PCR-Based Cloning, Characterization, and Stress-Induced Expression of Chitinase Genes in Kentucky Bluegrass (*Poa pratensis* L.)
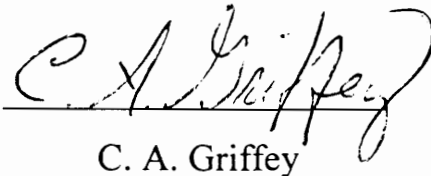
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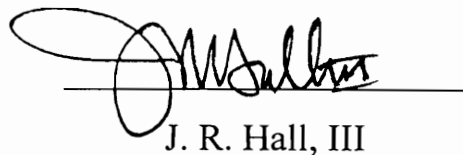
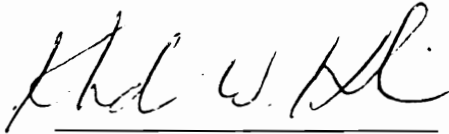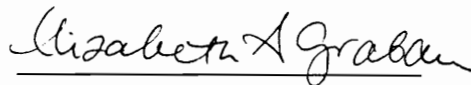DOCTOR OF PHILOSOPHY

in

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Crop and Soil Environmental Sciences

ABSTRACT

Chitinase is an enzyme that catalyzes the hydrolysis of chitin, an essential component of the cell walls of many fungi. Plant chitinases have been implicated in plant defense against pathogens. In plants, chitinase often exists as isoforms and three classes of chitinases have been proposed. In this study, isolation, characterization and expression of chitinase genes in Kentucky bluegrass (*Poa pratensis* L.) were studied.

With primers designed from conserved regions of chitinase genes from other plant species, a 710 bp fragment (CH710) containing a partial chitinase gene sequence was amplified from Kentucky bluegrass by PCR. Using cassette-ligation mediated PCR, we amplified four 5' and five 3' unknown sequences flanking CH710. The sequence information of these flanking fragments led us to the amplification of three genomic sequences from Kentucky bluegrass by PCR, KBCH1, KBCH2 and KBCH3, which contain full coding regions of chitinase genes. The chitinase genes carrying KBCH1, KBCH2 and KBCH3 were

designated as *chi1*, *chi2* and *chi3*, respectively. Southern blot hybridization indicated the presence of more than seven chitinase genes in the Kentucky bluegrass genome.

C*hi1* and *chi2* each contain an open reading frame with no introns, encoding polypeptides of 340 and 320 amino acids, respectively. Both CHI1 and CHI2, the predicted proteins encoded by *chi1* and *chi2*, are class I chitinases and share 94% amino acid identity. CHI1 has a short C-terminal extension, implicating that this protein may be a vacuolar protein. Although *chi3* has high sequence similarity to *chi1* and *chi2*, the potential open reading frame of *chi3* is interrupted by a translation termination codon at the 51st amino acid indicating that it does not encode a functional chitinase.

In this study, the expression of chitinase genes in Kentucky bluegrass under various stress conditions was also investigated. RNA blot hybridization showed that stresses such as cold, heat, salicylic acid and ethephon all induced an increased accumulation of chitinase mRNA in Kentucky bluegrass leaves with ethephon leading to the highest induction. After ethephon treatment, the accumulation of chitinase transcripts at a high level was observed from 2 days to 5 days. The expression of two individual chitinase genes, *chi1* and *chi2*, was shown to be stimulated, while being coordinately regulated, by ethephon.

To my beautiful daughter, Ning

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CHAPTER I

INTRODUCTION

Plants interact with pathogens in many different ways. Most plants are resistant to majority of pathogens. Nevertheless, many economic crops are susceptible to microbial pathogens, which can cause enormous crop losses. Thus, understanding the mechanisms of plant resistance is of great significance in developing novel pathogen control strategies and reducing the tremendous crop losses each year caused by disease problems.

Genetic studies of plants and pathogens have shown that a resistance gene (R) must be present in plants which are resistant to a particular pathogen race. But R genes are not the only plant genes required for a successful resistance response, as suggested by recent studies (Martin et al., 1993). In response to pathogen attack and other environmental stresses, plants also produce a variety of defense proteins, in addition to the activation of a particular R gene, to protect themselves. These inducible responses include synthesis of low-molecular-weight phytoalexins, production of hydrolytic enzymes such as chitinase and β-1,3-glucanase and other pathogenesis-related (PR) proteins, and deposition of new cell wall material including lignin and callose as a barrier to invading pathogens (Collinge and Slusarenko, 1987; Dixon and Harrison, 1990).

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Of these defense responses, the induction of chitinase in plants by microbial pathogens and other stresses is particularly interesting since plant chitinases have been shown to have an antifungal property (Mauch et al., 1988b).

Many chitinases and chitinase-encoding genes have been purified or isolated and characterized in numerous dicot plants, as well as in some monocots, such as barley (Swegle et al., 1989), rice (Huang et al., 1991; Nishizawa and Hibi, 1991; Nishizawa et al., 1993; Zhu and Lamb, 1991), wheat (Ride and Barber, 1990; Liao et al., 1994), maize (Huynh et al., 1992; Wu et al., 1994), and garlic (Van Damme et al., 1993). However, no studies on cloning of chitinase genes from turfgrass species have been reported. This research is aimed at learning about the structures of chitinase genes and understanding their regulation and expression in Kentucky bluegrass, an important turfgrass species. The results of this research should provide information useful for genetic engineering of turfgrasses with enhanced disease resistance.

LITERATURE REVIEW

Definition and Biochemical Properties of Chitinases

Chitin, an insoluble, linear β-1,4-linked polymer of N-acetylglucosamine (GlcNAc), is a structural component in a diverse array of organisms, including

fungi, insects, crustaceans, and nematodes (Cabib, 1987). These chitin-containing organisms produce chitinases which hydrolyze chitin. In other organisms which don't contain chitin, such as plants and bacteria, chitinases are also produced. Chitinases (EC 3. 2. 1. 14) are defined as enzymes which cleave a bond between the C1 and C4 of two consecutive N-acetylglucosamines of chitin. There are two types of chitinases: endochitinases and exochitinases. Cleavage by endochitinases occurs randomly at internal points of chitin and produces soluble, low-molecular weight multimers of GlcNAc such as chitotetraose and chitotriose and the dimer, di-acetylchitoboiose, with the dimer being predominant. On the other hand, exochitinases catalyze chitin in a different manner by progressively releasing di-acetylchitobiose, with no mono- or oligo-saccharides formed. Other related enzymes, such as β-N-acetylglucosaminidases and chitobiases, have also been characterized. Usually beta-N-acetylglucosaminidase is defined as an enzyme releasing N-acetylglucosamine (GlcNAc) monomers from its oligomers. Chitobiase hydrolyses chitobiose.

A number of assaying methods can be applied to measure both endo- and exo-chitinase enzyme activity. Among these are colorimetric, radiochemical, and gel electrophoresis-based assays (Collinge et al., 1993).

Generally, plant chitinases are endochitinases. Many purified plant endochitinases also display some degree of lysozyme activity (EC 3. 2. 1. 17)

(Boller, 1988), and can hydrolyze the glycosidic bond between the C1 of N-acetylmuramic acid (MurNAc) and the C4 of N-acetylglucosamine in bacterial peptidoglycan. The molecular weight of plant chitinases ranges from 25 kDa to 36 kDa.

Occurrence and Classification of Plant Chitinases

Plant chitinase proteins have been purified and studied in many plant species, including both dicotyledonous and monocotyledonous plants. Some of these plant species are bean (Awade et al., 1989; Boller, 1988; de Tapia et al., 1986), tobacco (Legrand et al., 1987; Meins and Ahl, 1989; Vogeli-Lange et al., 1988), tomato (Joosten and de Wit, 1989), cucumber (Metraux et al., 1988), melon (Roby and Esquerre-Tugaye, 1987), *Arabidopsis* (Verburg and Huynh, 1991), sugarbeet (Fleming et al., 1991), potato (Komrink et al., 1988), pea (Mauch et al., 1988a; Vad et al., 1991), rapeseed (Rasmussen et al., 1992a and 1992b), rice (Huang et al., 1991; Nishizawa and Hibi, 1991; Nishizawa et al., 1993; Zhu and Lamb, 1991), barley (Kragh et al., 1990; Leah et al., 1991), maize (Huynh et al., 1992; Nasser et al., 1988; Verburg et al., 1992), and wheat (Ride and Barber, 1990). Chitinases have been found in a wide variety of plant organs or tissues, including leaves, stems, roots, flowers, seeds, embryos, cotyledons, cell suspension cultures, calli, and protoplasts. The expression level of

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chitinases in healthy plants is generally very low, although it varies in different tissues.

Three classes of plant chitinases have been proposed based on the primary structure (Payne et al., 1990). Class I chitinases have an N-terminal cysteine-rich domain of approximately 40 amino acids in length and a highly conserved catalytic domain. The cysteine-rich domain is separated from the catalytic domain by a variable hinge region. Class I chitinases are usually basic and include most plant chitinases characterized so far, such as chitinases in bean, tobacco, *Arabidopsis*, potato, sugarbeet, pea, poplar, and barley.

Class II chitinases have high sequence similarity to class I chitinases but lack the N-terminal cysteine-rich domain. Class II chitinases are acidic and have been found in tobacco, petunia, and barley.

Class III chitinases show no amino acid sequence homology to those in class I or class II chitinases and also lack the N-terminal cysteine-rich domain. Class III chitinases are either acidic or basic and have been found in bean, tobacco, *Arabidopsis*, cucumber, and sugarbeet.

Some chitinases, such as basic sugarbeet chitinases (Mikkelsen et al., 1992), basic rapeseed chitinase ChB4 (Rasmussen et al., 1992a and 1992b), and acidic bean PR4 chitinase (Margis-Pingeiro et al., 1991), contain a cysteine-rich domain and a conserved catalytic domain like class I chitinases, but are

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significantly smaller due to four deletions. These chitinases may represent a new class of chitinases, class IV (Collinge et al., 1993).

The N-terminal cysteine-rich domain of class I chitinases is a chitin-binding domain and is also found in other proteins such as the wound-induced proteins in potato (Stanford et al., 1989), hevein proteins (Broekaert et al., 1990), wheat germ agglutinin (Raikhel and Wilkins, 1987) and nettle lectin (Broekaert et al., 1989). Comparison of their amino acid sequences suggests that these proteins may have evolved from the same ancestral gene by gene duplication or by gene fusion events (Chrispeels and Raikhel, 1991).

An essential tyrosine residue in the catalytic site is found crucial for the catalytic activity of most basic chitinases such as those from *Arabidopsis*, bean, tobacco, potato, barley and maize (Verburg et al., 1992). In rice, a phenylalanine residue, instead of tyrosine, was found essential for the catalytic mechanism (Zhu and Lamb, 1991).

Subcellular Localization of Plant Chitinases

Studies on localization of different chitinase isozymes by immunocytochemical techniques and cell fractionation approaches have shown that most class I chitinases are localized intracellularly in vacuoles, whereas other chitinases are localized in the extracellular space (Boller and Vogeli, 1984; Boller and Metraux, 1988; Mauch and Staehelin, 1989).

Some studies have demonstrated that a short C-terminal amino acid sequence present in a basic class I chitinase of tobacco is both necessary and sufficient for vacuolar targeting (Neuhaus et al., 1991b). A similar short C-terminal amino acid sequence for vacuolar targeting was also found in the basic chitinases from bean (Broglie et al., 1986), potato (Gaynor, 1988), *Arabidopsis* (Samac et al., 1990) and poplar (Parsons et al., 1989). However, there is no sequence homology between each other.

Regulation of Plant Chitinases

In general, chitinases are expressed at a very low basal level in healthy plants. However, chitinases accumulate to a high level upon the induction by various factors including pathogen infection, elicitor treatment, chemical stress, UV light treatment or wounding (for review, see Collinge et al., 1993).

Infection of plants with viruses, bacteria, or fungi can dramatically enhance chitinase induction. It has been found that the accumulation of the induced chitinase occurs earlier in resistant plant cultivars than in susceptible cultivars (Daugrois et al., 1990). In the later stages of infection, however, chitinase accumulates to a similar level in both resistant and susceptible plants (Daugrois et al., 1990).

Treatment of plants with ethylene induces the accumulation of basic or acidic chitinases in various plant species including bean (Boller et al., 1983;

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Broglie et al., 1986), tobacco (Keefe et al., 1990), *Arabidopsis* (Samac et al., 1990) and rice (Nishizawa and Hibi, 1991). Ethylene is considered an endogenous signal for chitinase induction since increased biosynthesis of ethylene in plants has been shown to accompany treatments with elicitors or pathogen infection, and an inhibitor of ethylene biosynthesis has been shown to reduce chitinase accumulation in some plant species (Broglie et al., 1989). However, the role of ethylene as a signal for chitinase induction is not yet clear and may be different from species to species. It has been demonstrated that chitinase induction is ethylene-independent in some plant species (Mauch et al., 1984).

Recently, salicylic acid has been suggested as an important endogenous signal for the induction of systemic acquired resistance in plants (Gaffeny et al., 1983; Ward et al., 1991; Yalpani et al., 1991). Acidic chitinases from Azuke bean (Ishige et al., 1993), cucumber (Metraux et al., 1989) and garlic (Van Damme et al., 1993) have been induced and characterized after treatment with salicylic acid.

Heavy metal compounds, such as mercuric chloride, are also inducers for plant chitinases. Two acidic chitinases from bean (Margis-Pingeiro et al., 1991; de Tapia et al., 1986) and four chitinases from maize (Nasser et al., 1988) have been induced by mercuric chloride.

Chitinases are also developmentally regulated in flowers, roots and basal leaves of tobacco (Lotan and Fluhr, 1989; Memelink et al., 1990; Neale et al., 1990) and *Arabidopsis* (Samac and Shah, 1991), as well as in pea pods (Mauch et al., 1988a) and in cereal grain (Jacobsen et al., 1990; Leah et al., 1991). It is not clear what roles chitinases play in plant development. However, recent observations that an extracellular chitinase can rescue a carrot mutant that fails to undergo somatic embryogenesis (de Jong et al., 1992) may imply such a function.

Molecular Cloning of Plant Chitinase Genes

Chitinase genes and complementary DNA clones for chitinases have been isolated in many plant species, especially in dicotyledonous plants. The general strategy for cloning genes encoding plant chitinases is *via* genomic DNA library or cDNA library screening with a homologous or heterologous probe. Cloning of genomic genes or cDNAs encoding chitinase has so far been reported in bean (Broglie et al., 1986; Margis-Pingeiro et al., 1991) tobacco (Lawton et al., 1992; Payne et al., 1990; Shinshi et al., 1990; Van Buuren et al., 1992), tomato (Danhash et al., 1993), cucumber (Lawton et al., 1994; Metuaux et al., 1989), potato (Gaynor and Unkenholz, 1989; Laflamme and Roxby, 1989), *Arabidopsis* (Samac et al., 1990), poplar (Davis et al., 1991), peanut (Herget et al., 1990), petunia (Linthorst et al., 1990), rapeseed (Rasmussen et al., 1992a), sugarbeet

(Nielsen et al., 1993), barley (Swegle et al., 1989), rice (Huang et al., 1991; Nishizawa and Hibi, 1991; Nishizawa et al., 1993; Zhu and Lamb, 1991), and garlic (Van Damme et al., 1993).

In most cases, plant chitinases are encoded by a small multigene family. However, in *Arabidopsis*, both basic and acidic chitinase genes are encoded by single-copy genes (Samac et al., 1990). Usually, the length of coding region of plant chitinases is about 1.0 kb. Chitinase genes in some plants, such as *Arabidopsis* (Samac et al., 1990) and tobacco (Shinshi et al., 1990), contain one or two introns, while most others do not contain any introns.

Roles of Chitinases in Plants

Since plant chitinases can be induced by pathogen attack and other various stresses, it has been speculated that they are involved in plant defense mechanisms. Responding to biotic or abiotic stresses, plants simultaneously produce many pathogenesis-related (PR) proteins, which form a complex defense system. Therefore, it is difficult to determine the specific role of chitinase. Evidence for the participation of plant chitinases in defense comes from the different responses of resistant (incompatible) and susceptible (compatible) cultivars to fungal infection. In general, chitinase accumulates more rapidly and in some instances at a higher concentration in resistant cultivars than in susceptible cultivars. In an incompatible interaction between plant and a

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pathogen, a hypersensitive reaction with very rapid, localized cell death usually occurs in the early stage of infection, which may act as a signal for the induction of PR-proteins, including chitinase, in the surrounding cells or tissues.

Various *in vitro* studies have demonstrated that purified plant chitinase can inhibit the growth of some fungi (Schlumbaum et al., 1986) and, when chitinase is in combination with β-1, 3-glucanase, the inhibition function is effective on more fungal species (Mauch et al., 1988b). *In vivo* studies with immunocytochemical techniques directly show that plant chitinase accumulates and interacts with the cell walls of the invading fungi in fungus-infected tomato (Benhamou et al., 1990).

With respect to their localization, it has been proposed that extracellular chitinases may be involved partly in an early disease response while vacuolar chitinases function in a delayed fashion following cell collapse (Graham and Sticklen, 1994).

As discussed before, chitinase genes are also expressed in a tissue-specific pattern in healthy plants, indicating that they may have a role in plant development. A recent study showed the importance of an acidic endochitinase in the early somatic embryo development of carrot (de Jong et al., 1992). The results demonstrated that this chitinase was essential for the completion of embryo development in a temperature-sensitive carrot mutant cell line at a non-

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permissive temperature. The activity of chitinase is much higher in roots, lower leaves, and flowers than in stems and upper leaves.

Genetic Engineering of Plants with Chitinase Genes

Transgenic techniques have been applied widely for assessing gene regulation and gene expression. They also provide a valuable tool for genetic engineering of disease resistant plants. With its antifungal property, plant chitinase is a potential candidate for genetically engineering plants for improved fungal disease resistance. Based on the fact that the production of chitinase in plants responding to pathogen attack is race-nonspecific, one can speculate that the over-expression of a chitinase gene in transgenic plants may result in enhanced resistance to more than one fungal pathogen.

Transgenic tobacco plants were constructed to constitutively express a basic bean chitinase gene at a high level under control of the cauliflower mosaic virus 35S promoter. These plants showed enhanced resistance to *Rhizoctonia solani* (Broglie et al., 1991). Studies on transgenic tobacco which expressed two bacterial chitinase genes at high levels demonstrated that the degree of resistance to *Rhizoctonia solani* increased with the level of chitinase gene expression. The reduction in disease severity obtained was up to 40% (Dunsmuir et al., 1993).

In another study, however, transgenic tobacco constitutively expressing a tobacco chitinase gene accumulated up to 120-fold more active chitinase than non-transformed plants, but did not exhibit increased resistance to the fungus *Cercospora nicotiana* (Neuhaus et al., 1991a). It is possible that the chitinase gene they used is not involved in the plant resistance to this particular fungus and in this instance, some other chitinase genes may be more effective on this disease. It may also indicate that chitinase alone may not be sufficient to provide enhanced disease resistance in this case and the over-expression of chitinase along with other enzymes like β-1,3-glucanase may provide better protection of plants against a wide range of pathogens.

A striking feature of the response of plants to pathogen attack is the induction of a battery of defenses, suggesting that different protective mechanisms may have complementary roles in overall disease resistance. It has been found that β-1,3-glucanase and chitinase are co-induced and act synergistically *in vitro* to inhibit fungal growth (Mauch et al., 1988b). Therefore, genetically engineered plants with coexpression of genes encoding chitinase and β-1,3-glucanase may be more efficient in protecting plants from pathogen attack. Transgenic tobacco exhibiting strong constitutive expression of a rice basic chitinase gene and an alfalfa acidic β-1,3-glucanase gene gave substantially greater protection against the fungus *Cercospora nicotiana* than transgenic

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tobacco expressing either gene alone (Zhu et al., 1994). In another study of transgenic tomato, similar results were obtained (Melchers et al., 1993).

OBJECTIVES

The goal of this research is to understand the structures of chitinase genes in Kentucky bluegrass and to understand the regulation and expression of these chitinase genes. To achieve this goal, the following specific objectives were pursued:

1. To clone chitinase genes from Kentucky bluegrass genome by PCR strategy and compare their nucleotide sequences to those from the other plant species.

2. To study the differential expression of chitinase genes in Kentucky bluegrass under different stress conditions such as ethylene, salicylic acid, cold and heat.

REFERENCES

Awade, A., de Tapia, M., Didierjean, L., and Burkard, G. 1989. Biological function of bean pathogenesis-related (PR3, PR4) proteins. Plant Sci. 63: 121-130.

Benhamou, N., Joosten, M.H.A.J., and De Wit, P.J.G.M. 1990. Subcellular localization of chitinase and of its potential substrate in tomato root tissues infected by *Fusarium oxysporum* f.sp. *radicislycopersici.* Plant Physiol. 92: 1108-1120.

Boller, T. 1988. Ethylene and the regulation of antifungal hydrolases in plants. Oxf. Surv. of Plant Mol. Cell Biol. 5: 145-174.

Boller, T., and Metraux, J.P. 1988. Extracellular localization of chitinase in cucumber. Physiol. Mol. Plant Pathol. 33: 11-16.

Boller, T., and Vogeli, U. 1984. Vacuolar localization of ethylene-induced chitinase in bean leaves. Plant Physiol. 74: 442-444.

Boller, T., Gheri, A., Mauch, F., and Vogeli, U. 1983. Chitinase in bean leaves: induction by ethylene, purification, properties, and possible function. Planta 157: 22-31.

Broekaert, W.F., Parijs, J.V., Leyns, F., Joos, H., and Peumans, W.J. 1989. A chitin-binding lectin from stinging nettle rhizomes with antifungal properties. Science 245: 1100-1102.

Broekaert, W.F., Lee, H.-I., Kush, A., Chua, N.-H., and Raikhel, N.V. 1990. Wounding induced accumulation of mRNA containing a hevein sequence in laticifers of rubber tree (*Hevea brasiliensis*). Proc. Natl. Acad. Sci. USA. 87: 7633-7637.

Broglie, K.E., Gaynor, J.J., and Broglie, R.M. 1986. Ethylene-regulated gene expression: Molecular cloning of the genes encoding an endochitinase from *Phaseolus vulgaris.* Proc. Natl. Acad. Sci. USA 83: 6820-6824.

Broglie, K.E., Biddle, P., Cressman, R., and Broglie, R. 1989. Functional analysis of DNA sequences responsible for ethylene regulation of a bean chitinase gene in transgenic tobacco. Plant Cell 1: 599-607.

Broglie, K.E., Chet, I., Holliday, M., Cressman, R., Biddle, P., Knowlton, S., Mauvais, C.J., and Broglie, R. 1991. Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani.* Science 254: 1194-1197.

Cabib, E. 1987. The synthesis and degradation of chitin. Adv. Enzymol. 59: 59-101.

Chrispeels, M.J., and Raikhel, N.V. 1991. Lectins, lectin genes, and their role in plant defence. Plant Cell 3: 1-9.

Collinge, D.B., and Slusarenko, A.J. 1987. Review: Plant gene expression in response to pathogens. Plant Mol. Biol. 9: 389-410.

Collinge, D.B., Kragh, K.M., Mikkelsen, J.D., Nielsen, K.K., Rasmussen, U., and Vad, K. 1993. Plant chitinases. Plant J. 3:31-40.

Danhash, N., Cornelia, A.M., Wagemakers, J.A.L. van K., and de Wit, P.J.G.M. 1993. Molecular characterization of four chitinase cDNAs obtained from *Cladosporium fulvum*-infected tomato. Plant Mol. Biol. 22: 1017-1029.

Daugrois, J.H., Lafitte, C., Barthe, J.P., and Touze, A. 1990. Induction of β-1,3-glucanase and chitinase activity in compatible and in incompatible interactions between *Colletotrichum lindemuthianum* and bean cultivars. J. Phytopathol. 130: 225-234.

Davis, J.M., Clarke, H.R.G., Bradshaw, H.D.,Jr., and Gordon, M.P. 1991. *Populus* chitinase genes: structure, organization, and similarity of translated sequences to herbaceous plant chitinases. Plant Mol. Biol. 17: 631-639.

de Jong, A., Cordewener, J., Lo Schiavo, F., Terzi, M., Vandekerckhove, J., Van Kammen, A., and De Vries, S.C. 1992. A carrot somatic embryo mutant is rescued by chitinase. Plant Cell 4: 425-433.

de Tapia, M., Bergmann, P., Awade, A., and Burkard, G. 1986. Analysis of acidic extractable bean leaf proteins induced by mercuric chloride treatment and Alfalfa Mosaic Virus infection. Partial purification and characterization. Plant Sci. 45: 167-177.

Dixon, R.A., and Harrison, M. 1990. Activation, structure and organization of genes involved in microbial defence in plants. Adv. Genet. 28: 165-234.

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Dunsmuir, P., Howie, W., Newbigin, E., Joe, L., Penzes, E., and Suslow, T. 1993. Resistance to *Rhizoctonia solani* in transgenic tobacco. *In*: Nester, E.W., and Verma, D.P.S. (*eds.*). Advances in Molecular Genetics of Plant-Microbe Interactions. Kluwer Academic Publishers. Netherlands.

Fleming, T.M., McCarthy, D.A., White, R.F., Antoniw, J.F., and Mikkelsen, J.D. 1991. Induction and characterization of some of the pathogenesis-related proteins in sugar beet. Physiol. Mol. Plant Pathol. 39: 147-160.

Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessmann, H., and Ryals, J. 1993. Requirement of salicylic acid for the induction of systemic acquired resistance. Science 261: 754-756.

Gaynor, J.J. 1988. Primary structure of an endochitinase mRNA from *Solanum tuberosum*. Nucleic Acids Res. 16: 5210.

Gaynor, J.J., and Unkenholz, K.M. 1989. Sequence analysis of a genomic clone encoding an endochitinase from *Solanum tuberosum*. Nucleic Acids Res. 17: 5855-5856.

Graham, L.S., and Sticklen, M.B. 1994. Plant chitinases. Can. J. Bot. 72: 1057-1083.

Herget, T., Schell, J., and Schrier, P.H. 1990. Elicitor-specific induction of one member of the chitinase gene family in *Arachis hypogaea*. Mol. Gen. Genet. 224: 469-476.

Huang, J.K., Wen, L., Swegle, M., Tran, H.C., Thin, T.H., Naylor, H.M., Muthakrishnan, S., and Reeck, G.R. 1991. Nucleotide sequence of a rice genomic clone that encodes a Class I endochitinase. Plant Mol. Biol. 16: 479-480.

Huynh, Q.K., Hironaka, C.M., Levine, E.B., Smith, C.E., Borgmeyer, J.R., and Shah, D.M. 1992. Antifungal proteins from plants. J. Biol. Chem. 267: 6635-6640.

Ishige, F., Mori, H., Yamazaki, K., and Imaseki, H. 1993. Cloning of a complementary DNA that encodes an acidic chitinase which is induced by ethylene and expression of the corresponding gene. Plant Cell Physiol. 34: 103-111.

Jacobsen, S., Mikkelsen, J.D., and Hejgaard, J. 1990. Characterization of two antifungal proteins from barley grain. Physiol. Plant. 79: 554-562.

Joosten, M.H.A.J., and de Wit, P.J.G.M. 1989. Identification of several pathogenesis-related proteins in tomato leaves inoculated with *Cladosporium fulvum* (syn. *Fulvia fulva*) as β-1,3-glucanases and chitinases. Plant Physiol. 89: 945-951.

Keefe, D., Hinz, U., and Meins, F. 1990. The effect of ethylene on the cell-type specific and intracellular localization of β-1,3-glucanase and chitinase in tobacco leaves. Planta 182: 43-51.

Kombrink, E., Schroder, M., and Hahlbrock, K. 1988. Several "pathogenesis-related" proteins in potato are β-1,3-glucanases and chitinases. Proc. Natl. Acad. Sci. USA 85: 782-786.

Kragh, K.M., Jacobsen, S., and Mikkelsen, J.D. 1990. Induction, purification and characterization of barley leaf chitinase. Plant Sci. 71: 55-68.

Laflamme, D., and Roxby, R. 1989. Isolation and nucleotide sequence of cDNA clones encoding potato chitinase genes. Plant Mol. Biol. 13: 249-250.

Lawton, K.A., Ward, E., Payne, G., Moyer, M., and Ryals, J. 1992. Acidic and basic class III chitinase mRNA accumulation in response to TMV infection of tobacco. Plant Mol. Biol. 19: 735-743.

Lawton, K.A., Beck, J., Potter, S., Ward, E., and Ryals, J. 1994. Regulation of cucumber class III chitinase gene expression. Mol. Plant-Microbe Interact. 7: 48-57.

Leah, R., Tommerup, H., Svendsen, I., and Mundy, J. 1991. Biochemical and molecular characterization of three barley seed proteins with antifungal properties. J. Biol. Chem. 266: 1564-1573.

Legrand, M., Kauffmann, S., Geoffroy, P., and Fritig, B. 1987. Biological function of pathogenesis-related proteins: Four tobacco pathogenesis-related proteins are chitinases. Proc. Natl. Acad. Sci. USA 84: 6750-6754.

Liao, Y.C., Kreuzaler, F., Fischer, R., Reisener, H.-J., and Tiburzy, R. 1994. Characterization of a wheat class Ib chitinase gene differentially induced in isogenic lines by infection with *Puccinia* graminis. Plant Sci. 103: 177-187.

Linthorst, H.J.M., van Loon, L.C., van Rossum, C.M.A., Mayer, A., Bol, J.F., van Roeckel, J.S.C., Meulenhoff, E.J.S., and Cornelissen, B.J.C. 1990. Analysis of acidic and basic chitinases from tobacco and petunia and their constitutive expression in transgenic tobacco. Mol. Plant-Microbe Interact. 3: 252-258.

Lotan, T., Ori, N.,and Fluhr, R. 1989. Pathogenesis-related proteins are developmentally regulated in tobacco flowers. Plant Cell 1: 881-887.

Margis-Pinheiro, M., Metz-Boutigue, M.H., Awade, A,. de Tapia, M., le Ret, M., and Burkard, G. 1991. Isolation of a complementary DNA encoding the bean PR4 chitinase: an acidic enzyme with an amino-terminus cysteine-rich domain. Plant Mol. Biol. 17: 243-253.

Martin, G.B., Brommonschenkel, S.H., Chunwongse, J., Frary, A., Ganal, M.W., Spivey, R., Wu, T., Earle, E.D., and Tanksley, S.D. 1993. Map-based cloning of a protein kinase gene conferring disease resistance in tomato. Science 262: 1432-1436.

Mauch, F., and Staehelin, A. 1989. Functional implications of the subcellular localization of ethylene-induced chitinase and β-1,3-glucanase in bean leaves. Plant Cell 1: 447-457.

Mauch, F., Hadwiger, L.A., and Boller, T. 1984. Ethylene; symptom, not signal for the induction of chitinase and β-1,3-glucanase in pea pods by pathogens and elicitors. Plant Physiol. 76: 607-611.

Mauch, F., Hadwiger, L.A., and Boller, T. 1988a. Antifungal hydrolases in pea tissue: I. Purification and characterization of two chitinases and two β-1,3-glucanases differentially regulated during development and in response to fungal infection. Plant Physiol. 87: 325-333.

Mauch, F., Mauch-Mani, B., and Boller, T. 1988b. Antifungal hydrolases in pea tissue: II. Inhibition of fungal growth by combinations of chitinase and β-1,3-glucanase. Plant Physiol. 88: 936-942.

Meins, F., and Ahl, P. 1989. Induction of chitinase and β-1,3-glucanase in tobacco plants infected with *Pseudomonas tabaci* and *Phytophthora parasitica* var. *nicotianae.* Plant Sci. 61: 155-161.

Melchers, L.S., Tigelaar, H., Sela-Buurlage, M.B., Ponstein, A.S., van Roekel, J.S.C., Bres-Vloemans, S.A., Cornelissen, B.J.C., Logemann, J., and Jongedijk, E. 1993. Synergistic activity of chitinases and β-1,3-glucanases enhances *Fusarium* resistance in transgenic tomato plants. Abstract No. 83, Molecular Genetics of Plant-Microbe Interactions Conference, Rutgers, NJ.

Memelink, J., Linthorst, H.J.M., Schilperoort, R.A., and Hoge, J.H.C. 1990. Tobacco genes encoding acidic and basic isoforms of pathogenesis-related proteins display different expression patterns. Plant Mol. Biol. 14: 119-126.

Metraux, J.P., Streit, L., and Staub, T. 1988. A pathogenesis-related protein in cucumber is a chitinase. Physiol. Mol. Plant Pathol. 33: 1-9.

Metraux, J.P., Burkhart, W., Boyer, M., Dincher, S., Middlesteadt, W., Williams, S.,Payne, G., Carnes, M., and Ryals, J. 1989. Isolation of a complementary DNA encoding a chitinase with structural homology to a bifunctional lysozyme/chitinase. Proc. Natl. Acad. Sci. USA. 86: 896-900.

Mikkelsen, J.D., Berglund, L., Nielsen, K.K., Christiansen, H., and Bojsen, K. 1992. Structure of endochitinase genes from sugarbeets. *In* Advances in Chitin and Chitosan. *Edited by* C.J. Brine, P.A. Sandford, and J.P. Zikakis. Elsevier Applied Science, London.

Nasser, W., de Tapia, M., Kauffman, S., Montasser-Kouhsari, S., and Burkhard, G. 1988. Identification and characterization of maize pathogenesis-related proteins. Four maize proteins are chitinases. Plant Mol. Biol. 11: 529-538.

Neale, A.D., Wahleithner, J.A., Lund, M., Bonnett, H.T., Kelly, A., Meeks-Wagner, D.R., Peacock, W.J., and Dennis, E.S. 1990. Chitinase, β-1,3-glucanase, osmotin, and extensin are expressed in tobacco explants during flower formation. Plant Cell 2: 673-684.

Neuhaus, J.-M., Ahl-Goy, P., Hinz, U., Flores, S., and Meins, F. 1991a. High-level expression of a tobacco chitinase gene in *Nicotiana sylvestris.* Susceptibility of transgenic plants to *Cercospora nicotianae* infection. Plant Mol. Biol. 16: 141-151.

Neuhaus, J.-M., Sticher, L., Meins, F., and Boller, T. 1991b. A short C-terminal sequence is necessary and sufficient for the targeting of chitinases to the plant vacuole. Proc. Natl. Acad. Sci. USA. 88: 10362-10366.

Nielsen, K.K., Mikkelsen, J.d.,Kragh,K.M., and Bojsen, K. 1993. An acidic class III chitinase in sugarbeet: induction by *Cercospora beticola,* characterization, and expression in transgenic tobacco plants. Mol. Plant-Microbe Interact. 6: 495-506.

Nishizawa, Y., and Hibi, T. 1991. Rice chitinase gene: cDNA cloning and stress-induced expression. Plant Sci. 76: 211-218.

Nishizawa, Y., Kishimoto, N., Saito, A., and Hibi, T. 1993. Sequence variation, differential expression and chromosomal location of rice chitinase genes. Mol. Gen. Genet. 241: 1-10.

Parsons, T.J., Bradshaw, H.D., and Gordon, M.P. 1989. Systemic accumulation of specific mRNAs in response to wounding in poplar trees. Proc. Natl. Acad. Sci. USA. 86: 7895-7899.

Payne, G., Ahl, P., Moyer, M., Harper, A., Beck, J., Meins, F., Jr., and Ryals, J. 1990. Isolation of complementary DNA clones encoding pathogenesis-related proteins P and Q, two acidic chitinases from tobacco. Proc. Natl. Acad. Sci. USA. 87: 98-102.

Raikhel, N.V., and Wilkins, T.A. 1987. Isolation and characterization of a cDNA clone encoding wheat germ agglutinin. Proc. Natl. Acad. Sci. USA. 84: 6745-6749.

Rasmussen, U., Bojsen, K., and Collinge, D.B. 1992a. Cloning and characterization of a pathogen-induced chitinase in *Brassica napus.* Plant Mol. Biol. 20: 227-287.

Rasmussen, U., Giese, H., and Mikkelsen, J.D. 1992b. Induction and characterization of chitinase in *Brassica napus* L. ssp. *oleifera* infected with *Phoma lingam.* Planta 187: 328-334.

Ride, J.P., and Barber, M.S. 1990. Purification and characterization of multiple forms of endochitinase from wheat leaves. Plant Sci. 71: 185-197.

Roby, D., and Esquerre-Tugaye, M.-T. 1987. Purification and some properties of chitinases from melon plants infected with *Colletotrichum lagenarium.* Carbohydr. Res. 165: 93-104.

Samac, D.A., and Shah, D.M. 1991. Developmental and pathogen-induced activation of the *Arabidopsis* acidic chitinase promoter. Plant Cell 3: 1063-1072.

Samac, D., Hironaka, C.M., Yallaly, P.E., and Shah, D.M. 1990. Isolation and characterization of the genes encoding basic and acidic chitinase in *Arabidopsis thaliana.* Plant Physiol. 93: 907-914.

Schlumbaum, A., Mauch, F., Vogeli, U., and Boller, T. 1986. Plant chitinases are potent inhibitors of fungal growth. Nature 324: 365-367.

Shinshi, H., Neuhaus, J.-M., Ryals, J., and Meins, F. 1990. Structure of a tobacco endochitinase gene: evidence that different chitinase genes can arise by transposition of sequences encoding a cysteine-rich domain. Plant Mol. Biol. 14: 357-368.

Stanford, A., Bevan, M., and Northcote, D. 1989. Differential expression within a family of novel wound-induced genes in potato. Mol. Gen. Genet. 215: 200-208.

Swegle, M., Huang, J.-K., Lee, G., and Muthukrishnan, S. 1989. Identification of an endochitinase cDNA clone from barley aleurone cells. Plant Mol. Biol. 12: 403-412.

Vad, K., Mikkelsen, J., and Collinge, D.B. 1991. Induction, purification, and characterization of chitinase isolated from pea leaves inoculated with *Ascochyta pisi.* Planta 184: 24-29.

Van Buuren, M., Neuhaus, J.-M., Shinshi, H., Ryals, J., and Meins, F., Jr. 1992. The structure and regulation of homeologous tobacco endochitinase genes of *Nicotiana sylvestris* and *N. tomentosiformis* origin. Mol. Gen. Genet. 232: 460-469.

Van Damme, E.J.M., Willems, P., Torrekens, S., Van Leuven, F., and Peumans, W.J. 1993. Garlic (*Allium sativum*) chitinases: characterization and molecular cloning. Physiol. Plant. 87: 177-186.

Verburg, J.G., and Huynh, Q.K. 1991. Purification and characterization of an antifungal chitinase from *Arabidopsis thaliana.* Plant Physiol. 95: 450-455.

Verburg, J.G., Smith, C.E., Lisek, C.A., and Huynh, Q.K. 1992. Identification of an essential tyrosine residue in the catalytic site of a chitinase isolated from *Zea mays* that is selectively modified during inactivation with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide. J. Biol. Chem. 267: 3886-3893.

Vogeli-Lange, R., Hansen-Gheri, A., Boller, T., and Meins, F. 1988. Induction of the defense-related glucanohydrolases, β-1,3-glucanase and chitinase, by tobacco mosaic virus infection of tobacco leaves. Plant Sci. 54: 171-176.

Ward, E.R., Uknes, S.J., Williams, S.C., Dincher, S.S., Widerhold, D.L., Alexander, D.C., Ahl-Goy, P., Metraux, J.-P., and Ryals, J.A. 1991. Coordinate gene activity in response to agents that induce systemic acquired resistance. Plant Cell 3: 1085-1094.

Wu, S., Kriz, A.L., and Widholm, J.M. 1994. Molecular analysis of two cDNA clones encoding acidic class I chitinase in maize. Plant Physiol. 105: 1097-1105.

Yalpani, N., Silverman, P., Wilson, T.M.A., Kleer, D.A., and Raskin, I. 1991. Salicylic acid is a systemic signal and an inducer of pathogenesis-related proteins in virus-infected tobacco. Plant Cell 3: 809-818.

Zhu, Q., and Lamb, C.J. 1991. Isolation and characterization of a rice gene encoding a basic chitinase. Mol. Gen. Genet. 226: 289-296.

CHAPTER II

PCR-BASED CLONING AND CHARACTERIZATION OF

CHITINASE GENES FROM

KENTUCKY BLUEGRASS (*Poa pratensis* L.)

ABSTRACT

In recent years, polymerase chain reaction (PCR) techniques have been widely used in cloning of genes of interest and new PCR techniques, such as inverse PCR and cassette-ligation mediated PCR, have been developed to amplify unknown sequences flanking a short stretch of known region. We have applied a PCR cloning method to amplify multiple chitinase genes from Kentucky bluegrass (*Poa pratensis* L.). Based on homologies found in plant class I chitinase genes isolated so far, a 710 bp fragment (CH710) containing a partial chitinase gene of Kentucky bluegrass was amplified by PCR. Using cassette-ligation mediated PCR, we amplified four 5' and five 3' unknown sequences flanking CH710. The sequence information from these flanking fragments led us to the amplification of three Kentucky bluegrass genomic sequences by PCR. These sequences, designated as KBCH1, KBCH2 and KBCH3, encode chitinases of Kentucky bluegrass and show sequence similarities to known chitinase genes in other plants. The putative chitinase genes for KBCH1,

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KBCH2 and KBCH3 were designated as *chi1*, *chi2* and *chi3*, respectively. *Chi1* and *chi2* contain an open reading frame with no introns, encoding a polypeptide of 340 and 320 amino acids, respectively. Both polypeptides consist of a signal peptide, a N-terminal cysteine-rich domain, a short hinge region, and a main catalytic domain, indicating they are class I chitinases. The predicted amino acid sequences from *chi1* and *chi2* are 94% identical and have high sequence similarity to a class I wheat chitinase. The chitinase encoded by *chi1* has a short C-terminal extension, implicating that this protein may be a vacuolar protein. Although *chi3* has high sequence similarity to *chi1* and *chi2*, the potential open reading frame of *chi3* is interrupted by a translation termination codon at the 51st amino acid. Southern blot hybridization indicated the presence of more than seven chitinase genes in the Kentucky bluegrass genome.

INTRODUCTION

Conventional gene cloning relies on genomic DNA or cDNA library construction and screening which can be tedious and time-consuming. The advent of polymerase chain reaction (PCR) provides a promising tool to implement the conventional methods for cloning genomic or cDNA sequences of interest. PCR amplifies a segment of DNA lying between two known sequences from which two oligonucleotide primers are chosen (Saiki et al., 1988). Through repeated cycles of high-temperature template denaturation, oligonucleotide primer annealing and DNA polymerase assisted extension, a given segment of DNA can be amplified exponentially. The reaction is so efficient that only extremely small amounts of DNA are needed. PCR has been widely used in molecular biology and clinical application. However, the requirement of two known specific primers limits the application of PCR to the amplification of unknown genes. Inverse PCR techniques have thus been developed to allow the amplification of unknown sequences flanking a known region (Ochman et al., 1988; Triglia et al., 1988). In inverse PCR, the target sequence is cut by a restriction enzyme, recircularized and amplified with two primers which are reversed in their direction in respect to their normal orientation for PCR. However, recircularization of linearized fragments is unreliable and often causes

concatamerization (Rosenthal and Jones, 1990). Recently, a new method has been developed for DNA walking and sequencing by a cassette-ligation strategy (Isegawa et al., 1992). This technique, referred to as cassette-ligation mediated PCR, requires only a single specific primer and is based on the ligation of oligo-cassettes with restriction fragments (Kalman et al., 1990; Rosenthal and Jones, 1990). Thus, it is a useful technique for amplification of adjacent unknown sequences when only a very short sequence is known and it also eliminates the problem caused by recircularization in inverse PCR. In addition, the cassette sequence can be used as a universal primer for direct sequencing of PCR products (Isegawa et al., 1992). Most importantly, this method eliminates the laborious cloning processes of cDNA or genomic DNA library construction and screening.

Cassette-ligation mediated PCR was successfully applied to the study of chitinase genes, a multigene family, in Kentucky bluegrass (*Poa pratensis* L.).

Plant chitinases have been implicated a role in defense against pathogens since they accumulate to a high level in response to pathogen infection. This is also supported by the fact that chitin which is the substrate of chitinase and an important component of fungal cell walls is absent in higher plants. To investigate the function of plant chitinases, chitinase genes have been isolated from many plant species (for review, see Collinge et al., 1993; Graham and Sticklen, 1994). Based on primary structures, plant chitinases have been divided

into three classes (Shinshi et al., 1990). Class I and II chitinases have high similarity in the catalytic domain but differ in that class I chitinases have a N-terminal cysteine-rich domain but class II chitinases do not. Class III chitinases share considerable sequence similarity to each other, but have no sequence similarity to class I or class II chitinases and do not contain a cysteine-rich domain.

Chitinases have been purified and characterized from a wide variety of dicotyledonous plants and their genomic or complementary DNA clones isolated. For monocots, however, most attention has only focused on chitinases from the limited number of major agronomic crops such as rice (Huang et al., 1991; Nishizawa et al., 1993; Zhu and Lamb, 1991), maize (Huynh et al., 1992; Wu et al., 1994), wheat (Liao et al., 1994; Ride and Barber, 1990), and barley (Leah et al., 1991; Swegle et al., 1989).

In many plants studied so far, chitinases are encoded by a multigene family (Shinshi et al., 1990; Zhu and Lamb, 1991). Cassette-ligation mediated PCR has been used for cDNA walking and sequencing of an entire 6.5 kb genome segment of hantavirus strain B1 with thirteen viral specific and three cassette primers (Isegawa et al., 1992). Here we demonstrate, for the first time, that this technique is also applicable to cloning multiple genes that are closely related members of a multigene family. We have successfully applied this cassette-ligation mediated PCR method to the efficient amplification of three chitinase

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genes from the Kentucky bluegrass (*Poa pratensis* L.) genome. To our knowledge this is the first report on cloning of chitinase genes from a turfgrass species.

MATERIALS AND METHODS

Plant Materials and Treatments

Kentucky bluegrass (*Poa pratensis* L. cv. Glade) seeds were sterilized in 70% ethanol for 2 min and then in Clorox bleach for 10 min, followed by four times of rinses with sterile distilled water. Sterilized seeds were germinated and grown in MS medium (Murashige and Skoog, 1962) without phytohormones at 25 °C in light at 16 h/day in a growth chamber.

In the experiment of chitinase induction for cDNA amplification by RT-PCR, Kentucky bluegrass seeds were planted in 4-inch pots with 0.3 gram seeds per pot. The plants were grown at 26 °C in a growth chamber and were watered once a day. Three-week-old seedlings were sprayed with 1 mg/ml ethephon to runoff twice a day and covered tightly with plastic bags. After four days, the plant materials were harvested, frozen in liquid nitrogen and stored at -70 °C until use.

Genomic DNA Isolation

Leaf tissue of Kentucky bluegrass seedlings grown on MS media for three weeks were used for genomic DNA isolation according to the CTAB method described by Murray and Thompson (1980) with slight modification. Briefly, 1 g of fresh plant tissue was ground to powder in liquid nitrogen. Genomic DNA was extracted in 10 ml of extraction buffer (0.7 M NaCl, 1% CTAB, 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 1% 2-mercaptoethanol) and finally, precipitated with equal volume of isopropanol.

RNA Isolation

Total cellular RNA was prepared with acid guanidinium thiocyanate-phenol-chloroform method essentially as described by Chomczynski and Sacchi (1987) from frozen Kentucky bluegrass leaves which had been stored at -70 °C. Poly (A)$^+$ RNA was purified with DYNABEADS mRNA Purification Kit (Dynal) according to the manufacturer's instruction.

First-Strand cDNA Synthesis

First-strand cDNA was synthesized from poly (A)$^+$ RNA with primer *Not* I-d(T)$_{18}$ and Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase according to the manufacturer's instruction (Pharmacia Biotech).

Polymerase Chain Reaction

Two oligonucleotide primers, primer A (5'-TTCGGCTGGTGCGGCTCCACC-3') corresponding to the amino acid sequence of FGWCGST in a rice class I chitinase (Zhu and Lamb, 1991) and primer B (5'-AGCCCGCCGTTGATGAT-GTT-3') corresponding to NIINGGL in the rice chitinase, were used to amplify a partial chitinase gene from Kentucky bluegrass genomic DNA. PCR amplification was carried out with a DNA Thermal Cycler (Perkin-Elmer Cetus) in a final volume of 50 ul containing 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 0.1 mg/ml bovine serum albumin (BSA), 200 uM of each dNTP, 0.4 uM of each primer, 100 ng genomic DNA of Kentucky bluegrass, 2.5 units of Ampli*Taq* DNA polymerase (Perkin-Elmer Cetus) and 2.5 units of *Taq* Extender PCR additive (Stratagene), for thirty cycles of denaturation at 94 °C, 1 min, annealing at 62 °C, 2 min, and extension at 72 °C, 2 min. The denaturation time for the first cycle and extension time for the last cycle were extended to 5 min and 10 min, respectively. The PCR products were cloned into pNoTA/T7 (5' → 3', Inc.) according to the manufacturer's instruction.

The same PCR amplification conditions as described above were used to amplify Kentucky bluegrass genomic DNA sequences containing the full coding region of chitinase, except that the annealing temperature was changed from 62 °C to 53 °C for optimal amplification.

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Cassette-Ligation Mediated PCR

Cassette-ligation mediated PCR was performed with PCR *in vitro* Cloning Kit (Takara Shuzo Co., Ltd.). Five ug of Kentucky bluegrass genomic DNA was digested to completion by incubating at 37 °C for 2 h with 50 units of each of ten restriction enzymes (i.e., *Hind*III, *Eco*RI, *Pst*I, *Bam*HI, *Bgl*II, *Nde*I, *Xho*II, *Sal*I, *Xho*I and *Xba*I) and then precipitated with ethanol. The DNA pellet was redissolved in 10 ul sterilized distilled water, 5 ul of which was used for ligation. The ligation mixture also contained 50 ng of a compatible cassette (Takara Shuzo Co., Ltd.), 2 units of T4 DNA ligase, 10 mM Tris-HCl, pH 7.3, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.005% (w/v) gelatin in a 10 ul total volume. After incubating overnight at 14 °C, ligated DNA was precipitated with ethanol and redissolved in 5 ul of sterilized distilled water, 1 ul of which was used as templates for PCR. Two rounds of PCR were performed. The primers for the first round PCR included a cassette primer C1 (5'-GTACATATTGTCGTTAGAACGCG-3') and a specific primer (the 5'S1 primer when amplifying 5' flanking sequences or the 3'S1 primer when amplifying 3' flanking sequences). The oligonucleotide sequences of primer 5'S1 and 3'S1 as well as primers 5'S2 and 3'S2 described later are indicated in Figure 1. One ul of the products of the first round PCR was used as templates for the second round PCR. The primers for the second round PCR included a cassette primer C2 (5'-TAATACGACTCACTATAGGGAGA-3')

and a nested specific primer (the 5'S2 primer when amplifying 5' flanking sequences or the 3'S2 primer when amplifying 3' flanking sequences). PCR amplification conditions were the same as described previously except that annealing temperature was 55 °C for cassette-ligation mediated PCR. Products of the second round PCR which showed distinct bands on agarose gel were recovered from the gel with GENECLEAN II Kit (Bio 101, Inc.). Recovered PCR products were used as templates in PCR with primer C2 and an internal primer corresponding to a short stretch of rice chitinase gene sequence to determine whether or not the PCR products recovered were partial chitinase sequences. The internal primers used to confirm the amplified 5' and 3' flanking sequence as a partial chitinase gene were 5'-GGTGGAGCCGCACCAGCCGAA-3' and 5'-ACCAACATCATCAACGGC-3', respectively. Gel-recovered PCR products which were confirmed as putative chitinase sequences by PCR were reamplified by PCR and cloned into pNoTA/T7 (5' to 3', Inc.) for further analysis.

Reverse Transcriptase-PCR (RT-PCR)

PCR amplification of cDNAs encoding Kentucky bluegrass chitinases was carried out with a DNA Thermal Cycler (Perkin-Elmer Cetus) in a final volume of 50 ul containing 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 0.1 mg/ml bovine serum albumin (BSA), 200 uM of each dNTP, 0.4 uM of the forward primer ch5'a (5'-TTGAGCGGGTTC-

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TGCTACATTG-3') and 0.4 uM of the reverse primer *Not* I-d(T)$_{18}$ (Pharmacia

Biotech), 5 *u*l of first-strand cDNA mixture (from the total volume of 15 ul), 2.5

units of Ampli*Taq* DNA polymerase (Perkin-Elmer Cetus) and 2.5 units of *Taq*

Extender PCR additive (Stratagene) for thirty cycles of denaturation at 94 °C, 1

min, annealing at 55 °C, 2 min, and extension at 72 °C, 2 min. The denaturation

time for the first cycle and extension time for the last cycle were extended to 5

min and 10 min, respectively. Two ul of the generated PCR products were used

for another round of PCR under the same conditions described above except the

nested reverse primer ch3'f1-a (5'-TTAGGAAGGAATTGACGAGATAG-3') or

ch3'f4-a (5'-TTGCCGGCCGATTGACAGA-3') replacing primer *Not* I-d(T)$_{18}$. The

PCR products were analyzed by agarose gel electrophoresis and the fragments

with expected size were recovered from the agarose gel and reamplified by PCR

for direct sequencing.

DNA Sequencing

Nucleotide sequences of the fragments cloned into plasmid vectors were

determined by the dideoxynucleotide chain-termination method (Sanger et al.,

1977) with Sequenase version 2.0 (Amersham).

For direct sequencing of PCR products, 5 ul of PCR amplification mixture was

incubated with 10 units of exonuclease I and 2 units of shrimp alkaline

phosphatase at 37 °C for 15 min to remove the remaining primers and

dephosphorylate the unincorporated nucleotides. The enzymes were then inactivated by heating at 80 °C for 15 min and the DNA was denatured by heating at 100 °C for 3 min, followed by the dideoxynucleotide chain-termination sequencing method (Sanger et al., 1977).

DNA Blot Hybridization

Fifteen ug of Kentucky bluegrass genomic DNA was digested at 37 °C for 8 h with 60 units of *Bam*HI, *Eco*RI, *Hind*III or *Xba*I in the buffer recommended by the manufacturer (Boehringer Mannheim). The digested DNA was fractionated by 0.8% agarose gel electrophoresis and transferred to a positively charged nylon membrane (Boehringer Mannheim) in 10X SSC with a vacuum blotter (Stratagene). Prehybridization was carried out in 5X SSC, 0.1% sodium-lauroylsarcosine, 0.02% SDS, 2% blocking reagent (Boehringer Mannheim), and 50% formamide at 55 °C for 2 h. Hybridization was performed in the same buffer at 55 °C overnight with DNA probe, a 710 bp Kentucky bluegrass chitinase sequence (CH710) labeled with digoxigenin-11-dUTP using the random-primed method. The probe concentration in the hybridization buffer was 25 ng/ml. After hybridization, the membrane was washed with constant agitation, twice in 2X SSC, 0.1% SDS for 5 min at 25 °C and twice in 0.5X SSC, 0.1% SDS for 15 min at 65 °C. Detection of hybridized probes was performed according to the manufacturer's instruction (Boehringer Mannheim).

RESULTS

Amplification, Cloning and Sequencing of a PCR-Derived Partial Chitinase Gene from Kentucky Bluegrass

The amino acid sequences of plant chitinases show a high degree of similarity. Based on an amino acid sequence alignment (Zhu and Lamb, 1991), several regions were found to be conserved among the class I chitinases from species including monocotyledonous rice (Zhu and Lamb, 1991) and dicotyledonous tobacco (Shinshi et al., 1990), bean (Broglie et al., 1986), and potato (Laflamme and Roxby, 1989). Two conserved amino acid sequences were selected to design a set of oligonucleotide primers to amplify an internal portion of a chitinase gene in Kentucky bluegrass by PCR. Primer A (5'-TTCGGCTGGTGCGGCTCCACC-3') corresponds to the amino acid sequence of FGWCGST of the rice chitinase (Zhu and Lamb, 1991) while primer B (5'-AGCCCGCCGTTGATGATGTT-3') corresponds to the sequence of NIINGGL in the rice chitinase. A 710 bp fragment (designated as CH710) was amplified. The size of these amplified products was expected based on the nucleotide sequence of the rice chitinase gene. The PCR products were cloned into the pNoTA/T7 vector and its nucleotide sequence was determined (Fig. 1). The nucleotide sequence of CH710 is 78% identical to the corresponding sequence of the rice chitinase gene (Zhu and Lamb, 1991), indicating that

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ttcggctggtgcggctccaccTCCGACTACTGAGGCACCGGCTGCCAGAG        50

CCAGTGCAACGGCTGCGGCGGTCCCACGCCGGTGACCCCGACTCCCTCCG       100
← primer 5'S2                        ← primer 5'S1

GCGGCGGCGTGTCCTCCATCATATCCCAGTCGCTCTTCGAACAGATGCTG       150

CTGCACCGCAACGACGCGGCGTGCCTGGCCAAGGGGTTCTACACCTACAA       200

CGCCTTCGTCGCCGCCGCCAACTCTTTCGCCGGCTTCGGGACCACGGGCG       250

GCACCGACGTCAGGAAGCGCGAGGTGGCCGCCTTCCTCGCTCAGACCTCC       300

CACGAGACCACCGGCGGGTGGCCCACGGCGCCCGACGGCCCCTACTCATG       350

GGGCTACTGCTTCAACCAGGAGAACGGCGCCACCTCCGACTACTGCTCGC       400

CGAGTTCACAGTGGCCGTGCGCGCCGGGGAAGAAGTACTTCGGCCGCGGG       450

CCCATCCAGATCTCCTACAACTACAACTACGGGCCGGCGGGACAGGCCAT       500

CGGCAGCGACCTGCTCAACAACCCGGACCTTGTGACCTCAGACCCGACCG       550

TGTCGTTCAAGACGGCGCTGTGGTTCTGGATGACGGCTCAGTCTCCCAAG       600

CCTTCGAGCCACGCCGTGATCACCGGCCAGTGGAGCCCCTCGGGCGCAGA       650
                 primer 3'S1 →

CCAGGCGGCGGGGAGGGTGCCCGGGTACGGCGTGATCACTaacatcatca       700
                 primer 3'S2 →

acggcgggct                                               710
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Fig.1. The nucleotide sequence of CH710, a PCR-amplified partial chitinase gene from Kentucky bluegrass. Sequences in lower case letters are primers used for PCR to amplify CH710. Oligonucleotide primers used for cassette-ligation mediated PCR to amplify 5'and 3' unknown regions flanking CH710 are *underlined* and marked. *Arrows* indicate directions of individual primers.

CH710 is a partial chitinase gene from Kentucky bluegrass.

Cloning of DNA Fragments Flanking CH710 by Cassette-Ligation Mediated PCR

Sequences flanking CH710 were amplified by cassette-ligation mediated PCR. The basic principle of cassette-ligation mediated PCR is outlined in Figure 2. Briefly, genomic DNA is digested with a restriction enzyme and then ligated to the cassette containing a compatible end. Since the 5' end of the cassette is not phosphorylated, a single stranded break will remain at the ligation site between the 5'-end of the cassette and the 3'-end of the restriction fragment. This prevents undesirable amplification of cassette dimers as well as non-specific amplification of cassette-ligated restriction fragments. Two rounds of PCR are performed to increase the specificity and yield of amplification. The first round PCR uses an outside cassette primer C1 and an outside specific primer, 5'S1 or 3'S1 depending on the amplification of 5' or 3' flanking sequence. The second round PCR uses an inside cassette primer C2 and another nested specific primer, 5'S2 or 3'S2.

To amplify the 5' unknown sequence flanking CH710, genomic DNA from Kentucky bluegrass was digested by each of the ten restriction enzymes including *Hind*III, *Eco*RI, *Pst*I, *Bam*HI, *Bgl*II, *Nde*I, *Xho*II, *Sal*I, *Xho*I and *Xba*I and

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Genomic DNA

↓ 1) Restriction Enzyme Digestion

Cassette

↓ 2) Ligation

5'S2 5'S1 Known Region   C2 C1

C1 C2   3'S1 3'S2

↓ PCR1 with Primers C1 and 5'S1 or 3'S1

↓ PCR2 with Primers C2 and 5'S2 or 3'S2

Amplified 5' Upstream   Amplified 3' Downstream

Unknown Region   Unknown Region
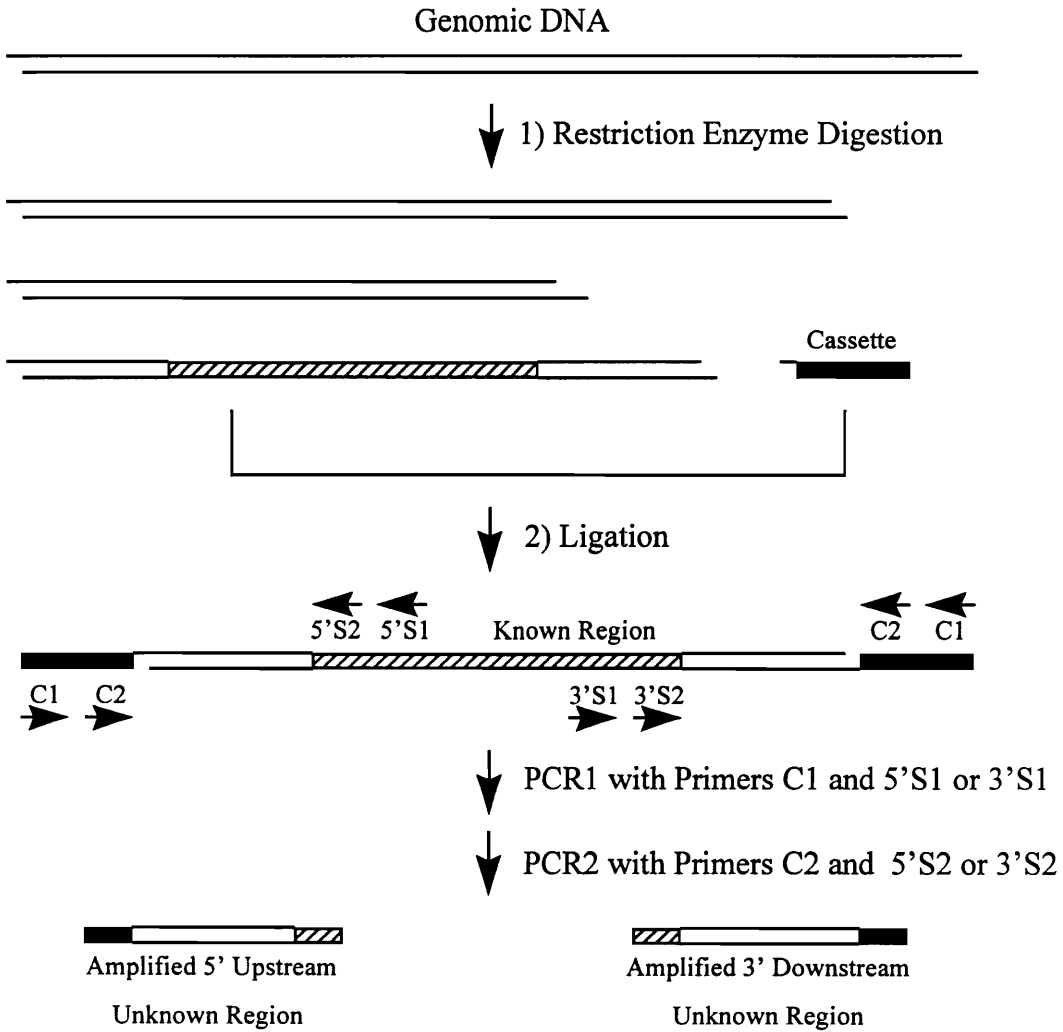
Fig. 2. Schematic diagram of cassette-ligation mediated PCR. C1 and C2 represent cassette primers. 5'S1, 5'S2, 3'S1 and 3'S2 are known region-specific primers. The 5' end of the cassette is not phosphorylated causing a nick within the ligation site.

ligated to a cassette with a compatible restriction end. Two gene-specific primers, 5'S1 and 5'S2, are shown in Figure 1. After the second round PCR, the amplified products were analyzed by agarose gel electrophoresis and distinct bands were recovered from the gel. Another PCR amplification with primer C2 and an internal primer was conducted to confirm whether these gel-recovered fragments were partial chitinase genes. Those fragments amplified from HindIII-, SalI-, BglII-, and XhoI-digested genomic DNA were demonstrated positive by PCR confirmation and thus, reamplified with primers of C2 and 5'S2 to increase the yield for subsequent subcloning. The nucleotide sequences of these fragments were determined and designated as CH5'F1 (HindIII), CH5'F2 (SalI), CH5'F3 (BglII) and CH5'F4 (XhoI) (Fig. 3). CH5'F1 has the largest length of 414 bp while CH5'F2, CH5'F3 and CH5'F4 are 362 bp, 355 bp, and 190 bp in length, respectively. CH5'F2 lacks the SalI restriction site at its 5' end and appears to be a truncated clone.

These four 5' flanking sequences share a high degree of similarity to each other. For example, the nucleotide similarities between CH5'F1 and CH5'F2, CH5'F1 and CH5'F3, CH5'F1 and CH5'F4, are 99%, 87%, and 92%, respectively. An ATG codon for translation initiation is present in all of the four sequences at the same positions (Fig. 3). Additionally, the nucleotide at the position -3 relative to the A (designated as position +1) of the ATG initiation codon in all of the four 5' flanking fragments is an adenine, which is highly

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```
CH5'F1   AAGCTTTAGCTAGGTCCCCTCCAATTTGCAGAGATCAGGCATGCATGAATC              51
         HindIII              CCAAT

CH5'F1   TACTAGAACTGCCGAACTTCTTGTAGATTAGGTTAGATCGATCGTGGGCTC             102
                  primer ch5'b
CH5'F2   ********************************************A*******             43
CH5'F3   ***T**************************************A****G**              47
           BglII

CH5'F1   ATGGCAAGTTGGATACGTCTGCTGAGCCCATGGCCATGGCTT--T------            145
CH5'F2   ******************************************--*------             86
CH5'F3   *********************CGG**A**********AT*GGCTTA              98

CH5'F1   GCTAGCTATATAAGAGCCTGCACAACACCAGCTTCTGCCACACACCACTAC            196
                 TATATAA                    Box P
CH5'F2   **************************************************             137
CH5'F3   *************GA*G******************G*******-**-**              147

CH5'F1   ACGAGACACCAGCTAGTATCTACAGCCAGTTGAGCGGGTTCTGCTACATTG            247
                                     primer ch5'a
CH5'F2   **************************************************             188
CH5'F3   -*ACC****G***A*C**-*-*GTAA**C*C*******************             195
CH5'F4                              C*C*******************              23
                                      XhoI

CH5'F1   CTACGATGAGAGGACTTGCTGTGGTGGCCATGCTGGTCGCGGCCTTCGCCG            298
CH5'F2   *****************************************************           239
CH5'F3   *C***★★★**********TG**AC**A****C*A*****************           246
CH5'F4   *C***★★★**********TG********G**********************            74

CH5'F1   TGTCTGCACACGCCGAGCAGTGCGGCTCGCAGGCAGGAGGGGCGACATGCC            349
CH5'F2   ********************************C********G****           290
CH5'F3   *******************************C**C********G****            297
CH5'F4   **********G*G******************C**C********G****            125

CH5'F1   CCAACTGCCTCTGCTGCAGCAAGTTCGGCTTCTGCGGCACCACCTCCGACT            400
CH5'F2   *****************************************T***********           341
CH5'F3   *****************************************************           348
CH5'F4   *******************A*************GA***********            176

CH5'F1   ACTGCGGCACCGGC                                                  414
CH5'F2   ****A*********                                                  355
CH5'F3   ***********A*                                                   362
CH5'F4   ***********A*                                                   190
```

Fig.3. Comparison of the PCR-amplified 5' flanking sequences of Kentucky bluegrass chitinase genes. The sequences of CH5'F2, CH5'F3 and CH5'F4 are aligned with CH5'F1 sequence. Only nucleotides differing from the sequence of CH5'F1 are shown. *Asterisks* indicate nucleotides that are identical to those of CH5'F1. Gaps are introduced for optimal alignment. Restriction enzyme sites used for digestion of Kentucky bluegrass genomic DNA for *cassette-ligation mediated PCR* and sequences used as forward primers for PCR to amplify chitinase genes containing full-length coding regions are *underlined* and marked. Putative CAAT box and TATA box are *double-underlined*. An elicitor- and light-responsive *cis*-acting element in parsley phenylalanine ammonia-lyase (*PAL*) promoter is *underlined* and indicated by *Box P*. Translation initiation codon is shown in *bold*.

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conserved in both animal and plant and has been found essential for protein synthesis (Lutcke et al., 1987), further supporting that this codon is probably used to initiate translation.

All of the four 5' flanking fragments except CH5'F4 contain a short upstream promoter sequence with variable length where some consensus sequences were found. In CH5'F1, a TATA-box sequence, TATATAA, is located at positions -95 to -101 (i.e., 95-101 bp upstream from the putative ATG translation initiation codon), while a CAAT-box sequence, CCAAT, is at positions -228 to -232. The same TATA-box sequence is also present in CH5'F2 at the same position as in CH5'F1. In CH5'F3, however, the sequence of TATATAA is located at positions -90 to -96. Another putative *cis*-acting element is also present in the 5' flanking regions. The sequence, AGCTTCTGCCACACACCACTACAC, which is present at positions -55 to -78 in CH5'F1 and in CH5'F2, is similar to Box P footprint, a sequence-specific protein (BPF-1) binding site in a parsley phenylalanine ammonia-lyase (PAL) promoter (Lois et al., 1989; Da Costa e Silva et al., 1993). It has been demonstrated that Box P is an elicitor and light responsive *cis*-acting element in parsley PAL promoter and sequences similar to Box P are also present in the promoters of several defense-related genes (Da Costa e Silva et al., 1993).

The same strategy of cassette-ligation mediated PCR was used to amplify the 3' flanking sequences of CH710. The specific primers, 3'S1 and 3'S2, are shown

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in Figure 1. Electrophoresis analysis of PCR products, recovery of distinct bands from gel and PCR confirmation with an internal primer were performed in the same way as used for 5' flanking sequences. After confirmation, the positive fragments amplified from genomic DNA digested by *Xba*I, *Pst*I, *Sal*I, *Bam*HI, and *Xho*II were cloned into vectors and their sequences were determined. All these five fragments, designated as CH3'F1 (*Xba*I), CH3'F2 (*Pst*I), CH3'F3 (*Sal*I), CH3'F4 (*Bam*HI), and CH3'F5 (*Xho*II), contain a partial chitinase coding sequence and a 3' untranslated region (Fig. 4). The nucleotide sequence comparison in Figure 4 shows that these five sequences share a high degree of similarity in their coding regions, while the nucleotide sequences in the 3' untranslated regions are not conserved among these five fragments. However, between CH3'F1 and CH3'F2 as well as between CH3'F3 and CH3'F4, the 3' untranslated sequences have high similarities (Fig. 4). CH3'F1 is of 527 bp, of which 213 bp encode 70 amino acids. A putative polyadenylation signal site is located approximately 280 bp downstream from the TAG codon and it fits the consensus polyadenylation sequence which is conserved in plants (Joshi, 1987; Heidecker and Messing, 1986). CH3'F2 shares a high similarity to CH3'F1 in its first 450 bp, however, CH3'F2 has a short deletion and a short addition compared to CH3'F1 (Fig. 4). The addition of the eight nucleotides shifts its reading frame.

```
CH3'F1 CACCAACATCATCAATGGCGGGCTCGAGTGCGGCAAAGGGCAGGACAACCGCGTCGCAGTCGCAGACCGGATCGGGTTCT  80
CH3'F2 ****************************************************----*-*************** 74
CH3'F3 CACCAACATCATCAACGGAGGGCTCGAGTGCGGCAAGGGGCAGGACAACCGCGTCGCCGACAGGATCGGGTTCTACAAGC  80
CH3'F4 **************T**C**********************************A******************** 80
CH3'F5 CACCAACATCATCAATGGCGGGCTCGAGCATTTGACAAATGGTACTACCGCAAATTCATATGGGAAT**TAG**GTGGTCACTT  80


CH3'F1 ACAAGCGCTACTGCGACCTCCTCGGCGTCAGCTACGGAGACAACCTGGACTGCTACAACGAGAGTCCGTTCGGTAGTTCT 160
CH3'F2 **************************************************************C************** 154
CH3'F3 GCTACTGCGACCTCCTCGGCGTCAGCTACGGAGACAACCTGGACTGCTACAACCAGAGGCCGTTCGGTGGT**TAA**GTTAAG 160
CH3'F4 *************************A*************G****************A****-----* 155
CH3'F5 CCTTTCGCCACAAACATATGCGATGGAATCGATGCAATGTTCAGCTTGTTTGCTATTGTGGCCGTTTGGGG__AATAAA__TAT 160


CH3'F1 --------GCCAAACGCAATATCAAACGCAATATCTATTATGTTAGCTGTTTTCCAACA**TAG**CTATCTCGTCAATTCCTT 232
                                                                       __primer ch3'fl-a__
CH3'F2 GTCAGTCG******************************C********************************* 234
CH3'F3 TCTGTCAATCGGCCGGCAACACAAGCAATTAATG__AATAAA__AGCTCTCTAAATACTGTAACTATGGTGTTGCAAACAGGAA 240
CH3'F4 *************-*---***C****************************C********G**GG 231
       __primer ch3'f4-a__                                                      primer
CH3'F5 TTTGCTGTTTACACTTTGCTCGGATGTGATATGGTGTTGGTGCACCTTTGTGATTGTTTGATTCTCGGCTGTCCTTCCAT 240


CH3'F1 __CCTAATTTCAGGGGGCGGGCCGATCGGATCGCCACCACCAGAATCGGGAGTTGACGGGCCAATCGGATCAACAACACCAG__ 312
CH3'F2 ***************************************************************************** 314
CH3'F3 __TGAATAA__TGGTGTGATCTATCGCAAGATATGCAGTCCATTAATGA__AATAAA__CAATTTGTGGCTATTACATTTTCACATGT 320
CH3'F4 __A__*************** 248
       ch3'f4-b
CH3'F5 TGAAACCATGCAACCAAGAAGGGAAGAAAGTGGAGTACTCTCATGCTTGGAGTATTAGACAACATTTAGCAGCATTTAGG 320


CH3'F1 TAGCAGCATGGGGAACTGACTCATCGTCGAAGAAGAGGATCAGAACATGTATGACAACTCTAATTACCTCTCTCTGACGT 392
CH3'F2 ********C*********G*****************************C******************* 394
CH3'F3 TACCTGGTCCACTCTATGCATTCTGAGCAAATGCCCACGC 360
CH3'F5 CGGATCT 327
       XhoII


CH3'F1 __TTATTCAACTAGCAGAGTCGCTCGCT__AAATCTGGGTTGGCTAAATTGGTTTGGTTATTCATATATGGAGTTTTTGAATAC 472
       __primer ch3'fl-b__
CH3'F2 *********************T*T**************************GGTGTCTTC*CGGACTCCG** 474


CH3'F1 TCTATTGCAATCTTATCCCATTA__AATAAA__CCGTGTAAGTATCTTTCGTT**TCTAGA** 527
                                                            XbaI
CH3'F2 AG*GACC*TC*G*C*C**T*CCTGT*CCT*CT***CGC*GA*G***TCCA*GG*CTCATCCGCTCCCACCCGG__CTGCAG__ 553
                                                                                  PstI
```

Fig.4. Comparison of five 3' flanking sequences of Kentucky bluegrass chitinase genes amplified by *cassette-ligation mediated PCR*. The sequences of CH3'F2 and CH3'F4 are aligned with CH3'F1 and CH3'F3 sequences, respectively. Only nucleotides differing from the reference sequence are shown. *Asterisks* indicate nucleotides that are identical to those of the reference sequence. Gaps are introduced for optimal alignment. Restriction enzyme sites used for digestion of Kentucky bluegrass genomic DNA for *cassette-ligation mediated PCR* and sequences used as reverse primers for PCR to successfully amplify chitinase genes containing full-length coding regions are *underlined* and marked. Putative polyadenylation signals are *double-underlined*. Translation stop codon is shown in *bold*.

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The length of the coding region of CH3'F4 is the same as that of CH3'F3. In untranslated regions, however, two small deletions were found in CH3'F4 when compared to CH3'F3. The coding region of CH3'F5 is much smaller than those of the other four 3' flanking sequences. Three putative polyadenylation signals were found at positions 41 bp, 87 bp, and 132 bp downstream from the TAA stop codon in CH3'F3, while only one was found at 32 bp downstream from the stop codon in CH3'F4 and one at 81 bp downstream in CH3'F5. All these sequences fit the consensus polyadenylation sequence found in plants (Heidecker and Messing, 1986; Joshi, 1987).

The putative amino acid sequences deduced from CH3'F1, CH3'F3, CH3'F4, and CH3'F5 are compared in Figure 5. In CH3'F3 and CH3'F4, translation termination occurs at the same position and only two amino acids were different when sequences encoding 50 amino acids were compared. Unlike the other three, CH3'F1 has a short C-terminal extension. CH3'F5 contains 28 fewer amino acid codons compared to CH3'F3 and CH3'F4.

PCR Amplification and Sequencing of Three Genomic Chitinase Genes from Kentucky Bluegrass

PCR techniques were also used to amplify genomic DNA sequences containing full coding regions of Kentucky bluegrass chitinases. Two forward

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```
CH3'F1     NLDCYNESPFGSSAKRNIKRNIYYVSCFPT*                    70
CH3'F3     ......QR...G*                                       50
CH3'F4     .....SQR...G*                                       50
CH3'F5     *                                                   22
```

Fig.5. Comparison of the putative amino acid sequences deduced from the sequences flanking CH710 at its 3' end. The amino acid sequences of CH3'F3, CH3'F4 and CH3'F5 are aligned with CH3'F1 amino acid sequence. Only amino acids differing from the sequence of CH3'F1 are shown. *Dots* indicate amino acids that are identical to those of CH3'F1. Gaps are introduced for optimal alignment. *Asterisks* indicate stop codon.

primers, ch5'a and ch5'b, were selected from the conserved regions of the 5'

flanking sequences (Fig. 3). Primer ch5'a was located at positions 6 bp to 27 bp

upstream from ATG in CH5'F1 and also conserved in CH5'F2 and CH5'F3 while

the primer ch5'b was located at positions 71 to 93 bp upstream from ATG in

CH5'F1 and conserved in all of the four sequences. We made efforts to amplify

multiple chitinase genes which would contain full coding regions by combining

one forward primer, either ch5'a or ch5'b, with one reverse primer selected from

the untranslated region of PCR amplified 3' flanking sequences. The PCR

primers which were used to successfully amplify three genomic chitinase

sequences, KBCH1, KBCH2 and KBCH3, from Kentucky bluegrass, are listed in

Table 1.

The sequences of KBCH1, KBCH2 and KBCH3 were determined by direct

sequencing of PCR products. Sequence analysis showed that there was a

perfect match between some PCR-amplified partial chitinase sequences and

KBCH1, KBCH2 or KBCH3. The sequences of CH3'F1 and CH3'F4, for

example, completely matched the corresponding sequences of KBCH1 and

KBCH2, respectively, while the sequences of CH5'F2 and CH710 completely

matched the corresponding sequences from KBCH3, suggesting that they were

amplified from the same individual gene and that the procedures of PCR

amplification and sequencing we used were reliable.

The nucleotide sequences of KBCH1, KBCH2 and KBCH3 are shown in Figure

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Table 1. **Summary of PCR primers used to successfully amplify three full-coding region containing chitinase genes in Kentucky bluegrass and their overlapping sequences**

		KBCH1 (*chi1*)	KBCH2 (*chi2*)	KBCH3 (*chi3*)
forward primer	name	ch5'a[1]	ch5'a[1]	ch5'b[2]
	sequence (5'→ 3')	TTGAGCGGGTT-CTGCTACATTG	TTGAGCGGGTT-CTGCTACATTG	CTGCCGAACTT-CTTGTAGATTAG
	position[3]	-27 to -6	-27 to -6	-93 to -71
reverse primer	name	ch3'f1-b[4]	ch3'f4-b[5]	ch3'f4-a[6]
	sequence (5'→ 3')	TATTCAACTAG-CAGAGTCGCTCG	GCAAACGGGGG-AGAATAATGGTGT	GTCTGTCAAT-CGGCCGGCAA
	position[7]	+180 to +202	+67 to + 90	+1 to +20
overlapping sequence		CH3'F1	CH3'F4	CH5'F2 CH710

[1]See Fig.3.
[2]See Fig.3.
[3]The position of the adenine in ATG, the translation initiation codon, is defined as +1. Therefore, the positions of upstream primers are relative to the translation initiation codon according to the sequence of CH5'F1.
[4]See Fig.4.
[5]See Fig.4.
[6]See Fig.4.
[7]The position of the first nucleotide after translation termination codon is defined as +1. Therefore, the positions of the downstream primers are relative to the translation termination codons.

6. The length of KBCH1 is 1252 bp which contains an open reading frame of 1023 bp and a 3' untranslated region of 202 bp. The length of KBCH2 is 1080 bp including an open reading frame of 963 bp and a 3' untranslated region of 90 bp with a polyadenylation signal (AATAAA) located at 32 bp downstream from the translation termination codon (TAA). The length of KBCH3 is 1223 bp including a short upstream promoter sequence. A TATA-box was found at 94 bp upstream from ATG. The open reading frames of KBCH1 and KBCH2 are 1023 bp and 963 bp, respectively, while that of KBCH3 is only 153 bp, which is terminated by a substitution of C for A at the position 346 in KBCH3 (Fig. 6). There are two 9 bp imperfect direct repeats located at the positions of 69-77 (CGCCGTGTC) and at the positions of 255-263 (CGGCGTGTC) in both KBCH1 and KBCH2. Imperfect direct repeats have also been found flanking the cysteine-rich domain in several class I chitinase genes from both dicotyledons (Shinshi et al., 1990) and monocotyledons (Liao et al., 1994). The second direct repeat is not complete in KBCH3 due to a deletion of three nucleotides (Fig. 6). These conserved sequences might be the target sites for transposable elements to excise the cysteine-rich domain and give rise to class II chitinases (Shinshi et al., 1990).

A nucleotide sequence comparison showed that the sequence identity in the coding region was 96% between KBCH1 and KBCH2 and 94% between KBCH2 and KBCH3 (Fig. 6). The putative chitinase genes for KBCH1, KBCH2 and

```
KBCH3                    ctgccgaacttcttgtagattagGTTAGATCGATCATGGGCTCATG    46

KBCH3 GCAAGTTGGATACGTCTGCTGAGCCCATGGCCATGGCTTTGCTAGCTATATAAGAGCCTG   106

KBCH3 CACAACACCAGCTTCTGCCACACACCACTACACGAGACACCAGCTAGTATCTACAGCCAG   166

KBCH1 ttgagcggggttctgctacattgCCACGATGAGAGGACTTGTGGTGGTGGCCATCCTGGTC    60
KBCH2 ttgagcggggttctgctacattg*************************A**********    60
KBCH3 TTGAGCGGGGTTCTGCTACATTG*T***************CT***********G******   226

KBCH1 GCGGCCTTCGCCGTGTCTGCGCACGCCGAGCAGTGCGGCTCGCAGGCCGGCGGGGCGACG   120
KBCH2 ******************A***************************************A************   120
KBCH3 ******************A*****************************A************   286

KBCH1 TGCCCCAACTGCCTCTGCTGCAGCAAGTTCGGTTTCTGCGGCAACACCTCCGACTACTGC   180
KBCH2 *************************A*****C**********C***************   180
KBCH3 ***************************C*********TC***************★★A   346

KBCH1 GGCACCGGCTGCCAGAGCCAGTGCAACGGCTGCAGCGGCCCCACGCCGGTGACCCCGACT   240
KBCH2 ************************************************************   240
KBCH3 *****************************G****T*******************   406

KBCH1 CCCTCCGGCGGCGGCGGCGTGTCCTCCCTCGTATCTCAGTCGCTCTTCGAACAGATGCTG   300
KBCH2 *****************************A**********************G********T   300
KBCH3 ******************---*********A**A****C****************   463

KBCH1 CTGCACCGTAACGACCCGTCGTGCCAGGCCAACGGGTTCTACACCTACAAAGCCTTCATC   360
KBCH2 ********C******G**G*A****T******G**T***************C*********   360
KBCH3 ********C******G**G******T******G****************C******G**   523

KBCH1 GCCGCTGCCAACTCTTTCGCCGGTTTCGGGACCACGGGCAGCACCGACGTCAGGAAGCGC   420
KBCH2 *****C****************************************************   420
KBCH3 *****C***************C*****************G*****************   583

KBCH1 GAGGTGGCCGCCTTCCTCGCTCAGACCTCCCACGAGACCACCGGCGGGTGGCCGACGGCG   480
KBCH2 ************************************************************   480
KBCH3 ****************************************************C******   643

KBCH1 CCCGACGGCCCCTACTCCTGGGGCTACTGCTACAAACAGGAGCAAGGCGCCACCTCCGAC   540
KBCH2 *****************************T****************************   540
KBCH3 ****************A***********T***C*****A*C**************   703

KBCH1 TACTGCTCGCCGAGCTCGCAGTGGCCGTGCGCGCCGGGGAAGAAGTACTTCGGCCGGGGG   600
KBCH2 ************************************************************   600
KBCH3 **************T**A***************************************C***   763

KBCH1 CCCATCCAGATCTCCTTCAACTACAACTACGGGCCAGCGGGGCAGGCCATTCAAACTGAC   660
KBCH2 ***************A***********************A*******C*********   660
KBCH3 ***************A*****************G*****A********CGGC*GC***   823
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50

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KBCH1 CTGCTCAACAACCCGGACCTTGTGGCCTCGAACGCGACCGTGTCGTTCAGGACGGCGCTG   720
KBCH2 ***********************A*AG**C****************A*********        720
KBCH3 **********************A****AG**C****************A*********      883

KBCH1 TGGTTCTGGATGACGGCTCAGTCGCCCAAGCCGTCGAGCCACGCCGTGATCACCGGCCAG   780
KBCH2 ************************************************************   780
KBCH3 *********************T********T*****************************   943

KBCH1 TGGAGCCCCTCGAGCGCAGACCAGGCGGCGGGGAGGGTGCCCGGGTACGGCGTGATCACC   840
KBCH2 ********************A*************************************** 840
KBCH3 ***********G*********************************************T 1003

KBCH1 AACATCATCAATGGCGGGCTCGAGTGCGGCAAAGGGCAGGACAACCGCGTCGCAGTCGCA   900
KBCH2 ***************************************************----- 895
KBCH3 **********C*******************G******************C*----- 1058

KBCH1 GACCGGATCGGGTTCTACAAGCGCTACTGCGACCTCCTCGGCGTCAGCTACGGAGACAAC   960
KBCH2 -**********************************************A*****  954
KBCH3 -**A***********************************************G******  1167

KBCH1 CTGGACTGCTACAACGAGAGTCCGTTCGGTAGTTCTGCCAAACGCAATATCAAACGCAAT  1020
KBCH2 *************G*C****G************AA*----------*C*GTC*GT*GGC  1004
KBCH3 ***************C****G******AA*G***AAgtctgtcaatcggccggcaa  1223

KBCH1 ATCTATTATGTTAGCTGTTTTCCAACATAGCTATCTCGTCAATTCCTTCCTAATTTCAGG  1080
KBCH2 CGGC*AC*CAG*TAAC*AA*-AA**----**C***---A***A*-*--G***C-*AT**  1052

KBCH1 GGGCGGGCCGATCGGATCGCCACCACCAGAATCGGGAGTTGACGGGCCAATCGGATCAAC  1140
KBCH2 C*TTgcaaacggggagaataatggtgt                                  1080

KBCH1 AACACCAGTAGCAGCATGGGGAACTGACTCATCGTCGAAGAAGAGGATCAGAACATGTAT  1200

KBCH1 GACAACTCTAATTACCTCTCTCTGACGTTtattcaactagcagagtcgctcg          1252
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Fig.6. Comparison of the nucleotide sequences of Kentucky bluegrass chitinase genes. Sequences used as PCR primers are indicated by lower case letters. The sequences of KBCH2 and KBCH3 are aligned with that of KBCH1. Only nucleotides differing from the sequence of KBCH1 are shown. *Asterisks* indicate nucleotides that are identical to those of KBCH1. Gaps are introduced for optimal alignment. Putative TATA box and polyadenylation signal are *double-underlined*. Two imperfect direct repeats flanking the cysteine-rich domain are *underlined*. Codons for translation initiation and termination are showed in *bold*.

KBCH3 were designated as *chi1*, *chi2* and *chi3*, respectively.

Cloning and Sequencing of the cDNAs of chi1 and chi2 by Reverse Transcriptase-PCR (RT-PCR)

Whether chitinase genes *chi1* and *chi2* contain introns was also studied. RT-PCR was performed to amplify complementary DNA sequences of *chi1* and *chi2* from Kentucky bluegrass leaves pretreated with ethephon for 4 days. The forward primer ch5'a (Fig. 3) and the reverse primer ch3'f1-a (Fig. 4) were used for amplification of cDNA sequence of *chi1,* and the primers, ch5'a and ch3'f4-a (Fig. 4), for *chi2* cDNA amplification. Ch3'f1-a and ch3'f4-a were *chi1*- and *chi2*-specific primer, respectively. The fragments shown on agarose gel with expected sizes were purified from gel and reamplified by PCR for direct sequencing to determine their nucleotide sequences. The length of the PCR-amplified cDNA sequence of *chi1* is 1073 bp, including the coding sequence of 1020 bp. The length of the cDNA fragment of *chi2* is 1010 bp including the full coding region of 960 bp in length. The cDNA sequences of *chi1* and *chi2* completely match their genomic counterparts, indicating that these two genes have no introns.

Primary Structures of Kentucky Bluegrass Chitinases and Amino Acid Sequence Similarity to Other Chitinases

The Kentucky bluegrass chitinase propeptides encoded by *chi1* and *chi2* are 340 and 320 amino acids in length, respectively. The G+C contents of the coding region are 64.5% for *chi1* and 66.0% for *chi2*. Both *chi1* and *chi2* open reading frames exhibit strong codon bias (87.1% and 90.0%, respectively) for G or C in the third nucleotide in all codons, as found in chitinase genes from other monocots, such as rice (Nishizawa and Hibi, 1991) and maize (Wu et al., 1994), but not in chitinase genes from dicots. Both CHI1 and CHI2, the polypeptides corresponding to *chi1* and *chi2*, contain a signal peptide of 20 amino acids at the N-terminus (Fig. 7). The putative cleavage site for the signal peptides is between an alanine and a glutamic acid at positions of +20 and +21 according to the rules for protein processing proposed by Von Heijne (1986). The signal peptides are composed of a large portion of hydrophobic residues (80%) and represent a typical signal peptide including a splicing motif (Perlman and Halvorson, 1983). The signal peptides of CHI1 and CHI2 share a high amino acid sequence identity of 95%. The signal peptides of Kentucky bluegrass chitinases also show a high degree of sequence similarity (85%) to a wheat chitinase, WCH1 (Liao et al., 1994), although the similarities of the signal peptides of chitinases between Kentucky bluegrass and other plants are very low.

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              <       Signal peptide       ><       N-Cysteine-rich
CHI1       M---R--GLVVVAILVAAF-AV-SAHAEQCGSQAGGATCPNCLCCSKFGFCGNTSDYCGT      53
CHI2       --- --      T        - -                              T          53
WHEAT      --- -- V   M A       - -                              S       N  53
RICE       --- --A A    MVARP L A-AV            V          Q W  S       A   54
BEAN       KKN MMMMIWSVGV WMLLL GGSYG     R        L  GGN  Q W  S T     P    60
TOBACCO    --- LCKFTALSS LFSLLLL-  S            R   SG   K W    N       P    56


              domain>< Hinge region    ><       Catalytic domain
CHI1       G-CQSQCNGCSG-PTPV-TPTPSGGGGVSSLVSQSLFEQMLLHRNDPSCQANGFYTYKAF      110
CHI2       -          -        -         I                 AA L K      N     110
WHEAT      -          GG     PV    T-      II      D        AA   K    N G    111
RICE       -        SRLRR-RR --DASGG  S  A I   R    DL      AA P SN     D    110
BEAN       -        G P P------A -----DL A I R T D     K    GA P K      D    108
TOBACCO    N        P -- -    T-P    P    DLG II S M D    K  NA GK    S N    112


CHI1       IAAANSFAGFGTTGSTDVRKREVAAFLAQTSHETTGGWPTAPDGPYSWGYCYKQEQGATS      170
CHI2                                                      F               170
WHEAT      V      S    A    GA                            FN    R  A      171
RICE       V     SA P  AAA DA TN                    A      T    F E N GAG  170
BEAN             KAYPS   N  D AT    I     G          A        A  FVR RNPST  168
TOBACCO    N   R  P    S D TA     I   F           WA        A    WLR   SPG  172


CHI1       -DYCSPSSQWPCAPGKKYFGRGPIQISFN̲Y̲NYGPAGQAIQTDLLNNPDLVASNATVSFRT      229
CHI2       -                           Y                   TDP      K       229
WHEAT      -      N                    Y        R  G        TD      K       230
RICE       P   QQ A     A    Y       L Y F          GA  GD      D      D    230
BEAN       --   ATP F     QQ  Y         W      QC R  GV    K      SVI   KS   226
TOBACCO    -   T  G       R            H         C R  GV       TDPVI   KS   231


CHI1       ALWFWMTAQSPKPSSHAVITGQWSPSSADQAAGRVPGYGVITNIINGGLECGKGQDNRVA      289
CHI2                                                                     289
WHEAT               P        D   R    G                       R    G     290
RICE       F        P       CN  A    T  AD  R                  H E D I    290
BEAN                        D    SR T    V  R L     TV         R     S Q  286
TOBACCO             P       C D    I R Q  AG R   N L   F        R T S Q   291


CHI1       VADRIGFYKRYCDLLGVSYGDNLDCYNESPFGSSAKRNIKRNIYYVSCFPT*           340
CHI2       --                 N      SQR      *                          320
WHEAT      --              T          QR  A*                             320
RICE       --          I       A      SQR SAPPKL LPSFHTVINNH*            336
BEAN       --       F       G  N      SQT    N LLLSDLVTSQ*               328
TOBACCO    --      R   SI    P      G QRS  NGLLVDTM*                     329
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Fig.7. Comparison of the deduced amino acid sequences of chitinases from Kentucky bluegrass and from wheat (Liao et al., 1994), rice (Zhu and Lamb, 1991), bean (Broglie et al., 1986), and tobacco (Shinshi et al., 1990). The amino acid sequence of CHI1 which is predicted from Kentucky bluegrass chitinase gene *chi1* is shown on the top line. Amino acid sequences of CHI2 of Kentucky bluegrass, as well as wheat, rice, bean, and tobacco chitinases are aligned with the CHI1 sequence. Only amino acids differing from the sequence of CHI1 are shown. *Dashes* indicate gaps which are introduced for optimal alignment. *Asterisks* indicate stop codons. The regions of the signal peptides, the N-terminal cysteine-rich domains, the hinge regions, and the start of catalytic domains are marked. The putative site for catalytic activity of chitinase is shown in a *bold* letter and *double-underlined*.

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Molecular weights of the predicted mature chitinase proteins encoded by *chi1* and *chi2* are approximately 34.0 kDa and 31.7 kDa, respectively. The computer-calculated isoelectric points are 7.55 for CHI1 and 6.95 for CHI2. The identity of the amino acid sequences between CHI1 and CHI2 is 94%. Both of them have a cysteine-rich domain containing 39 amino acids followed by a proline- and glycine-rich hinge region (Fig. 7), indicating that they are class I chitinases. CHI1 chitinase has a C-terminal extension of 18 amino acids while it is absent in CHI2 chitinase.

The deduced amino acid sequences of the Kentucky bluegrass chitinases, CHI1 and CHI2, show a high similarity to other plant chitinases (Table 2). Similarity in mature proteins between Kentucky bluegrass chitinases and a wheat chitinase (Liao et al., 1994) is 86% with 72% similarity between Kentucky bluegrass chitinases and chitinases of rice (Zhu and Lamb, 1991), bean (Broglie et al., 1986), and tobacco (Shinshi et al., 1990). An amino acid sequence of NYNYG which is conserved in most chitinases was also found at positions of 198-202 in CHI1 as well as in CHI2 (Fig. 7). The first tyrosine (Y) of this sequence has been demonstrated to be the catalytic activity site in a maize chitinase (Verburg et al., 1992).

Table 2.   Comparison of aligned amino acid sequences of mature chitinases

Chitinase[b]	Percentage of Identity[a]					
	CHI1	CHI2	Wheat	Rice	Bean	Tobacco
Kentucky bluegrass CHI1	100	94	85	72	69	71
Kentucky bluegrass CHI2		100	87	74	72	73
Wheat			100	74	72	73
Rice				100	66	65
Bean					100	74
Tobacco						100

[a]Percentage of positions with identical amino acids
[b]References for sequences cited in Fig.7

Genomic Organization of Kentucky Bluegrass Chitinase Genes

To estimate the number of chitinase genes in the Kentucky bluegrass genome, Southern blot hybridization was performed with genomic DNA isolated from Kentucky bluegrass leaves. Digoxigenin-labeled CH710, a PCR-amplified 710 bp fragment representing a partial chitinase coding sequence (Fig. 1) was used as a probe to hybridize Kentucky bluegrass genomic DNA which was digested to completion with restriction enzymes, such as *Bam*HI, *Eco*RI, *Hind*III and *Xba*I. There are no restriction sites for these enzymes in either CH710, KBCH1, KBCH2 or KBCH3. As shown in Figure 8, the probe hybridized to at least seven restriction fragments of Kentucky bluegrass genomic DNA digested with *Bam*HI, *Eco*RI, *Hind*III or *Xba*I, indicating that Kentucky bluegrass chitinase is encoded by a multigene family.

DISCUSSION

The present chapter describes a PCR-based approach for cloning genes which are closely related members of a multigene family. The method comprises the following three steps: 1) PCR amplification and sequencing of a partial chitinase gene from Kentucky bluegrass using two primers from conserved regions, 2) amplification of 5' and 3' unknown sequences flanking the known
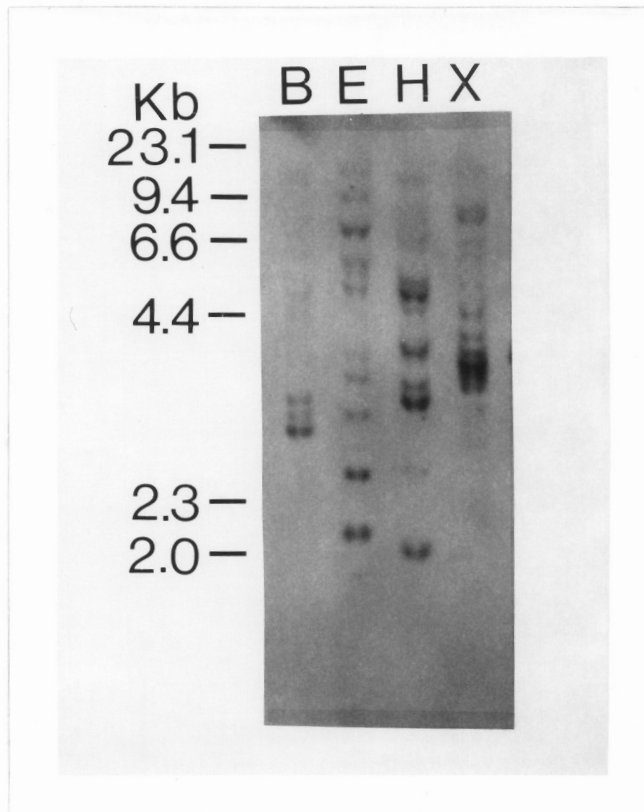
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Fig. 8. Southern blot analysis of chitinase genes in Kentucky bluegrass. Kentucky bluegrass genomic DNA was digested with *Bam*HI (B), *Eco*RI (E), *Hind*III (H) or *Xba*I (X) and hybridized with biotin-labeled CH710, a 710 bp fragment containing a conserved partial chitinase sequence.

partial chitinase gene by using cassette-ligation mediated PCR, 3) amplification of chitinase genes containing full coding region by using primers selected from 5' and 3' flanking fragments amplified by cassette-ligation mediated PCR. Using this approach, we have amplified three genomic DNA sequences encoding chitinases in Kentucky bluegrass.

Cassette-ligation mediated PCR has been used to amplify cDNA sequence of a single gene (Isegawa et al., 1992). It has been demonstrated that this and other similar techniques are very useful for cloning an unknown gene if only a single known sequence that is just long enough to design a specific primer for PCR is available (Shyamala and Ames, 1989). In this report we show that this technique is also applicable to cloning genes that are closely related members of a multigene family. With cassette-ligation mediated PCR, four 5' flanking fragments and five 3' flanking fragments were amplified. The sequence similarity among the four 5' flanking fragments was very high. This is not surprising because plant chitinases are usually encoded by a multigene family and many members share a high sequence similarity. In potato, for example, more than 10 bands were detected with a chitinase cDNA sequence as probes by DNA blot hybridization, and sequence comparison of the isolated chitinase cDNAs showed an identity as high as 95% between these cDNAs (Beerhues and Kombrink, 1994). In Kentucky bluegrass, the existence of more than seven chitinase genes is also indicated by DNA blot hybridization (Fig. 8).

Cassette-ligation mediated PCR is a simple technique for genome walking into unknown regions and it only requires a short stretch of sequence to be used to design a gene-specific primer (Isegawa et al., 1992). The primer can be potentially selected anywhere in the known region. The four 5' flanking sequences amplified by cassette-ligation mediated PCR were regarded as partial sequences of genomic genes encoding class I chitinases in Kentucky bluegrass since the two specific primers, primer 5'S1 and primer 5'S2 (Fig. 1), were selected from the amino acid regions within hinge region and N-terminal cysteine-rich domain of CH710, two regions present only in class I chitinases. In addition to the sequences of class I chitinases, 5' flanking sequences of class II chitinases could also be amplified by cassette-ligation mediated PCR if specific primers were selected in the main catalytic domain, a region in which class I and class II chitinases share high similarity.

Restriction of genomic DNA or cDNA is required by cassette-ligation mediated PCR for ligation to the cassette (Isegawa et al., 1992). Since no restriction mapping information is available for the unknown region before the amplification, it is possible that the selected restriction enzyme may produce a flanking fragment whose size is out of the limit of the standard PCR amplification. Thus, multiple restriction enzymes should be used. Furthermore, it is desirable to optimize the PCR conditions for the amplification of long fragments. Among all of the nine flanking sequences we amplified by cassette-ligation mediated

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PCR, the largest fragment was only about 600 bp. Recently, successful amplification of large DNA fragments by long-distance PCR has been demonstrated (Barnes, 1994; Cheng et al., 1994), which could greatly expand the effectiveness of cassette-ligation mediated PCR on chromosome walking. Long-distance PCR also allows more freedom for selection of specific primers for cassette-ligation mediated PCR in regard to the locations of primers. For example, the specific primer for amplification of 3' sequences can be at a location upstream from the specific primer for amplification of 5' sequences. Thus, the 5' and 3' flanking sequences amplified by cassette-ligation mediated PCR would have overlapping regions, which would facilitate the alignment of these sequences.

Genes *chi1* and *chi2* have single open reading frames encoding class I chitinases of 340 amino acids and 320 amino acids, respectively. Although *chi3* has a high sequence similarity at nucleotide level to *chi1* and *chi2* (approximately 93%), a single nucleotide substitution of C for A in *chi3* changes the cysteine at position 51 to a translation termination codon and therefore, the open reading frame of *chi3* encodes a peptide of only 50 amino acids. It is unlikely that the substitution was introduced during PCR amplification because it was also present in CH710 (Fig. 1) and CH5'F2 (Fig. 3), two fragments which were amplified in independent PCR reactions with different primers.

Computer-calculated isoelectric points for the predicted mature chitinases encoded by *chi1* and *chi2* are 7.55 and 6.95, respectively. The latter (6.95) is close to that of a wheat class I chitinase (pI 6.8) encoded by *Wch1* as described by Liao et al. (1994). Interestingly, the amino acid sequence of CHI2 also shows a higher identity with WCH1 (87%) than CHI1 does. Although class I chitinases are mostly basic forms, acidic chitinases with a N-terminal cysteine-rich domain have also been found in other monocotyledonous plants, such as garlic (Van Damme et al., 1993), maize (Huynh et al., 1992; Wu et al., 1994) and rice (Nishizawa et al., 1993).

CHI1 contains a C-terminal extension of 18 amino acids in length (Fig. 7). The C-terminal extension is necessary and sufficient for targeting the barley lectin (Bednarek and Raikhel, 1991) and a tobacco chitinase (Neuhaus et al., 1991) to the vacuole. Thus, Kentucky bluegrass CHI1 is likely to be targeted to the vacuole by its C-terminal extension, whereas CHI2 is presumably localized extracellularly since it lacks a C-terminal extension. Other known vacuolar proteins such as wheat germ agglutinin (Raikhel and Wilkins, 1987), rice lectin (Wilkins and Raikhel, 1989), tobacco β-1,3-glucanase (Shinshi et al., 1988), and bean chitinase (Broglie et al., 1986) also have C-terminal extensions. C-terminal extension has also been found in certain monocot chitinases including CHT2 of rice (Nishizawa et al., 1993), RCH10 of rice (Zhu and Lamb, 1991), and CH11 of

maize (Wu et al., 1994). However, there is no sequence similarity among these C-terminal extensions.

Although Kentucky bluegrass CHI1 and CHI2 may have different cellular localization, both are class I chitinases and have N-terminal cysteine-rich domains of 39 amino acids which differ at only one position between CHI1 and CHI2 (Fig. 7). The cysteine-rich domain not only is shared by class I chitinases but also is present in hevein, chitin-binding lectins and wheat germ agglutinin, implicating its chitin-binding function. Figure 9 shows the comparison of the cysteine-rich domains of CHI1 and CHI2 with those of wheat (Liao et al., 1994), rice (Zhu and Lamb, 1991), bean (Broglie et al., 1986) and tobacco (Shinshi et al., 1990) chitinase, as well as rubber hevein (Broekaert et al., 1990), barley lectin (Lerner and Raikhel, 1989), rice lectin (Wilkins and Raikhel, 1989), and wheat germ agglutinin (Raikhel and Wilkins, 1987). Certain positions within the cysteine-rich domains of different proteins are highly conserved (Fig. 9). Particularly well conserved are the eight cysteines that are present at the same positions in all of the sequences compared in Figure 9.

Kentucky bluegrass CHI1 and CHI2 show high identity in amino acid sequence with other chitinases from monocotyledons and dicotyledons (Fig. 7). A wheat class I chitinase, WCH1 (Wu et al., 1994), has the highest identity with the amino acid sequences of CHI1 and CHI2. In signal peptide, N-terminal cysteine- rich domain, hinge region and main catalytic domain, the identity

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CHI1            EQCGSQAGGATCPNCLCCSKFGFCGNTSDYCGTG--CQS-QC 39
CHI2            ...........................T........--...-.. 39
WHEAT           ...........................S......N.--...-.. 39
RICE            ..........V........Q..W..S......A.--...-.. 39
BEAN            ....R.....L..GGN...Q..W..S.T....P.--...-.. 39
TOBACCO         ..........R..SG....K..W....N....P.-N...-.. 40
HEVEIN          ....R....KL...N....QYQY..S.DE..SPDHN...-N. 41
BARLEY LECTIN   QR..E.GSNME...N....QY.Y..MGG....K.--..NGA. 40
RICE LECTIN     QT..K.ND.MI..HN....Q..Y..LGR......--...GA. 40
WGA             QR..E.GS.ME...N....QY.Y..MGG....K.--..NGA. 40
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Fig.9. Comparison of the amino acid sequences of the N-terminal cysteine-rich domains of CHI1 and CHI2 with the same domains in other proteins. The amino acid sequence of the N-terminal cysteine-rich domain of CHI1 is used as reference. Aligned amino acid sequences are from CHI2, wheat Wch1 (Liao et al., 1994), rice RCH10 (Zhu and Lamb, 1991), bean CH18 (Broglie et al., 1986), tobacco (Shinshi et al., 1990), as well as rubber tree hevein (Broekaert et al., 1990), barley lectin (Lerner and Raikhel, 1989), rice lectin (Wilkins and Raikhel, 1989) and wheat germ agglutinin (Raikhel and Wilkins, 1987). The *dots* indicate the identical amino acid residues. The gaps are introduced for optimal alignment.

between CHI1 or CHI2 and WCH1 are 80-85%, 95%, 65%, and 85-87%, respectively. Neither CHI2 or WCH1 has a C-terminal extension. The two class I chitinases of Kentucky bluegrass also show high identity in the catalytic domain with a barley class II chitinase which does not have the cysteine-rich domain (Leah et al., 1991). CHI1 and CHI2 are less similar to a rice class I chitinase, RCH10, than to the wheat chitinase.

The two Kentucky bluegrass chitinase genes, *chi1* and *chi2*, have high sequence identity and both encode class I chitinases whose cellular localization is predicted to be different. Whether these two genes are regulated in the same way or differently is yet clear. To gain in our understanding of the regulation of Kentucky bluegrass chitinase genes, the overall expression of chitinase genes, as well as the gene-specific expression of *chi1* and *chi2*, has been studied under various stress conditions. The results will be discussed in chapter III.

REFERENCES

Barnes, W.M. 1994. PCR amplification of up to 35-kb DNA with high yield from l bacteriophage templates. Proc. Natl. Acad. Sci. USA 91: 2216-2220.

Bednarek, S.Y., and Raikhel, N.V. 1991. The barley lectin carboxyl-terminal propeptide is a vacuolar protein sorting determinant in plants. Plant Cell 3: 1195-1206.

Broekaert, W.F., Lee, H.-I., Kush, A., Chua, N.-H., and Raikhel, N.V. 1990. Wounding induced accumulation of mRNA containing a hevein sequence in laticifers of rubber tree (*Hevea brasiliensis*). Proc. Natl. Acad. Sci. USA 87: 7633-7637.

Broglie, K.E., Gaynor, J.J., and Broglie, R.M. 1986. Ethylene-regulated gene expression: Molecular cloning of the genes encoding an endochitinase from *Phaseolus vulgaris.* Proc. Natl. Acad. Sci. USA 83: 6820-6824.

Cheng, S., Fockler, C., Barnes, W.M., and Higuchi, R. 1994. Effective amplification of long targets from cloned inserts and human genomic DNA. Proc. Natl. Acad. Sci. USA 91: 5695-5699.

Chomczynski, P., and Sacchi, N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162: 156-159.

Collinge, D.B., Kragh, K.M., Mikkelsen, J.D., Nielsen, K.K., Rasmussen, U., and Vad, K. 1993. Plant chitinases. Plant J. 3:31-40.

Da Costa e Silva, O., Klein, L., Schmelzer, E., Trezzini, G.F., and Hahlbrock, K. 1993. BFP-1, a pathogen-induced DNA-binding protein involved in the plant defense response. Plant J. 4: 125-135.

Heidecker, G., and Messing, J. 1986. Structural analysis of plant genes. Annu. Rev. Plant Physiol. 37: 439-466.

Huang, J.K., Wen, L., Swegle, M., Tran, H.C., Thin, T.H., Naylor, H.M., Muthakrishnan, S., and Reeck, G.R. 1991. Nucleotide sequence of a rice genomic clone that encodes a Class I endochitinase. Plant Mol. Biol. 16: 479-480.

Huynh, Q.K., Hironaka, C.M., Levine, E.B., Smith, C.E., Borgmeyer, J.R., and Shah, D.M. 1992. Antifungal proteins from plants. J. Biol. Chem. 267: 6635-6640.

Isegawa, Y., Sheng, J., Sokawa, Y., Yamanishi, K., Nakagomi, O., and Ueda, S. 1992. Selective amplification of cDNA sequence from total RNA by cassette-ligation mediated polymerase chain reaction (PCR): Application to sequencing 6.5 kb genome segment of hantavirus strain B-1. Mol. Cellular Probes 6: 467-475.

Joshi, C.P. 1987. Putative polyadenylation signals in nuclear genes of higher plants: a compilation and analysis. Nucleic Acids Res. 15: 9627-9640.

Kalman, M., Kalman, E.T., and Cashel, M. 1990. Polymerase chain reaction (PCR) amplification with a single specific primer. Biochem. Biophysic. Res. Commun. 167: 504-506.

Laflamme, D., and Roxby, R. 1989. Isolation and nucleotide sequence of cDNA clones encoding potato chitinase genes. Plant Mol. Biol. 13: 249-250.

Leah, R., Tommerup, H., Svendsen, I., and Mundy, J. 1991. Biochemical and molecular characterization of three barley seed proteins with antifungal properties. J. Biol. Chem. 266: 1564-1573.

Lerner, D.R., and Raikhel, N.V. 1989. Cloning and characterization of root-specific barley lectin. Plant Physiol. 91: 124-129.

Liao, Y.C., Kreuzaler, F., Fischer, R., Reisener, H.-J., and Tiburzy, R. 1994. Characterization of a wheat class Ib chitinase gene differentially induced in isogenic lines by infection with *Puccinia* graminis. Plant Sci. 103: 177-187.

Lois, R., Dietrich, A., Hahlbrock, K., and Schulz, W. 1989. A phenylalanine ammonia-lyase gene from parsley: structure, regulation and identification of elicitor and light responsive *cis*-acting elements. EMBO J. 8: 1641-1648.

Lutcke, H.A., Chow, K.C., Mickel, F.S., Moss, K.A., Kern, H.F., and Schede, G.A. 1987. Selection of AUG initiation codons differs in plants and animals. EMBO J. 6: 43-48.

Murashige, T., and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

Murray, M.G., and Thompson, W.F. 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. 8: 4321-4325.

Neuhaus, J.-M., Sticher, L., Meins, F., and Boller, T. 1991. A short C-terminal sequence is necessary and sufficient for the targeting of chitinases to the plant vacuole. Proc. Natl. Acad. Sci. USA. 88: 10362-10366.

Nishizawa, Y., and Hibi, T. 1991. Rice chitinase gene: cDNA cloning and stress-induced expression. Plant Sci. 76: 211-218.

Nishizawa, Y., Kishimoto, N., Saito, A., and Hibi, T. 1993. Sequence variation, differential expression and chromosomal location of rice chitinase genes. Mol. Gen. Genet. 241: 1-10.

Ochman, H., Gerber, A.S., and Martl., D.L. 1988. Genetic applications of an inverse polymerase chain reaction. Genetics 120: 621-623.

Perlman, D., and Halvorson, H.O. 1983. A putative signal peptidase recognition site and sequence in eukaryotic and prokaryotic signal peptides. J. Mol. Biol. 167: 391-409.

Raikhel, N.V., and Wilkins, T.A. 1987. Isolation and characterization of a cDNA clone encoding wheat germ agglutinin. Proc. Natl. Acad. Sci. USA. 84: 6745-6749.

Ride, J.P., and Barber, M.S. 1990. Purification and characterization of multiple forms of endochitinase from wheat leaves. Plant Sci. 71: 185-197.

Rosenthal, A., and Jones, D.S.C. 1990. Genomic walking and sequencing by oligo-cassette mediated polymerase chain reaction. Nucl. Acids Res. 18: 3095-3096.

Saiki, R.K., Gelfand, D.H., and Stoffel, S. 1988. Primer-directed enzymatic amplification of DNA with a thermmostable DNA polymerase. Science 239: 487-491.

Sanger, F., Nicklen, S., and Coulson, A.R. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74: 5463-5467.

Shinshi, H., Wenzler, H., Neuhaus, J.-M., Felix, G., and Hofsteenge, J. 1988. Evidence for N- and C-terminal processing of a plant-defense related enzyme. Primary structure of tobacco prepro-β-1,3-glucanase. Proc. Natl. Acad. Sci. USA 85: 5541-5545.

Shinshi, H., Neuhaus, J.-M., Ryals, J., and Meins, F. 1990. Structure of a tobacco endochitinase gene: evidence that different chitinase genes can arise by transposition of sequences encoding a cysteine-rich domain. Plant Mol. Biol. 14: 357-368.

Shyamala, V., Ames, G.F.-L. 1989. Genome walking by single-specific-primer polymerase chain reaction: SSP-PCR. Gene 84: 1-8.

Swegle, M., Huang, J.-K., Lee, G., and Muthukrishnan, S. 1989. Identification of an endochitinase cDNA clone from barley aleurone cells. Plant Mol. Biol. 12: 403-412.

Triglia, Y., Peterson, M.G., and Kemp, D.J. 1988. A procedure for *in vitro* amplification of DNA segments that lie outside the boundaries on known sequences. Nucleic Acids Res. 16: 8188.

Van Damme, E.J.M., Willems, P., Torrekens, S., Van Leuven, F., and Peumans, W.J. 1993. Garlic (*Allium sativum*) chitinases: characterization and molecular cloning. Physiol. Plant. 87: 177-186.

Verburg, J.G., Smith, C.E., Lisek, C.A., and Huynh, Q.K. 1992. Identification of an essential tyrosine residue in the catalytic site of a chitinase isolated from *Zea mays* that is selectively modified during inactivation with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide. J. Biol. Chem. 267: 3886-3893.

Von Heijne, G. 1983. Patterns of amino acids near signal-sequence cleavage sites. Eur. J. Biochem. 133: 17-21.

Wilkins, T., and Raikhel, N.V. 1989. Expression of rice lectin is governed by two temporally and spatially regulated mRNAs in developing embryos. Plant Cell 1: 541-549.

Wu, S., Kriz, A.L., and Widholm, J.M. 1994. Molecular analysis of two cDNA clones encoding acidic class I chitinase in maize. Plant Physiol. 105: 1097-1105.

Zhu, Q., and Lamb, C.J. 1991. Isolation and characterization of a rice gene encoding a basic chitinase. Mol. Gen. Genet. 226: 289-296.

CHAPTER III

EXPRESSION AND REGULATION OF CHITINASE GENES IN

KENTUCKY BLUEGRASS (*Poa pratensis* L.) INDUCED BY STRESSES

ABSTRACT

Plant chitinase, while normally expressed at a low level in healthy plants, accumulates to a high level in response to pathogen attack. Similarly, other stresses such as fungal cell wall elicitors, wounding, ethylene, salicylic acid, heavy metals, UV light, heat shock and cold temperature also induce chitinases, implicating the role of plant chitinase in defense. In present report, the overall expression of chitinase genes and the specific expression of two class I chitinase genes, *chi1* and *chi2*, were investigated in Kentucky bluegrass (*Poa pratensis* L.) under various stress conditions. Kentucky bluegrass plants were treated with either water, cold, heat, salicylic acid (SA) or ethephon. RNA blot hybridization showed that cold, heat, SA and ethephon treatments induced the accumulation of chitinase transcripts at a higher level compared to the untreated control or water treatment. Among the four different treatments that induced the accumulation of chitinase transcripts, ethephon incited the highest induction. In a time course study, increased accumulation of chitinase transcripts was

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observed in ethephon-treated Kentucky bluegrass leaves from 2 days to 5 days after the initiation of the treatment. RNA blot hybridization with gene-specific probes demonstrated that the expression of *chi1* and *chi2* is stimulated, while being coordinately regulated, only by ethephon.

INTRODUCTION

Plants possess a wide variety of defense mechanisms to protect themselves from pathogen infection and other environmental stresses. These mechanisms involve physical and biochemical changes in host plants, which are the result of the activation of an array of host defense genes. Physical changes include reinforcement of plant cell walls, a physical barrier against pathogen attack, by accumulation of hydroxyproline-rich glycoproteins (Showalter et al., 1985), enhanced lignification (Vance et al., 1980) and callose deposition (Ride, 1980). Among the biochemical changes are biosynthesis and accumulation of phytoalexins which are low molecular-weight anti-microbial compounds (Dixon et al., 1983), production of proteinase inhibitors (Dixon and Lamb, 1990), and accumulation of hydrolytic enzymes such as chitinase and β-1,3-glucanase (Van Loon, 1985).

Chitinases catalyze the hydrolysis of chitin, an essential cell wall component in many fungi but absent in higher plants. The role of chitinases in host defense against pathogen infection is implicated by the fact that chitinase activities are induced and accumulate to a significantly high level in response to pathogen infection, compared to the undetectable or extremely low basal level of chitinase activities in healthy plants. Furthermore, *in vitro* studies provide direct evidence

for the antifungal properties of plant chitinases by showing that purified plant chitinase inhibits fungal growth (Mauch et al., 1988; Schlumbaum et al., 1986).

Three classes of plant chitinases have been proposed based on a comparison of the predicted amino acid sequences of several plant chitinases (Shinshi et al., 1990). Class I chitinases have a N-terminal cysteine-rich domain and a main catalytic domain, which are linked by a small variable glycine- or proline-rich hinge region. Class II chitinases show high amino acid sequence similarity to class I chitinases in the catalytic domain but lack the N-terminal cysteine-rich domain. Class III chitinases share considerable amino acid sequence similarity to each other, but have no similarity to class I or class II chitinases in their amino acid sequences. Some class I chitinases are targeted to the vacuole by a short C-terminal extension (Neuhaus et al., 1991). Class II and class III chitinases are usually localized in the extracellular space.

It has been demonstrated by many studies that plant chitinases are induced by various biotic or abiotic factors, such as fungal, viral or bacterial infection, fungal cell wall elicitor treatment, chemical treatment including ethylene and salicylic acid, heavy metal treatment, plant hormone treatment, environmental changes such as heat shock and cold temperature treatment, as well as wounding (Graham and Sticklen, 1994). There is evidence that the responses of chitinase genes to various induction treatments differ among individual groups of chitinases. For example, salicylic acid (SA) is a strong inducer for induction of

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acidic class II or III chitinases (Lawton et al., 1994; Linthorst et al., 1990; Metraux et al., 1989) but not for induction of vacuolar class I chitinases. Ethylene or ethephon (2-chloroethylphosphonic acid), an ethylene-releasing compound, have been shown to be effective in inducing class I vacuolar chitinases (Boller and Vogeli, 1984; Broglie et al., 1986; Samac et al., 1990) but not effective in inducing most class II or III chitinases (Samac et al., 1990).

Most plant chitinases are encoded by a multigene family (Broglie et al., 1986; Legrand et al., 1987). Expression of chitinase genes which are members of a multigene family can be either coordinately or differentially regulated during plant development or pathogen infection (Mauch et al., 1988). In tobacco, class I chitinases are encoded by four genes (Van Buuren et al., 1992). These genes are regulated in the same way by ethylene treatment but respond differently to infection by the pathogen *Cercospora nicotianae*.

We have isolated two chitinase genes, *chi1* and *chi2*, from Kentucky bluegrass (*Poa pratensis* L.). The polypeptides encoded by *chi1* and *chi2*, CHI1 and CHI2, share 94% identity and both are class I chitinases because of the presence of a N-terminal cysteine-rich domain (Chapter II). However, they differ in that CHI1 contains a C-terminal extension while CHI2 does not. Thus, it is predicted that CHI1 is a vacuolar protein while CHI2 is an extracellular protein.

Like chitinases in most plant species studied so far, Kentucky bluegrass chitinases are also encoded by a multigene family. While the regulation of

chitinase genes from dicotyledons has been well studied, only limited information is available on the regulation of chitinase genes from monocotyledons. To understand how the expression of chitinase genes in Kentucky bluegrass is regulated, we studied overall and gene-specific expression of chitinase genes in Kentucky bluegrass under various stress conditions. Using Northern hybridization, we show evidence that the overall expression of chitinase genes in Kentucky bluegrass is stimulated by cold, heat, salicylic acid and ethephon, while the expression of *chi1* and *chi2* is induced only by ethephon. We also show that *chi1* and *chi2* are coordinately regulated under these stress conditions.

MATERIALS AND METHODS

Plant Materials and Treatments

Kentucky bluegrass (*Poa pratensis* L. cv. Glade) seeds were planted in 4-inch pots with 0.3 gram seeds per pot. The plants were grown at 26 °C in a growth chamber and watered once a day.

The following stress conditions were applied to 3-week-old Kentucky bluegrass plants. For cold temperature treatment, plants were exposed to 4 °C for 12 h every 24 h. For heat shock treatment, plants were exposed to 45 °C for 1 h with an interval of 11 h. For ethephon or salicylic acid treatment, plants were

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sprayed with 1 mg/ml ethephon or 10 mM salicylic acid to runoff twice a day and covered tightly with plastic bags. For water treatment, plants were sprayed with water twice a day and covered tightly with plastic bags. Control plants with no treatment were covered with plastic bags. After five days, the leaves from different treatments were harvested, frozen in liquid nitrogen and stored at -70 °C until use.

In an experiment to determine the time course of expression of Kentucky bluegrass chitinases genes, 3-week-old Kentucky bluegrass plants were sprayed with 1 mg/ml ethephon every 12 h for five days, and covered with plastic bags. Samples were taken at 6 h, 12 h, 1 day, 2 days, 3 days, 4 days and 5 days after initiation of the treatment. For water treatment, plants were sprayed with water twice a day, covered tightly with plastic bags and harvested at the same time course used in the ethephon treatment. Control plants with no treatment were covered with plastic bags.

RNA Isolation

Total cellular RNA was prepared from frozen Kentucky bluegrass leaves which had been stored at -70 °C as described by Chomczynski and Sacchi (1987).

RNA Probe Labeling

Biotin-labeled antisense RNA probes for Northern hybridization were synthesized by *in vitro* transcription with T7 RNA polymerase (Ambion) according to the manufacturer's instruction. Unincorporated nucleotides were removed from the biotin-labeled RNA probes by LiCl precipitation. The RNA probes were quantified with a spectrophotometer and stored at -70 °C until use.

A non-specific RNA probe was generated from CH710 which had been cloned into pNoTA/T7 vector (5'→ 3', Inc.). CH710 is a 710 bp fragment corresponding to a conserved region in Kentucky bluegrass chitinases (Fig. 1 in chapter II). Gene-specific antisense RNA probes for *chi1* and *chi2* were also synthesized by *in vitro* transcription with T7 RNA polymerase by using PCR-amplified fragments as templates. The PCR-amplified templates corresponded to the region of +901 to +1252 in KBCH1 or the region of +895 to +1080 in KBCH2 (Fig. 6 in chapter II), respectively.

A 90 bp human 18 S ribosomal DNA template with T7 promoter sequence was purchased from Ambion and labeled with the same *in vitro* transcription method as described above.

Northern Hybridization

Ten ug of total RNA dissolved in 4 ul of DEPC-treated water was mixed with four volumes of freshly prepared gel loading buffer (250 ul deionized formamide;

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83 ul formaldehyde, 37%; 50 ul 10X MOPS buffer; bromophenol blue, 0.01%, adjusted to 500 ul with H_2O) and denatured by heating at 65 °C for 10 min, followed by cooling in an ice bath.

To prepare a 1% formaldehyde-containing agarose gel, 10 ml of 10X MOPS and 75 ml H_2O were added to 1 gram agarose. The mixture was heated to melt the agarose and allowed to cool down to 60 °C before 16.5 ml of formaldehyde, 37% (v/v) was added. Then the gel mixture was poured to a gel tray.

RNA samples were loaded onto the gel and the gel was run in 1X MOPS running buffer for 3 h at 80 V. Fractionated RNA was transferred from the gel onto a positively charged nylon membrane (Boehringer Mannheim) by the downward alkaline transfer method described by Chomczynski (1992). The transfer was completed in 1.5 hours. The nylon membrane was subsequently neutralized in sodium phosphate buffer (200 mM, pH 6.8) for 10 min and dried for 15 min at 80 °C.

Prehybridization was carried out in 5X SSC, 0.1% sodium-lauroylsarcosine, 0.02% SDS, 2% blocking reagent (Boehringer Mannheim), and 50% formamide for 2 hr at 68 °C. Hybridization was performed overnight at 68 °C in the same buffer with the RNA probe added at the concentration of 30 ng/ml. After hybridization, the membrane was washed with constant agitation, twice in 2X SSC, 0.1% SDS for 15 min at room temperature and twice in 0.1X SSC, 0.1% SDS for 15 min at 68 °C.

Detection of biotin-labeled probe hybridized to the membrane-bound RNA was carried out according to the manufacturer's instruction manual (Ambion).

RESULTS

Expression of Kentucky Bluegrass Chitinase Genes in Response to Cold, Heat, Salicylic Acid or Ethephon Treatment

To understand the expression pattern of chitinase genes in Kentucky bluegrass under various stress conditions, we extracted total RNA from leaf tissue treated with water, cold, heat, salicylic acid (SA) or ethephon for 5 days and examined chitinase mRNA accumulation by RNA blot hybridization. Biotin-labeled CH710, a 710 bp fragment covering a conserved region of Kentucky bluegrass chitinase genes, was used as the probe to detect the overall chitinase gene expression. A considerable basal level of chitinase transcripts was detected in the leaves of Kentucky bluegrass control plants (Fig. 1). Figure 1 also shows that the accumulation level of chitinase mRNAs increased in Kentucky bluegrass leaves treated with cold, heat, SA or ethephon compared to that in untreated control or water-treated leaves. The highest accumulation level was observed in ethephon-treated Kentucky bluegrass leaves (Fig. 1).

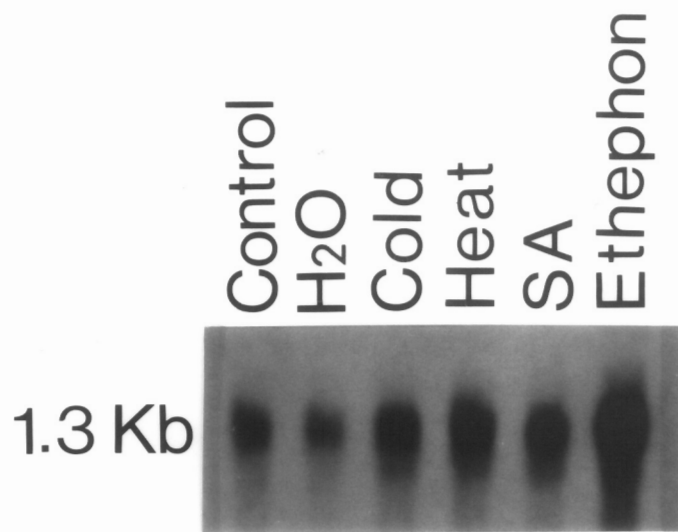To investigate the expression of two individual chitinase genes, *chi1* and *chi2*,

Fig. 1. Northern blot analysis showing the accumulation of chitinase mRNA in Kentucky bluegrass leaves after treatment with various stresses for 5 days. Total RNA (10 ug) isolated from each sample was fractionated by formaldehyde agarose gel electrophoresis. The blot was probed with biotin-labeled CH710, a 710 bp fragment from a conserved region of Kentucky bluegrass chitinases. SA, salicylic acid.

under various stress conditions, gene-specific probes derived from the 3' untranslated regions of *chi1* and *chi2* were used in RNA blot hybridization. Compared to the undetectable or low level of *chi1* and *chi2* mRNA in untreated and water-treated leaves, increased accumulation of *chi1* mRNA and *chi2* mRNA was detected only in ethephon-treated leaves (Fig. 2), suggesting that both *chi1* and *chi2* were induced by ethephon. Surprisingly, the accumulation of *chi1* or *chi2* mRNA in cold, heat and SA treated leaves was similar to that in the untreated control or water-treated leaves, indicating that neither *chi1* nor *chi2* was induced by cold, heat or SA. This result suggests that some chitinase genes in Kentucky bluegrass other than *chi1* and *chi2* may be responsible for the overall expression of chitinase genes induced by cold, heat or SA treatment.

Since ethephon induced a higher level of chitinase mRNA accumulation in Kentucky bluegrass than cold, heat or salicylic acid, ethephon treatment was used for a time course study of chitinase gene expression in Kentucky bluegrass.

Time-Course Study of Chitinase Gene Expression in Kentucky bluegrass Treated with Ethephon

To examine the time-course induction of chitinase genes following ethephon treatment, Kentucky bluegrass leaves were sprayed to runoff with 1 mg/ml ethephon twice a day and harvested at 6 h, 12 h, 1 day, 2 days, 3 days, 4 days and 5 days after initiation of the ethephon treatment. Total RNA isolated
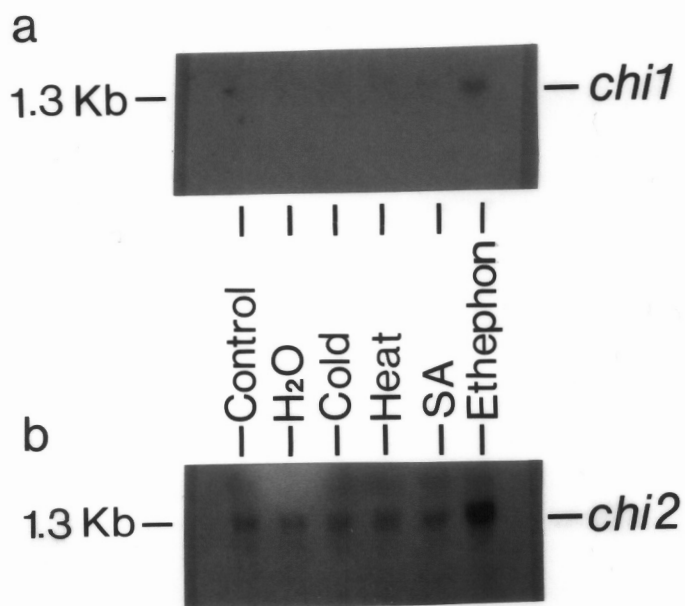
Fig. 2. Northern blot analysis showing the accumulation of *chi1* and *chi2* mRNA in Kentucky bluegrass leaves after treatment with various stresses for 5 days. Total RNA (10 ug) isolated from each sample was fractionated by formaldehyde agarose gel electrophoresis. The blot was probed with either biotin-labeled *chi1*-specific probe (a) or biotin-labeled *chi2*-specific probe (b). SA, salicylic acid.

from ethephon-treated leaves was hybridized with either CH710 probe, or *chi1*-
or *chi2*-specific probe. When hybridized with CH710 probe, a basal level of
chitinase transcripts was detected in untreated controls (Fig. 3a). Compared to
this basal level observed in untreated control plants, the accumulation of
chitinase transcripts in plants treated with ethephon for 6 h to 1 day was
decreased, while increased accumulation of chitinase transcripts was detected
within 2 days after ethephon treatment. From 3 days to 5 days after the
treatment, an even higher level of chitinase mRNA was observed (Fig. 3a).
When the same RNA samples were hybridized to 18 S ribosomal RNA probes, a
band with equal intensity was observed in each lane (Fig. 3b), confirming equal
loading of RNA samples.

With *chi1*- and *chi2*-specific probes, the time-course induction of these two
individual genes by ethephon was investigated. Like the overall ethephon-
induced expression of chitinase genes detected by CH710 (Fig. 3a), the
accumulation of *chi1* and *chi2* transcripts at a higher level compared to control
could be detected within 2 days and a similar level continued up to 5 days after
the treatment (Fig. 4). With the specific probes we used, the level of *chi2*
transcripts appeared to be higher than that of *chi1* transcripts. Since different
probes were used to detect *chi1* and *chi2* transcripts and their probe
specificity may vary, the expression levels of *chi1* and *chi2* cannot be compared
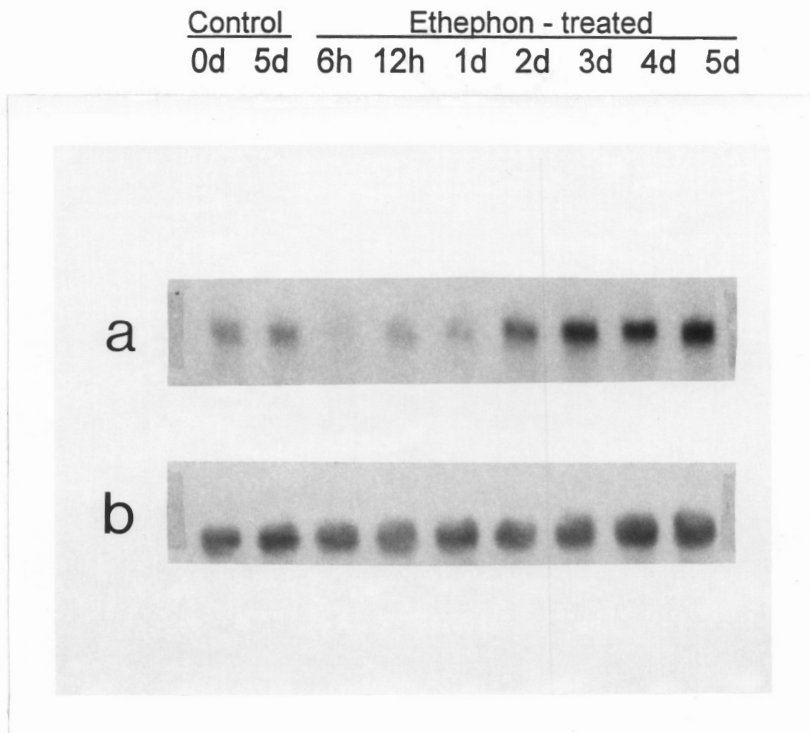
Fig. 3. Northern blot analysis showing the time course of the accumulation of chitinase transcripts in Kentucky bluegrass in response to ethephon (a). Plants were grown in pots in a growth chamber and at about 3-week-old, they were sprayed with 1 mg/ml ethephon, twice a day, and then covered with plastic bags. Control plants were untreated. Leaves were harvested at the times indicated and total RNA was isolated. Ten ug of total RNA from each sample was subjected to Northern blot analysis with biotin-labeled CH710 fragment as probe. In panel b, total RNA from ethephon-treated Kentucky bluegrass leaves were hybridized to 18 S ribosomal RNA probes to monitor equal loading.
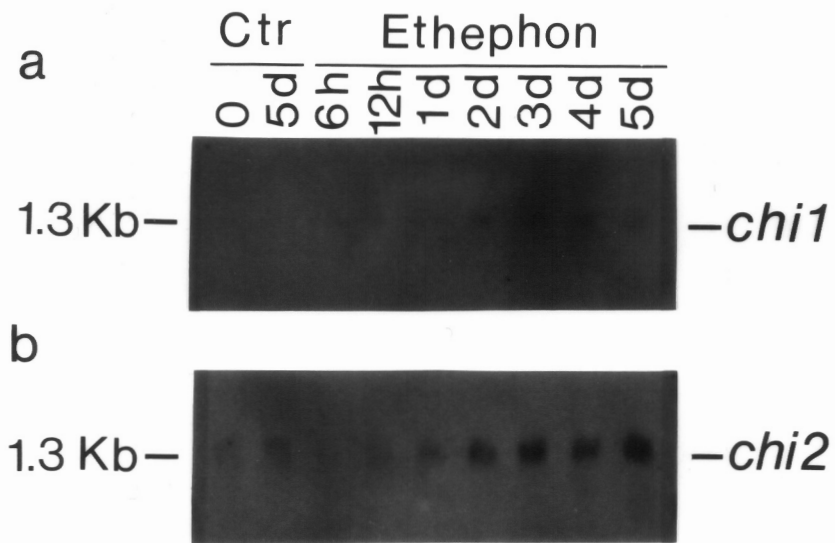
Fig. 4. Northern blot analysis showing the time course of the accumulation of *chi1* or *chi2* mRNA in Kentucky bluegrass in response to ethephon. Plants were grown in pots in a growth chamber and at about 3-week-old, they were sprayed with 1 mg/ml ethephon, twice a day, and then covered with plastic bags. Control plants were untreated. Leaves were harvested at the times indicated and total RNA was isolated. Ten ug of total RNA from each sample was subjected to Northern blot analysis with biotin-labeled *chi1*-specific probe (a) or *chi2*-specific probe (b). h,hour. d,day.

at a quantitative level.

DISCUSSION

The major role of plant chitinases has been implicated in plant defense reactions against pathogen infection (Boller, 1988). Three classes of chitinases have been proposed and they differ in structure, activity, subcellular localization, expression, regulation, and function (Shinshi et al., 1990). We have isolated two class I chitinase encoding genes, *chi1* and *chi2*, from Kentucky bluegrass by a PCR-based approach (Chapter II). In this study, we demonstrated that the overall expression of chitinase genes, as well as the gene-specific expression of *chi1* and *chi2,* was regulated by stresses such as ethephon, salicylic acid, cold and heat.

The overall expression of chitinase genes in Kentucky bluegrass leaves was induced by cold, heat, SA and ethephon, while the expression of *chi1* and *chi2* was induced only by ethephon.

The expression of plant chitinase genes in response to ethylene or ethephon has been shown to be regulated at transcriptional level. In potato, A remarkably increased mRNA accumulation for a basic class I chitinase was observed within 12 h and continued up to 48 h after ethylene treatment (Beerhues and Kombrink,

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1994). A detailed time-course study has not been reported in monocotyledons. However, a 2-fold increase in the accumulation of chitinase mRNA was observed in rice at 48 h after ethephon treatment (Nishizawa and Hibi, 1991). This induction level is much lower than that in potato. Our results showed that ethephon treatment induced an increased accumulation of chitinase mRNA in Kentucky bluegrass within 2 days with a level similar to that detected in rice. Thus, induction of chitinase transcripts occurs more rapidly and more efficiently in dicots than in monocots. Additional studies at the protein level have also shown supporting evidence. In bean, chitinase activity in ethylene-treated plants started to increase after a lag of 6 h and was induced by 30 fold within 24 h (Boller et al., 1983), indicating that ethylene is a potent inducer of chitinase gene expression in dicotyledonous plants. However, ethylene is not a strong inducer in monocotyledonous plants. In wheat, chitinase activity was not induced by ethylene (Boller et al., 1983). With ethephon, only slight induction of chitinase activity by 4 fold was observed in garlic at 5 days after treatment (Van Damme et al., 1993).

In Kentucky bluegrass treated with ethephon, suppression of chitinase mRNA accumulation was observed from 6 h to 1 day (Fig. 3a). In the same period, 18 S ribosomal RNA was synthesized at the same level as in other periods of time (Fig. 3b) indicating that ethephon inhibits the expression of chitinase genes but not other genes such as ribosomal genes. Ethephon breaks down in water

releasing ethylene and forming hydrochloric acid and phosphonic acid. Whether the suppression of chitinase mRNA accumulation is caused by ethylene or hydrochloric or phosphonic acid is yet clear. The similar suppression pattern has not been reported in either dicots or monocots. In dicots, increased accumulation of chitinase mRNA has been observed as early as 12 h after treatment (Beerhues and Kombrink, 1994). In monocots, however, induction of chitinase mRNA accumulation is either undetectable or occurs much more slowly than in dicots. Whether this slow induction process is related to the early suppression found in Kentucky bluegrass is not known. Nevertheless, the difference in induction level and lag period for turning on the expression of chitinase genes by ethylene or ethephon between dicotyledons and monocotyledons, as shown by analysis of chitinase mRNA accumulation pattern or activity induction pattern, indicate that the signal transduction pathways involved in monocotyledons may be different from that in dicotyledons.

Increased ethylene synthesis was observed in plants infected with pathogens (de Laat and Van Loon, 1982; Elad, 1990), elicitors (Mauch et al., 1984; Toppan and Esquerre-Tugaye, 1984; Roby et al., 1985 and 1986), and wounding (Glazer et al., 1986). It was also found that increased ethylene synthesis coincided with induced expression of a wide range of plant defense genes (Ecker and Davis, 1987; Metraux and Boller, 1986) implicating that ethylene may be a signal molecule in plant defense mechanisms. An ethylene-inducible class I bean

chitinase gene has been demonstrated to be responsible for enhanced resistance to *Rhizoctonia solani* in transgenic tobacco plants (Broglie et al., 1991). It would be interesting to test whether the constitutive over-expression of the ethephon-inducible chitinase genes from Kentucky bluegrass, such as *chi1* and *chi2*, can also confer disease resistance to transgenic plants.

When treated with cold, heat, or salicylic acid for five days, Kentucky bluegrass plants showed slight induction of chitinase gene expression, much lower than that induced by ethephon. In garlic, chitinase activity was induced at a similar level by ethephon, SA, cold, or heat by 4- to 7-fold higher than that in control (Van Damme et al., 1993). In rice, salicylic acid induced higher chitinase gene expression than ethephon (Nishizawa and Hibi, 1991). In this study of Kentucky bluegrass, ethephon induced chitinase gene expression at a higher level than cold, heat or SA (Fig. 4). Different results were obtained from maize, where neither ethephon nor salicylic acid induced chitinase gene expression (Wu et al., 1994). Taken together, these results indicate that the expression of chitinase genes in different plant species may be regulated differently under a similar stress condition and that different signal pathways may be involved in the induction of chitinase gene expression in different plant species.

With gene-specific probes, we could detect the specific expression of *chi1* and *chi2*, two individual chitinase genes from Kentucky bluegrass, under various stress conditions (Fig. 2) and in a time-course expression following ethephon

treatment (Fig. 4). In both cases, the expression pattern of *chi1* transcripts was similar to that of *chi2*. Thus, we speculate that these two genes may be coordinately regulated. Similar coordinated regulation of chitinase genes has been found in other plant species as well. Two genes encoding class I chitinase from rice, *Cht-1* and *Cht-3*, respond in similar ways to different elicitors (Nishizawa et al., 1993). These two genes have 88% amino acid sequence similarity and sequence comparison of their 5' flanking regions has revealed a conserved upstream region near the transcription site. In tobacco, the mRNAs for two class III chitinases, one basic form and one acidic form, were also shown to be coordinately regulated in response to pathogen infection (Lawton et al., 1992). In other cases, differential regulation of chitinase genes during plant development and in response to pathogen infection has been reported in tobacco (Van Buuren et al., 1992) and maize (Wu et al., 1994). The coordinated regulation of *chi1* and *chi2* shown in this study does not necessarily exclude the possibility that they may be differentially regulated by other stresses, e.g., pathogen infection. DNA blot hybridization indicates that there appears to be more than seven chitinase genes in Kentucky bluegrass. This study provides indirect evidence that other chitinase genes in this family may be regulated differently from *chi1* and *chi2* because the overall induced expression of chitinase genes by cold, heat and SA treatments could not be accounted for by *chi1* or *chi2* which showed no induction under these treatments. To increase our

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understanding of the regulation of chitinase genes in monocotyledons, further investigation is needed on isolation, characterization, and expression studies of more individual chitinase genes in Kentucky bluegrass.

REFERENCES

Beerhues, L., and Kombrink, E. 1994. Primary structure and expression of mRNAs encoding basic chitinase and 1, 3-β-glucanase in potato. Plant Mol. Biol. 24: 353-367.

Boller, T. 1988. Hydrolytic enzymes in plant disease resistance. In *Plant-Microbe Interactions, Molecular and Genetic Perspectives,* Volume 3 (Koshuge, T. and
Nester, E.W., eds). New York: Macmillan, pp. 385-411.

Boller, T, and Vogeli, U. 1984. Vacuolar localization of ethylene-induced chitinase in bean leaves. Plant Physiol. 74: 442-444.

Boller, T., Gheri, A., Mauch, F., and Vogeli, U. 1983. Chitinase in bean leaves: induction by ethylene, purification, properties, and possible function. Planta 157: 22-31.

Broglie, K.E., Gaynor, J.J., and Broglie, R.M. 1986. Ethylene-regulated gene expression: Molecular cloning of the genes encoding an endochitinase from *Phaseolus vulgaris.* Proc. Natl. Acad. Sci. USA 83: 6820-6824.

Broglie, K.E., Chet, I., Holliday, M., Cressman, R., Biddle, P., Knowlton, S., Mauvais, C.J., and Broglie, R. 1991. Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani.* Science 254: 1194-1197.

Chomczynski, P. 1992. One-hour downward alkaline capillary transfer for blotting of DNA and RNA. Anal. Biochem. 201: 134-139.

Chomczynski, P., and Sacchi, N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162: 156-159.

de Laat, A.M.M., and Van Loon, L.C. 1982. Regulation of ethylene biosynthesis in virus-infected tobacco leaves: II. Time course of levels of intermediates and *in vivo* conversion rates. Plant Physiol. 69: 240-245.

Dixon, R.A., and Lamb, C.J. 1990. Molecular communication in plant : microbial pathogen interactions. Annu. Rev. Plant Physiol. 41: 339-367.

Dixon, R.A., Dey, P.M., Lawton, M.A., and Lamb, C.J. 1983. Phytoalexin induction in French bean. Intercellular transmission of elicitation in cell suspension cultures and hypocotyl sections of *Phaseolus vulgaris*. Induction of enzyme activities. Plant Physiol. 71: 251-256.

Ecker, J.R., and Davis, R.W. 1987. Plant defense genes are regulated by ethylene. Proc. Natl. Acad. Sci. USA. 84: 5202-5206.

Elad, Y. 1990. Production of ethylene by tissues of tomato, pepper, French bean and cucumber in response to infection by *Botrytis cinerea*. Physiol. Mol. Plant Pathol. 36: 277-287.

Glazer, I., Epstein, E., Orion, D., and Apelbaum, A. 1986. Interactions between auxin and ethylene in root-knot nematode (*Meloidogyne javanica*) infected tomato roots. Physiol. Mol. Plant Pathol. 28: 171-179.

Graham, L.S., and Sticklen, M.B. 1994. Plant chitinases. Can. J. Bot. 72:1057-1083.

Lawton, K.A., Ward, E., Payne, G., Moyer, M., and Ryals, J. 1992. Acidic and basic class III chitinase mRNA accumulation in response to TMV infection of tobacco. Plant Mol. Biol. 19: 735-743.

Lawton, K.A., Beck, J., Potter, S., Ward, E., and Ryals, J. 1994. Regulation of cucumber Class III chitinase gene expression. Mol. Plant-Microbe Interact. 7: 48-57.

Legrand, M., Kauffmann, S., Geoffroy, P., and Fritig, B. 1987. Biological function of pathogenesis-related proteins: Four tobacco pathogenesis-related proteins are chitinases. Proc. Natl. Acad. Sci. USA 84: 6750-6754.

Linthorst, H.J.M., van Loon, L.C., van Rossum, C.M.A., Mayer, A., Bol, J.F., van Roeckel, J.S.C., Meulenhoff, E.J.S., and Cornelissen, B.J.C. 1990. Analysis of acidic and basic chitinases form tobacco and petunia and their constitutive expression in transgenic tobacco. Mol. Plant-Microbe Interact. 3: 252-258.

Mauch, F., Hadwiger, L.A., and Boller, T. 1984. Ethylene: symptom, not signal for the induction of chitinase and beta-1,3-glucanase in pea pods by pathogens and elicitors. Plant Physiol. 76: 607-611.

Mauch, F., Mauch-Mani, B., and Boller, T. 1988. Antifungal hydrolases in pea tissue: II. Inhibition of fungal growth by combinations of chitinase and beta-1,3-glucanase. Plant Physiol. 88: 936-942.

Metraux, J.P., and Boller, T. 1986. Local and systemic induction of chitinase in cucumber plants in response to viral, bacterial, and fungal infections. Physiol. Mol. Plant Pathol. 28: 161-169.

Metraux, J.P., Burkhart, W., Boyer, M., Dincher, S., Middlesteadt, W., Williams, S., Payne, G., Carnes, M., and Ryals., J. 1989. Isolation of a complementary DNA encoding a chitinase with structural homology to a bifunctional lysozyme/chitinase. Proc. Natl. Acad. Sci. USA 86: 896-900.

Neuhaus, J.-M., Sticher, L., Meins, F., and Boller, T. 1991. A short C-terminal sequence is necessary and sufficient for the targeting of chitinases to the plant vacuole. Proc. Natl. Acad. Sci. USA. 88: 10362-10366.

Nishizawa, Y., and Hibi, T. 1991. Rice chitinase gene: cDNA cloning and stress-induced expression. Plant Sci. 76: 211-218.

Nishizawa, Y., Kishimoto, N., Saito, A., and Hibi, T. 1993. Sequence variation, differential expression and chromosomal location of rice chitinase genes. Mol. Gen. Genet. 241: 1-10.

Ride, J.P. 1980. The effect of induced lignification on the resistance of wheat cell walls to fungal degradation. Physiol. Plant Pathol. 16: 187-196.

Roby, D., Toppan, A., and Esquerre-Tugaye, M.-T. 1985. Cell surfaces in plant-microorganism interactions. V. Elicitors of fungal and plant origin trigger the synthesis of ethylene and of cell wall hydroxyproline-rich glycoprotein in plants. Plant Physiol. 77: 700-704.

Roby, D., Toppan, A., and Esquerre-Tugaye, M.-T. 1986. Cell surfaces in plant-microorganism interactions. VI. Elicitors of ethylene from *Colletotrichum lagenarium* trigger chitinase activity in melon plants. Plant Physiol. 81: 228-233.

Samac, D.A., Hironaka, C.M., Yallaly, P.E., and Shar, D.M. 1990. Isolation and characterization of the genes encoding basic and acidic chitinase in Arabidopsis thaliana. Plant Physiol. 93: 907-914.

Schlumbaum, A., Mauch, F., Vogeli, U., and Boller, T. 1986. Plant chitinases are potent inhibitors of fungal growth. Nature 324: 365-367.

Shinshi, H., Neuhaus, J.-M., Ryals, J., and Meins, F. 1990. Structure of a tobacco endochitinase gene: evidence that different chitinase genes can arise by transposition of sequences encoding a cysteine-rich domain. Plant Mol. Biol. 14: 357-368.

Showalter, A.M., Bell, J.N., Cramer, C.L., Bailey, J.A., Varner, J.R., and Lamb, C.J. 1985. Accumulation of hydroxyproline-rich glycoprotein mRNAs in biologically stressed cell cultures and hypocotyls. NATO Cell Biol. 1: 235-244.

Toppan, A., and Esquerre-Tugaye, M.T. 1984. Cell surfaces in plant-microbe interactions. IV. Fungal glycopeptides which elicit the synthesis of ethylene in plants. Plant Physiol. 75: 1133-1138.

Van Buuren, M., Neuhaus, J.-M., Shinshi, H., Ryals, J., and Meins, F., Jr. 1992. The structure and regulation of homeologous tobacco endochitinase genes of *Nicotiana sylvestris* and *N. tomentosiformis* origin. Mol. Gen. Genet. 232: 460-469.

Van Damme, E.J.M., Willems, P., Torrekens, S., Van Leuven, F., and Peumans, W.J. 1993. Garlic (*Allium sativum*) chitinases: characterization and molecular cloning. Physiol. Plant. 87: 177-186.

Van Loon, L.C. 1985. Pathogenesis-related proteins. Plant Mol. Biol. 4: 111-116.

Vance, C.P., Kirk, T.K., and Sherwood, R.T. 1980. Lignification as a mechanism of disease resistance. Annu. Rev. Phytopathol. 18: 259-288.

Wu, S., Kriz, A.L., and Widholm, J.M. 1994. Molecular analysis of two cDNA clones encoding acidic class I chitinase in maize. Plant Physiol. 105: 1097-1105.

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