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CHARACTERIZATION OF AN ELM CHITINASE GENE AS A POSSIBLE RESISTANCE AGENT OF GRASSES AGAINST INSECTS

By

Donald Stuart Warkentin

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ABSTRACT

CHARACTERIZATION OF AN ELM CHITINASE GENE AS A POSSIBLE RESISTANCE AGENT OF GRASSES AGAINST INSECTS

By

Donald Stuart Warkentin

The purpose of this research project was to investigate the possible role of plant chitinase in resistance against phytophagous insects. A total of 17 cDNA clones that hybridized with a WIN6 poplar chitinase gene probe were obtained from an elm cDNA library. The clone designated pHS2 was most complete; so it was chosen for more detailed study. The gene was characterized and found to be highly homologous with other plant chitinases. This elm chitinase contains a chitinase catalytic region and a chitin binding domain, and is a typical class I chitinase.

The *hs2* elm chitinase gene contains 1225 nucleotides, with a 951 nucleotide open reading frame (ORF). Using the deduced 317-amino acid sequence from this ORF, a homology search using TFastA showed 56 of the 57 best matches to be with chitinases. It has strong homology to broad bean, poplar, and tobacco class I chitinases. The predicted protein product was named ECH2 (elm chitinase 2).

The *hs2* deduced amino acid sequence contains two chitinase class I signatures and a chitin binding domain signature. The first signature contains one of the six cysteines conserved in most chitinases. The second chitinase signature is at position 215 in ECH2. A chitin recognition or binding domain signature is found in ECH2 at position 33. The first 21 residues of ECH2 comprise a hydrophobic signal peptide. Residues 312-

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317 may be a C-terminal extension routing peptide to direct the protein into vacuoles. Residues 22-311 compose the main, catalytic domain.

The *hs2* gene was expressed in *Escherichia coli*. ECH2 from *E. coli* was injected into a rabbit to produce an antiserum.

A plasmid, designated pKYLX71-hs2, was constructed for plant transformations with *hs2*. It contained *hs2* controlled by the CaMV 35S promoter, a kanamycin resistance gene for selection of putatively transformed plants, a tetracycline resistance gene for selection in bacteria, and T-DNA borders for integration into plant chromosomes via *Agrobacterium tumefaciens* mediated plant transformation. Tobacco transformed by pKYLX71-hs2 expressed the *hs2* gene. Creeping bentgrass transformed by pKYLX71-hs2 via a biolistic method also expressed the *hs2* gene.

Transgenic turfgrass plants expressing the *hs2* gene were used in bioassays to study the potential of chitinase for plant resistance to insects. Feeding bioassays were done with Japanese beetle larvae. There were very few statistically significant differences (P=0.05) between the larval growth rates, pupal weights, survival, or pupation of insects that fed on Penncross nontransgenic turfgrass, a transgenic turfgrass line expressing the *bar* herbicide resistance gene but not the *hs2* gene, or four different transgenic turfgrass lines expressing both *bar* and *hs2*.

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DEDICATION

Dedicated to my parents, Joel and Mary Jane Warkentin, who have continually encouraged and supported me throughout my entire life.

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GENERAL INTRODUCTION

Introduction

Several reviews on plant chitinases have been published recently (Flach et al. 1992; Collinge et al. 1993; Graham and Sticklen, 1994). Chitinases (poly (1,4-(N-acetyl-β-D-glucosaminide)) glycanohydrolase, EC 3.2.1.14) are enzymes that hydrolyze chitin (Figure 0.1). They occur in many plants, and have been called pathogenesis-related (PR) proteins (Linthorst 1991). There is evidence that chitin may have antifungal activity and possibly also insecticidal activity. Many fungi contain chitin as a component of cell walls (Mauch et. al. 1988a). The activity of chitinase in many plants can be increased by wounding or by infection with bacterial or fungal pathogens (Majeau et al. 1990; Roby et al. 1990), or by induction with fungal cell wall material (Herget et al. 1990; Kurosaki et al. 1987b) or plant cell wall material (Hadwiger and Beckman 1980). Ethylene can also be an inducer of chitinase activity (Roby et al. 1985, 1991). Ethylene may be produced in plants following infection by plant pathogens (de Laat and van Loon 1982). Many plants contain chitin in their cell walls (Mauch et al. 1988a). Chitinase, either alone or in combination with β-1,3-glucanase, can inhibit the growth of fungi at the hyphal tips and can degrade fungal cell walls (Arlorio et al. 1992; Boler et al. 1983; Mauch et al. 1988b; Schlumbaum et al. 1986). Chitinase can associate with hyphal walls (Wubben et al. 1992). Some chitinases that have lysozymal activity (Majeau et al. 1990; Mauch et al. 1988a) may be defensive proteins against bacterial pathogens.

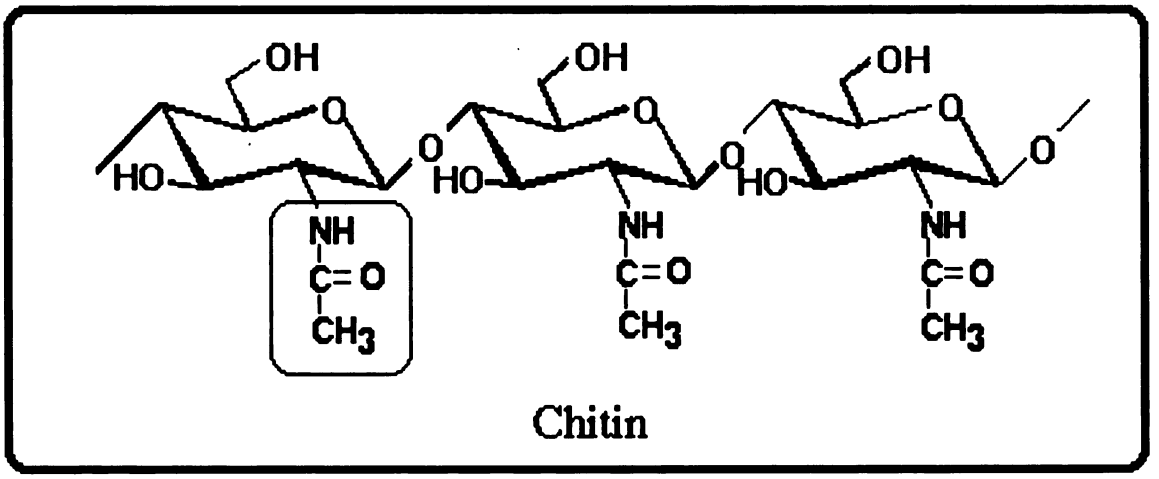

Figure 0.1 Chemical structure of chitin.

Plant chitinase may be involved in embryo development (De Jong et al. 1992), sexual reproduction, and pollination (Leung 1992). Some lipophilic chitin derivatives that occur in plant cell walls (Benhamou and Asselin 1989) and extracts of cell walls (Spaink et al. 1993) may be substrates for chitinase.

Classification and properties

Most plant chitinases that have been characterized are endochitinases that hydrolyze chitin internally. Chitin is a polymer of N-acetyl-glucosamine (NAG). Often the chitin is hydrolyzed with the release of NAG polymers two to five residues long (NAG_{2-5}) (Koga et al. 1989). Chitinase from yam (*Dioscorea opposita*) hydrolyzed NAG_3 to NAG_1 and NAG_2. NAG_6 was hydrolyzed to NAG_5 and NAG_1, NAG_4 and NAG_2, or 2 NAG_3. The most common products were NAG_4 and NAG_2. The second or third β-1,4 linkage from the reducing terminus was the preferential enzymatic site.

Some plant chitinases are also exochitinolytic. These include chitinases from melon (Roby and Esquerré-Tugayé 1987a), sugarbeet (Nielsen et al. 1993), carrot (Kurosaki et al. 1989), and chitinase-lysozymes from *Hevea brasiliensis* (Martin 1991). NAG_2 is the predominant product of carrot and melon exochitinases. Melon and *H. brasiliensis* exochitinases also release NAG_1. A melon chitinase also produced NAG monomer (Roby and Esquerré-Tugayé 1987a).

Chitinases have been isolated and characterized from many plants including dicots and monocots (Graham and Sticklen, 1994). The genomic, cDNA, and protein sequences for many chitinases have been reported (Graham and Sticklen, 1994).

Chitinases are most commonly monomers, but may be dimers (Ary et al. 1989). Their pI may be basic or acidic. They contain a catalytic domain and may also contain a chitin-binding domain. Some are glycoproteins, including chitinases from carrot (De Jong et al. 1992), bean (Margis-Pinhiero et al. 1991), and yam (Tsukamoto et al. 1984). Activity of chitinases is retained after dialysis, indicating that cofactors are not required (Jeuniaux 1966). Most chitinases have maximum activity at acidic pH, but some prefer more neutral or basic conditions. For example, a yam chitinase was reported to have a pH optimum of 3.8 (Tsukamoto et al. 1984), while a bean chitinase had a pH optimum of 6.5 (Boller et al. 1983). Yam chitinases were found to have dual pH optima, one basic and one acidic (Tsukamoto et al. 1984). Chitinase activity assays should be done at the pH optimum for the chitinase being assayed (Koga et al. 1989; Boller and Mauch 1988; Jeuniaux 1966; Pan et al. 1990; Molano et al. 1977).

The 3-D structure of a crystallized barley chitinase has been reported (Hart et al. 1993). It is α-helix rich and has a cleft that may be the catalytic site. There are three disulfide bridges.

There is a highly conserved region of the catalytic domain common to class I and class II chitinases. A tyrosine in this region may be indirectly involved in catalysis (Verburg et al. 1992). It is present in most chitinases, except that beet, bean, rice, and tobacco chitinases have serine, phenylalanine, or asparagine at that position.

The level of chitinase activity in many plants is inducible, and many plants also have a constitutive level of chitinase activity (Beerhues et al. 1990). Chitinase mRNA levels increase upon induction (Boller 1988; Broglie et al. 1986; Hedrick et al. 1988; Gerget et al. 1990; Nishizawa and Hibi 1991).

Chitinases have been classified into three classes (Shinshi et al. 1990). Class I chitinases are basic and have a cysteine-rich N-terminal domain with putative chitin-binding properties. Class II chitinases are acidic, do not have a cysteine-rich N-terminal domain, and have sequence similarity with class I chitinases in a catalytic domain. Class III chitinases have no serological cross reactivity with class I or II chitinases. They have a high level of homology at the amino acid level with the chitinase-lysozyme from *Hevea brasiliensis*. They include chitinases from cucumber and Azuki bean. Class IV chitinase are structurally similar to class I chitinases, but are different from them in sequence (Collinge et al. 1993).

Class I Chitinases

Class I chitinases occur in bean (Boller et al. 1983), pea (Mauch et al. 1988a; 1988b), tobacco (Legrand et al. 1987), and tomato (Pegg and Young 1982). Class I chitinases may occur in vacuoles or may be extracellular (Dore et al. 1991; Keefe et al. 1990; Mauch and Stahelin 1989). The barley class I chitinase, chitinase T had specific activity three times that of the barley class II chitinase, chitinase C (Jacobsen et al. 1990). Class I chitinases from tobacco had from six to fifteen times the specific activity of class II PR-P and PR-Q chitinases (Legrand et al. 1987; Sela-Burlage et al. 1993). Three class I chitinases, CHN50, CHN48, and CHN14 were expressed in tobacco when induced (van Buren et al. 1992).

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Gene and Protein Structures

Class I chitinases have a hydrophobic signal peptide that may route the protein into the endoplasmic reticulum, a cysteine-rich domain of about 40 amino acids, a hypervariable region of about 20 amino acids, a catalytic domain, and possibly a C-terminal extension that may target the protein to the vacuole. The signal peptide is removed from the protein to create the mature enzyme (von Heijne 1983). The length of the signal peptide is 23 amino acids in tobacco (Shinshi et al. 1990), 26 in rice (Zhu and Lamb 1991), 26 in potato (Gaynor 1988), and 27 in bean (Broglie et al. 1986). These signal peptides are hydrophobic.

A cysteine-rich domain of about 40 amino acids occurs in most basic chitinases, wheat germ agglutinin, hevein, and chitin-binding lectins. A 4.7 kDa hevein has a cysteine-rich chitin-binding domain (Van Parijs et al. 1991). Chitinases from tobacco (Shinshi et al. 1990), potato (Gaynor 1988), *Arabidopsis thaliana* (Samac et al. 1990), rice (Nishizawa and Hibi 1991; Zhu and Lamb 1991), rapeseed (Rasmussen et al. 1992a), and bean (Broglie et al. 1986; Margis-Pinhiero et al. 1991) contain chitin-binding domains. Also, hevein (Lucas et al. 1985) and nettle lectin (Stanford et al. 1989) contain cysteine-rich chitin binding domains.

Most chitin-binding domains contain eight cysteines whose positions are conserved in many chitinases. Possibly these cysteines may be required to obtain proper folding of the domain (Graham and Sticklen 1994). Other sequences within this domain that are conserved among several chitinases are an LCCSQFGWC sequence and a

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CQSQC sequence. The function of these sequences is unknown, but it has been speculated that they may be important for substrate binding (Graham and Sticklen 1994).

A proline-rich hinge region separates the catalytic domain from the chitin-binding domain in most chitinases, although it is absent in the *Populus win8* chitinase (Davis et al. 1991). In the tobacco chitinases CHN-A and CHN-B, most of the hinge prolines are hydroxylated (Sticher et al. 1992). These chitinases are hydroxyproline-containing proteins (HCPs).

Direct repeats are found flanking the chitin-binding domain and hinge region of chitinases from tobacco, potato, and bean (Shinshi et al. 1990). The 9 bp perfect repeats found in tobacco gene 48 chitinase are CCTCGGCAG. Other basic chitinase genes from tobacco, potato, and bean have 9 or 10 bp imperfect repeats. These repeats may be indicative of transposons. Possibly the chitin-binding domains were introduced into chitinase genes by transposition. Class II chitinases that lack the chitin-binding domain may have lost this domain by excision. One acidic chitinase gene that lacks a chitin-binding domain does have imperfect 9 bp repeats.

The chitinase catalytic domain contains a 20 amino acid hypervariable region and several conserved cysteines that may form a loop (Meins et al. 1992). This domain is capable of hydrolyzing chitin even without the chitin-binding domain, although in combination with the chitin-binding domain, the enzymatic activity is greater (Legrand et al. 1987).

The class I chitinase genes have less than 3 kb in the open reading frame. They have no introns in bean chitinase gene CH5B (Broglie et al. 1989) and rice chitinase gene RCH10 (Zhu and Lamb 1991). They have one intron in an *Arabidopsis* chitinase gene

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(Samac et al. 1990), two introns in tobacco chitinase genes CHN14, CHN48, and CHN50 (Shinshi et al. 1990; van Buuren et al. 1992; Meins et al. 1992), and two introns in *Populus* chitinase gene *win6* (Davis et al 1991).

Class I chitinases may be encoded by small, tightly-linked families of genes. In *Populus*, three chitinase genes (*win6.2a-c*) were on a single 15 kb genomic clone (Davis et al. 1991). A fourth gene, *chiX*, was found between *win6.2b* and *win6.2c*. It is probably a chitinase pseudogene. In tobacco, seven different class I chitinase genes have been found (Fukuda et al. 1991; Hooft van Huidsduijnen et al. 1987; Neale et al. 1990; Shinshi et al. 1987, 1990). More than one class I chitinase gene has also been found in potato, pea, garlic, and bean. Based upon sequence comparisons and DNA blotting analyses, the class I chitinase genes CHN14' and CHN50 from *Nicotiana tabacum* may have come from *Nicotiana sylvestris*, and the *Nicotiana tabacum* chitinase genes CHN14 and CHN48 may have come from *Nicotiana tomentosiformis* (Fukuda et al. 1991; van Buuren et al. 1992).

Chitinase Localization and Function

Basic chitinases accumulate in the vacuoles of bean leaf cells (Boller and Vögeli 1984; Dore et al. 1991; Mauch and Stahelin 1989) and tobacco leaves (Keefe et al. 1990). Chitinases are targeted to vacuoles through the secretory pathway (Dorel et al. 1989). C-terminal extensions that occur in basic chitinases but not in acidic chitinases are responsible for vacuolar targeting (Chrispeels and Raikel 1992; Margis-Pinhiero et al. 1991; Neuhaus et al. 1991b). Chitinase released from the vacuoles of plant cells that lyse

as a result of fungal infection may cause the lysis of fungal hyphae (Keefe et al. 1990; Mauch and Stahelin 1989).

A pumpkin chitinase is excreted into liquid medium during cell culture (Esaka et al. 1990). Two tobacco basic chitinases accumulated in the extracellular space (Legrand et al. 1987). A barley chitinase (Swegle et al. 1990) and a pea chitinase (Vad et al. 1991) do not contain a C-terminal extension.

Class II Chitinases

Class II chitinases contain catalytic domains with high sequence homology to the catalytic domains of class I chitinases. They do not contain cysteine-rich domains. They are usually acidic. They may occur extracellularly in apoplastic washing fluids or in protoplast culture media.

The tobacco PR-P and PR-Q proteins are class II chitinases. They are pathogenesis-related proteins that accumulate in Samsun NN tobacco in response to infection by tobacco mosaic virus (TMV) (van Loon 1985). PR-P and PR-Q are serologically related (Hooft van Huidsduijnen et al. 1987; Legrand et al. 1987).

A substrate gel system was used to detect six proteins with chitinase activity in TMV-infected Zanthi-nc tobacco (Trudel et al. 1989; Trudel and Asselin 1989). Also, PR-O, an acidic β-1,3-glucanase, was present (Memelink et al. 1990).

Transcripts for PR proteins have been categorized into nine groups labeled A-I (Hooft van Huidsduijnen et al. 1986). PR-P and PR-Q mRNAs were categorized as class D, and class I chitinase mRNAs were categorized as class F (Memelink et al. 1990).

Gene and Protein Structure

Class II chitinases contain, in addition to a catalytic domain with homology to those of class I chitinases, a hydrophobic signal peptide of approximately 23 amino acids (Linthorst et al. 1990). A gene for PR-P, CHA18, as well as three genes encoding class I tobacco chitinases, contain pairs of intervening sequences in similar positions (Meins et al. 1992). An acidic tobacco chitinase (Linthorst et al. 1990) and the CHN17 basic chitinase clone (Shinshi et al. 1990) also contain two introns.

Chitinase Localization and Function

Class II chitinases are typically located extracellularly (Benhamou et al. 1990; Dore et al. 1991). These chitinases may release elicitors from invading fungal hyphae (Mauch and Stahelin 1989). These elicitors may induce plant genes for additional chitinases (Herget et al. 1990; Kombrink et al. 1988; Kurosaki et al. 1987b; Roby et al. 1987) and phytoalexins (Ham et al. 1991; Ken and Yoshikawa 1983; Keen et al. 1983).

When tested in combination with β–1,3 glucanases, the antifungal activities of tobacco class I chitinases were greater than those of tobacco class II chitinases, and only tobacco class I chitinases, but not tobacco class II chitinases, had antifungal activity alone without β–1,3 glucanases (Sela-Burlage et al. 1993). One possibility that might explain this difference in antifungal activity is the presence of chitin-binding domains in class I chitinases. Class II chitinases do not possess chitin-binding domains.

There is some evidence that a chitinase may have a role in plant development. A carrot chitinase rescued a temperature-sensitive carrot mutant that could not otherwise develop past the globular stage at the nonpermissive temperature (De Jong et al. 1992).

Serological Cross-Reactivities between Class I and Class II Chitinases

Rabbit polyclonal antisera raised against either class I or class II chitinases commonly cross-reacted with both class I and class II chitinases. For example, antibodies versus tobacco PR-P and PR-Q reacted with both class I and class II tobacco chitinases (Hooft van Huidsduijnen et al. 1987; Legrand et al. 1987). Antibodies versus a bean leaf class I chitinase adsorbed with six potato basic chitinases (Kombrink et al. 1988). An antibody versus a class II chitinase from tomato leaves adsorbed to three other tomato leaf chitinases (Joosten et al. 1989). Two of these were suggested to be class I, and one was suggested to be class II.

Class III Chitinases

The amino acid sequences of the catalytic domains of class III chitinases are different from those of class I and II chitinases. Some class III chitinases have a low level of amino acid sequence homology with chitinase D of *Bacillus circulans* (Watanabe et al. 1992). A class III chitinase from *Parthenocissus quinquifolia* (Virginia creeper) has strong homology with a lysozyme from *Hevea brasiliensis* (Bernasconi et al. 1987). An acidic cucumber chitinase has strong homology to this *Parthenocissus quinquifolia* class

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III chitinase (Métraux et al. 1988, 1989). This cucumber chitinase accumulates in the intercellular leaf fluid in response to infections by tobacco necrosis virus (TNV), *Pseudomonas lachrymans*, *Pseudoperonospora cubensis*, *Colletotrichum lagenarium*, or to salicylic acid. This cucumber class III acidic chitinase gene is one of three tightly linked chitinase genes with high sequence homology to each other (Lawton et al. 1994). Only one of the three genes is expressed.

An acidic chitinase gene from *Arabidopsis thaliana* was discovered and isolated with the help of degenerate oligonucleotide probes whose sequences were based on the conserved sequences of class III chitinases from cucumber, rubber, and Virginia creeper (Samac et al. 1990). This chitinase contains a 30 amino acid signal peptide.

A 27 kDa acidic chitinase from Azuki bean is a class III chitinase (Ishige et al. 1991). It accumulates intercellularly in response to ethylene.

The cDNA clones for two class III chitinases, one basic and one acidic, were isolated from tobacco (Lawton et al. 1992). Both of these chitinases were inducible by TMV infection. Three tightly linked chitinase genes were found in cucumber (Lawton et al. 1994). Only one of the genes appears to be expressed, and it is an acidic class III chitinase.

Class IV Chitinases

Three chitinases have been designated class IV (Collinge et al. 1993). They are the bean PR4 chitinase (Margis-Pinhiero et al. 1991), the rapeseed ChB4 chitinase (Rasmussen et al. 1992a), and the sugarbeet chitinase (Mikkelsen et al. 1992). These

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class IV chitinases are similar to class I chitinases in that that have chitin-binding domains, hinge regions, and catalytic domains. They have cysteines at seven of the eight conserved positions that occur in the chitin-binding domains of class I chitinases. However, there is not a high level of sequence homology between class I and class IV chitinases in the remainder of the domains. Class IV chitinases are smaller than class I chitinases, because they have four deletions, on in the chitin-binding domain, and three in the catalytic domain. They also lack the C-terminal extension found in most class I chitinases.

Chitinases Not Classified

Several chitinases do not seem to fit well in any of these four classes. Two potato basic chitinases may be extracellular rather than vacuolar (Kombrink et al. 1988). A basic chitinase from barley endosperm lacks a chitin-binding domain (Leah et al. 1987). The deduced protein sequences of two class I chitinase genes from poplar have net charges of -15 and -17 (Davis et al. 1991). Two acidic chitinases from garlic appear to be class I (Van Damme et al. 1993).

Urtica dioica agglutinin (UDA) is an 8.5 kDa protein in nettle rhizomes and flowers (Lerner and Raikhel 1992). It contains a C-terminal chitinase-like catalytic domain and two tandem N-terminal chitin-binding domains. Class I chitinases have only one chitin-binding domain. The chitin-binding and catalytic domains have a high level of amino acid homology with class I chitinases. The chitinase domain may be removed by

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post-translational processing. UDA is synergistic with chitinases in antifungal activity. UDA may crosslink chitin chains at hyphal tips (Broekaert et al. 1989).

An endochitinase from *Croix lachrymosa-jobi* is a 52.5 kDa dimer that also has insect α-amylase inhibitor activity (Ary et al. 1989). The dimer may be stabilized by disulfide bridges.

Not all plant chitinases clearly fit into one of the four classes (Shinshi et al 1990). Some plant chitinases are bifunctional with lysozyme or α-amylase inhibitor activity in addition to chitinase activity.

Comparison of Chitinases to Lysozymes

The substrate for chitinase is chitin, a polymer of N-acetylglucosamine. The substrate for lysozyme is a polymer of alternating NAG and N-acetylmuramic acid that occurs in bacterial cell walls. Some plant enzymes have both chitinase and lysozyme activities. Class III chitinases in particular, and some class I chitinases as well may have lysozyme activity (Mauch et al. 1988a). These bifunctional enzymes vary in substrate preference, and in pH and salt concentration optima (Bernasconi et al. 1987; Bernier et al. 1971; Howard and Glazer 1969), as well as in tolerance to a range of pH (Howard and Glazer 1969).

The optimal ionic concentrations for fig lysozyme (Glazer et al. 1969) and for cucumber seed basic chitinase (Majeau et al. 1990) is lower than that for hen egg white lysozyme (HEWL). Compared with HEWL, several of the bifunctional enzymes that have been characterized have lower substrate affinity for bacterial cell walls but higher

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substrate affinity for chitin or chitin oligosaccharides (Bernasconi et al. 1987; Boller et al. 1983; Glazer et al. 1969; Goward and Glazer 1969). N-Acetylglucosamine is an inhibitor of lysozymes (Bernier et al. 1971) including HEWL as well as bifunctional enzymes (Bernier et al. 1971; Boller et al. 1983; Glazer et al. 1969; Goward and Glazer 1969). Bernasconi et al. (1987) proposed that bifunctional enzymes could be classified as either chitinases or lysozymes depending upon their relative maximal chitinase and lysozymal activities.

Gene Expression and Enzymatic Activity

Chitinase gene expression and enzyme activity in plants vary in a developmental and organ-specific manner. Roots and flowers display higher activity than other organs, and some chitinase isoforms are present only in specific organs. The variation in chitinase activity may indicate a priority for protection of critical or easily infected tissues. The tissue specificity and localization of some chitinases, especially those with high levels of floral expression, suggest that these chitinases may have roles other than as antifungal agents. Chitinase activity and distribution of isoforms also may vary between species.

Roots often have chitinase activity. Up to 4% of the soluble protein in tobacco roots may be chitinases (Shinshi et al. 1987). Six acidic chitinases were found in the roots of 11-day-old cucumber seedlings, and two of the chitinases were unique to the roots (Majeau et al. 1990). The activities of the other four chitinases were higher in roots than in cotyledons or stems. Several basic chitinases not specific to roots were also found.

In the roots of tobacco, basic chitinase activity was higher than acidic chitinase activity (Trudel et al. 1989).

A positive correlation was found between the levels of chitinase activity in roots and the levels of transcriptional activity of chitinase genes. Northern blot results demonstrated that a class I tobacco chitinase was expressed at a higher level in roots than in leaves, internodes, or flowers (Neale et al. 1990). Class I chitinase mRNA was more abundant in roots than in other tissues examined, but class III acidic chitinase mRNA was not detected in *Arabidopsis* roots (Samac et al. 1990). A β-glucuronidase (GUS) gene controlled by an *Arabidopsis* class III chitinase promoter resulted in a high level of expression of GUS mRNA in both *Arabidopsis* and tomato roots (Samac and Shah 1991). It is probable that there may be differences between the half lives of the GUS message and the class III chitinase message.

Chitinase may kill or slow the growth of soil-borne fungal and/or bacterial root pathogens. Roots may have a high constitutive level of chitinase activity. Levels of chitinase activity in tobacco seedling roots were similar in plants grown either axenically or in a greenhouse (Shinshi et al. 1987). The high constitutive level of chitinase activity in roots may be an effective defense against some soil-borne pathogens.

Chitinase activity is higher in flowers than in most other organs. The level of chitinase activity varies depending on the flower age. Class I chitinase mRNA in tobacco flowers increases with flower maturity (Neale et al. 1990). In tobacco, the level of expression of class I chitinase mRNA was highest in roots, and second highest in flowers (Neale et al. 1990). Chitinase levels may vary between different floral organs. Tobacco flowers contain at least five different acidic chitinases, and the only organ with more was

apical leaves (Trudel et al. 1989). Two acidic chitinases have their highest levels of expression in sepals and petals. Several chitinases were found in sepals, petals, pistils, and stamens (Trudel et al. 1989). Trudel et al. (1989) found the highest level of chitinase activity in sepals. In tobacco, PR-P and PR-Q were found in sepals, ovaries, and anthers, but not in petals, styles, and stigmas. Elicitors caused an increase in the levels of tobacco PR-P and PR-Q in styles (Lotan et al. 1989). Transgenic *Arabidopsis* plants containing a class III chitinase gene promoter controlling a GUS gene expressed GUS activity in anthers, but not in other flower tissues (Samac and Shah 1991). In cucumber, class III chitinase mRNA accumulated only in petals, sepals, and stamens, and the highest levels were found just prior to senescence in the open-flower stage (Lawton et al. 1994). One chitinase is expressed only in the stigma of petunia flowers, and its activity increases five hundred percent following anther dehiscence. Possibly this chitinase may be involved in the germination of pollen grains (Leung 1992). There has been some speculation that chitinase may play a role in sexual reproduction. This speculation is based upon the differential accumulation of chitinase seen in the flowers of some species, however, a specific role for chitinase in sexual reproduction has not been determined, and the differential accumulation of chitinase in flowers seems to be also consistent with the priority of protecting valuable tissues that are especially vulnerable to pathogens.

There does not seem to be any particular class of chitinase that is specific to any particular flower organ. Both basic and some acidic chitinases may have activity in all parts of tobacco flowers, but some acidic chitinases are restricted to particular floral organs.

Chitinases have been frequently purified from leaf tissues. The levels of chitinase and chitinase mRNA in leaves varies, depending on age. In uninduced plants, chitinase activity is usually lower in leaves than in roots or flowers. Senescent tobacco leaves had no detectable acidic chitinase activity, but apical leaves had at least seven chitinases (Trudel et al. 1989). Senescent and apical leaves had similar levels of basic chitinase activity (Trudel et al. 1989). Higher levels of class I chitinases and chitinase mRNA were found in basal, older tobacco leaves compared with the levels found in apical, younger leaves. For example, almost 20-times as much class I tobacco chitinase was present in the third leaf as was in the thirteenth leaf (Shinshi et al. 1987). A class I tobacco chitinase mRNA was present at higher levels in the first two leaves than in the sixth leaf (Neale et al. 1990). The levels of chitinase mRNA rose steadily as the leaves aged.

Fruits and seeds also contain chitinases. Class I chitinases were purified from wheat germ (Molano et al. 1979), barley seeds (Kragh et al. 1990; Leah et al. 1987, 1991),

and pea pods (Mauch et al. 1988a). The pea pod chitinases, CH1 and CH2, accumulate as the pods mature. The level of CH1 increased upon induction with *Fusarium solani* f. sp. *phaseoli*, but the level of CH2 did not change when induced with the same fungus. In mature uninduced pods the level of CH2 was higher than that of CH1 (Mauch et al. 1988a).

Mature seed may contain no detectable levels of chitinase. A class II barley seed chitinase mRNA accumulated during seed development but persisted for only four days after germination (Leah et al. 1991). A basic chitinase in cucumber seeds disappeared

almost completely within three days postgermination (Majeau et al. 1990). Barley Chit A mRNA was present in shoots, roots, and seeds, and the levels were highest in imbibed seeds (Huynh et al. 1992). Seeds are required to perpetuate many plant species, and therefore they warrant protection against fungal pathogens. Seed-specific chitinases may be developmentally regulated.

Stems generally are low in chitinase activity and gene expression relative to those found in other tissues. In cucumber, the level of class III chitinase mRNA increases in basal stems during flowering (Lawton et al. 1994). Lower levels were observed in middle stems and none in top stems. This mRNA was not detectable in stems before flowering.

Rhizobium meliloti is a symbiotic bacterium that produces nodulation (Nod) factors that induce nodulation in alfalfa. These Nod factors contain chitin (Lerouge et al. 1990), therefore chitinase may be involved in this symbiosis. The development of nodules is directed by Nod factors, which may mimic an uncharacterized plant hormone. In this proposed model, there would have to be an endogenous substrate for chitinase. Butanol extracts of *Lathyrus* plants contain lipophilic compounds that migrate on reverse phase thin layer chromatography (TLC) similarly to rhizobial Nod factors. Treatment of these compounds with an acidic chitinase that can degrade Nod factors reveals digestion products similar to Nod factor digestion products, suggesting that there may be a substrate for chitinase in the plants.

It is not known whether there is an organ-specific function for any class of chitinase, although several chitinases are organ-specific in distribution. Class I and class II chitinases are present in many tissues in at least some stage of development, and their

activities are inducible by infection by plant pathogens. Several chitinases occur constitutively in seeds and roots, and may be defensive proteins against fungal colonization by plant pathogens. Some plants have developmentally regulated chitinases present in flowers or fruits in addition to inducible chitinases. There is some speculation that these chitinases, for example the carrot embryo class II chitinase, may contribute to the development of the embryo or other reproductive structures rather than being antifungal proteins, but there is no direct evidence for this, and an antifungal role seems more likely (De Jong et al. 1992).

Chitinase Gene Induction

The inducibility of chitinase genes is well documented. Inducing agents include fungal, bacterial, and viral infections, wounding, ethylene, fungal and plant cell wall components (elicitors), salicylic acid (SA), and heavy metal salts. Auxins and cytokinins modulate the induction of chitinase genes. As much as a 600-fold increase in chitinase activity could be induced by infection (Metraux and Boller 1986), and chitinase mRNA was increased by as much as 100-fold by ethylene induction (Broglie et al. 1986).

Infection by Pathogens

The infection of a host plant by a pathogen initiates a complex series of events that includes the elicitation of defense responses. Infections may cause a rapid and enduring change in the soluble proteins observed on polyacrylamide gels (Coutts 1978;

Majeau et al. 1990; Metraux and Boller 1986; Trudel et al. 1989; van Loon 1985). Chitinase activity can be induced in plants by infection with fungi, bacteria, or viruses. Infections may induce an increase in mRNA levels (Roby and Esquerre-Tugaye 1987b; Roby et al. 1990). A portion of the response of a plant to infection may be nonspecific because infections by different pathogens can induce production of the same PR proteins. The same tobacco chitinases and β-1,3-glucanases may be induced by infection with either fungi, bacteria, or TMV (Meins and Ahl 1989). There may be a gradient in level of induction declining with greater distance from the point of infection (Dore et al. 1991). The *Colletotrichum lagenarium*-infected areas of cucumber first leaves had a 600-fold increase in chitinase activity relative to first leaves from uninfected plants (Metraux and Boller 1986). Uninfected areas of the same infected leaves had only a 60-fold increase in the level of chitinase induction. Second leaves from the same plant had a smaller level of chitinase induction.

Chitinase activity remained at an elevated level for several days after induction by infection. Elevated chitinase activities were induced in cucumber cotyledons infected with *Colletotrichum lagenarium*, and the maximum activity level occurred at seven days postinfection (Roby et al. 1991). In melon, the maximum level of induction of mRNA coding for two chitinases occurred at 5.5 days postinfection, and chitinase mRNA synthesis returned to near control levels at 6.5 days postinfection (Roby and Esquerre-Tugaye 1987b). Apoplastic chitinase activity increased for 10 days postinfection, then plateaued in tomato plants infected by *Cladosporium fulvum* (Joosten et al. 1989). Chitinase mRNA levels may increase in response to pathogen infections. TMV infection of tobacco leaves induced an increase in the local accumulation of chitinase mRNA, and

maximum levels occurred three days postinfection (Brederode et al. 1991). Tobacco plants infected with *Phytophthora parasitica* had a 230-fold increase in chitinase mRNA levels within seven days, and the majority of the increase occurred between the fourth and seventh days (Meins and Ahl 1989).

Induction of a chitinase promoter was observed with a chitinase promoter linked to a reporter gene (Roby et al. 1990). Tobacco was transformed with the bean basic chitinase 5B promoter linked to the *Escherichia coli gus A* gene. The leaves of these transgenic plants were inoculated on one-half with *Botrytis cinerea*. A high level of GUS activity was observed at the periphery of the infection sites in the inoculated half of the leaves. There was a smaller increase in GUS activity in the uninoculated halves of the leaves.

The *Arabidopsis* class III chitinase promoter controlled the expression of GUS in transgenic *Arabidopsis* plants. GUS activity was detected in mesophyll cells near *Rhizoctonia solani* infection sites (Samac and Shah 1991).

The pathogenicity and compatibility of an infection may be factors in determining the levels of induction of chitinase activity. No induction of chitinase activities was observed upon compatible *Puccinia* infections of oat, but incompatible *Puccinia* infections resulted in rapid inductions of chitinase activities (Fink et al. 1990). A pathogen and a nonpathogen of pea were similarly inhibited by a pea chitinase *in vitro*, suggesting that defensive mechanisms in addition to chitinase may have been important in this plant-pathogen interaction (Mauch et al. 1988b).

The time course of the induction of chitinase and the levels of chitinase induction may vary in French bean leaves infected with virulent or avirulent races of *Pseudomonas*

syringae pv. *phaseolicola* (Vöisey and Slusarenko 1989). Infection by an avirulent race resulted in increases in chitinase leaf activity within six to nine hours post-infection, and chitinase activity rose to 19 times the level in uninfected leaves by 48 hours post-infection. Infection by the virulent race led to increases in chitinase leaf activity 24 hours post-infection, and by 48 hours post-infection, the levels of chitinase leaf activity were only 75% of the levels in plants infected by the avirulent race.

After inoculation of tobacco leaves with compatible, incompatible, or avirulent strains of *Pseudomonas solanacearum*, there were no significant differences in the accumulation of chitinase mRNA as detected by the labeled probe, pCHN50 (Godiard et al. 1990, Shinshi et al. 1987). However, the infiltration inoculation technique used resulted in an equivalent chitinase response when water only was used as the inoculum; so it may be that the inoculation technique caused wounding that resulted in the observed response.

Heat-killed virulent and avirulent *Pseudomonas syringae* cells induced chitinase activity in French bean leaves (Vöisey and Slusarenko 1989). Induction of phytoalexin synthesis and the hypersensitive response required a living pathogen. Components of fungal cell walls may be inducers of chitinase.

Chitinase induction in response to pathogen infection may be localized or systemic (Uknes et al. 1992). Inoculation of cucumber first leaves with tobacco necrosis virus (TNV) resulted in resistance to infection of second leaves by *Colletotrichum lagenarium* 48 hours later. There was a positive correlation between induced resistance and levels of chitinase activity (Métraux and Boller 1986). In tobacco, resistance against blue mold infection was correlated with the accumulation of chitinase (Tuzun et al.

1989). However, the blue mold pathogen lacks chitin in its cell walls; so chitinase may not have been involved in the resistance. Chitinase accumulation was not correlated with resistance to tobacco mosaic virus (TMV) in tobacco (Ye et al. 1990).

Arabidopsis plants that acquired systemic resistance in response to the chemical inducers 2,6-dichloroisonicotinic acid or salicylic acid (SA) had increased levels of the proteins PR-1, PR-2, and PR-5, but chitinase levels were not elevated (Métraux et al. 1991; Uknes et al. 1992). Rice plants induced by infection *with Pseudomonas syringae* pv. *syringae* developed systemic resistance to *Pyricularia oryzae*, but there was no systemic induction of chitinase activity (Smith and Métraux 1991).

There may be a lack of systemic induction of chitinase in tobacco plants susceptible to certain fungal pathogens (Meins and Ahl 1989). In tobacco plants (Havana 425), there was local but not systemic induction of basic chitinase and basic chitinase mRNA in response to infection by *Pseudomonas syringae* or *Phytophthora parasitica* var. *nicotianae*.

The accumulation of mRNA for class I and class II chitinases was induced by infections of tobacco by TMV or yellow spot mosaic virus (Hooft van Huidsduijnen et al. 1987; Legrand et al. 1987; Memelink et al. 1990). A class I chitinase accumulated only locally, but a class II chitinase accumulated both locally and systemically in tobacco (Brederode et al. 1991; Ward et al. 1991). This response occurred only in plants that possessed the dominant N gene, and these plants exhibited a hypersensitive response to infections. Chitinase has no known anti-viral activity, and its induction as a response to viral infection may be explained by saying that it is part of a general hypersensitive

response (Vögeli-Lange et al. 1988). Two classes of chitinase are induced in cucumber in response to infections by *Colletrorichum lagenarium* (Métraux and Boller 1986).

Chitinase and β-1,3-glucanase are induced in tobacco (Meins and Ahl 1989; Pan et al. 1991) and maize (Cordero et al. 1994) in response to infections. In tobacco infected with TMV, the levels of induction of both chitinase and β-1,3-glucanase is higher in Samsun NN plants compared with the levels of induction in Samsun nn plants (Vögeli-Lange et al. 1988).

Three acidic chitinases and a single β-1,3-glucanase were induced in maize. The accumulation of these proteins differed between radicles, coleoptiles, and embryo tissues of seedlings (Cordero et al. 1994). Both chitinase and β-1,3-glucanase were induced in tomato plants infected with *Cladosporium fulvum.*

Colonization of soybean roots by *Bradyrhizobium japonicum* does not induce chitinase activity in the central region of nodules (Stahelin et al. 1992). Chitinase activity is induced in the cortex of nodules.

The mycorrhizal organism, *Glomus versiforme*, induces chitinase activity in leek (*Allium porrum*) (Spanu et al. 1989). Once the mycorrhizal colonization of roots is completed after 30-90 days, the chitinase levels drop to less than the levels in non-colonized plants. This fungus has proteins and carbohydrates on the hyphal surface that protect the chitins in the cell walls from chitinases. Perhaps the reductions in chitinase levels may be related to the observation that auxins and cytokinins may suppress chitinase levels (Shinshi et al. 1987).

Induction of Chitinase by Ethylene

The biosynthesis of ethylene involves the conversion of *S*-adenosyl methionine (SAM) to the intermediate, 1-aminocyclopropane-1-carboxylic acid (ACC) catalyzed by ACC synthase (Adams and Yang 1979). ACC is converted to ethylene by an ethylene-forming enzyme (EFE). The conversion of SAM to ACC may be a rate-limiting step in ethylene biosynthesis (Boller 1988). An increase in available ACC was required to obtain an increase in ethylene in TMV-infected tobacco (de Laat and van Loon 1983b).

Ethylene is involved in the wound response of plants. The genes for the enzymes 1-phenylalanine ammonia-lyase (PAL; EC 4.3.1.4) and 4-coumarate:CoA ligase (EC 6.2.1.12) in the phenylpropanoid pathway and chalcone synthase in the flavonoid glycoside pathway are induced by ethylene. Also induced by ethylene are the genes for hydroxyproline-rich glycoprotein (Ecker and Davis 1987), a component of plant cell walls (Esquerré-Tugayé and Lamport 1979). In addition, ethylene induces the genes for several unidentified proteins (Ishige et al. 1991). Ethylene also induces the genes for class I (Boller et al. 1983) and class III (Métraux and Boller 1986) chitinases.

After treatment with elicitors, ethylene production increases rapidly (Roby et al. 1985, 1986; Toppan and Esquerré-Tugayé 1984). Ethylene levels reach a maximum five to seven hours after treatment of pea pods with fungal elicitors, then fall to near-initial levels after 15 hours (Mauch et al. 1984). Ethylene production increased for six hours after treatment of parsley cells with *Phytophthora megasperma* elicitor (Chappell et al. 1984). ACC synthase was induced about ten times in the first hour after treatment of parsley cells with *Phytophthora megasperma* elicitor.

In tomato suspension-cultured cells, a yeast cell wall preparation resulted in the stimulation of the activity of ACC synthase and EFE (Felix et al. 1991). Ethylene production may be induced by endogenous elicitors (Roby et al. 1985). Pear cell wall material added to a pear cell culture induced ethylene production for two hours (Tong et al. 1986). Ethylene production was induced by *Trichoderma viridae* xylanases (Fuchs et al. 1989).

Ethylene production may be induced by viral infections (de Laat and van Loon 1982). Ethylene production may be associated with a hypersensitive response (de Laat and van Loon 1983b). Infection of hypersensitive Samsun NN tobacco leaf discs with TMV induces an increase in ethylene production.

Ethylene production may be induced by wounding caused by nematode feeding (Glazer et al. 1986). Aminoethoxyvinylglycine (AVG) is an inhibitor of endogenous ethylene biosynthesis, and it blocks elicitor-induced ethylene production (Chappell et al. 1984).

Exogenously applied ethylene induces transcription of chitinase genes (Table 6). In rice suspension cells, chitinase was induced four fold by ethylene (Nishizawa and Hibi 1991). The induction was 36-fold in bean leaf tissue (Boller et al. 1983). The maximum induction of chitinase mRNA occurred 30 hours after treatment with ethylene, and maximum chitinase activity occurred 48 hours after ethylene treatment (Boller et al. 1983; Boller 1988).

Deletion analysis of a bean chitinase (CH5B) promoter and production of CH5B mRNA in transgenic tobacco was used to identify promoter regions involved in ethylene induction (Broglie et al. 1989). In another study of ethylene induction, a bean CH5B

promoter fused to a GUS gene was used in a bean protoplast transient assay system (Roby et al. 1991). Results were obtained that indicated a region of the promoter that may contain an element responsive to ethylene induction.

Ethylene induces β-1,3-glucanase activity in the primary leaves of bean seedlings (Vögeli et al. 1988). A model was proposed that involved the action of antifungal proteins on hyphal cell walls containing chitin and β-1,3-glucan.

Ethephon, a plant growth regulator, induced the accumulation of mRNA encoding acidic and basic tobacco chitinases (Brederode et al. 1991). Fungal elicitors stimulated ethylene production in melons, and AVG inhibited ethylene production in melons (Roby et al. 1986). AVG plus fungal elicitor-treated leaves had chitinase activity levels intermediate between those in leaves treated only with fungal elicitors and those in untreated leaves. The presence of AVG depressed chitinase activities (Boller 1988).

Freshly excised, split immature pea pods were treated with AVG, then chitinase activity was induced by various elicitors including autoclaved *Fusarium solani* spores, chitosan (a polymer of β-1,4-D-glucosamine), auxin, and $CdCl_2$ (Mauch et al. 1984). AVG reduced ethylene production in pods by 80-85% compared with the production in pods treated with any of these elicitors except auxin but not treated with AVG, yet the induced chitinase activities were reduced by only about 15% by AVG. Induction of chitinase production is not linearly correlated with ethylene concentration (Boller et al. 1983). Induction of chitinase by elicitors occurred even when AVG reduced ethylene concentrations to levels lower than those in non-induced pea pods (Mauch et al. 1984). *Rubus hispidus* calli produced only low amounts of ethylene and exogenously applied ethylene did not induce chitinase activity, but elicitors did induce chitinase activity,

suggesting that ethylene may not be the only chemical signal that can induce chitinase activity (Bernasconi et al. 1986).

Elicitors of Chitinase

Wounded fungal and plant cells may release elicitors of chitinase such as β-1,3-glucans, chitin oligomers, chitosan, plant α-1,4-D-oligogalacturonides, and xylans (Ryan 1987).

Extracellular β-1,3-glucanases may release polysaccharides to signal the presence of fungal cells (Maunch and Stahelin 1989). The fungal cell wall material, β-1,3-glucan, has chitinase elicitor activity (Herget et al. 1990; Keen and Yoshikawa 1983; Kombrink et al. 1988). Glucans with 3-, 6-, and 3-6 linkages may also be chitinase elicitors (Ryan 1987).

Chitin oligosaccharides can elicit chitinase activity (Kurosaki et al. 1987b; Roby et al. 1987). In melon plants, the maximal induction of chitinase activity occurred with chitin oligosaccharides of 6-9 residues in length (Roby et al. 1987). Chitinase activity was induced in a carrot cell culture by inoculation with a solution of *Chaetomium globosum* mycelial cell walls.

Seeds of black pine, soybean, radish, rice, and milk vetch had increased levels of chitinase when coated with chitosan derivatives (Hirano et al. 1990). Rice suspension cultures had increases in chitinase production when induced with chitosan (Masuta et al. 1991). In carrot suspension cultures, chitosan, crude mycelial walls, or purified chitin elicited chitinase activity (Kurosaki et al. 1987a). The induced chitinase was detected

extracellularly (Kurosaki et al. 1987b). In pea tissue, chitosan released by chitinase and β-1,3-glucanase initiated a chain of cytological changes local to the site of infection by *Fusarium solani* (Hadwiger et al. 1988). Chitosan elicited systemic changes in tomato plants, resulting in increased resistance to *Fusarium oxysporum* f. sp. *radicis-lycopersici.*(Benhamou and Thériault 1992). Fungal growth was restricted to the epidermis and cortex, and hyphae had altered cell wall morphologies and cytoplasmic changes.

Pectic fragments of plant cell wall material may be elicitors of chitinase in pea leaves (Hadwiger and Beckman 1980). Phytoalexins (Davis et al. 1986) and proteinase inhibitors (Ryan 1987) were elicited by α-1,4-D-oligogalacturonides. Xylanase from *Trichoderma viridae* elicited PR-P and PR-Q in tobacco leaves (Lotan and Fluhr 1990). Xylose chains released by xylanase from tobacco cell walls could be inducers of PR-P and PR-Q (Eda et al. 1976).

Phytophthora megasperma elicitor or yeast extract induced an increase in chitinase mRNA levels in peanut (Herget et al. 1990). Chitinase mRNA was inducible in bean (Hedrick et al. 1988). In bean protoplasts, a bean chitinase promoter fused to a GUS gene was inducible by elicitors (Roby et al. 1991).

Chitinase activity peaked by 30 hours postinjection in melon seedling injected under the epidermis with chitin oligosaccharides (Roby et al. 1987). Bean chitinases may be stored in vacuoles (Boller and Vögeli 1984).

Ethylene induced four chitinases (28.7, 23.0, 17.5, and 15.5 kDa) in carrot cell cultures (Kurosaki et al. 1989). Fungal mycelial walls induced four chitinases (29.0, 20.5, 17.5, and 12.5 kDa) in carrot cell cultures (Kurosaki et al. 1986).

Induction of Chitinase by Wounding

Mechanical wounding of lower leaves with pliers induced a systemic accumulation of chitinase-like mRNAs in *Populus* (Bradshaw et al. 1991; Parsons et al. 1989).

Chitinase enzyme (Bronner et al. 1991; Ban Damme et al. 1993) and chitinase mRNA (Bradshaw et al. 1991; Brederode et al. 1991; Hedrick et al. 1988; Mehta et al. 1991; Parsons et al. 1989) were induced by wounding. Wounding of tomato pericarp resulted in chitinase induction lasting less than four hours (Mehta et al. 1991). Excision of bean hypocotyls resulted in accumulation of chitinase mRNA for up to 25 hours (Hedrick et al. 1988). Accumulations of basic tobacco chitinase mRNA peaked at approximately 27 hours (Brederode et al. 1991). Gall mite feeding on *Solanum dulcamara* L. resulted in chitinase inductions as part of a hypersensitive response lasting over five days (Bronner et al. 1991). Cutting tobacco leaves induced basic chitinases, PR-P, and PR-Q. Accumulation of chitinases resulted after induction with ethylene, TMV infection, or UV light (Brederode et al. 1991).

Induction of Chitinase by Organic Molecules and Salts

Salicylic acid (SA) induced chitinase activity in cucumber (Métraux et al. 1989), rice suspension cultures (Nishizawa and Hibi 1991), tobacco (Linthorst 1991), and sunflower leaves (Jung et al. 1993). SA induced GUS activity controlled by a chitinase promoter in transgenic *Arabidopsis* plants (Samac and Shah 1991). In tobacco, SA

induced mRNAs involved in systemic acquired resistance (SAR) (Gaffney et al. 1993), including those for PR-P, PR-Q, and acidic and basic class III chitinases (Lawton et al. 1994; Ward et al. 1991). The amino acid analog, α-aminobutyrate, induced PR-P and PR-Q in tobacco leaf discs (Lotan and Fluhr 1990).

The inorganic salt, K_2HPO_4, induced chitinase activity in cucumber (Irving and Kúc 1990). Sodium chloride, nickel chloride, barium chloride, and selenium oxide induced expression of cucumber acidic and basic endochitinases (Métraux and Boller 1986). Cr_2O_4, but not chromium chloride, induced chitinase activity in barley or rapeseed (Jacobsen et al. 1992). Mercuric chloride or brome mosaic virus induced the activity of a class I chitinase in maize (Nasser et al. 1988, 1990).

Induction of Chitinase by Growth Regulators

Auxins such as indole acetic acid (IAA) are plant hormones that are involved in the maintenance of apical dominance, xylem differentiation, and cell elongation (Kuhlemeier et al. 1987). Cytokinins such as kinetin and zeatin are plant hormones that induce cytokinesis and are involved in cell division and tissue differentiation (Horgan 1987). IAA increases production of ethylene in tomato roots, possibly by increasing synthesis of ACC catalyzed by ACC synthase (Glazer et al. 1986).

Auxin (IAA) increased chitinase levels in bean leaves in cultivars resistant or susceptible to *Colletotrichum lindemuthianum*.(Hughes and Dickerson 1991). Elicitors also increased chitinase levels in bean leaves, but IAA in addition to elicitors resulted in chitinase induction no higher than that obtained with elicitors alone. AVG blocked IAA-

mediated induction of chitinase, suggesting that IAA-mediated induction of chitinase may involve ethylene.

Tobacco tissue was cultured on media containing two hormones, auxin (α-naphthalene) and cytokinin (kinetin) for seven days (Shinshi et al. 1987). Then the tissues were transferred to media containing either no hormone, auxin only, cytokinin only, or both hormones. The tissues subcultured on hormone-free medium produced levels of chitinase five times the amount that they produced during the primary culture on media containing both hormones. Tissues on medium with only one hormone, either auxin or cytokinin, produced four times the amount of chitinase compared with the amount that they had produced in the primary culture on media with both hormones. Tissues subcultured on medium with both hormones produced an amount of chitinase equal to the amount that they had produced during the prior culture on medium with both hormones. One possible interpretation of these results might be that the tissues produced chitinase in response to stress. A change in hormones in the subculture medium compared to what was present in the primary media caused stress to the tissues, resulting in increased chitinase production.

In *Picea abies*, a mixture of four hormones (2,4-dichlorophenoxyacetic acid, IAA, 1-naphthylacetic acid, and kinetin) depressed chitinase levels in suspension cells induced by cell wall fraction from *Amanita muscaria* (Sauter and Hager 1989). Uninduced *Picea abies* suspension cells also had depressed chitinase levels when these four hormones were in the medium.

Overproduction of cytokinin in tobacco plants transformed with a cytokinin gene (T-cyt from *Agrobacterium tumefaciens*) resulted in the induction of six mRNAs, one of

which encoded a class I chitinase (Memelink et al. 1987, 1990). Untransformed tobacco shoots cultured on medium with kinetin produced more chitinase mRNA than shoots on medium without kinetin.

Some of these results may seem contradictory, however, it may be that there is an optimal level of hormones, and any change from that may have unpredictable results, possibly either elevating or depressing levels of chitinase (Hughes and Dickerson 1991).

Effects of Environmental Conditions on Chitinase Production

There was in increase in chitinase activity in temperature-stressed garlic (Van Damme et al. 1993). Photoperiod induction of flowering in tobacco resulted in a decrease in root chitinase levels (Neale et al. 1990).

Signaling

Membrane fractions of soybean roots bound tritiated β-1,3-glucan (Schmidt et al. 1987). Pronase eliminated the binding, suggesting that the binding site was a protein. Possibly this protein was a β-1,3-glucan receptor that was involved in a signaling pathway.

Cultured tomato cells reacted to chitin and xylanase with a change in the culture medium pH (Felix et al. 1993). This may have indicated the cellular perception of signaling compounds.

Plant hormones such as ethylene and auxin may have response cascades mediated by membrane-bound or soluble binding proteins (Jones 1995). Protein phosphorylation may be involved in the accumulation of chitinase in tobacco leaves in response to ethylene (Raz and Fluhr 1994). Two inhibitors of mammalian protein kinase C, H-7 and K-252a, inhibited the accumulation of chitinase in tobacco leaves. Inhibition of endogenous phosphatases resulted in the increased accumulation of chitinase and other pathogenesis-related (PR) proteins. Phosphatases and kinases may be involved in the ethylene induction of chitinase. The induction of chitinase by xylanase was not affected by protein kinase inhibitors.

Summary of Chitinase Inducers

A plant under attack by pathogenic fungi or phytophagous insects may utilize several signal molecules to activate its defensive mechanisms. These signal molecules may include ethylene, elicitors from fungal and plant cell walls, growth regulators, and salicylic acid (SA).

Ethylene, an inducer of chitinase activity, is induced by pathogens and elicitors. Elicitors of chitinase activity may be released from fungal cell walls by chitinases and β-1,3-glucanases or from plant cells by xylanases and galacturonases. During pathogen attack, released elicitors may induce ethylene production, elicitors and ethylene may induce chitinase activity, and chitinase may release additional elicitors. During certain developmental processes including flower and fruit formation, ethylene production is

increased. This increased ethylene production may result in the induction of chitinase, which may provide increased protection of especially valuable tissues.

Elicitors may provide local signals to the presence of fungal infections, while salicylic acid (SA) may be a systemic signal. In rice suspension cultures, chitosan induced chitinase activity and a hypersensitive response (Masuta et al. 1991). The addition of catalase or free radical scavengers reduced or eliminated the hypersensitive response. The production of O^{2-} and H_2O_2 are involved in the hypersensitive response in tobacco (Keppler et al. 1989) and soybean (Haga et al. 1986). The addition of SA with chitosan reduced the hypersensitive response. In a proposed model, the cells nearest the site of pathogen infection might exhibit a hypersensitive response, while cells slighter farther from the site of infection would produce SA to induce a systemic response. Cells farther from the site of infection would produce PR proteins. Class II chitinases of tobacco may not kill fungi directly, but they may release elicitors from fungi (Sela-Burlage et al. 1993).

Heavy metals and salts may induce chitinase activity. Possibly these heavy metals and salts are toxic to plants, killing cells and releasing elicitors from plant cell walls. Crushing by pliers of lower leaves of poplar trees systemically induced two chitinase encoding mRNAs, *win6* and *win8* (Parsons et al. 1989). UV light induced the accumulation of PR-P and PR-Q mRNAs in tobacco (Brederode et al. 1991).

Chitinase Transgenic Plants

Although some have speculated that chitinase may have an endogenous function (De Jong et al. 1992), there is convincing evidence that chitinases are antifungal proteins. Chitinases are induced by infection by pathogens or by pathogen cell wall material, and chitinases are involved in pathogenesis responses by plants. Chitinases hydrolyze chitin, a polysaccharide found in fungal cell walls but not in plants, and purified chitinases lyse fungal hyphae *in vitro* (Arlorio et al. 1992; Boller et al. 1983; Dunsmuir and Suslow 1989; Mauch et al. 1988b). The antifungal activity of wheat germ agglutinin may be caused by chitinase contamination (Schlumbaum et al. 1986).

A tobacco hybrid that expresses high levels of chitinase, *Nicotiana glutinosa* X *Nicotiana debneyi*, has equal or greater resistance compared with either parent against fungal and bacterial pathogens (Ahl Goy et al. 1992). Chitinase genes have been expressed from a rubisco small subunit promoter (Lund et al. 1989) and from the cauliflower mosaic virus (CaMV) 35S promoter.

Transgenic tobacco with the bean CH5B chitinase gene controlled by the CaMV 35S promoter were produced (Broglie et al. 1989). These transgenic plants expressed chitinase at levels up to 50 times higher than in control untransformed plants. Grown in soil inoculated with the root pathogen *Rhizoctonia solani*, transgenic plants expressing elevated levels of chitinase grew faster, lost less root weight, and had lower seedling mortality compared with control untransformed tobacco seedlings. *Rhizoctonia solani* that infected roots of canola plants transformed with the same CaMV 35S-CH5B chitinase construct had hyphae that appeared physically damaged and had increased

vacuolization and cell lysis compared with fungi that infected roots of untransformed control plants, and the extent of the infections was reduced to within the cortex (Benhamou et al. 1993).

Bacterial chitinase has been expressed in plants (Lund et al. 1989; Suslow et al. 1988). A *Serratia marcescens* exochitinase gene (ChiA) was expressed in tobacco controlled by CaMV 35S promoter, *Agrobacterium tumefaciens* nopaline synthase (nos) promoter, petunia ribulose bisphosphate carboxylase (rubisco) promoter, or petunia chlorophyll *a/b* binding protein promoter (Jones et al. 1988; Lund et al. 1989). Transgenic tobacco expressing ChiA had increased resistance to *Alternaria longipes* evidenced by reduced necrotic lesions and chlorosis during infections. The resistance faded as the plants matured (Suslow et al. 1988).

Hyphae of *Trichoderma viridae* are susceptible to lysis by a pea basic chitinase (Mauch et al. 1988b). Other pathogens, including *Fusarium solani*, *Alternaria solani*, and *Fusarium oxysporum*, were susceptible only to a combination of chitinase and β-1,3-glucanase. A class I chitinase from *Arabidopsis* was active against *Trichoderma reesei* but not against *Fusarium oxysporum*, *Alternaria solani*, *Sclerotinia sclerotiorum*, *Gaeummanomyses graminis*, or *Phytophthora megasperma* (Verburg and Huynh 1991).

Not all plant genetically engineered with chitinase genes had increases in resistance to fungal pathogens. Transgenic tobacco plants with the SE2 class III chitinase gene from sugarbeet were not resistant against the frog eye disease pathogen, *Cercospora nicotianae* (Nielsen et al. 1993). *Nicotiana sylvestris* plants transgenic with a class I tobacco chitinase controlled by the CaMV 35S promoter had inconsistent resistance against *Cercospora nicotianae* (Neuhaus et al. 1991a).

Different pathogens may be differentially susceptible to various chitinase species. The effectiveness of some chitinases against certain fungal pathogens may depend upon synergistic activity with β-1,3-glucanase. The barley chitinase, Chit A, had ten times greater activity than Chit B against *Fusarium oxysporum*, *Alternaria solani*, and *Trichoderma reesei* (Huynh et al. 1992). Chitinases from barley, wheat, and maize had greater activity than chitinases from *Serratia marcescens* and *Streptomyces griseus* against *Trichoderma reesei* and *Phycomyces blakesleeanus* (Roberts and Selitrennikoff 1988). It seems that various chitinases differ in their substrate specificity, and various fungi differ in their susceptibility to specific chitinases. This may complicate the selection of a chitinase gene for use in transgenic plant production for resistance to pathogenic fungi, but it may also open a large number of possibilities for producing plants with resistance to various fungal pathogens.

Although there has been extensive research into the involvement of plant chitinases in resistance to fungal pathogens, there has been considerably less research into the possibility that plant chitinases may be involved in resistance against phytophagous insects. The main focus of this research project was to characterize and study a chitinase gene from elm. This chitinase gene was used to transform tobacco and creeping bentgrass. The transgenic creeping bentgrass expressing elm chitinase was screened and found to have an elevated level of resistance against the turfgrass pathogen, *Rhizoctonia solani*. Screening of the turfgrass was done to determine if the elm chitinase transgenic creeping bentgrass had enhanced resistance to Japanese beetles. So far, the elm chitinase has not been found to provide enhanced resistance against insects, but the transgenic plants produced in this project will make possible additional insect bioassays.

CHAPTER I

ELM CHITINASE GENE CHARACTERIZATION

SUMMARY

Dutch elm disease, caused by the fungus *Ophiostoma ulmi*, has killed the majority of the American elm trees in the United States. Recently, an elm tree with resistance to Dutch elm disease was discovered in the monument area in Washington, D.C. The tree was designated *Ulmus americana* NPS3-487. The mechanism of resistance in this tree is unknown. To investigate the possibility that chitinase may be involved in the resistance, Hajela et al. (1993) screened a cDNA library of *U. americana* NPS3-487 with a poplar chitinase clone, and recovered a gene (*hs2*) that hybridized with this probe. This *hs2* gene was characterized and found to be highly homologous with chitinases from a variety of other species. This cloned elm chitinase contains a chitinase catalytic region and a chitin binding domain, and is a typical class I chitinase. Discovery and characterization of this chitinase gene from *U. americana* NPS3-487 is a first step toward understanding its potential role as a defensive gene against plant-pathogenic fungi and possibly as a defensive gene versus insects as well.

INTRODUCTION

The chitinase gene studied for this dissertation was cloned from American elm (Hajela et al. 1993). American elm trees (*Ulmus americana,* L.) were once widely planted in urban areas for the shade provided by their spreading branching habit. However, Dutch elm disease, caused by the ascomycetous fungus *Ophiostoma ulmi* (Buis.) Nannf. (formerly *Ceratocystis ulmi*) has killed the majority of the American elm trees in the United States (Stipes and Campana 1981).

Therefore, suitable trees are needed to replace American elm trees which are killed by Dutch elm disease. Certain elm species, such as Japanese elm, *Ulmus japonica* and Siberian elm, *Ulmus pumila,* are resistant to Dutch elm disease (Karnosky and Mickler 1986). However, these species lack the characteristic branching habit of the American elm, and therefore are not widely used as replacements (Townsend and Schreiber 1975).

An individual elm tree resistant to Dutch elm disease and with size, shape, and branching habit similar to American elm was discovered in the monument area of Washington, D.C. (Sherald et al. 1994). Rooted cuttings from this tree, designated *Ulmus americana* NPS3-487, are also resistant to the disease.

In an effort to determine the parent species of *U. americana* NPS3-487, Sherald et al. 1994 examined its chromosomes. *U. americana* NPS3-487 is a triploid, suggesting that it may be a hybrid between American elm and one of the Asian elm species resistant to Dutch elm disease (Santamour 1970). American elm is a tetraploid, but the resistant Asian elm species are diploids (Sax 1933). The mechanism of resistance of *U. americana* NPS3-487 to Dutch elm disease is unknown. One possible mechanism may involve chitinase.

Chitinase is an enzyme that catalyzes the hydrolysis of chitin, which is located in the cell walls of many fungi, including *Ophiostoma ulmi* (Mauch et al. 1988). The *in*

vitro growth of several fungi can be inhibited by chitinase (Mauch et al. 1988). Wounding or fungal infection induces chitinase synthesis in several herbaceous plants. (Majeau et al. 1990; Roby et al. 1990).

Chitinase genes have been cloned and characterized from several different plant species (for review, see Graham and Sticklen 1994). A chitinase gene from broad bean, ***Phaseolus vulgaris***, was transferred via *Agrobacterium* to tobacco, where it conferred resistance to several tobacco fungal pathogens (Broglie et al. 1991).

The presence of a chitinase gene in *U. americana* NPS3-487 suggests a possible resistance mechanism against Dutch elm disease. This chapter describes the characterization of an elm chitinase gene that was cloned from an *U. americana* NPS3-487 cDNA library (Hajela et al. 1993).

MATERIALS AND METHODS

Source of *hs2* Elm Chitinase Gene

A chitinase gene cloned from an elm cDNA library was used for these studies. A total of 17 cDNA clones that hybridized with a WIN6 poplar chitinase gene probe were obtained from an elm cDNA library. The clone designated pHS2 was most complete; therefore it was chosen for use in this project.

Elm Chitinase Gene (*hs2*) DNA Sequence Determination

The complete DNA sequence of the elm chitinase gene *hs2* was determined by Sanger dideoxy sequencing (Sanger et al. 1977).

Elm Chitinase Gene (*hs2*) DNA Sequence Analysis

Sequence analysis of the elm chitinase gene *hs2* was performed with the Genetics Computer Group Sequence Analysis Software Package Version 7.0-UNIX, September 1991 (Devereux et al. 1984). The deduced 327 amino acid sequence from the open reading frame (ORF) of *hs2* was used to conduct a homology search of the GeneBank and EMBL databases using TFastA (Altschul et al. 1990). A search for protein consensus sequences described in the "PROSITE dictionary of sites and patterns in proteins" (Bairoch 1993) was done using the *hs2* deduced amino acid sequence.

RESULTS

The complete DNA nucleotide sequence of a full length, 1225 bp cDNA clone, containing a 951 nucleotide open reading frame (ORF) was determined (Fig. 1).

Using the deduced 317-amino acid sequence from this ORF, a homology search using TFastA (Altschul et al. 1990) showed 56 of the 57 best matches to be with chitinases; the remaining one was a lectin, which shares a chitin-binding domain with the pHS2 translation product. This product revealed strong homology to broad bean, poplar, and tobacco class I chitinases. The predicted protein product of this clone was designated ECH2 (elm chitinase 2). The amino acid sequence of ECH2 has 71.2% sequence identity to that of the translation product of the broad bean chitinase clone pCH18 (Broglie et al. 1986), 70.5% to the tobacco CHN50 chitinase (Fukuda et al. 1991).and 70% to the poplar WIN6 (Parsons et al. 1989) translation product.

The pHS2 deduced amino acid sequence contained two chitinase class I signatures and the chitin binding domain signature described in the PROSITE dictionary of sites and patterns in proteins (Bairoch 1993). The first highly conserved signature pattern is located in the N-terminal section and contains one of the six cysteines conserved in most, if not all chitinases. The consensus pattern is C-x(4)-F-Y-[ST]-x(3)-[FY]-[LIVMF]-x-A-x(3)-[YF]-x(2)-F-[GSA]; in the pHS2 deduced amino acid sequence, it occurs at amino acid number 89 as CPAKGFYTYDAFIAAAKAFPAFG. The second chitinase signature sequence is [LIVM]-[GSA]-F-x-[STA](2)-[LIVMFY]-W-[FY]-W-[LIVM]. It occurs in the pHS2 deduced amino acid sequence at position 215 as ISFKTALWFWM.

The chitin recognition or binding domain signature found in several plant and fungal proteins exhibiting binding specificity for oligosaccharides of N-acetylglucosamine (Meins et al. 1992; Wright et al. 1991) is C-x(4,5)-C-C-S-x(2)-G-x-C-G-x(4)-[FYW]-C. It is found in the pHS2 deduced amino acid sequence at position 33 as CPVGLCCSKFGWCGSTNEYC. The pHS2 deduced amino acid sequence contains eight conserved cysteine residues in a putative chitin binding domain.

The 3' untranslated region of pHS2 reveals three putative polyadenylation signals (Joshi 1987). A classic AATAAA signal is not present; however, an AATAAG and an AACAAA are present. The AATAAG motif alone is present in another chitinase-like clone from this elm. Immediately 5' to the AATAAG is a YAYTG-like sequence (CAATG). Finally, 10 nucleotides downstream, a TGTGTGCACT is present with high identity to a third polyadenylation motif (Joshi 1987).

ECH2 has an overall charge of +10 and a deduced pI of 8.49. The first 21 residues of ECH2 comprise a signal peptide; this peptide has the characteristic hydrophobicity coupled with a positively charged residue proximal to the initial methionine (von Heijne 1983). Residues 312-317 appear to compose a second routing peptide known as a C-terminal extension. It has been established that similar C-terminal extensions are sufficient and necessary to direct a protein into the vacuole (Chrispeels and Raikhel 1993). Residues 22-311 compose the main, catalytic domain.

```
   1 CGGGATTAAC GAGATTCCCA CTGTCCCTGT CTACTACCCA GCGAAACCAC
  51 AGCCAAGGGA ACGGGCTTGG CAGAATCAGA ACTAAACATG AGGTTTTGGG
 101 CATTGACGAC ACTTTCTCTT CTATTGTCCA TTATCCAAGG AGGCTGGGCA
 151 GAGCAATGTG GAAGCCAAGC TGGGGGTGCA GTGTGTCCCG TTGGGCTCTG
 201 CTGCAGCAAA TTTGGGTGGT GTGGGAGCAC AAACGAGTAC TGTGGTGATG
 251 GCTGCCAAAG CCAATGTGGC GGCAGCGGTA GCGATGACAT TGGCGGTCTC
 301 ATATCAAGCT CCGCCTTTAA TGACATGCTT AAGCATCGTA ACGACGGTGG
 351 TTTTCCTGCC AAGGGGTTTT ACACCTATGA TGCTTTTATT GCGGCTGCCA
 401 AGGCTTTCCC TGCATTTGGC TCCACCGGCG ATGATACCAC CCGTAAAAGG
 451 GAGATTGCTG CTTTCTTAGG TCAAACTTCC CATGAAACTA CAGGTGGGTG
 501 GGCAAGTGCA CCCGACGGTC CATACTCTTG GGGATACTGC TACAATAGGG
 551 AGCAAAACCC TTCTTCCGAT TATTGTTCTT TTAGTCCTAC TTGGCCTTGT
 601 GCTTCCGGAA AGAGATACTT TGGCCGTGGT CCCATTCAAC TCTCCTGGAA
 651 CTACAACTAT GGACAGTGTG GAAGGGCCAT AGGAGCCAAC CTATTAAACA
 701 ACCCTGATCT CGTAGCAACT GACCCTGTCA TTTCCTTCAA AACGGCCTTA
 751 TGGTTCTGGA TGACCCCACA GTCACCAAAG CCCTCGTGCC ATGACGTCAT
 801 CACCGGAAGA TGGAGTCCTT CCGGCACCGA CCAGTCGGCC GGCCGAGTTG
 851 CGGGCTACGG CGTGATCACC AACATTATCA ACGGTGGGAT AGAATGCGGG
 901 AAAGGTCAGG TTCCTCAGGT GGTGGATCGG ATTGGATTCT ACAAGAGGTA
 951 CTGTGATATC CTTAGAGTTG GCTATGGGAA CAATCTTGAT TGCTATAACC
1001 AGAGGCCTTT TGGGAATGGA CTCTTGTTGG ACGCCATGTA ACGACTTGTC
1051 GTAGTTGTTA TACATATCTG TGTTTTGAGG GTTTGGCCGT CGTTTGTCGT
1101 CACTTCTTCG GTGACAATTT CGTTGTGTTA TAGTTACTAT ATATGTCAAT
1151 GGAATAAGAA CAAAGGATGT GTGCACTACA ACAAAAAAAA AAAAAAAAAA
1201 AAAAAAAAAA AAAAAAAAAA AAAAA
```

Figure 1.1. Elm chitinase gene *hs2* DNA nucleotide sequence.

CGGGATTAACGAGATTCCCACTGTCCCTGTCTACTACCCAGCGAAACCACAGCCAAGGGA

ACGGGCTTGGCAGAATCAGAACTAAACATGAGGTTTTGGGCATTGACGACACTTTCTCTT
MetArgPheTrpAlaLeuThrThrLeuSerLeu

CTATTGTCCATTATCCAAGGAGGCTGGGCAGAGCAATGTGGAAGCCAAGCTGGGGGTGCA
LeuLeuSerIleIleGlnGlyGlyTrpAlaGluGlnCysGlySerGlnAlaGlyGlyAla

GTGTGTCCCGTTGGGCTCTGCTGCAGCAAATTTGGGTGGTGTGGGAGCACAAACGAGTAC
ValCysProValGlyLeuCysCysSerLysPheGlyTrpCysGlySerThrAsnGluTyr

TGTGGTGATGGCTGCCAAAGCCAATGTGGCGGCAGCGGTAGCGATGACATTGGCGGTCTC
CysGlyAspGlyCysGlnSerGlnCysGlyGlySerGlySerAspAspIleGlyGlyLeu

ATATCAAGCTCCGCCTTTAATGACATGCTTAAGCATCGTAACGACGGTGGTTTTCCTGCC
IleSerSerSerAlaPheAsnAspMetLeuLysHisArgAsnAspGlyGlyPheProAla

AAGGGGTTTTACACCTATGATGCTTTTATTGCGGCTGCCAAGGCTTTCCCTGCATTTGGC
LysGlyPheTyrThrTyrAspAlaPheIleAlaAlaAlaLysAlaPheProAlaPheGly

TCCACCGGCGATGATACCACCCGTAAAAGGGAGATTGCTGCTTTCTTAGGTCAAACTTCC
SerThrGlyAspAspThrThrArgLysArgGluIleAlaAlaPheLeuGlyGlnThrSer

CATGAAACTACAGGTGGGTGGGCAAGTGCACCCGACGGTCCATACTCTTGGGGATACTGC
HisGluThrThrGlyGlyTrpAlaSerAlaProAspGlyProTyrSerTrpGlyTyrCys

TACAATAGGGAGCAAAACCCTTCTTCCGATTATTGTTCTTTTAGTCCTACTTGGCCTTGT
TyrAsnArgGluGlnAsnProSerSerAspTyrCysSerPheSerProThrTrpProCys

GCTTCCGGAAAGAGATACTTTGGCCGTGGTCCCATTCAACTCTCCTGGAACTACAACTAT
AlaSerGlyLysArgTyrPheGlyArgGlyProIleGlnLeuSerTrpAsnTyrAsnTyr

```
GGACAGTGTGGAAGGGCCATAGGAGCCAACCTATTAAACAACCCTGATCTCGTAGCAACT
GlyGlnCysGlyArgAlaIleGlyAlaAsnLeuLeuAsnAsnProAspLeuValAlaThr

GACCCTGTCATTTCCTTCAAAACGGCCTTATGGTTCTGGATGACCCCACAGTCACCAAAG
AspProValIleSerPheLysThrAlaLeuTrpPheTrpMetThrProGlnSerProLys

CCCTCGTGCCATGACGTCATCACCGGAAGATGGAGTCCTTCCGGCACCGACCAGTCGGCC
ProSerCysHisAspValIleThrGlyArgTrpSerProSerGlyThrAspGlnSerAla

GGCCGAGTTGCGGGCTACGGCGTGATCACCAACATTATCAACGGTGGGATAGAATGCGGG
GlyArgValAlaGlyTyrGlyValIleThrAsnIleIleAsnGlyGlyIleGluCysGly

AAAGGTCAGGTTCCTCAGGTGGTGGATCGGATTGGATTCTACAAGAGGTACTGTGATATC
LysGlyGlnValProGlnValValAspArgIleGlyPheTyrLysArgTyrCysAspIle

CTTAGAGTTGGCTATGGGAACAATCTTGATTGCTATAACCAGAGGCCTTTTGGGAATGGA
LeuArgValGlyTyrGlyAsnAsnLeuAspCysTyrAsnGlnArgProPheGlyAsnGly

CTCTTGTTGGACGCCATGTAACGACTTGTCGTAGTTGTTATACATATCTGTGTTTTGAGG
LeuLeuLeuAspAlaMetEnd

GTTTGGCCGTCGTTTGTCGTCACTTCTTCGGTGACAATTTCGTTGTGTTATAGTTACTAT

ATATGTCAATGGAATAAGAACAAAGGATGTGTGCACTACAACAAAAAAAAAAAAAAAAAA

AAAAAAAAAAAAAAAAAAAAAAAAA
```

Figure 1.2. Elm chitinase gene *hs2* DNA nucleotide sequence with deduced protein amino acid sequence.

DISCUSSION

The elm cDNA clone pHS2 containing the *hs2* chitinase gene was sequenced and characterized. The coding sequence is predicted to code for a class I chitinase according to Shinshi nomenclature (Shinshi et al. 1990).

The deduced amino acid sequence of this full length class I chitinase cDNA clone (pHS2) from *Ulmus americana* NPS3-487 had 56 of the 57 best matches from a TFastA (Altschul et al. 1990; Pearson and Lipman 1988) amino acid homology search with chitinases isolated from various plants. The deduced amino acid sequence of the pHS2 elm chitinase also contains two class I chitinase signatures, a chitin binding domain signature, and eight conserved cysteine residues in a putative chitin binding domain (Van Parijs et al. 1991).

Chitinases are pathogenesis response-related proteins found in a wide variety of plants (Meins et al. 1992). Evidence strongly suggests that chitinases are antifungal proteins (Ahl Goy et al. 1992).

Accumulation of chitinase (Meins and Ahl 1989; Pegg and Young 1981; Roby et al. 1990; Rasmussen et al. 1992) and mRNA encoding chitinase (Meins and Ahl 1989; Métraux and Boller 1986; Roby et al. 1990; Roby and Esquerré-Tugayé 1987) are induced strongly during fungal infection. The induction of chitinase occurs when plant tissue is treated with fungal cell wall material (Kurosaki et al. 1987; Roby and Esquerré-Tugayé 1987). Ethylene, a gaseous plant hormone normally produced during fungal infection, induces chitinase activity (Boller et al. 1983; Broglie et al. 1986). In addition, chitinase induction results from wounding (Parsons et al. 1989).

Transgenic tobacco plants expressing a broad bean class I chitinase gene were resistant to *Rhizoctonia solani* (Broglie et al. 1991). Furthermore, transgenic tobacco plants expressing a rice class I chitinase gene were resistant to *Cercospora nicotianae* (Zhu et al. 1994).

Therefore, these results suggest that the *hs2* elm chitinase gene may be involved in resistance to fungal pathogens, possibly including *O. ulmi*, the causal agent of Dutch elm disease. It may also be effective at conferring resistance to other fungal pathogens in other hosts when successfully expressed in transgenic plants.

Although the mechanisms of resistance of *U. americana* NPS3-487 to Dutch elm disease are still unknown, chitinase may be involved in this resistance. The cDNA sequence determination and gene characterization of the *hs2* elm chitinase gene were the first steps toward studying the possible roles of the gene in plant resistance versus plant pathogenic fungi and/or phytophagous insects.

CHAPTER II

PLASMID CONSTRUCTION

SUMMARY

Plasmids containing the *hs2* elm chitinase gene were constructed for expression of the elm chitinase HS2 in *Escherichia coli* bacteria and in transgenic plants.

Plasmid for Bacterial Expression of Elm Chitinase

The chitinase coding region of pHS2 was transferred to pGEX-4T-1 (Pharmacia), a prokaryotic expression vector designed to express proteins as fusions with glutathione-S-transferase (Smith and Johnson 1988). The pHS2 clone contained approximately 100 nucleotides before the translational initiation codon for *hs2*. To make a construct without the 5' leader sequence, it was necessary to insert a restriction site immediately adjacent to the translational initiation codon. Polymerase chain reaction (PCR) (Mullis and Faloona 1987) was used to create an *Eco* RI site. A primer designed to insert an *Eco* RI site immediately before the initiation codon of the chitinase gene contained the sequence AAGAATTCATGAGGTTTTGGGCATTG. The PCR reaction used the *Eco* RI primer and a -20 primer that annealed to a sequence in the multicloning site of the pBluescript KS- vector (Stratagene, La Jolla, CA). The PCR reaction product was restricted with *Eco* RI and *Xho* I, and ligated into *Eco* RI and *Xho* I linearized pGEX-4T-1. The resulting plasmid, designated pGEX-4T-1-pHS2, was used for expression of elm chitinase in *E. coli* bacteria.

Plasmid for Plant Expression of Elm Chitinase

The cDNA portion of pHS2 was removed by digestion with restriction enzymes *Eco* RI and *Xho* I and the *Eco* RI cohesive terminus was filled in. After agarose gel

purification of the cDNA fragment, it was ligated into pKYLX71 plasmid (Schardl et al. 1987) opened with *Hind* III and *Xho* I with the *Hind* III cohesive terminus filled in. This plasmid construct, designated pKYLX71-pHS2 was used to transform tobacco and turfgrass for plant expression of elm chitinase.

INTRODUCTION

A gene such as the *hs2* elm chitinase gene that is obtained from a cDNA library must be modified by adding the appropriate promoters and terminators before it can be used for bacterial or plant transformation and expression. A cDNA clone typically contains a 5' noncoding leader sequence, the gene coding region, a 3' noncoding region, and a poly A 3' terminus. The *hs2* elm chitinase gene cloned as the pHS2 cDNA plasmid contained all of these elements.

A commonly used strategy for expression of genes in bacteria is to express them as fusion proteins. Foreign genes have been expressed in bacteria such as *Escherichia coli* to form fusion proteins with glutathione-S-transferase (Smith and Johnson 1988). There are several advantages to expressing foreign genes in bacteria as fusion proteins rather than as native proteins. Native foreign proteins, especially if they are from eukaryotes, are often insoluble when expressed in bacteria, because bacteria do not possess the chaperonins required for proper folding of eukaryotic proteins. Foreign proteins expressed in bacteria as fusion proteins with glutatione-S-transferase are more likely to be soluble, although they are still often insoluble.

Expression of foreign proteins in bacteria as fusion proteins with glutathione-S-transferase may facilitate their purification and serological detection. Antisera are available to detect the glutathione-S-transferase domain of the fusion protein. If desired, the glutathione-S-trasnferase domain may be removed by digestion with the appropriate proteinase.

Expression of a foreign protein in plants requires the construction of a plasmid with all of the required components. Plant transformation is dependant upon use of a properly designed and constructed plasmid containing the gene desired to be expressed in transgenic plants (Gruber and Crosby 1993). A plasmid designed for plant transformation should contain a plant promoter, the foreign gene whose expression is desired in transgenic plant material, and a transcription terminator (Morrish et al. 1993). The choice of a plant promoter is critical (Hensgens et al. 1993). Cauliflower mosaic virus (CaMV) 35S promoter (Guilley et al. 1982) was chosen for this project because it is very well characterized and provides a constitutive high level of expression in most plant tissues. Its expression levels are usually higher in dicots than in monocots, but it still provides an easily detectable level of expression in both dicots and monocots (Fromm et al. 1985; Hauptmann et al. 1987; Zhang et al. 1991; Last et al. 1991; Taylor et al. 1993). The 35S promoter would be adequate for expression of a foreign gene in transgenic plants, whether they be dicots or monocots, depending upon the desired level of expression (McElroy et al. 1990; Koziel et al. 1993; Fujimoto et al. 1993).

When designing a plasmid for plant transformation by a biolistic method, a decision must be made whether to try a co-transformation strategy or to try transformation with two linked genes on the same plasmid (Lyznik et al. 1989). Either method has advantages and disadvantages. The co-transformation strategy utilizes a mixture of two separate plasmids bombarded together by a biolistic transformation technique into plant tissues (Peng et al. 1990). One of the plasmids should contain the foreign gene of interest, and the second plasmid should contain a selectable marker gene

(Kyozuka and Shimamoto 1993). This technique eliminates the time and expense of constructing a single plasmid containing both genes.

Another consideration in plasmid design for plant transfmation is which technique will be used for plant transformation. If *Agrobacterium* will be used, then at least one T-DNA border sequence should be present. If biolistics will be used then no T-DNA border sequence is required. A plasmid containing a T-DNA border sequuence can be used for both *Agrobacterium*-mediated plant transformation and biolistic plant transformation.

MATERIALS AND METHODS

Plasmid constructions were done according to standard protocols (Sambrook et al. 1989). Expression vectors pKK223-3 and pGEX4T-1 (Smith and Johnson 1988) were from Pharmacia (Piscataway, NJ). Polymerase chain reaction (PCR) (Saiki et al. 1988) was done in a Perkin-Elmer-Cetus GeneAmp PCR System model 9600 thermocycler using 35 cycles of 94, 48, and 72^{o} C. The *Escherichia coli* strain used for bacterial propagation of plasmids was XL1-Blue (Stratagene Cloning Systems, La Jolla, CA).

RESULTS

Source of *hs2* Elm Chitinase Gene

The pHS2 plasmid was the source of the *hs2* elm chitinase gene used for this project. This was a cDNA clone selected from an elm cDNA library. The *hs2* chitinase gene was cloned into the pBluescript SK- cloning vector from *Eco* RI to *Xho* I.

Plasmid pGEX-4T-1-hs2 for Bacterial Expression of Elm Chitinase

In order to express the *hs2* elm chitinase gene in *Escherichia coli* bacteria, it was necessary to clone the gene into a prokaryotic expression vector. The plasmid pGEX-4T-1 was chosen for this purpose. The pGEX-4T-1 plasmid contained the glutathione-S-transferase gene with a multicloning site downstream so that a foreign gene could be cloned there to create a gene fusion between glutathione-S-transferase and the foreign gene.

To obtain expression of *hs2* elm chitinase in bacteria, it would be necessary to clone it into pGEX-4T-1 in such a way that the 5' noncoding sequences were not present. To accomplish this, a polymerase chain reaction (PCR) primer, designated MB45, was designed with the nucleotide sequence, "5' AAGAATTCATGAGGTTTTGGGCATTG 3'". This 26-mer PCR primer contained an *Eco* RI restriction site (GAATTC) near its 5' terminus. Two extra As were added 5' to this *Eco* RI site to provide room for binding of the *Eco* RI restriction enzyme. Immediately 3' to the *Eco* RI restriction site is the ATG

initiation codon, followed by an additional 15 bases of the nucleotide sequence of the *hs2* elm chitinase gene.

The MB45 PCR primer was designed to work in combination with an M13 -20 sequencing primer with the nucleotide sequence of "5' GTAAAACGACGGCCAGT 3'" to amplify a portion of the *hs2* elm chitinase gene from the ATG initiation codon past the poly A 3' region and past the *Xho* I cloning site and into the pBluescript SK- cloning vector. The expected PCR product should contain an *Eco* RI restriction site immediately upstream of the ATG initiation codon of the *hs2* elm chitinase gene.

The annealing temperatures of the two PCR primers were calculated using the formula, "[4X(G+C)]+[2X(A+T)]-4°C". The calculated annealing temperature for MB45 was 52°C, and it was 48°C for the M13 -20 primer. Therefore, the lower annealing temperature of 48°C was used for the PCR reaction.

The template DNA used for the PCR reaction was pHS2 linearized with *Bam* HI. The PCR reaction mixture contained: 20 µl of 10X PCR buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3); 16 µl of dNTP mixture (2.5 mM of each of the four dNTPs); 2 µl of primer M13 -20 (20 pmoles/µl); 2 µl of primer MB45 (20 pmoles /µl); 160 µl water; and 1 µl *Taq* DNA polymerase (Boehringer Mannheim Corporation, Indianapolis, Indiana). The PCR reaction was run with an initial temperature of 94°C for 5 minutes, followed by 35 cycles (94°C for 15 seconds to denature the DNA, 48°C for 15 seconds for annealing, and 72°C for one minute for polymerization), followed by 72°C for 5 minutes for polymerization, and then 4°C for preservation until the reaction was removed from the thermocycler. A 5 µl sample of the PCR reaction products was

electrophoresed in a 1% agarose gel. The approximately 1.1 kb reaction product was clearly visible in the ethidium bromide-stained gel (Figure 2.4).

The 1.1 kb PCR product was cut from the gel and purified using Geneclean II Kit (Bio 101, La Jolla, California) according to the manufacturer's directions. The purified PCR product was digested with Eco RI and Hind III restriction enzymes (Boehringer Mannheim Corporation, Indianapolis, Indiana). The digestions products were electrophoresed in an agarose gel, and the 1.1 kb restriction fragment was cut from the gel and purified with Geneclean II Kit.

One μg of the plasmid, pGEX-4T-1 was digested with the restriction endonucleases *Eco* RI and *Xho* I. The digestion products were electrophoresed in an agarose gel. The approximately 4900 bp fragment was cut from the gel and purified with Geneclean II Kit.

To clone the *hs2* PCR product into the pGEX-4T-1 cloning vector, the ligation reaction was done with T4 DNA ligase (Boehringer Mannheim Corporation, Indianapolis, Indiana). The ligation products were used to transform *E. coli* XL1 Blue bacteria (Stratagene, La Jolla, California), and the bacteria were plated on LB agar medium. Sixteen resulting colonies were selected for screening. Plasmids from these colonies were purified and screened by digestion with *Eco* RI and *Xho* I restriction enzymes. The digestion products were electrophoresed in an agarose gel stained with ethidium bromide, and visualized with ultraviolet illumination. Five of the sixteen colonies picked (#2, 3, 12, 15, 16) contained the desired construct (Figure 2.5). These plasmids were used to transform *E. coli* JM105 (Amersham Pharmacia Biotech Inc., Piscataway, New Jersey).

Plasmid pKYLX71-hs2 for Plant Expression of Elm Chitinase

For plant expression of elm chitinase, a plasmid was constructed that could be used for both *Agrobacterium*-mediated plant transformation and biolistic plant transformation. This plasmid, designated pKYLX71-hs2 (Figure 2.3), contains an RK2 origin of replication, which is functional in both *E. coli* and *Agrobacterium tumefaciens*, a tetracycline resistance gene with a prokaryotic promoter for selection in bacteria, both the right and left T-DNA borders to facilitate integration of the T-DNA into the plant genome, the cauliflower mosaic virus (CaMV) 35S promoter driving the expression of the *hs2* elm chitinase gene with the pea rubisco 3' transcription terminator, and a kanamycin resistance gene with a plant promoter, *nos-Aph(3')II*, for selection of putative transgenic plants.

The plasmid pKYLX71 was derived from pKYLX7 (Schardl et al. 1987) by insertion of the polylinker, "5' AAGCTTGGATCCTCGAGCTGCAGGAGCTCGAATTGATCCTCTAGA 3'", between the *Hind* III and *Xba* I sites, which occurred between the CaMV 35S promoter and the pea rubisco 3' transcription terminator. The inserted polylinker contained *Hind* III and *Xho* I restriction sites. This plasmid contained all of the elements listed above with the exception of the *hs2* elm chitinase gene.

To insert the *hs2* elm chitinase gene into the plasmid pKYLX71 between the CaMV 35S promoter and the pea rubisco 3' transcription terminator, pKYLX71 was first digested with *Hind* III restriction enzyme. The cohesive termini thus generated were

filled in with the Klenow fragment of DNA polymerase I (Boehringer Mannheim Corporation). After 15 minutes reaction time, the enzymes were inactivated by heating to 75°C for 10 minutes. The plasmid was then digested with *Xho* I restriction enzyme, and the enzyme was inactivated by heating to 75°C for 20 minutes.

The plasmid pHS2 was digested with *Eco* RI restriction enzyme, filled in with the Klenow fragment of DNA polymerase I, heat inactivated, digested with *Xho* I restriction enzyme, and heat inactivated again.

The prepared pKYLX71 and pHS2 were mixed together and ligated with T4 DNA ligase (Boehringer Mannheim Corporation). Because the *Hind* III site of pKYLX71 and the *Xho* I site of pHS2 had been converted to blunt ends, while the cohesive *Xho* I termini of both plasmids were left intact, directional cloning of the *hs2* elm chitinase gene into the pKYLX71 vector was achieved, with the insert in the desired orientation. The *hs2* elm chitinase gene was cloned into pKYLX71 from a *Hind* III/*Eco* RI fusion to the *Xho* I site. The ligation reaction products were used to transform *E. coli* DH5α bacterial cells, and the cells were plated on LB agar. Nine of the resulting colonies were selected for screening.

For screening of the pKYLX71-hs2 constructs, plasmids were purified, digested with *Pst* I and *Xho* I restriction enzymes, and electrophoresed in an agarose gel. Ultraviolet visualization of the ethidium bromide stained gel revealed that two of the constructs (#2 and #6) contained the desired elements. *E. coli* DH5α bacterial cells were transformed with the correctly constructed pKYLX71-hs2 plasmids.

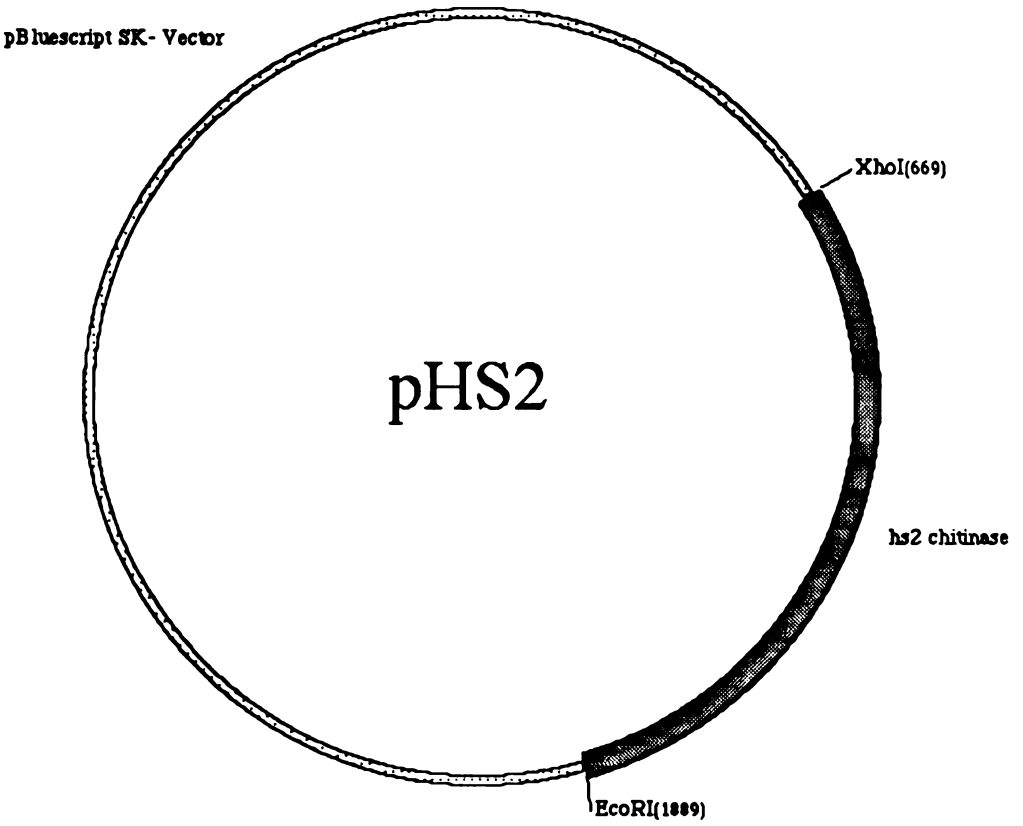

Figure 2.1 Plasmid pHS2 containing the *hs2* elm chitinase gene cloned into the pBluescript SK- vector between the *Eco* RI and *Xho* I restriction sites.

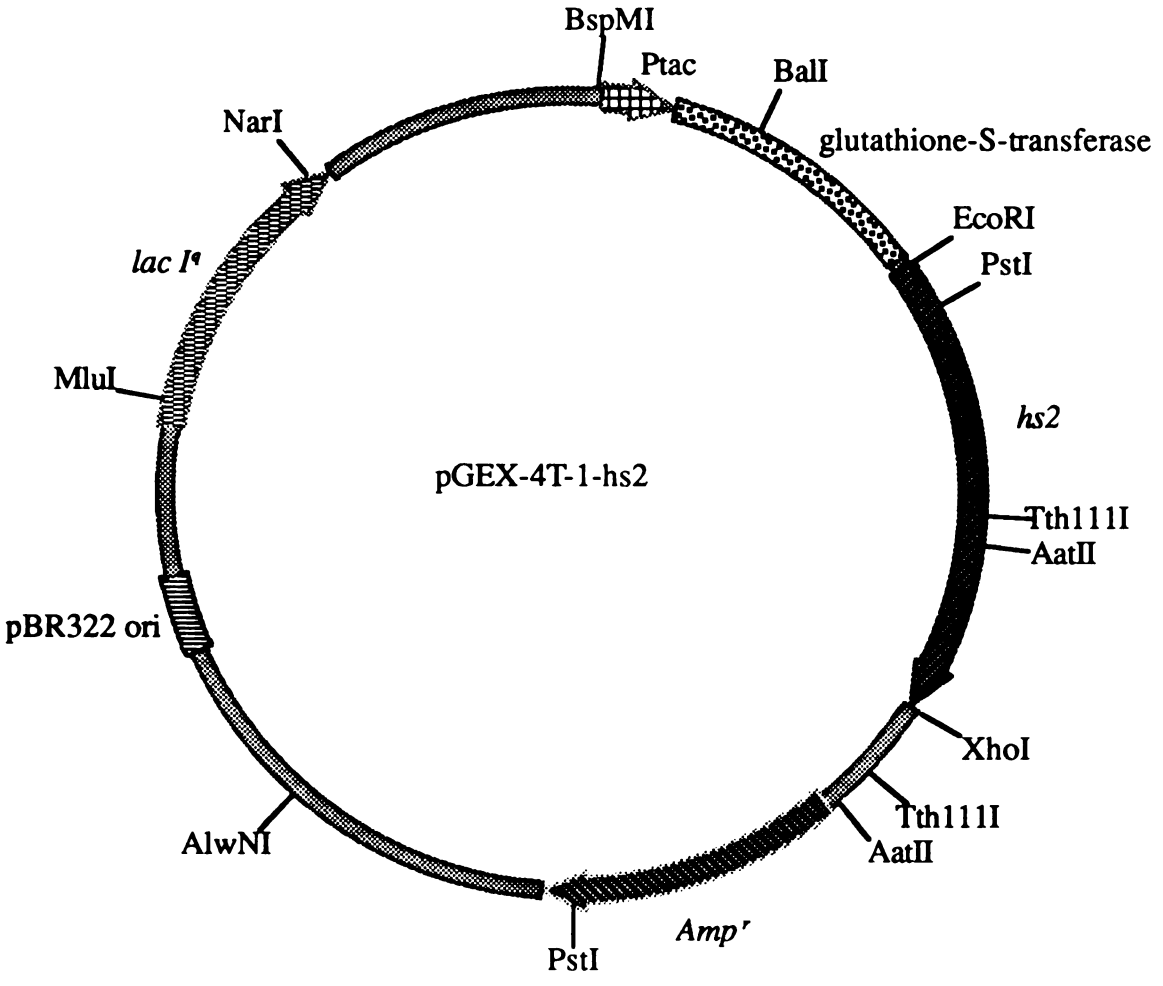

Figure 2.2 Plasmid pGEX-4T-1-hs2. Ptac: tac promoter; glutathione-S-transferase: glutathione-S-transferase gene; *hs2*: *hs2* elm chitinase gene; *Amp*^{r}: ampicillin resistance gene; pBR322 ori: pBR322 origin of replication; *lac I*^{q}: *lacI*^{q} gene.

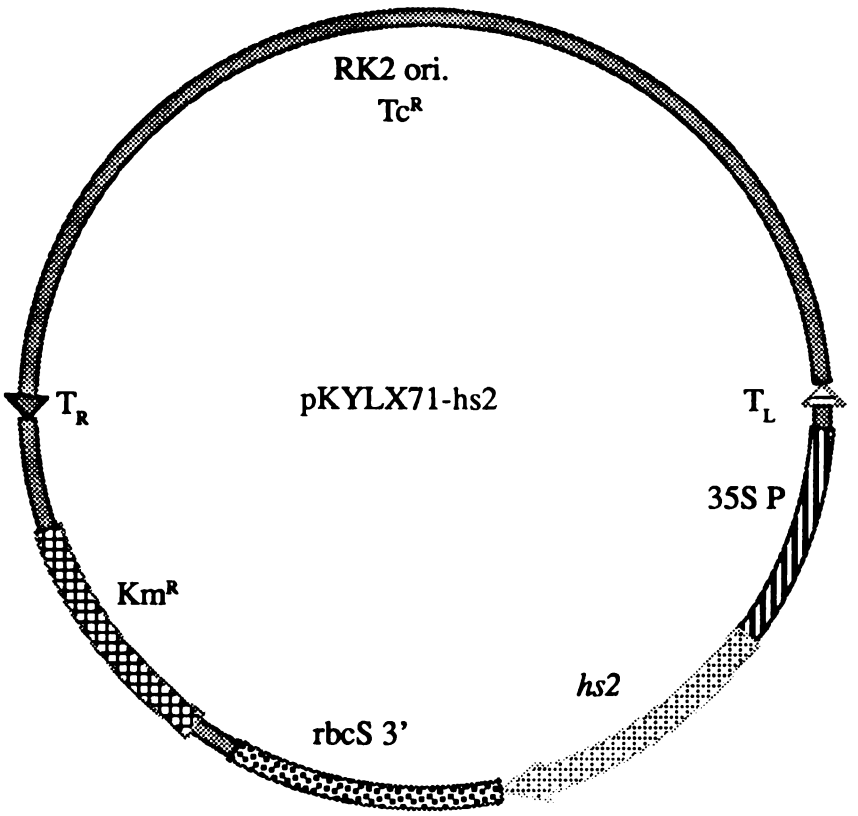

Figure 2.3. Plasmid pKYLX71-hs2. RK2 ori: RK2 wide host range origin of replication; Tc^R: tetracycline resistance gene; T_L: T-DNA left border; T_R: T-DNA right border; 35S P: cauliflower mosaic virus (CaMV) 35S promoter; *hs2*: *hs2* elm chitinase gene; rbcS 3': pea rubisco 3' transcription terminator.; KmR: *nos-Aph(3')II* kanamycin resistance gene.

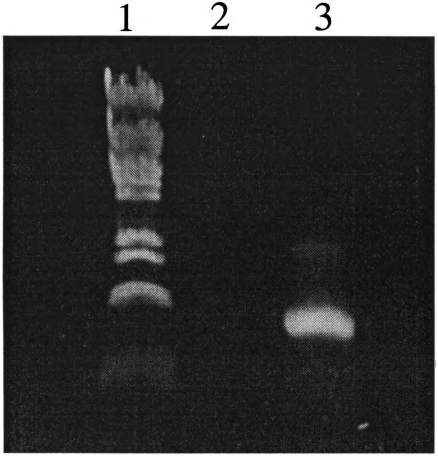

Figure 2.4. Agarose gel electrophoresis of *hs2* PCR products. Lane 1: λ/*Bst* EII molecular weight markers; Lane 2: PCR reaction with no template DNA; Lane 3: PCR product with pHS2/*Bam* HI template DNA.

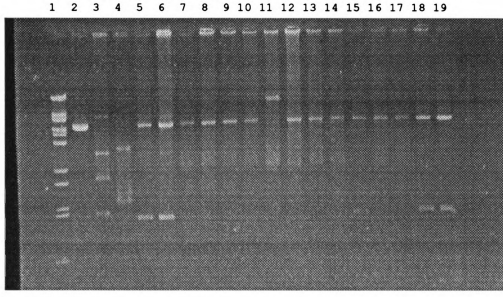

Figure 2.5. Screening of pGEX-4T-1-hs2 plasmid constructs by restriction digestion and agarose gel electrophoresis. Lane 1: λ/*Bst* EII molecular weight markers; Lane 2: pGEX4T-1/*Eco* RI/*Xho* I; 3: pHS2/*Eco* RI/*Xho* I; Lanes 4-19: pGEX4T-1-hs2 constructs #1-16/*Eco* RI/*Xho* I. Correctly constructed pGEX-4T-1-hs2 plasmids with the expected restriction patterns are in lanes 5, 6, 15, 18, and 19.

DISCUSSION

Plasmid pGEX-4T-1-hs2 for Bacterial Expression of Elm Chitinase

The pGEX-4T-1 prokaryotic expression vector chosen for construction of the pGEX-4T-1-hs2 plasmid for bacterial expression of the *hs2* elm chitinase gene is part of an integrated system for the expression, purification, and detection of fusion proteins produced in *Escherichia coli* . The pGEX plasmids were designed for inducible, high-level intracellular expression of genes as fusions with *Schistosoma japonicum* glutathione-S-transferase (GST) (Smith and Johnson 1988). The pGEX plasmid expression vectors were constructed to direct the synthesis of foreign polypeptides in *E. coli* as fusions with the C terminus of Sj26, a 26-kDa glutathione S-transferase (GST; EC 2.5.1.18) encoded by the parasitic helminth *Schistosoma japonicum.* In some cases, fusion proteins are soluble in aqueous solutions and can be purified from crude bacterial lysates under non-denaturing conditions by affinity chromatography on immobilized glutathione. Using batch wash procedures, fusion proteins have been purified with yields of up to 15 ng protein/ml of culture. The pGEX-4T-1 vector was engineered so that the GST carrier can be cleaved from fusion proteins by digestion with the site-specific protease, thrombin, following which, the carrier and any uncleaved fusion protein can be removed by adsorption on glutathione-agarose. This system has been used for the successful expression and purification of more than 30 different eukaryotic polypeptides.

The pGEX-4T-1 vector has: a *tac* promoter for chemically inducible, high-level expression; an internal *lac* I^q gene; and a thrombin protease recognition site for cleaving

the foreign protein from the fusion product. Downstream from the thrombin site, the pGEX-4T-1 expression vector has a multicloning site containing the *Eco* RI and *Xho* I sites used for the directional insertion of the *hs2* elm chitinase gene. Protein expression from a pGEX plasmid is under the control of the *tac* promoter, which is inducible by the lactose analog isopropyl β-D-thiogalactoside (IPTG).

Plasmid pKYLX71-hs2 for Plant Expression of Elm Chitinase

The plasmid pKYLX71-hs2 contained the *hs2* elm chitinase gene driven by the CaMV 35S promoter (Odell et al. 1985). This promoter has strong constitutive activity in dicots, and also has constitutive activity in monocots (Fromm et al. 1986; Horsch et al. 1985; Odell et al. 1985; Ou-Lee et al. 1986, Sanders et al. 1987). The 3' end of the pea *rbcS*-E9 gene was used to obtain predictable, efficient polyadenylation of the mRNA (Nagy et al. 1985; Fluhr and Chua 1986; Morelli et al. 1985).

The pKYLX71-hs2 plasmid is based on pGA472 (An et al. 1985). It has the RK2 origin of replication for maintenance in *E. coli* and *Agrobacterium tumefaciens*. It also has the necessary sequences for conjugative transfer into *A. tumefaciens* using pRK2013 (Ditta et al. 1980) as helper in tri-parental matings. It has the tetracycline resistance gene from RK2 for selection of bacterial transconjugates, and a chimeric *nos-Aph(3')II* gene for selection of transformed plant cells with kanamycin (An et al. 1985). The kanamycin resistance gene, the 35S promoter, the *hs2* elm chitinase gene, and the rubisco transcription terminator are between the left and right borders of T-DNA from the pTiT37 tumor-inducing plasmid of *A. tumefaciens*.

The tetracycline resistance gene in pKYLX71-hs2 must be induced. This can be done by growing the bacteria containing the plasmid at 37°C for 30 minutes in broth without the antibiotic, then adding tetracycline to one μg/ml. After another 30 minutes of growth at 37°C with shaking, then the bacteria should be plated on agar medium containing 12.5 μg/ml tetracycline. After the colonies grow overnight, they should be restreaked on agar medium containing 12.5 μg/ml of tetracycline and 10 μg/ml of kanamycin. The *nos* promoter driving the kanamycin resistance gene has a low level of expression in bacteria, and adding kanamycin to the bacterial medium helps to ensure that the plasmid has an intact kanamycin resistance gene for selection of transformed plant cells. The pRK2-based plasmids are low copy number, and attempts to amplify them have not been successful.

The size of pKYLX71-hs2 is approximately 13.2 kb. Satisfactory transgene stability was reported with plasmids of 9.0 and 11.0 kb (Potrykus et al. 1985; Vasil et al. 1991; Gruber and Crosby 1993). Most plant transformation vectors are between 4.0 to 7.0 kb (Gruber and Crosby 1993). The selectable markers on an 18.7 kb plasmid were not expressed in all transformants (Gruber and Crosby 1993). Because larger vectors may tend to be more unstable than smaller ones, the use of 10 μg/ml of kanamycin in the bacterial medium may be especially important to select for plasmids that have an intact kanamycin resistance gene. The other alternative would be to use the plasmid pKYLX71-hs2 as one of two plasmids in cotransformation. The second plasmid should have the selectable marker gene, such as *bar*. In that case, it would not matter if the kanamycin resistance gene in pKYLX71-hs2 was intact or not, since it would not be used for selection of transformed plant cells.

Linear plasmid DNA has been reported to be more efficient than supercoiled plasmid DNA for biolistic plant transformation (West et al. 1991). The pKYLX71-hs2 can be linearized by *Eco* RI restriction endonuclease.

The plasmid pKYLX71-hs2 can be used for plant transformation by both the biolistic method and by *Agrobacterium*. *A. tumefaciens* strain A281 was reported to be capable of infecting rice (Raineri et al. 1990). *A. tumefaciens* strain A856 caused extensive root proliferation in rice. Transgenic rice tissues were obtained from immature rice embryos using *Agrobacterium*-mediated transformation (Raineri et al. 1990). *Agrobacterium* plasmid DNA has been directly electroporated into wheat callus cells (Zaghmout and Trolinder 1993). Some efforts have been expended in attempts to develop an *Agrobacterium*-mediated transformation system for maize (Grimsley et al. 1987; Boulton et al. 1989; Gould et al. 1991; Schlapp and Hohn 1992; Raineri et al. 1993). *Agrobacterium* is thought to have good potential for rice transformation (Cao et al. 1991).

CHAPTER III

EXPRESSION OF CHITINASE IN BACTERIA

SUMMARY

The ECH2 elm chitinase was expressed in *Escherichia coli* JM105 bacteria as a fusion protein with glutathione-S-transferase (GST) from *Schistosoma japonicum*. This was accomplished by IPTG induction of a culture of the *E. coli* JM105 bacteria containing the plasmid pGEX-4T-1-hs2. Bacterial cultures that were not induced, or that did not contain the plasmid did not express the GST-chitinase fusion protein. Coomassie brilliant blue-stained electrophoresis gels revealed the presence of the approximately 56 kd GST-chitinase fusion protein in induced bacterial cultures containing the pGEX-4T-1-hs2 plasmid, but not in cultures not induced or without the plasmid.

INTRODUCTION

The ECH2 elm chitinase-glutathione-S-transferase (GST) fusion protein was produced in *Escherichia coli* bacteria containing the pGEX-4T-1-hs2 recombinant plasmid. Protein expression was under the control of the *tac* promoter, which was induced using the lactose analog, isopropyl β-D-thiogalactoside (IPTG). Induced cultures were allowed to express GST-chitinase fusion proteins for up to four hours, after which time the cells were harvested, lysed, and electrophoresed in polyacrylamide gels. The bacterial cultures were grown in 2% glucose medium to decrease the basal expression level of the very strong *tac* promoter.

The rationale for expressing elm chitinase in bacteria was three fold. First, if the chitinase had been expressed in an enzymatically active form, it could have been used to test its activity against various plant pathogenic fungi in growth inhibition assays. Second, chitinase enzyme assays could have been done to confirm that the *hs2* gene actually is capable of being expressed as an enzymatically active protein. Third, regardless of whether the expressed protein had enzymatic activity, it could be injected into a rabbit for production of antiserum versus ECH2 elm chitinase.

Chitinase is capable of degrading the cell walls of certain plant pathogenic fungi (Shapira et al. 1989; Elad et al. 1982; Ordentlich et al. 1988). Chitin is a major structural component of the cell walls of many fungi. Many organisms that produce chitinases that are able to hydrolyze chitin to chitobiose dimers have been implicated in biocontrol processes (Deshpande 1986). *Serratia marcescens*, a Gram-negative bacterium that produces chitinase, is a biocontrol agent of *Sclerotium rolfsii* under greenhouse

conditions (Ordentlich et al. 1987). *S. marcescens* also reduced damping-off of bean, caused by *Rhizoctonia solani* Kühn, but was not effective against *Pythium aphanidermatum* (Edson) Fitzp. in cucumber. Because chitinases seem to be effective at lysing the cell walls of some fungi, but are ineffective against others, fungal growth inhibition assays on agar media in Petri dishes using enzymatically-active chitinase expressed in bacteria may be valuable for identifying which fungi may be susceptible to a certain chitinase.

S. marcescens can secrete several chitinolytic enzymes (Fuchs et al. 1986). *E. coli* bacteria containing a plasmid with the *chiA* gene, coding for a chitinase produced by *S. marcescens*, secreted chitinase. Partially purified chitinase expressed in *E. coli* bacteria protected bean seedling against the plant pathogens *S. rolfsii* and *R. solani* (Shapira et al. 1989).

Chitinases and β-1,3-glucanases were produced by *Stachybotrys elegans*, a mycoparasite of *R. solani* (Tweddell et al. 1994). *S. elegans* (Pidopl.) W. Gams had a strong *in vitro* antagonistic activity against *R. solani* (Benyagoub and Jabaji-Hare 1992; Turhan 1990). Ultrastructural studies of the sites of intersection between *S. elegans* and *R. solani* revealed partial degradation of *R. solani* mycelial and sclerotial cell walls, and suggested the production by *S.* elegans of hydrolytic enzymes such as chitinases and β-1,3-glucanases (Benyagoub and Jabaji-Hare 1992).

A chitinase gene from a marine bacterium, *Alteromonas* sp. strain O-7, was cloned, sequenced, and expressed in *E. coli* bacteria (Tsujibo et al. 1993). The chitinase protein was purified, assayed and found to have chitinase enzymatic activity. Several chitinase genes from terrestrial and marine bacteria such as *S. marcescens* (Harpster and

Dunsmuir 1989; Jones et al. 1986), *Bacillus circulans* (Watanabe et al. 1990b, 1992), *Vibrio harveyi* (Soto-Gil et al. 1984), and *Vibrio vulnificus* (Wortman et al. 1986) have been cloned and sequenced.

Chitin is the second most abundant biopolymer in nature, second only to cellulose (Tracey 1957). Many bacteria, including *Aeromonas* (Mitsutomi et al. 1990), *Bacillus* (Takayanagi et al. 1991; Watanabe et al. 1990a), *Clostridium* (Pel et al. 1990), *Norcardia* (Nanjo et al. 1990), and *Streptomyces* (Beyer and Diekmann 1985; Hara et al. 1989; Robbins et al. 1988; Tarentino and Maley 1974), secrete chitinases.

MATERIALS AND METHODS

Escherichia coli JM105 bacterial cultures either with or without the plasmid pGEX-4T-1-hs2 were grown at 37°C with 200 rpm shaking in 2X YT-G liquid medium with 100 μg/ml ampicillin for 12-15 hours, then diluted 1:10 into fresh pre-warmed 2X YT-G medium with 100 μg/ml ampicillin and grown at 37°C with 200 rpm shaking until the A_{600} reached 1-2, then induced with 0.1 mM IPTG or left uninduced (Sambrook et al. 1989). Incubation was continued for 2-6 hours after induction, and in some cases, bacterial samples were collected at one hour intervals. Bacterial cell extracts were electrophoresed on 10% polyacrylamide SDS gels and stained with Coomassie brilliant blue (Sambrook et al. 1989).

RESULTS

When induced with IPTG, *E. coli* JM105 cultures containing the pGEX4T-1-hs2 plasmid construct expressed a 58 kD fusion protein consisting of glutathione-S-transferase (GST) and ECH2 elm chitinase (Fig. 3.1). Each pair of lanes from 6-15 contained pairs of separate constructions of the pGEX4T-1-hs2 plasmid. The constructs in lanes 7, 11, and 13 expressed the 58 kD GST-ECH2 fusion protein upon induction with IPTG. Cultures that were not induced or that did not contain the pGEX-4T-1-hs2 plasmid, either induced or uninduced, did not express the 58 kD GST-ECH2 fusion protein. An expression construct containing the *hs2* elm chitinase gene, previously made by Dr. Soleman and designated pSoleman, did not result in the expression of the ECH2 elm chitinase either induced with IPTG or uninduced.

To test the timing of IPTG induction of GST-ECH2 chitinase expression, ten 4 ml cultures of YT medium with 500 μg/ml penicillin were inoculated from an overnight culture of *E.* coli JM105 containing pGEX-4T-1-hs2 plasmid grown in the same medium. The cultures were grown at 37°C with 250 rpm shaking for 2 hours, then five of the ten cultures were induced with 1 mM IPTG. At 1, 2, 3, 4, and 5 hours after induction, one induced and one uninduced culture were removed from the incubator and placed in ice. Samples of all ten cultures were boiled in gel loading buffer and electrophoresed in a polyacrylamide gel. The gel was stained with Coomassie brilliant blue and visualized (Fig. 3.2). There was no visible expression of the GST-ECH2 chitinase fusion protein in any of the cultures that were not induced. There was visible expression of GST-ECH2 chitinase fusion protein in all of the induced cultures. The amount of GST-ECH2 chitinase fusion protein increased between each time interval up to 5 hours after induction.

To test the potential of scaling up the production of GST-ECH2 chitinase in bacteria, four 1 liter cultures of *E. coli* JM105 containing the plasmid pGEX-4T-1-hs2

were grown in 2YT medium at 37°C. The cultures were induced with 1 mM IPTG. A sample of each induced culture was electrophoresed in a polyacrylamide gel (Fig. 3.3). As controls, additional lanes of the gel contained protein molecular weight marker (GIBCO BRL Life Technologies, Gaithersburg, MD) or samples from 4 ml cultures that contained either *E. coli* JM105 with no plasmid, either induced or uninduced, or *E. coli* JM105 with the plasmid pGEX-4T-1-hs2 either induced or uninduced. All four 1 liter cultures expressed the 58 kD GST-ECH2 chitinase fusion protein at levels that were visibly even higher than the amount expressed in the induced 4 ml culture containing pGEX-4T-1-hs2. The uninduced culture and the cultures without plasmid, either induced or uninduced, produced no visible GST-ECH2 chitinase fusion protein.

To test the fractionation of GST-ECH2 chitinase fusion protein expressed in *E. coli* bacteria, three 500 ml cultures were inoculated with 1/10 volume from overnight cultures. The three cultures contained *E. coli* JM105 with either no plasmid, plasmid pGEX-4T-1, or plasmid pGEX4T-1-hs2. After 1 hour of incubation, IPTG to 0.1 mM was added to induce expression of the fusion protein. Cells were collected 4 hours after induction. Cells were resuspended in 15 ml of phosphate buffered saline (PBS) with 0.2 mM AEBSF and 1 mM EDTA. Cells were treated with lysozyme for lysis. Samples were collected from the supernatant after cells were pelleted; after lysis before pelleting; after spinning down cell debris, and from the pellet with cell debris after resuspension in an equal volume of PBS. These samples were electrophoresed in a polyacrylamide gel. The gel was stained with Coomassie brilliant blue and visualized (Fig. 3.4). The supernatant after cells were pelleted contained no visible proteins from any of the three cultures.

The cells after lysing before pelleting contained a 28 kD GST protein from the culture that contained the pGEX-4T-1 plasmid. This 28 kD protein was not expressed in the cultures containing no plasmid or containing the plasmid pGEX-4T-1-hs2. The cells after lysing before pelleting contained a 58 kD GST-ECH2 chitinase fusion protein from

the culture containing the plasmid pGEX-4T-1-hs2. This 58 kD fusion protein was not expressed in the cultures containing no plasmid or containing the plasmid pGEX-4T-1.

The supernatant after spinning down cell debris contained the 28 kD GST protein in the culture containing the plasmid pGEX-4T-1. This 28 kD protein was not contained in the supernatant after spinning down cell debris from the cultures containing no plasmid or containing the plasmid pGEX-4T-1-hs2. The supernatant after spinning down cell debris from the culture containing the plasmid pGEX-4T-1-hs2 did not contain the 58 kD GST-ECH2 chitinase fusion protein, nor was it in the supernatants after spinning down cell debris from the cultures containing no plasmid or containing the plasmid pGEX-4T-1.

The pellet with cell debris resuspended in an equal volume of PBS from the culture containing the plasmid pGEX-4T-1 contained only a very small amount of the 28 kD GST protein. This 28 kD protein was not contained in the pellets with cell debris resuspended in an equal volume of PBS from the cultures containing no plasmid or containing the plasmid pGEX-4T-1-hs2. The pellet with cell debris resuspended in an equal volume of PBS from the culture containing the plasmid pGEX-4T-1-hs2 contained the 58 kD GST-ECH2 chitinase protein. This 58 kD protein was not in the pellets with cell debris from the cultures containing no plasmid or containing the plasmid pGEX-4T-1.

The GST-ECH2 elm chitinase fusion protein was likely contained in insoluble inclusions and was recoverable from the insoluble fraction of bacterial cell extracts.

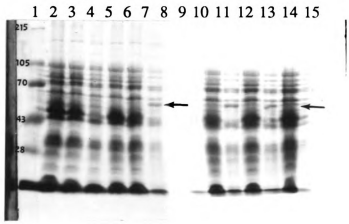

Figure 3.1. Expression of glutathione-S-transferase-ECH2 elm chitinase fusion protein in *E. coli* bacteria.

E. coli JM105 bacteria containing no plasmid, pSoleman, or pGEX-4T-1-hs2 were grown in 2YT-G medium at 37°C with 200 rpm shaking. Induction was with 1 mM IPTG. Samples were harvested 4 hours after induction and electrophoresed in a polyacrylamide gel. The gel was stained with Coomassie brilliant blue. Lane 1: molecular weight markers; Lanes 2, 4, 6, 8, 10, 12, 14: not induced; Lanes 3, 5, 7, 9, 11, 13, 15: induced with IPTG; Lanes 2, 3: no plasmid; Lanes 3, 4: pSoleman; Lanes 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15: pGEX-4T-1-hs2. Arrows show the position of the 58 kd glutathione-S-transferase-ECH2 elm chitinase fusion protein.

1 2 3 4 5 6 7 8 9 10 11

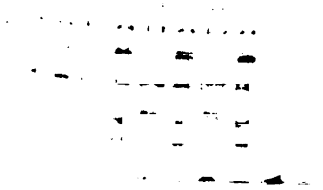

Figure 3.2. Expression of glutathione-S-transferase-ECH2 elm chitinase fusion protein in *E. coli* bacteria 1-5 hours after induction with 1 mM IPTG.

Lane 1: molecular weight markers; Lanes 2, 4, 6, 8, 10: not induced; Lanes 3, 5, 7, 9, 11: induced with IPTG; Lanes 2, 3: 1 hour after induction: Lanes 4, 5: 2 hours after induction; Lanes 6, 7: 3 hours after induction Lanes 8, 9: 4 hours after induction; Lanes 10, 11: 5 hours after induction. The arrow marks the position of the 58 kd glutathione-S-transferase-ECH2 elm chitinase fusion protein.

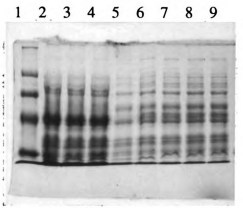

Figure 3.3. Expression of glutathione-S-transferase-ECH2 elm chitinase fusion protein in one liter cultures.

E.coli JM105 cultures either containing pGEX4T-1-hs2 plasmid or no plasmid were either induced with IPTG or left uninduced. Samples from each culture were electrophoresed in an agarose gel and stained with Coomassie brilliant blue. Lane 1: molecular weight markers; Lane 2: No plasmid uninduced; Lane 3: No plasmid induced; Lane 4: pGEX-4T-1-hs2 plasmid uninduced; Lane 5: pGEX-4T-1-hs2 plasmid induced; Lanes 6-9: pGEX-4T-1-hs2 plasmid induced in 4 liter cultures #1-4. The arrow marks the position of the 58 kd glutathione-S-transferase-ECH2 elm chitinase fusion protein.

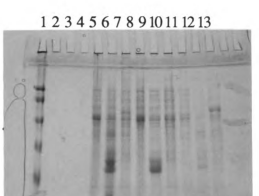

Figure 3.4. Expression of glutathione-S-transferase-ECH2 elm chitinase in *E. coli* bacteria and fractionation of cell extracts.

E. coli JM105 bacteria either with no plasmid, with pGEX4T-1 plasmid, or with pGEX4T-1-hs2 plasmid were grown and induced with 0.1 mM IPTG. Bacterial cells were harvested 4 hours after induction, lysed with lysozyme, fractionated by centrifugation, and electrophoresed in an agarose gel. The gel was stained with Coomassie brilliant blue for visualization of proteins. Lane 1: molecular weight markers; Lanes 2, 5, 8, 11: no plasmid; Lanes 3, 6, 9, 12: plasmid pGEX-4T-1; Lanes 4, 7, 10, 13: plasmid pGEX-4T-1-hs2; Lanes 2, 3, 4: supernatant after cells were pelleted; Lanes 5, 6, 7: cell extract after lysis but before pelleting; Lanes 8, 9, 10: supernatant after spinning down cell debris; Lanes 11, 12, 13: pellet of cell debris resuspended in an equal volume of phosphate buffered saline. The arrows mark the locations of the 28 kD glutathione-S-transferase and the 58 kD glutathione-S-transferase-ECH2 elm chitinase fusion protein.

DISCUSSION

The GST-ECH2 elm chitinase was successfully expressed from the pGEX-4T-1-hs2 plasmid in *E. coli* bacteria . The accumulation of the GST-ECH2 chitinase increased hourly from 1-5 hours after induction with IPTG. Either 1 mM or 0.1 mM IPTG was effective for induction of GST-ECH2 chitinase expression. A test of the induction of GST-ECH2 chitinase in scaled up cultures of 1 liter showed that the expression was high and reliable in cultures of that size. Fractionation of the bacterial cultures containing GST-ECH2 chitinase demonstrated that the fusion protein could be recovered from the insoluble fraction of lysed cells with no detectable degradation of the fusion protein. The proteinase inhibitor AEBSF was used at 0.2 mM and the divalent cation chelating agent EDTA was used at 1 mM in PBS to protect the proteins from proteases that might be released from bacterial cells upon lysis of the cells with 1 mg/ml of lysozyme.

The *hs2* elm chitinase gene contains 5' coding sequences for a hydrophobic amino terminal signal peptide. Presumably this signal peptide may be removed by proteolytic processing in the plant after targeting of the chitinase protein to the vacuoles. *E. coli* bacteria are unable to perform this processing; so it may be necessary to produce the protein without the signal peptide to obtain soluble, enzymatically active chitinase protein from bacterial expression.

Insolubility is a frequent characteristic of foreign proteins expressed in *E. coli*, especially eukaryotic proteins (Marston, 1986). Insolubility of GST fusion proteins is sometimes associated with the presence of strongly hydrophobic regions such as signal peptides, and elimination of these regions may increase stability and/or solubility. Insolubility may (Cheng, et. al, 1981) or may not (Schoemaker, et. al., 1985) be

associated with increased protein stability in *E. coli*. Both insoluble and soluble GST fusion proteins are usually stable. The stability of a soluble GST fusion protein is comparable to that or an insoluble β-galactosidase fusion protein (Smith and Johnson, 1988). The stability of GST-ECH2 chitinase was sufficient for bands to be visible in Coomassie brilliant blue-stained polyacrylamide gels.

The pGEX-4T-1 vector contains the over-expressed *lacI*^{q} allele of the *lac* repressor; so expression of GST fusion proteins from the strong *tac* promoter is efficiently repressed until induction with IPTG, regardless of the *lacI* status of the *E. coli* host. There was never any visible expression of GST or GST-ECH2 chitinase in any cultures that were not induced with IPTG.

CHAPTER IV

PLANT TRANSFORMATION

SUMMARY

The pKYLX71-hs2 plasmid construct containing the *hs2* elm chitinase gene was used to transform both tobacco (*Nicotiana tabacum*) and creeping bentgrass (*Agrostis palustris* Huds.). The tobacco transformation was done via *Agrobacterium*-mediated transformation, and the creeping bentgrass transformation was performed via biolistic transformation. The pKYLX71-hs2 plasmid proved its versatility and effectiveness by successfully transforming both a dicot by *Agrobacterium*-mediated transformation and a monocot by biolistic transformation. The tobacco transformation was done in cooperation with Dr. Lan-Ying Wen, and the creeping bentgrass transformation was done in cooperation with Dr. Benli Chai.

INTRODUCTION

Two plant species were transformed in this project to test the plasmid construct, pKYLX71-hs2. Tobacco (*Nicotiana tabacum*) was transformed via *Agrobacterium*-mediated transformation in cooperation with Dr. Lan-Ying Wen in Dr. Mariam Sticklen's laboratory (Crop and Soil Sciences Department, Michigan State University). Creeping bentgrass (*Agrostis palustris* Huds.) was transformed via biolistic transformation in cooperation with Dr. Benli Chai in Dr. Mariam Sticklen's laboratory (Crop and Soil Sciences Department, Michigan State University).

Creeping bentgrass is one of the most widely used turf-type bentgrass species for golf course putting greens, tees, and closely mowed fairways in the USA. It is a cool-season turfgrass in cool and transitional climatic regions and in cooler portions of warm climatic regions, especially in arid zones (Beard 1982). Its susceptibility to multiple fungal diseases such as brown patch (*Rhizoctonia solani*), *Pythium* blight (*Pythium graminicola*), dollar spot (*Sclerotinia homeocarpa*), and take-all patch (*Gaeumannomyces graminis* var. *avenae*) (Beard 1973) is a major problem in turfgrass culture. Conventional control of fungal diseases in creeping bentgrass culture often depends on the use of chemically synthesized fungicides, which raises increasing concerns over the environmental impact. Breeding for host resistance to these fungal diseases would be an effective and environmentally sound approach to minimizing the damage to turfgrass culture caused by fungal diseases. It could lead to improvements in the ease and economy of cultural aspects, such as establishment, persistence, durability, and maintenance requirements.

Plant resistance to pathogens involves the accumulation of pathogenesis-related proteins (PR-proteins) that are active in natural defense mechanisms (Collinge and Sluzarenko 1987). Defense mechanisms involving PR-proteins may sometimes be too weak or too slow to be effective in protecting host plants from pathogens. The progress achieved in the genetic manipulation of plants and the ability to transfer foreign DNA from a variety of sources to plants has facilitated testing the effects of constitutive overexpression of PR-proteins. This may enhance natural defense systems in plants and may significantly extend what could be achieved by traditional breeding methods. Plant transformation using chitinase transgenes for host resistance to fungal diseases has been successfully conducted in tobacco, carrot, and *Brassica napus* (Broglie et al. 1991; Zhu et al. 1994; Stuiver et al. 1996; Grison et al. 1996).

Creeping bentgrass was chosen for transformation because it has economically important fungal pathogens for which genetically modified plants could potentially become part of a breeding program to produce resistant cultivars. Tobacco was chosen not because of any interest in producing resistant cultivars, but because tobacco is relatively quick and easy to transform via *Agrobacterium*, and could potentially serve as part of a model system for testing the efficacy of elm chitinase for development of plant cultivars resistant to fungal plant pathogens and/or phytophagous insects.

Success in regeneration of creeping bentgrass has paved the way for its genetic manipulation. Two types of transformation systems have been tested successfully for developing creeping bentgrass transgenic plants; direct DNA uptake using protoplasts mediated by polyethylene glycol (PEG) or electroporation (Lee et al. 1995); and direct DNA delivery to embryogenic callus via microprojectile bombardment (Zhong et al.

1993; Hartman et al. 1994; Lee et al. 1995; Liu 1996). The embryogenic callus system is superior over the protoplast system because it is more simplified, less genotype-dependent in regenerability, and prone to less risk of somatic variations, which result from genetic instability as a consequence of the stress of protoplast isolation (Karp 1994).

To efficiently produce transgenic cells after gene transfer, selectable marker genes are usually introduced for positive selection of transformed cells. A phosphinothricin (PPT) acetyltransferase (PAT) gene has been shown to be an excellent selectable marker in creeping bentgrass transformation (Hartman et al. 1994; Lee et al. 1995; Liu 1996). Resistance to the herbicide PPT or bialaphos is conferred by PAT, which inactivates PPT by acetylation. PPT or bialaphos selection has also been used successfully in the transformation of potato, tobacco (DeBlock et al. 1987), *Brassica* species (DeBlock et al. 1989), *Populus* hybrids (DeBlock 1990), maize (Fromm et al. 1990; Gordon-Kamm et al. 1990), and wheat (Vasil et al. 1992).

Chitinases are PR-proteins that are found in a wide variety of plants. Evidence suggests strongly that chitinases function as antifungal proteins, and possibly as insecticidal proteins as well. Accumulation of chitinases (Meins and Ahl 1989a,b; Rasmussen et al. 1992a,b) and their encoding mRNAs (Metraux and Boller 1986; Roby and Esquerre-Tugaye 1987b; Meins and Ahl 1989a,b; Roby et al. 1990) is significantly induced during fungal infection. The induction of chitinase also occurs when plant tissue is treated with fungal cell wall material (Kurosaki et al. 1987b; Roby and Esquerre-Tugaye 1987a). Ethylene, a gaseous plant hormone that is normally produced during fungal infection, also induces chitinase activity (Boller et al. 1983; Broglie et al. 1986). Chitinase is also induced by wounding (Parsons et al. 1989). Although chitinases and

other PR-proteins have been found to play an active role in natural defense mechanisms, their effectiveness could be greatly compromised or even overcome by pathogens that have evolved mechanisms to evade or inactivate antifungal gene products. As a common strategy in transformation, foreign genes are chosen as transgenes in order to delay the development of PR-protein tolerance in fungal pathogens. The effective antifungal activity of chitinase genes has been reported in several species of transgenic plants that constitutively produce exogenous chitinases. Transgenic tobacco plants expressing dry bean chitinase were resistant to *Rhizoctonia solani* (Broglie et al. 1991). Transgenic tobacco plants expressing both a rice chitinase and a glucanase gene were also resistant to *Cercospora nicotianae*, the causal agent of frogeye (Zhu et al. 1994). Transgenic carrot plants with both chitinase-I and glucanase-I showed resistance to *Alternaria radicina* and *C. carotae* (Stuiver et al. 1996). Elevated field tolerance to fungal pathogens was first reported in *Brassica napus* transgenic plants that contained a chimeric chitinase gene from bean (Grison et al. 1996).

Genetic transformation of turfgrass has been successful in recent years with the development of efficient regeneration systems and the use of reliable selectable markers and DNA delivery technologies such as biolistic bombardment. However, chitinase genes or other PR-protein genes have only recently been used as transgenes for turfgrass species (Warkentin et al. 1998). Progress in transferring exogenous chitinase genes to turfgrass may greatly benefit turfgrass breeding programs for fungal disease resistance and possibly for insect resistance also. It would also facilitate our understanding of gene expression and plant-pathogen and/or plant-insect interactions.

MATERIALS AND METHODS

The plasmid pKYLX71-hs2 was introduced into *Agrobacterium tumefaciens* LBA4404 by triparental mating with *Escherichia coli* containing pGEX-4T-1-hs2 and *E. coli* containing the mobilization helper plasmid pRK2013. The *A. tumefaciens* LBA4404 containing pKYLX71-hs2 was then used for transformation of tobacco (*Nicotiana tabacum*) by the leaf disk method (Horsch 1985).

Creeping bentgrass was transformed with pKYLX71-hs2 plasmid by a biolistic method. Embryogenic callus was induced from mature seeds (caryopses) of creeping bentgrass (*Agrostis palustris*) cultivar Penncross (Zhong et al. 1993). Seeds were surface sterilized with 50% commercial Clorox bleach solution containing 1% Tween 20 with a vacuum applied and then soaked in 70% ethanol for 5 minutes. The sterilized seeds were rinsed with sterilized distilled water three times, then transferred to a semi-solid medium comprised of MS basal salts supplemented with 500 mg/L enzymatic casein hydrolysate, 3% sucrose, 30 μM Dicamba (3,6-dichloro-*o*-anisic acid), 2.25 μM BA (6-benzyladenine), and 7 g/L phytagar. Cultures were incubated in the dark at 25°C and subcultured every two weeks. Light yellow, friable calli were selected and placed on a 2 cm^2 area in a single layer in the center of Petri dishes containing callus induction medium for microprojectile bombardment.

A Biolistic PDS-1000/He system (DuPont/Bio-Rad) was used to deliver tungsten particles coated with plasmid DNA. Physical parameters were optimized to increase the numbers of transiently GUS-expressing cells. The following conditions were found to be superior and used as a standard bombardment protocol: rupture disk pressure, 1,550 psi;

gap distance from rupture disk to macrocarrier, 6 mm; macrocarrier travel distance, 16 mm; microcarrier travel distance, 6 cm. Prior to bombardment, plasmid DNA was precipitated onto tungsten particles (0.9-1.2 μm in diameter) (Zhong et al. 1993), using a precipitation mixture that included 1.5 mg tungsten, 30 μg plasmid DNA, 1.1 M $CaCl_2$, and 8.7 mM spermidine free base. The plasmid construct pJS101 carrying the selectable marker coding sequence (*bar* gene) driven by the CaMV 35S promoter was provided by Dr. Ray Wu of Cornell University, while the *hs2* gene (elm chitinase gene) driven by the CaMV 35S promoter was in the plasmid pKYLX71-hs2. To optimize the conditions for co-transformation, the pKYLX71-hs2 and pJS101 plasmid DNAs were coprecipitated in different ratios. Embryogenic callus was bombarded three times and transferred to fresh medium immediately after bombardment to minimize contamination.

Selection for transformants was conducted in three stages: callus, seedlings, and greenhouse plants with an increasing concentration of the selective agent. Two weeks after bombardment, calli were subcultured onto selection medium containing 3 mg/L bialophos. Additional selections at the callus stage were conducted at a 4 week subculture interval under 5 mg/L and 10 mg/L bialophos in consecutive order. The surviving calli on 10 mg/L bialophos were transferred to MS medium containing 10 mg/L bialophos and incubated under lights for regeneration. Thousands of these putatively transformed plantlets were regenerated via somatic embryogenesis and were transferred to the greenhouse one month after culturing in regeneration medium. Selection in the greenhouse was done by spraying the putatively transformed plants with 1% Ignite® solution (Hoechst-Roussel Agri-Vet Company, Leland, MS) containing 180 mg/L PPT.

Two sprays were applied 2 weeks and 8 weeks after transfer to the greenhouse. The effects of sprays were evaluated 7 days after herbicide application.

RESULTS

The pKYLX71-hs2 was introduced into *Agrobacterium tumefaciens* LBA4404 by triparental matings. A restriction digestion of the pKYLX71-hs2 plasmid DNA purified from *A. tumefaciens* LBA4404 displayed the identical restriction pattern as the same plasmid purified from *Escherichia coli* (Figure 4.1).

The *A. tumefaciens* LBA4404 containing pKYLX71-hs2 was used for transformation of tobacco (*Nicotiana tabacum*) by the leaf disk method. The putatively transformed tobacco plants were regenerated and transferred to the greenhouse in potting medium.

Thousands of putatively transformed creeping bentgrass plantlets from seven out of twelve bialophos-resistant callus colonies were regenerated. All of the calli and plantlets from these colonies are consistent in resistance to increasing concentrations of bialophos. Molecular analysis confirmed the presence of the *hs2* elm chitinase gene in both the putatively transgenic tobacco and the putatively transgenic creeping bentgrass. Polymerase chain reaction (PCR) was used to screen for the putative transformants that contained the *hs2* elm chitinase gene. Southern blots confirmed the presence of the *hs2* chitinase gene in the putatively transgenic tobacco and creeping bentgrass plants.

Bioassays using the turfgrass fungal pathogen *Rhizoctonia solani* revealed that at least one of the transgenic turfgrass lines had increased resistance to this pathogen.

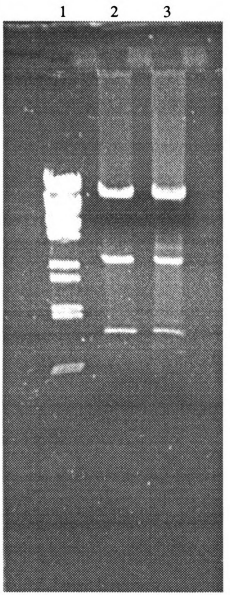

Figure 4.1. *Pst* I/*Xho* I restriction patterns for pKYLX71-hs2 plasmid DNA purified from *Escherichia coli* or *Agrobacterium tumefaciens*.

Lane 1: lambda/*Bst* EII molecular weight markers; Lane 2: pKYLX71-hs2/*Pst* I/*Xho* I from *E. coli*; Lane 3: pKYLX71-hs2/*Pst* I/*Xho* I from *A. tumefaciens*.

DISCUSSION

The triparental mating successfully introduced the plasmid pKYLX71-hs2 into *Agrobacterium tumefaciens* LBA4404. The restriction patterns of the plasmids purified from either *Escherichia coli* or *A. tumefaciens* were identical, indicating that the plasmid had been successfully transferred from *E. coli* into *A. tumefaciens*. The plasmid was stable and provided DNA with no visible degradation from both bacteria. The RK2 origin of replication was functional in both bacteria.

The transformation and regeneration of tobacco was routine, as it is a procedure that has been well established in many laboratories. Kanamycin was used for selection of putative transformants. Polymerase chain reaction (PCR) and Southern blots confirmed the integration of the *hs2* gene into the tobacco genome.

The pKYLX71-hs2 plasmid demonstrated its versatility by successfully transforming creeping bentgrass by the biolistic system as well as tobacco by *Agrobacterium*-mediated transformation.

Bioassays conducted by Dr. David Green in Dr. Joe Vargas' laboratory (Botany and Plant Pathology Department, Michigan State University) indicated that the *hs2* elm chitinase expressed in transgenic creeping bentgrass was effective in increasing the level of resistance versus the fungal pathogen *Rhizoctonia solani*. This transgenic turfgrass line (711) might be useful for breeding resistance to *R. solani*, the causal agent of brown patch of turfgrass, into commercial cultivars.

CHAPTER V

BIOASSAYS FOR INSECT RESISTANCE

SUMMARY

Bioassays were conducted to test transgenic turfgrass expressing the *hs2* elm chitinase gene to determine if they had elevated levels of resistance versus Japanese beetle grubs. These beetle grubs possess peritrophic membranes partially composed of chitin. The hypothesis to be tested was whether elm chitinase expressed in transgenic turfgrass could inhibit the growth or increase the mortality of Japanese beetles that feed on the roots of these plants. One possibility might be that the elm chitinase would disrupt or permeabilize the peritrophic membranes of these beetles, thereby reducing their efficiency of digestion of food or increasing their susceptibility to intestinal pathogens.

INTRODUCTION

Most insect groups possess peritrophic membranes (PM) (Du Porte 1959). The term peritrophic envelope (PE) is synonymous. The peritrophic membrane extends through the mid-gut and into the hindgut. It is a thin chitinous tube free from the epithelial wall. It encloses the food and protects the delicate epithelial cells from contact with hard or rough partticles. In many insects, the membrane is secreted by a ring of specialized cells at the anterior end of the mid-gut. In others, it is formed, at least partially, by desquamation of sections of the striated border of the epithelial cells. The sections adhere to form a continuous tube.

An insect's body surface is entirely enclosed within its integumental exoskeleton, which also covers the lumeral face of the foregut, hindgut, and salivary glands, and lines the trachea. The midgut endothelium does not secrete a cuticle, but is protected by the peritorphic membrane (Reynolds and Samuels 1996). The peritrophic membrane is a chitin-containing structure that has some similarities with cuticle, but is distinct from it (Spence 1991).

The peritrophic membrane, which surrounds the food bolus in the midgut (MG) of various arthropods, was described by a number of authors in the nineteenth century, dating at least as far back as 1843 (Richards and Richards 1977). Balbiani (1890) apparently gave it its present name, which means surrounding the food.

Most insects have peritrophic membranes in both larval and adult instars (Peters 1969). Peritrophic membranes are found in insects whose diets contain harsh particles, as

well as in many insects, such as adult butterflies and bloodsucking flies, that have diets with no rough particulate matter (Waterhouse 1953).

There are two types of peritrophic membrane, formed in two different ways (Wigglesworth 1972). The type i membrane occurs in Phasmids, Acridiids, Ephemeroptera, Odonata, caterpillars, *Tenebrio*, *Hydrophilus*, and other beetles, and *Apis*, *Vespa*, and their larvae. The type i membrane is composed of concentric lamellae, independent or loosely attached to one another. It is produced by the separation of thin sheets from the surface of cells throughout the length of the midgut.

The type ii peritrophic membrane occurs in the larvae and adults of Diptera, in the earwig, and perhaps in termites. The type ii peritrophic membrane consists of a single uniform layer. It is secreted in viscous form by a group of cells at the anterior limit of the mid-gut. It passes through an annular cleft between the oesophageal invagination and the midgut, and solidifies to form a homogeneous tube.

In *Polisted*, *Bombus*, *Apis*, and other insects, the peritrophic membrane may have a double origin, combining the features of type i and type ii. It may represent a stage in the evolution of type ii from type i.

In the larvae of Neuroptera and ants, in adult *Similium*, and adult Culicidae, the entire mid-gut epithelium produces a viscous secretion enveloping entirely the gut contents. The resulting peritrophic membrane has been regarded as belonging to a third type.

It seems that since there are insects with types of peritrophic membrane that are intermediate bertween type i and type ii, and even some that don't seem to exactly fit either type, that there is not good evidence for more than one origin of peritrophic

membrane. Some may conclude that there are two or even three origins since there seem to be two or even three different types, but the observation of types that are intermediate between the types seems to indicate that a single origin is more likely, and that the second type and probably the third arose from the first type.

The peritrophic membrane may function to protect the mid-gut cells from abrasion by hard fragments in the food, replacing in this respect the mucous involucrum which lubricates the gut of vertebrates. This belief seems to be consistent with the fact that insects that feed only on fluids, such as Hemiptera, adult Lepidoptera, and many blood sucking insects such as Tabanids, fleas, and lice lack a peritrophic membrane. In Culicids and *Phlebotomus* it is extremely tenuous (Wigglesworth 1972).

The peritrophic membrane is not found in many carnivorous Coleoptera (Carabids and Dytiscids), in which the mid-gut cells degrade during secretion. It is reported to be absent in *Gryllotalpa*, in the ant, and in the larva of *Anthrenus* (Dermestidae). A poorly organized PM occurs in *Gryllotalpa*. One occurs in certain Corixids and in *Anthrenus* and other Dermestrids. A type i membrane is in adult *Pieris*, and a type ii in other families of Lepidoptera.

The permeability of PM has been tested with various dyes in *Carausius*, termites, *Apis*, *Vanessa* larva, and *Calliphora*. Large colloidal particles such as congo red or Berlin blue are arrested by PM. Colloidal gold particles 2-4 nm diameter diffuse through PM of mosquito larvae, but 20 nm particles do not pass through. The excised *Manduca* sexta PM was found to be freely permeable to proteins of molecular weights up to 10^5 Da (Wolfesberger et al. 1986). The PM may serve as an ultra filter. Digestive enzymes and the products of digestion pass through it.

Baines (1978) estimates pore size in *Locusta* to be 200 nm. Skaer (1981) estimates the pore size of locust and cockroach membranes to be about 150 nm. About 60% of the membrane area is pore; so PM is a very efficient high-flux sieve. These pores provide free passage to all but the largest molecules, while protecting the midgut from bacterial attack or abrasion by food particulates (Dow 1986). Terra and Ferreira (1983) have asserted that large digestive enzymes are unable to cross the peritrophic membrane, allowing biochemical specialization of the endo- and ecto- peritrophic spaces in a fruit-eating Dipteran larva, *Rhynchosciara americana.*

In solid feeders, the midgut is protected by a strong peritrophic membrane, but peritrophic membranes are rarely present in liquid feeders, as the diet is not abrasive (Dow 1986). A delaminated peritrophic membrane is secreted in the Collembolan, *Tomocerus minor*; so this seems to be a primitive characteristic of insect midgut (Humbert 1979). The PM in mosquito larva seems to be an effective barrier to protozoan parasites in the midgut (Dodd 1971).

Santos et al. (1983, 1984) claim that the PM is a sufficient barrier to allow the enzyme and ionic contents of the endo- and ecto- peritrophic spaces to differ significantly, allowing a counter-current to operate. However, Dow (1986) claims that there is some conflict in the data, which could only be resolved by a careful study of the permeability of excised peritrophic membrane. The counter current model was outlined by Berridge (1970). It was suggested that there was an endo/ectoperitrophic circulation of fluid, and that the anterior midgut is specialized for absorption of nutrients, and the posterior midgut for secretion of water. Dow (1986) suggested that the countercurrent model undoubtedly occurs in locusts, mosquito larvae, and other Dipteran larvae, but was

not convinced that it occurred in Lepidopteran larvae. His scepticism focused around the observations that movement of food in Lepidoptera, particularly *Erinnys ello*, the species studied by Santos et al. (1983), was very rapid, and dye passage is uniformly aborally (posteriorly) directed in larvae (Hukuhara et al. 1981).

In cases where countercurrents of fluid occur, there is a morphologically specialized uptake site, in caeca at the anterior border of the foregut and midgut. This site does not occur in Lepidoptera (Dow 1986). The posterior region of the midgut is the most highly convoluted membrane in Lepidoptera. No significant water fluxes have been observed concommitant with potassium transport in Lepidopteran midgut (Nedergaard 1972; Zerahn 1985). The gastric caeca is the major site of nutrient uptake in the locust (Treherne 1957, 1958a,b, 1959), but such data is not yet available for the caterpillar.

Dow (1986) suggested that the observation of Santos et al. (1984) that midgut enzymes do not appear rapidly in the faeces could be explained by a purely mechanical function; that the solid material entering the hindgut is compressed in the ileum, forming faecal pellets, and the fluid squeezed out is retained in the midgut. Dow (1986) also suggested that this observation could at least partially explain the apparent difference in concentrations of midgut enzymes between the endo- and ecto- peritrophic spaces. Discrete faecal pellets are produced by locusts, because an S bend in the hindgut serves to snap the peritrophic membrane and solid materials in the food bolus (Goodhue 1963).

According to Summers and Felton (1996), it is reasonable to assume that the peritrophic membrane or peritrophic envelope (PE), as they prefer to call it, protects the mid-gut epithelium from abrasive food particles within the lumen, and may act as a lubricant to facilitate the passage of the food bolus (Wigglesworth 1974) considering the

tensile strength of the chitin microfibril mesh and the relative smoothness of the PM relative to the food particles in the lumen. Other suggested functions of the PM include compartmentalization of midgut function, exclusion of pathogens from the midgut epithelium, and binding or exclusion of toxins (Abedi and Brown 1961; Spence 1991; Peters 1992; Barbehenn and Martin 1992, 1994).

PM may be an ultrafilter with pore sizes from 2-650 nm (Peters and Wiese 1986; Adang and Spence 1983). PM may serve to exclude toxins from the epithelial surface. Abedi and Brown (1961) reported that a DDT-resistant strain of *Aedes aegypti* produced nine times as much PE as did a DDT-susceptible strain.

Bernays and Chamberlin (1980) reported that PM was important in protection against dietary tannins. PM could prevent tannin toxicity by adsorption (Bernays and Chamberlin, 1980), ultrafiltration (Barbehenn and Martin, 1992), and/or ionic exclusion (Barbehenn and Martin, 1994). Felton and Summers (1995) suggested an additional function for PM: protecting the midgut epithelium from oxidative damage by dietary prooxidants.

Spence (1991) lists five functions of the PM: influencing the absorption of nutrients; compartmentalizing the midgut; interfering with the penetration of invading microbes; protecting the gut from abrasive food particles; and serving as structural material for cocoons.

The suggestion that the peritrophic membrane protects the endodermal cells against abrasion seems to be axiomatic. In other words, it has been generally accepted as being true for a number of years, and no one seems to have seriously challenged the idea. There doesn't seem to have been any conclusive experiments done that would provide

strong evidence one way or the other regarding the truth of this axiom. The only evidence seems to be the observations that the peritrophic membrane is composed of chitin, protein, and polysaccharide similar to mucus; so it seems to have the adequate strenth and slipperyness to facilitate the passage of food through the midgut while protecting the epithelial cells from abrasion. Also there is the observation that insects that feed on rough, hard diets seem to have peritrophic membranes, while Homoptera, which feed on plant sap, which is not abrasive, do not have peritrophic membranes. The exception to liquid feeders not having peritrophic membranes is the blood-feeding Dipterans, which do have peritrophic membranes. In this case, the function of the peritrophic membrane may be other than protecting from abrasion, although it is true that the blood-feeding dipterans dehydrate thier meal before digesting it; so it is possible that the dried blood is more abrasive than the liquid diet to Homopterans, who do not dehydrate their meals before digestion, at least not to the extent of the Dipterans. Some Hompterans have filter chambers that remove some of the water and concentrate the meal, but it is not dehydrated to the extent that it is in the blood-feeding Dipterans.

There is good evidence that the peritrophic membrane protects against insect pathogens. Indeed, some insect pathogens produce chitinase and/or protease to disrupt the peritrophic membrane to allow them to achieve access to the epithelial cells (Huber et al. 1991; Chen and Thiem 1997; Lepore et al. 1996; Derksen and Granados 1988; Wang et al. 1994). Plant sap feeders do not need this protection, because most plant viruses do not replicate in insects and are not pathogenic to them, although there are some exceptions. In addition, it may be advantageous to Homopterans to transmit plant viruses, because virus-infected plants have a higher concentration of protein in their

phloem compared to that found in uninfected plants; so they are more nutritious to Homopterans that feed on plant sap, which is ordinarily very high in carbohydrate concentration but very low in protein concentration compared with what would be the ideal nutritional diet for Homopterans. Not all plant viruses need access to the epithelial cells in order to be transmitted, but some do.

Because of the evidence that peritrophic membranes may function to protect the epithelial cells of the digestive tract from abrasive damage, may function in digestion of food, and may protect the epithelial cells from pathogens, a bioassay was conducted to determine if the ingestion of chitinase in the diet of insects could inhibit the growth of insects and/or affect the mortality rate of insects by interfering with one or more of these functions.

MATERIALS AND METHODS

Transgenic Turfgrass

The plasmid pKYLX71-hs2, containing the *hs2* elm chitinase gene controlled by the CaMV 35S promoter, was used to transform creeping bentgrass (*Agrostis palustris* Huds.) by the biolistic method. The cultivar transformed was Penncross, which is a synthetic cultivar that is very heterogeneous. Biolistics and regeneration were done by Dr. Benli Chai. The turfgrass plants were cotransformed with the plasmid, pJS101, which has the *bar* gene to give bialaphos herbicide resistance. The plasmid pJS101 has the *bar* gene controlled by the CaMV 35S promoter. The gene *mtlD* for mannitol dehydrogenase for drought resistance was also on the plasmid pJS101. The *mtlD* gene was controlled by the rice actin promoter. The vector control plant line was bombarded with a plasmid vector that did not contain the chitinase gene. It was bombarded with pJS101 only, but not with pKYLX-HS2. It was screened for bialaphos resistance, as were the transgenic lines. The transgenic plant lines were: 711; 9601; 9603; and 9606. The nontransgenic control plant line was Penncross. The vector control transgenic plant line was 7201.

Experimental Design

Five Japanese beetle grubs were placed on the surface of the turfgrass growing as a lawn in each four inch square plastic pot. The planting mix was Baccto High Porosity

Professional Planting Mix. The plants were fertilized every fourteen days with 20-20-20 NPK fertilizer. The beetles were collected from the campus of Michigan State University, East Lansing, Michigan from 4-5 pm on May 21, 1998. There were stored in soil overnight at 10^0C. They were weighed and put on the turfgrass on May 22, 1998 (Table 5.2). The beetle grubs were placed on the surface of the grass and then covered with soil. They were allowed to burrow into the grass. A wet paper towel was placed over each pot to prevent desiccation until the grubs had burrowed under the surface of the grass. Five pots were used for each treatment. The treatments were: four different transgenic plant lines (711, 9601, 9603, and 9606); one nontransgenic control plant line (Penncross), and one vector control plant line (7201). In addition, five pots of each plant line were kept without grubs.

The data were collected on June 22, 1998 (Table 5.3). The grubs were collected from each pot, counted, and weighed. The numbers of living larvae, living pupae, and dead larvae, and dead pupae in each pot were counted and weighed. The data was analyzed by ANOVA.

RESULTS

The turfgrass on which grubs had fed for five weeks was compared in appearance with the turgrass on which no grubs had fed. There was no visible difference in appearance of the turfgrass on the surface. There was no visible feeding damage on the grass surface. The roots of the turfgrass on which grubs had fed were eaten, but the surface of the grass was unaffected.

The data on mean larval growth rates, mean pupal weights, survival, and pupation of the Japanese beetles is summarized in Table 5.1. The data were statistically analyzed by ANOVA.

There were no statistically significant differences for larval growth rates between the Penncross nontransgenic cultivar, the 7201 transgenic line, which had been transformed with the *bar* gene for herbicide resistance but not with the *hs2* elm chitinase gene, and any of the transgenic lines (711, 9601, 9603, or 9606) that had been transformed with the hs2 elm chitinase gene. There were differences, but due to high variability within the lines, they were not statistically significant at P=0.05 (Pr > F was 0.21).

For pupal weights, the only statistically significant difference at P=0.05 was between the Penncross nontransgenic cultivar and the 711 chitinase transgenic line. The pupae that fed on the 711 transgenic line were larger than the ones that fed on Penncross. Pr > F was 0.002. There were no insects that pupated on the 7201 transgenic line with *bar* only but not the *hs2* elm chitinase gene.

All of the turfgrass lines were statistically the same at P=0.05 for insect survival. The insects that fed on Pencross and 7201 transgenic line without chitinase had greater survival than the ones that fed on any of the transgenic lines, but the differences were not significant at P=0.05. The Pr > F was 0.74.

The only statistically significant difference (P=0.05) for pupation was between Pencross and 7201 transgenic line. This was the transgenic line withthe *bar* gene but without the *hs2* chitinase gene. The Pr > F was 0.05. There was no pupation in any of the pots containing the 7201 line.

The tables of raw data for the Japanes beetle bioassays are shown in the appendix to this chapter. Table 5.2 lists the initial Japanese beetle larval weights. Table 5.3 lists the final Japanese beetle larval weights after five weeks of feeding on turfgrass lines. Table 5.4 lists the final Japanese beetle pupal weights. Table 5.5 lists the survival and pupation of Japanese beetles on turfgrasses.

Table 5.1 Larval growth rate, pupal weight, survival, and pupation of Japanese beetles on turfgrasses.

Turfgrass Line	Mean Larval Growth Rate (mg/day)[1]	Mean Pupal Weight (gm)[2]	Mean Survival[3]	Mean Pupation[4]
Penncross	0.40 a	0.12 ab	3.0 a	1.6 b
7201	0.74 a	NA[5]	2.8 a	0.0 a
9601	1.71 a	0.24 bc	2.6 a	0.4 ab
9603	-0.33 a	0.19 abc	2.4 a	1.0 ab
9606	-1.36 a	0.10 a	2.0 a	0.4 ab
711	0.87 a	0.26 c	2.6 a	0.6 ab

[1]Growth measured after 35 days of feeding on turfgrass lines and calculated to growth per day.

[2]Pupal weights measured 35 days after placing larvae on turfgrass lines for feeding.

[3]Mean number of insects per pot (combined larvae and pupae) that were still alive 35 days after placing larvae on turfgrass lines for feeding. Five larvae were placed in each pot initially.

[4]Number of live pupae per pot 35 days after five larvae were placed in each pot of turfgrass.

[5]No insects pupated in any pots with turfgrass line 7201.

DISCUSSION

There were no statistically significant differences (P=0.05) in larval growth rates between the Penncross nontransgenic turfgrass cultivar, the 7201 transgenic line with *bar* but without the *hs2* chitinase gene, and any of the transgenic turfgrass lines with hs2 elm chitinase. There were differences that were not statistically significant at P=0.05. The lack of statistical significance was because of too much variability within each turfgrass line. Possibly this amount of variability could be reduced if the experiment were to be repeated by carefully selecting larvae that were very similar in initial weights. Also, by increasing the number of replications, possibly statistical significance at P=0.05 could be achieved.

The only transgenic turfgrass line that had a statistically significant difference (P=0.05) from Penncross for pupal weights was 711. The pupal weights were greater for the insects on 711 than for the insects on Penncross. It is uncertain whether this result is due to chitinase, somaclonal variation, undefined sources of experimental variability, or some possible combination of unexplained physiological responses. There were no pupae in any of the pots containing 7201 transgenic line with *bar* herbicide resistance gene but not with *hs2* chitinase gene. The possible reasons for this lack of pupation on 7201 are unknown.

All of the turfgrass lines were statistically the same (P=0.05) for insect survival. The insects on Penncross and 7201 had greater survival than the insects that were on any of the transgenic lines with *hs2* chitinase, but the differences were not statistically

significant at P=0.05. If this bioassay were to be repeated, a greater number of replications could possibly result in statistically significant differences in insects survival.

The only statistically significant difference in insect pupation was between Penncross and 7201. The turfgrass line 7201 had the *bar* herbicide resistance gene, but not the *hs2* chitinase gene. Somaclonal variation is one possible explanation for this result. All of the transgenic turfgrass lines with chitinase had lower pupation compared with the pupation of insects on Penncross, but these results were not statistically significant at P=0.05. Possibly a greater number of replications might result in statistical significance at P=0.05 if the bioassay were to be repeated.

With only a few exceptions, these results were not statistically significant at P=0.05 although there were differences that were not statistically significant at that level. One possible explanation for these results may be that the peritrophic membranes of Japanese beetle grubs are not highly susceptible to significant damage by the levels of elm chitinase that were expressed in the transgenic turfgrass used for this study. Many nontransgenic plants naturally produce chitinase; so it may be that Japanese beetle populations have been naturally selected for those that are resistant to certain plant chitinases.

One goal of this poject was to determine if insects may be more susceptible to chitinases from plants other than the ones on which they commonly feed. Insect populations may be selected to be resistant to the chitinases from the plants on which they commonly feed, but they may be more susceptible to chitinases from plants on which they do not feed. Yet this limited feeding study did not provide statistically significant evidence to support that possibility.

This study did not provide statistically significant results to answer the question of whether or not plant chitinases may be involved in defense against phytophagous insects. Many plants produce chitinase, and insects have had opportunities to develop resistant to plant chitinases. This does not rule out the possibility that certain plant chitinases may be effective in controlling certain insects. More tests are warranted with other insects and with greater numbers of replications to determine if chitinases may be effective for plant resistance against insects.

APPENDIX

TABLES OF RAW DATA FROM INSECT BIOASSAYS

Table 5.2 Initial Japanese beetle grub weights (gms)

Pot Number	Turfgrass Line	Grub #1	Grub #2	Grub #3	Grub #4	Grub #5	Average Weight	S.D.[1]
1	Penncross	0.21	0.32	0.22	0.29	0.19	0.25	0.06
2	Penncross	0.26	0.20	0.21	0.25	0.25	0.23	0.03
3	Penncross	0.22	0.23	0.20	0.22	0.19	0.21	0.02
4	Penncross	0.26	0.23	0.19	0.18	0.21	0.21	0.03
5	Penncross	0.20	0.26	0.30	0.22	0.28	0.25	0.04
6	9606	0.10	0.21	0.22	0.24	0.23	0.20	0.06
7	9606	0.20	0.19	0.20	0.25	0.26	0.22	0.03
8	9606	0.07	0.16	0.15	0.04	0.22	0.13	0.07
9	9606	0.22	0.10	0.28	0.21	0.22	0.21	0.07
10	9606	0.23	0.25	0.24	0.23	0.23	0.24	0.01
11	7201	0.23	0.24	0.15	0.20	0.19	0.20	0.04
12	7201	0.26	0.19	0.30	0.24	0.17	0.23	0.05
13	7201	0.21	0.19	0.22	0.26	0.06	0.19	0.08
14	7201	0.18	0.24	0.24	0.22	0.21	0.22	0.02
15	7201	0.22	0.22	0.20	0.31	0.26	0.24	0.04
16	9603	0.20	0.21	0.21	0.25	0.07	0.19	0.07
17	9603	0.16	0.25	0.26	0.06	0.26	0.20	0.09
18	9603	0.24	0.20	0.28	0.07	0.04	0.17	0.11
19	9603	0.20	0.22	0.07	0.23	0.19	0.18	0.06
20	9603	0.21	0.21	0.22	0.22	0.23	0.22	0.01
21	711	0.31	0.29	0.18	0.25	0.22	0.25	0.05
22	711	0.20	0.06	0.20	0.20	0.21	0.17	0.06
23	711	0.22	0.18	0.22	0.20	0.21	0.21	0.02
24	711	0.25	0.17	0.20	0.23	0.23	0.22	0.03
25	711	0.25	0.21	0.27	0.22	0.27	0.24	0.03
26	9601	0.28	0.21	0.20	0.23	0.24	0.23	0.03
27	9601	0.21	0.26	0.24	0.07	0.20	0.20	0.07
28	9601	0.30	0.17	0.18	0.07	0.23	0.19	0.08
29	9601	0.23	0.21	0.29	0.28	0.11	0.22	0.07
30	9601	0.22	0.22	0.22	0.24	0.21	0.22	0.01

[1] Standard deviation.

Table 5.3 Final Japanese beetle larval weights (gm)

Pot Number	Turfgrass Line	Larva #1	Larva #2	Larva #3	Larva #4	Larva #5	Average Weight	S.D.[1]
1	Penncross						######	####
2	Penncross	0.23	0.35	0.14	0.25		0.24	0.09
3	Penncross	0.21	0.32				0.27	0.08
4	Penncross	0.19	0.20				0.20	0.01
5	Penncross						######	####
6	9606	0.10	0.19	0.14			0.14	0.05
7	9606	0.20	0.16				0.18	0.03
8	9606						######	####
9	9606	0.22	0.15	0.15			0.17	0.04
10	9606	0.21	0.14				0.18	0.05
11	7201	0.05	0.11				0.08	0.04
12	7201	0.24	0.26	0.25			0.25	0.01
13	7201	0.28	0.28	0.26	0.20		0.26	0.04
14	7201	0.32	0.38	0.25			0.32	0.07
15	7201	0.20	0.42				0.31	0.16
16	9603	0.18					0.18	####
17	9603	0.22					0.22	####
18	9603	0.17	0.18				0.18	0.01
19	9603	0.09	0.21				0.15	0.08
20	9603	0.17					0.17	####
21	711	0.35	0.27	0.20			0.27	0.08
22	711	0.19					0.19	####
23	711	0.27	0.35	0.23	0.33		0.30	0.06
24	711	0.17	0.25				0.21	0.06
25	711						######	####
26	9601	0.18	0.18				0.18	0.00
27	9601	0.20	0.20	0.17	0.27		0.21	0.04
28	9601	0.37					0.37	####
29	9601	0.25					0.25	####
30	9601	0.43	0.37	0.26			0.35	0.09

[1] Standard deviation.

Table 5.4 Final Japanese beetle pupal weights (gm)

Pot Number	Turfgrass Line	Pupa #1	Pupa #2	Pupa #3	Pupa #4	Pupa #5	Average Weight	S.D.[1]
1	Penncross	0.12					0.12	####
2	Penncross						######	####
3	Penncross	0.16					0.16	####
4	Penncross	0.13	0.12				0.13	0.01
5	Penncross	0.13	0.09	0.12			0.11	0.02
6	9606						######	####
7	9606						######	####
8	9606	0.07					0.07	####
9	9606						######	####
10	9606	0.12					0.12	####
11	7201						######	####
12	7201						######	####
13	7201						######	####
14	7201						######	####
15	7201						######	####
16	9603	0.20					0.20	####
17	9603						######	####
18	9603						######	####
19	9603	0.22	0.22				0.22	0.00
20	9603	0.17	0.15				0.16	0.01
21	711						######	####
22	711	0.16					0.16	####
23	711						######	####
24	711						######	####
25	711	0.30	0.31				0.31	0.01
26	9601						######	####
27	9601						######	####
28	9601	0.29					0.29	####
29	9601						######	####
30	9601	0.18					0.18	####

[1] Standard deviation.

Table 5.5 Survival and pupation of Japanese beetles on turfgrasses[1].

Pot Number	Turfgrass Line	Number of Live Larvae	Number of Live Pupae	Total Number of Live Insects
1	Penncross	0	1	1
2	Penncross	4	0	4
3	Penncross	1	2	3
4	Penncross	2	2	4
5	Penncross	0	3	3
6	9606	2	0	2
7	9606	2	0	2
8	9606	0	1	1
9	9606	2	0	2
10	9606	2	1	3
11	7201	2	0	2
12	7201	3	0	3
13	7201	4	0	4
14	7201	3	0	3
15	7201	2	0	2
16	9603	1	1	2
17	9603	1	0	1
18	9603	2	0	2
19	9603	2	2	4
20	9603	1	2	3
21	711	3	0	3
22	711	1	1	2
23	711	4	0	4
24	711	2	0	2
25	711	0	2	2
26	9601	2	0	2
27	9601	4	0	4
28	9601	1	1	2
29	9601	1	0	1
30	9601	3	1	4

[1] Five larvae were initially placed on the surface of a lawn of turfgrass growing in each pot. The numbers of live larvae and live pupae were counted one month later.

GENERAL CONCLUSIONS

The American elm NPS3-487 chitinase gene *hs2* was characterized and found to be a typical class I chitinase gene (Shinshi et al., 1990) encoding the elm chitinase ECH2. According to a TFastA amino acid homology search (Altschul et al., 1990; Pearson and Lipman, 1988) of the GeneBank, the deduced amino acid sequence of ECH2 has a high level of homology to the deduced amino acid sequences of class I chitinases from other plants including tobacco (Fukuda et al., 1991), broad bean (Broglie et al., 1986), and poplar (Parsons et al., 1989). The deduced amino acid sequence of ECH2 contains several elements that are typical of class I chitinases including two class I chitinase signatures, a chitin binding domain signature, and eight conserved cysteine residues in a putative chitin binding domain (Van Parijs et al., 1991).

There is strong evidence that chitinases from a variety of plants are antifungal proteins (Ahl Goy et al., 1992). Chitinases are pathogenesis response-related proteins that have been found in a wide variety of plants (Meins et al., 1992). Transgenic tobacco plants expressing a broad bean class I chitinase were resistant to the fungal plant pathogen *Rhizoctonia solani* (Broglie et al., 1991). Transgenic tobacco plants expressing a rice class I chitinase were resistant to the fungal plant pathogen *Cercospora nicotianae* (Zhu et al., 1994).

Two plasmids were constructed for expression of *hs2* elm chitinase gene in bacteria and in transgenic plants. The plasmid pGEX-4T-1 was chosen as the vector for bacterial expression of elm chitinase. The *hs2* elm chitinase gene was removed from the plasmid pHS2 and ligated into linearized pGEX-4T-1 with compatible cohesive termini.

The resulting plasmid, designated pGEX-4T-1-hs2, contained the *hs2* elm chitinase gene as a fusion with a *Schistosoma japonicum* glutathione-S-transferase (GST) gene under the control of the IPTG-inducible *tac* promoter. The pGEX vectors have been used for the successful bacterial expression of over thirty different eukaryotic polypeptides.

The plasmid pKYLX71-hs2 was constructed for expression of *hs2* elm chitinase in transgenic plants. It was made by ligating the *hs2* elm chitinase gene from the plasmid pHS2 into the plant expression vector pKYLX71 (Schardl et al., 1987). The pKYLX71-hs2 plasmid contained the *hs2* elm chitinase gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Odell et al., 1985). The 3' terminus of the pea *rbcS*-E9 gene was used to obtain transcription termination and polyadenylation of the *hs2* mRNA transcript (Nagy et al., 1985; Fluhr and Chua, 1986; Morelli et al., 1985). The RK2 origin of replication from the plasmid pGA472 (An et al., 1985) allowed the plasmid to replicate in both *Escherichia coli* and *Agrobacterium tumefaciens*. It contained the sequences required for conjugative transfer of the plasmid from *E. coli* to *A. tumefaciens* with the mobilization helper plasmid pRK2013 (Ditta et al., 1980) by triparental mating. It has a tetracycline resistance gene for selection in bacteria, and a *nos-Aph(3')II* kanamycin resistance gene for selection of putative transgenic plant material (An et al., 1985). The plasmid has left and right borders of T-DNA to facilitate genomic integration of the gene cassette by *Agrobacterium*-mediated transformation.

The plasmid pKYLX71-hs2 is suitable for plant transformation by either of two methods, *Agrobacterium*-mediated plant transformation, or biolistic plant transformation. The plasmid can be linearized by *Eco* RI restriction endonuclease to increase the efficiency of biolistic plant transformation (West et al., 1991).

The GST-ECH2 fusion protein was successfully expressed in *E. coli* bacteria from the plasmid pGEX-4T-1-hs2. This fusion protein contained the glutathione-S-transferase amino terminus and the ECH2 elm chitinase carboxy terminus. The GST-chitinase fusion protein accumulated in induced bacterial cultures for up to 5 hours after induction. The GST-chitinase fusion protein was contained in the insoluble fraction of lysed bacterial cells, and was probably contained in inclusion bodies. Insolubility is a common characteristic of eukaryotic proteins expressed in *E. coli* bacteria (Marston, 1986). Removal of the highly hydrophobic signal peptide of *hs2* may be a future option to obtain expression of soluble elm chitinase in bacteria.

Triparental mating of *E. coli* containing pKYLX71-hs2 with *E. coli* containing the mobilization helper plasmid pRK2013 and *A. tumefaciens* LBA4404 was successful to transfer the pKYLX71-hs2 plasmid into *A. tumefaciens* LBA4404. The plasmid appeared to be stable in *A. tumefaciens* LBA4404, and restriction digestion and gel electrophoresis of the plasmid purified from either *A. tumefaciens* LBA4404 or *E. coli* showed that the restriction patterns were the same from both bacteria, and there was no visible degradation from either bacterium.

Tobacco was successfully transformed by the *Agrobacterium* leaf disk method with the plasmid pKYLX71-hs2. The transformation and regeneration was routine according to well-established procedures.

Creeping bentgrass (*Agrostis palustris* Huds.) was transformed with pKYLX71-hs2 by a biolistic plant transformation system. Polymerase chain reaction (PCR) and Southern blots confirmed the integration of the *hs2* elm chitinase gene into the plant genomes. The plasmid pKYLX71-hs2 proved its versatility by successfully transforming

both a dicot by *Agrobacterium*-mediated transformation and a monocot by biolistic transformation.

Preliminary bioassays indicated that creeping bentgrass expressing ECH2 elm chitinase had elevated levels of resistance versus the fungal plant pathogen *Rhizoctonia solani*. Potentially, these transgenic plants could be used in a future breeding program to develop *R. solani*-resistant turfgrass cultivars.

The transgenic creeping bentgrass expressing ECH2 elm chitinase did not possess significantly enhanced resistance versus Japanese beetle grubs. The Japanese beetles are native to Japan (Davis and Smitley, 1998). They have spread to every state in the USA east of the Mississippi River. Irrigated turf may be severely damaged by Japanese beetle feeding. The grubs feed on grass roots from April to June and from August to November. The larvae damage grass roots, which may interfere with water uptake. Elm chitinase in transgenic creeping bentgrass did not prove to be useful for control of Japanese beetles. A second major pest of turfgrass is European chafers. Future experimentation with transgenic creeping bentgrass or other plants expressing elm chitinase may indicate whether or not elm chitinase is effective in controlling European chafer or other turfgrass and plant pests.

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