves and roots.

**Keywords:** ZIP gene; Strawberry (*Fragaria x ananassa* Duch); Genome walking; RLM-RACE PCR; Southern blot; Gene expression.

*This part of research work will be published in DNA sequence (Shi and Shih, 2005 in press) ([http://www.tandf.co.uk](http://www.tandf.co.uk)).
INTRODUCTION

Transition metals are essential micronutrients for all organisms. They take part in many different physiological and biochemical processes and are important for growth and developmental processes. Among these metals, zinc is non-redox-active but has a key structural and/or catalytic role in many proteins and enzymes, because it tends to form stable tetrahedral complexes (Berg et al. 1996). Zinc is an essential catalytic component of over 300 enzymes, including alkaline phosphatase, alcohol dehydrogenase, Cu-Zn superoxide dismutase, and carbonic anhydrase (Grotz et al. 1998). It has been shown that more than 3 percent of the protein sequences predicted from the Caenorhabditis elegans genome contained sequence motifs characteristic of zinc-binding structural domains (Clarke and Berg, 1998). In plants, zinc deficiency is a widespread micronutrient deficiency which decreases crop production (Ruel and Bouis, 1998).

Zinc transporters are needed to regulate uptake and storage of zinc in plants. Different zinc transporters are located in cellular or organellar membranes to make it possible to transport zinc from soil into the root, and from root to other plant tissues (Grotz et al. 1998; Hall and Williams, 2003). Many zinc transporters are members of the ZIP [ZRT(Zn-regulated transporter), IRT (Fe-regulated transporter) -like protein] family (Guerinot, 2000; Mäser et al. 2001; Hall and Williams, 2003). These proteins are involved in the transport of Fe, Zn, Mn, and Cd ions. ZIP family is structurally distinct from other metal ion transporters, such as the cation antiporters (Gaxiola et al. 2002) and the CDF family (Paulsen and Saier, 1997; Williams et al. 2000). After the first ZIP gene was identified in Arabidopsis (Eide et al. 1996), ZIP family has become one of the rapidly growing families of eukaryotic proteins. In recent years, with the use of genetic and
molecular techniques, ZIP genes have been identified in many different plants. These include *Arabidopsis thaliana* (Grotz et al. 1998; Mäser et al. 2001), *Thlaspi caerulescens* (Pence et al. 2000), *Lycopersicon esculentum* (Eckhardt et al. 2001), *Glycine max* (Moreau et al. 2002), *Oryza sativa* (Ramesh et al. 2003), and *Medicago truncatula* (Burleigh et al. 2003).

Strawberry (*Fragaria × ananassa* Duch) is a member of the *Rosaceae* family, which is a large plant family comprising of approximately 3000 members (Baumgardt, 1982). This plant family includes other important fruit crops such as apples, peaches, pears, raspberries and blackberries. In this communication, we report the isolation and partial characterization of a strawberry zinc transporter gene. A genomic DNA clone and a closely related cDNA clone were obtained and completely sequenced. To our knowledge, this is the first ZIP gene to be identified in a *Rosaceae* family member.

**MATERIALS AND METHODS**

**Isolation of RNA and DNA**

Total nucleic acids were extracted from strawberry tissues according to the method described by Manning (1991). RNase-free plasticware and reagents (Ambion, Austin, TX) were used in all procedures. For genomic DNA preparation, RNA was removed from total nucleic acid samples by treating them with RNase A (final concentration 0.1 mg/ml) at 37°C for 2h. For RNA preparation, nucleic acid samples were first purified by using RNeasy Mini Kit (QIAGEN, Valencia, CA). This was followed by treating the samples with DNase I using the DNA-free™ kit (Ambion, Austin, TX) to completely remove any residual contaminating DNA.
3’ and 5’ RLM-RACE PCR

In an experiment to isolate a strawberry β-1, 3-glucanase cDNA, using a 3’ RNA ligase mediated-rapid amplification of cDNA ends (RLM-RACE) procedure, we accidentally obtained a cDNA which exhibited sequence homology to plant zinc transporter genes. This 3’ RLM-RACE experiment was carried out as follows. Strawberry leaf RNA was reverse transcribed by using the FirstChoice RLM-RACE kit (Ambion, Austin, TX). The 20 µl reaction mixture contained 1µg of RNA as template, 500 µM of each dNTP, 2 µl of 3’ RACE adapter (5’-GCGAGCACAGAATTAATACGACTCACTATAGGT 12 VN-3’) which served as the primer, 1 x reverse transcription buffer, 1U of placental RNase inhibitor, and 10 U of molony murine leukemia virus reverse transcriptase. The reaction was carried out at 42°C for 1h. A PCR was then performed in a 25 µl PCR reaction mixture containing 400 µM of each dNTP, 2 µl of 3’ RACE Outer Primer (5’-GCGAGCACAGAATTAATACGACT-3’) and 400 nM β-1, 3-glucanase gene specific primer (5’-TCCAAACGGAAGCAAAGTCAACT-3’), 3mM MgCl₂, 1 x PCR buffer, and 1 U DNA polymerase mix of the FailSafe PCR system (Epicenter Technologies, Madison, WI). A touch-down PCR program was used with one step for 2 min at 94°C, followed by 10 cycles of 1 min at 94°C, 1 min at 62°C (decreasing 1°C/cycle), 2 min at 72°C, 28 cycles of 1 min at 94°C, 1 min at 52°C, 2 min at 72°C, and a final step of 10 min at 72°C.

When the amplification product was fractionated on an agarose gel, several DNA bands were obtained. Each DNA band was purified and subsequently cloned into the pGEM T-easy vector (Promega, Madison, WI). Sequence analysis of the recombinant plasmids revealed that one of the cDNA sequences was homologous to some plant zinc
transporter genes. We postulate that the reason for obtaining such a cDNA is probably due to the presence of an oligo (U) sequence located immediately before the coding region of the putative strawberry zinc transporter mRNA. The complimentary oligo (A) sequence present in the first strand cDNA then could anneal with the oligo (dT) part of the 3′ RACE adapter used in the reverse transcription step, thus, fortuitously allowing the adapter to serve as the primer for the synthesis of the second cDNA strand.

To obtain the complete sequence of the putative strawberry zinc transporter mRNA, a 5′ RLM-RACE was performed using again the FirstChoice RLM-RACE kit (Ambion, Austin, TX). Briefly, 10 µg of total RNA was treated with calf-intestinal phosphatase to remove the 5′-phosphate of any degraded RNA fragments. After removal of the phosphatase by phenol: chloroform extraction, the RNA sample was treated with tobacco acid pyrophosphatase to remove the cap structure of intact mRNA molecules. Using RNA ligase, a synthetic RNA adapter was then ligated to each RNA chain bearing a 5′-phosphate. The ligated RNA was reverse transcribed in a 20 µl reaction mixture containing 2 µl of the RNA as template, 500 µM of each dNTP, 5 µM of random decamers which serve as primers, 1 × reverse transcription buffer, 1 U of placental RNase inhibitor, and 10 U of molony murine leukemia virus reverse transcriptase. The reaction was carried out at 42°C for 1h. PCR was than used to amplify the zinc transporter cDNA from the reverse transcription products. Two nested gene-specific reverse primers (5′-CGATTGCACCGCCACGAGGATG-3′ and 5′-TATTTTAGGGETTCGCTTCTGTTACTG-3′) along with two adaptor-specific primers were used in the PCR amplification. Primary and secondary PCR reactions were performed in 25 µl PCR reaction mixtures containing 400 µM of each dNTP, 400 nM of
each primer, 3 mM MgCl₂, 1 x PCR buffer, and 2.5 U Taq DNA polymerase (Promega, Madison, WI). A touch-down PCR program was used with one step for 2 min at 94°C, followed by 5 cycles of 1 min at 94°C, 1 min at 60°C (decreasing 1°C/cycle), 1 min at 72°C, 30 cycles of 35s at 94°C, 35s at 55°C, 1 min at 72°C, and a final step of 10 min at 72°C. A DNA fragment of 193-bp was obtained. The fragment was cloned into the pGEM T-easy vector and sequenced. By combining with the cDNA sequence obtained from the 3’ RLM-RACE experiment, a composite sequence of 1276 bp, representing the complete sequence of a ZIP mRNA, was obtained. This 1276-bp cDNA sequence was designated as *FaZIPc*.

**Isolation of a genomic clone of a Strawberry ZIP Gene**

Based on the cDNA sequences derived from 3’ and 5’ RLM-RACE PCR, a genome walking procedure was performed using the Universal GenomeWalker™ Kit (Clontech, Palo Alto, CA). Two nested reverse primers (primer 1: 5’-CGATTGCACCGGCCACGAGGATG-3’, primer 2: 5’-TATTTTAGGCTTGCTTCTGTTACTG-3’) were used to anneal to the 5’ coding region of the putative zinc transporter gene. After two runs of PCR following the conditions recommended by the manufacturer, a PCR product of 813 bp containing the upstream sequence of the gene was obtained. The PCR product was cloned and sequenced. In the same way, two nested forward primers (primer 1: 5’-GCTTCAAGTTGGGGCTAATGTTTCAC-3’, primer 2: 5’-TTTGGAGCTGGTTGCATGTCTCTAATA-3’) were used to anneal to the 3’ coding region of the putative zinc transporter gene. A DNA fragment of 428 bp was obtained and its sequence was determined. These additional sequence data from the genome walking
experiments allowed the design of a final set of primers to obtain a genomic clone encompassing the complete coding region together with its 5′ and 3′ flanking sequences. These terminal specific primers were: forward primer: 5′-
CTTTTGAATAGATGGGAGATGAGATGAT-3′, reverse primer: 5′-
ATGTTGCAGGTGATAAGTAGAAGTAAAGTT-3′. PCR was performed in a 25µl PCR reaction mixture containing 20ng of strawberry genomic DNA, 400µM of each dNTP, 3mM MgCl₂, 400nM of each primer, 1 x PCR buffer, and 1 U DNA polymerase mix of the FailSafe PCR system (Epicenter Technologies, Madison, WI). A touch-down PCR program was used with one step for 2 min at 94°C, followed by 10 cycles of 1 min at 94°C, 1 min at 62°C (decreasing 1°C/cycle), 3 min at 72°C, 30 cycles of 1 min at 94°C, 1 min at 52°C, 3 min at 72°C, and a final step of 10 min at 72°C. The amplification reaction yielded a DNA fragment of 2659 bp. This DNA fragment, designated as FaZIP1, was purified and cloned into the pGEM T-easy vector. Both strands of the insert of the resulting recombinant plasmid, designated as pFaZIP1, were sequenced.

**Sequence Analyses.**

Nucleotide sequences were determined with an Applied Biosystems model 373A Automated Sequencer (Gene and Probe Laboratory, School of Veterinary Medicine, Lousiana State University, Baton Rouge, LA), using the Ready-reaction Dye-Terminator kit with AmpliTaq FS (Applied Biosystems). Standard protein-protein BLAST (blastp) was used for finding similar sequences in protein databases (Altschul et al. 1990). Potential promoter sites were determined by using the Neural Network Promoter Prediction (Reese, 2001). Potential transmembrane domains were identified by using SMART (Schultz et al. 1998, 2000), TMMTOP (Tusnády and Simon, 1998, 2001),
TMHMM (Sonhammer et al., 1998) and PSORT (Nakai and Kanehisa, 1992). Potential N-glycosylation sites were identified by using NetNGlyc (Gupta et al. 2004). Multiple sequence alignment was performed by using Clustal (Higgins and Sharp 1989; Thompson et al. 1997) and modified by using GeneDoc (Nicholas et al. 1997). Molecular mass and isoelectric point (pI) were calculated by using the Compute pI/Mw tool (Bjellqvist et al. 1993, 1994).

**Southern Blotting**

Leaf DNA (10 µg) was digested with *Eco* RI, *Eco* RV or *Hind* III. The digested DNA samples were subjected to electrophoresis on a 0.8% agarose gel, and the separated DNA fragments were transferred to a Zeta-probe-GT nylon membrane (BioRad, Hercules, CA) by downward capillary transfer with 20 x SSC buffer. The DNA fragments were crosslinked to the membrane by UV-irradiation in a Stratalinker Crosslinker (Stratagene, La Jolla, CA) on auto setting delivering 1.2 x 10^5 µj of energy. The membrane was pre-hybridized with 10 ml/100 cm² of DIG Easy Hyb solution (Roche Applied Science, Indianapolis, IN) containing 200 µg of fragmented salmon sperm DNA for 1h at 45°C. A DIG-labeled probe (see below) was boiled with 800 µg of salmon sperm DNA for 10 min and cooled on ice immediately. The probe was added to fresh DIG Easy Hyb solution which contained 200 µg of fragmented salmon sperm DNA. The membrane was incubated with the probe solution at 45°C overnight. The blot was washed twice with low stringency wash buffer (2 x SSC, 0.1% SDS) for 10 min each time at room temperature and twice with high stringency buffer (0.1 x SSC, 0.1% SDS) for 30 min each time at 45°C. The *FaZIP1* was detected by using the DIG High Prime DNA
Labeling and Detection Starter Kit I (Roche Applied Science, Indianapolis, IN) according to the manufacturer’s recommended conditions.

A random priming method was used for preparing the probe. The template DNA for the random priming reaction was synthesized using PCR with the following primers: forward primer: 5′-TCCTCTCCTTCTTCTGCATCCTCTT-3′, reverse primer: 5′-CCTCATGCTCTCCAACCTTTCTCCTCA-3′. pFaZIP1 (10 ng) was used as the template. The resulting 477-bp PCR product, that spans nucleotide positions 594-1070 in the first exon of FaZIP1 and FaZIPc, was labeled with digoxigenin-dUTP by using the DIG High Prime DNA Labeling and Detection Starter Kit I according to the manufacturer’s recommended conditions.

**Gene Expression Analysis using Reverse Transcriptase-polymerase Chain Reaction (RT-PCR)**

Complimentary DNA synthesis was carried out using the Omniscript RT Kit (QIAGEN Valencia, CA). RNA was reverse transcribed in 20 µl reaction mixtures containing 1 µg of RNA as template, 500 µM of each dNTP, 1 µM of oligo-dT primer, 1 x reverse transcription buffer, 10 U RNase inhibitor, 4 U Omniscript Reverse Transcriptase. In the control reactions, reverse transcriptase was omitted. Reverse transcription reaction was carried out at 37°C for 1h. An aliquot of 2 µl of this reverse transcription reaction solution was used in a PCR to detect the presence of FaZIP1 or related gene transcript. Two gene specific primers for FaZIP1 (and FaZIPc) were used. (These two primers were the same as those used for preparing the probe for Southern Blotting experiment). PCR was performed in a 25 µl PCR reaction mixture containing 2 µl of this reverse transcription reaction solution, 400 µM of each dNTP, 3 mM MgCl2,
400 nM of each primer, 1 x PCR buffer, and 2.5 U Taq DNA polymerase (Promega, Madison, WI). A touch-down PCR program was used with one step for 2 min at 94°C, followed by 10 cycles of 1 min at 94°C, 1 min at 62°C (decreasing 1°C/cycle), 1 min at 72°C, 30 cycles of 1 min at 94°C, 1 min at 52°C, 1 min at 72°C, and a final step of 10 min at 72°C.

RESULTS AND DISCUSSION

Cloning and Analysis of *FaZIP1* and *FaZIPc*

Complementary DNA fragments of a strawberry ZIP gene were isolated by using 3’ and 5’ RLM-RACE PCR. A cDNA fragment isolated by the 3’ RLM-RACE PCR is 1226 bp long, which contains the entire coding region and the poly-A tail of a ZIP mRNA. The cDNA fragment isolated by 5’ RLM-RACE PCR is 193 bp long, which contains the 5’end sequence of the mRNA and has a 143-bp overlap with 3’cDNA fragment. The sequences of the 143-bp overlap region of the 3’ fragment and the 5’ fragment are completely identical, indicating that the two cDNA fragments are indeed derived from the same mRNA. The resulting composite cDNA sequence is 1276 bp in length (GenBank accession number AY805423).

Based on the cDNA sequence information, a PCR- based genome walking procedure was applied to isolate a 2659-bp DNA fragment (*FaZIP1*) (GenBank accession number AY805422). Fig. 1 shows nucleotide sequence and a deduced amino acid sequence of the encoded protein. *FaZIP1* is comprised of three exons (nucleotide positions 569 to 1174, 1721 to 1870, and 2028 to 2333) and two introns of 546 bp and 157 bp. Both introns had the conserved G/gt and ag/G sequences at the splice junctions.
FIGURE 1  Nucleotide sequence and deduced amino acid sequence of *FaZIP1*. Intron sequence is shown in lower case letters. TATA box is underlined. The transcriptional...
start site is indicated by a rightward arrow. The signal peptide cleavage site is indicated by a vertical arrow. Numbers on the left and right represent nucleotide and amino acid positions, respectively.

(Fig. 1) (Shapiro and Senapathy, 1987), and contained 64% and 71% of A+T for the first and the second intron, respectively. In comparison, the A+T content of the coding region is 52%. Similar higher intron A+T content was also observed with two strawberry class II chitinase genes (Khan and Shih, 2004) and one strawberry \( \beta-1, 3\)-glucanase gene (Khan et al. 2003). It has been proposed that A+T rich introns can be more efficiently spliced (Ko et al. 1998). The positions of the splice junctions were confirmed by comparing the genomic DNA sequence with the cDNA (FaZIPc) sequence.

Based on the 5′ RLM-RACE result, a G residue at nucleotide position 508, which is 61 nucleotides upstream of the predicted translational start site (Fig. 1), was assigned as the transcriptional start site. This start site assignment is only one base different from a start site predicted by using the computer program Neural Network Promoter Prediction (Reese, 2001). The latter predicated a G residue at nucleotide position 509. In addition, using this software program, a TATA box-like sequence, TACATAA, (nucleotide positions 478-484 in Fig.1) was identified. Furthermore, CAAT box-like signals were identified in the upstream region of the gene (Shirsat et al. 1989).

Comparison of FaZIPc with FaZIP1 revealed one nucleotide substitution and one base gap in the 5′-UTR of FaZIP1, and 30 nucleotide substitutions in the coding region resulting in 19 amino acids changes. It seems likely that FaZIPc is derived from a gene related to FaZIP1.

FaZIP1 has a potential to code for a protein of 353 amino acids with a molecular mass of 37.6 kDa and a pI of 6.0. The predicted product of FaZIPc is a protein of 353
amino acids with a molecular mass of 37.8 kDa and a pI of 6.4. For both proteins, the N-terminal 26 amino acids may constitute a signal peptide (Schultz et al. 1998, 2000) that would be cleaved to produce a mature protein of 34.7 kDa with a pI of 6.1 for FaZIP1 and 34.9 kDa with a pI of 6.3 for FaZIPc (Bjellqvist, et al. 1993, 1994).

The predicted FaZIP1 and FaZIPc sequences each contains eight potential transmembrane domains, designated as TM I-VIII (amino acid residues 46-65, 78-96, 123-142, 203-222, 231-250, 261-280, 301-320, 333-350) (Tusnády and Simon, 1998, 2001; Schultz et al. 1998, 2000). FaZIP1 and FaZIPc each have a potential signal sequence (amino acids residues 1-26) (Schultz, et al. 1998, 2000) and both are predicted to be plasma membrane proteins (Nakai and Kanehisa, 1992). Like other ZIPS, both FaZIP1 and FaZIPc have a short carboxyl-terminus predicted to be situated on the outer surface of the plasma membrane (amino acids residues 351-353) (Sonnhammer et al., 1998; Guerinot, 2000). A potential N-glycosylation site (NRS) was predicted at amino acid residue 37 (Gupta et al. 2004). This site is located between the signal peptide and the first transmembrane domain. Like the carboxyl-terminus, this site is expected to be situated on the outer surface of the plasma membrane.

In zinc transporter proteins, the region between transmembrane domains III and IV is predicted to be located on the cytoplasmic side and is a potential metal-binding domain rich in histidine residues (Grotz et al. 1998; Guerinot, 2000). Both FaZIP1 and FaZIPc have this histidine rich region, containing 10 and 11 histidine residues, respectively, between amino acid residues 148 and 197 (Fig. 2). In addition, three highly conserved histidine residues are found in different regions of ZIP proteins (Grotz et al. 1998). These histidine residues are also present in FaZIP1 and FaZIPc: amino acid
FIGURE 2. Comparison of the deduced amino acid sequences of FaZIP1 and FaZIPc with the sequences of related proteins. Fully conserved residues are shaded in black while semi-conservative substitutions are shaded in gray. Putative transmembrane domains of FaZIP1 and FaZIPc are indicated with bars and Roman numbers. The signal peptides of FaZIP1 and FaZIPc are indicated with bar. The conserved histidine residues are indicated by asterisks (*). Numbers on the left represent zinc transporter or related proteins from the following sources: (1) FaZIP1 (GenBank accession No. AY805422); (2) FaZIPc (AY805423); (3) M. truncatula (AAR08414); (4) G. max (AAK37761) (5) A. thaliana (AAL38432); and (6) L. esculentum (AAD30549). Numbers on the right represent amino acid positions.
residues 97, 211, and 238 (Fig. 2). For all the ZIPS, the most conserved region lies in transmembrane domain IV and is predicted to form an amphipathic helix containing a fully conserved histidine (residue 211 in FaZIP1 and FaZIPc) that may form part of an intramembranous metal binding site involved in transport (Guerinot, 2000; Mäser et al. 2001). The transport function is eliminated when these conserved histidines or certain adjacent residues are replaced by mutations (Rogers et al. 2000). Based on these comparisons of conserved histidines, FaZIP1 and FaZIPc are expected to be functionally active.

FaZIP1 shows 95% sequence identity to FaZIPc. A GenBank Blastp search revealed that FaZIP1 has highest homology, 60% identity and 70% similarity, to a Medicago truncatula metal transport protein (GenBank accession number AAR08414). It has 57% identity and 70% similarity to the Glycine max zinc transporter protein ZIP1 (AAK37761), and 49% identity and 66% similarity to the Arabidopsis thaliana metal transporter ZIP5 (AAL38432). FaZIP1 also shows relatively high homology to the Lycopersicon esculentum iron-regulated transporter 2 (AAD30549) with 48% identity and 64% similarity. Fig. 2 shows an alignment of FaZIP1 and FaZIPc with these ZIPS or related proteins.

**Southern Blot Analysis**

To determine the copy numbers of FaZIP1 or related genes in strawberry, a southern blot hybridization analysis was performed. As shown in Fig. 3, multiple bands were detected, suggesting that the FaZIP gene is present in several copies in the strawberry genome. This observation is in agreement with ZIP genes in other plants. For most of these ion transporters, the gene families are quite large. For example, in
Arabidopsis, there are 15 ZIPs (Mäser et al. 2001). Different zinc transporters may be needed for different transport pathways, for example, transporting from soil into the root, from root into other tissues, and for transporting across different cellular and organellar membranes within the plant. Specific zinc transporters may also be needed in respond to a different stress conditions (Hall and Williams, 2003; Grotz et al. 1998).

FIGURE 3  Southern blot analysis of FaZIP1. Lane 1, Eco RI digest; Lane 2, Eco RV digest; lane 3, Hind III digest; Lane 4, positive control (100 pg of FaZIP1 DNA fragment); lane 5, negative control (100 pg of a DNA fragment containing a strawberry β-1, 3-glucanase gene).

Gene Expression Analysis

FaZIPc was derived by reverse transcription of strawberry leaf RNA. This obviously indicated that the ZIP gene encoding the FaZIPc mRNA is constitutively expressed in strawberry leaves. To further examine the expression of strawberry ZIP
genes, we chose to analyze the expression of *FaZIP1* (and/or *FaZIPc*) in roots, the organ involved in zinc uptake from the soil. Root RNA samples, along with leaf RNA samples, were isolated from strawberry plants and these samples were used in RT-PCR experiments. The agarose gel patterns in Fig. 4 show the presence of *FaZIP1* (and/or *FaZIPc*) transcripts in all the leaf (lanes 2 and 4) and root samples (lanes 6 and 8). Negative controls (lanes 3, 5, 7, and 9), where reverse transcriptase was omitted at the cDNA synthesis step, did not show any product, confirming the absence of DNA contaminations in the test samples. This result indicated that *FaZIP1* (and/or *FaZIPc*) is constitutively expressed in strawberry roots.

Zinc uptake activities of some members of the ZIP family from other plants have been investigated by several laboratories (Grotz et al. 1998; Moreau et al. 2002; Ramesh et al. 2003). In future experiments, it would be interesting to determine the zinc uptake
activities of FaZIP1 and FaZIPc by expressing the genes encoding them in yeast. Also, it is known that some ZIPs may express preferentially in specific organs, for example, a soybean ZIP gene, *GmZIP1*, was specifically expressed in the nodules and not in roots, stems or leaves (Moreau et al. 2002). It would be interesting to determine the specific expression of *FaZIP1* and *FaZIPc* and their relative quantity in different plant organs using real time PCR.

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**References**


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