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Plant Chitinases and Their Roles in Resistance to Fungal Diseases[1]

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Abstract: Chitinases are enzymes that hydrolyze the N-acetylglucosamine polymer chitin, and they occur in diverse plant tissues over a broad range of crop and noncrop species. The enzymes may be expressed constitutively at low levels but are dramatically enhanced by numerous abiotic agents (ethylene, salicylic acid, salt solutions, ozone, UV light) and by biotic factors (fungi, bacteria, viruses, viroids, fungal cell wall components, and oligosaccharides). Different classes of plant chitinases are distinguishable by molecular, biochemical, and physicochemical criteria. Thus, plant chitinases may differ in substrate-binding characteristics, localization within the cell, and specific activities. Because chitin is a structural component of the cell wall of many phytopathogenic fungi, extensive research has been conducted to determine whether plant chitinases have a role in defense against fungal diseases. Plant chitinases have different degrees of antifungal activity to several fungi in vitro. In vivo, although rapid accumulation and high levels of chitinases (together with numerous other pathogenesis-related proteins) occur in resistant tissues expressing a hypersensitive reaction, high levels also can occur in susceptible tissues. Expression of cloned chitinase genes in transgenic plants has provided further evidence for their role in plant defense. The level of protection observed in these plants is variable and may be influenced by the specific activity of the enzyme, its localization and concentration within the cell, the characteristics of the fungal pathogen, and the nature of the host–pathogen interaction. The expression of chitinase in combination with one or several different antifungal proteins should have a greater effect on reducing disease development, given the complexities of fungal–plant cell interactions and resistance responses in plants. The effects of plant chitinases on nematode development in vitro and in vivo are worthy of investigation.

Key words: antifungal protein, biotechnology, chitinase, disease resistance, enzyme, fungus, genetic engineering, hydrolase, nematode.

Chitin, a β-1,4-linked polymer of N-acetylglucosamine, is a structural component in a diverse array of organisms, including fungi, insects, various crustaceans, and nematode eggs (32,47,53,149,157). In nature, chitin forms a complex with various other substances, such as polysaccharides and proteins (149). Chitin can also be found in agricultural and noncultivated soils. It has not, however, been reported as a constituent of higher plant cell walls. The enzyme chitinase (poly [1,4-(N-acetyl-β-D-glucosaminide] glycanhydrolase, EC 3.2.1.14) hydrolyzes the chitin polymer to release N-acetyl glucosamine oligomers, following either endo or exo cleavages of the β-1,4 bond. Other enzymes such as chitosanases act on the related substrate chitosan (a polymer of β-1,4-D-glucosamine) (55,137).

Various techniques for assaying for chitinases have been described (15,47,138, 197). Higher plants produce endochitinases, either constitutively or following induction, and the possible functions of these enzymes within the plant have generated much interest and speculation. Chitinases are also secreted by a number of different microorganisms, including actinomycetes, soil bacteria, and various fungi (30,67,101,136), and in many cases appear to be involved in the biological control of fungal pathogens (30,67,101,136,166). One of the roles attributed to chitinases in higher plants is a defense mechanism against attack by pathogens, especially fungi, because the expression of chitinases is significantly enhanced following infection. Furthermore, chitinases have antifungal activity and cause hyphal tips to lyse in vitro (113,165,166). Some chitinases also have lysozymal activity and can hydrolyze the peptidoglycans in bacterial cell walls (10,35,74,106,110,150,183), whereas others have exohydrolytic activity (110,112,

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154). The expression of chitinases could therefore be speculated to have a defensive role during both the early and the late stages of the infection process, depending on the levels of constitutive enzyme and the rapidity of induction.

Much of the evidence for the suggested roles of chitinases in plant defense has been based on dramatic and rapid enhancement of enzyme levels in hypersensitive reactions, during induced host resistance (i.e., in association with several other pathogenesis-related [PR] proteins), and in tissues following infection by a pathogen. These observations, however, are not conclusive for the roles of these enzymes in resistance, because a cause-and-effect relationship cannot be discerned. The availability of techniques in molecular biology now permits the isolation of specific genes and their reintroduction into plants, providing a powerful tool to elucidate the roles of specific enzymes in plants. The objectives of this paper are to review the occurrence of chitinases in plants and factors enhancing chitinase expression, to summarize the evidence for the possible roles of chitinases in plants, and to review the application of genetic engineering to study the role of chitinases in plant defense against pathogens. This area of research has gained much attention, which is apparent from the vast recent published literature (for reviews, see also 34,47); therefore, only relevant articles published during 1987–93 are reviewed here.

Occurrence and Induction of Plant Chitinases

Chitinases have been reported from over 41 monocotyledonous and dicotyledonous plant species and occur in widely different tissues, including embryos (23,106), seeds (66,72,83,94,95,106,130, 179,200), cotyledons (33,106,202), leaves and stems (16,69,71,82,114,115,147,183, 186), roots (106,120,129,162,180), flowers (98,102,129,183), leaf abscission zones (52), tissue-cultured calli (167,196), cell suspension cultures (10,44,50,61,62, 80,83,85–88,111,135,162,191,192,196), and protoplasts (56,152). Among cultivated crop species, chitinases occur in adzuki bean (69,70), barley (71,72,82,83,94, 95,178), bean (4,25,37,61,73, 105,108,109,114,115,179,193), cabbage (29), cacao (170), carrot (39,85–88,201), celery (84), chickpea (191,192), corn (63, 66,128,130,188), cucumber (16,68,106, 122,123,201,202), garlic (186), leek (171), melon (153–155), oat (45,46), onion (40,196), pea (40,79,112,113,185), peanut (5,62), potato (51,81,89,142,164), pumpkin (44), rapeseed (58,71,144,145), rice (65,111,135,169,204), rye (200), soybean (174), sugarbeet (48,124,156), sunflower (77), tobacco (12,22,23,28,43,50,56,64,78, 93,96,116,120,139,141,163,167,168,183, 184,190,194,199), tomato (20,76), turnip (35), wheat (23,147), and yam (1,2). In addition, there are reports of chitinases in noncrop species, including *Arabidopsis* (159,187), bentgrass (80), chestnut (33), Job's Tears (3), petunia (98), poplar (38,140), rubber (74,110), spruce (162), stinging nettle (97), tall fescue (148), thornapple (23), and Virginia creeper (10).

Many plant chitinases are expressed constitutively, generally at a low level. Some evidence exists for the developmental regulation of chitinase expression in specific tissues and at specific stages during plant development (95,98,102, 106,112,120,129,142,159,160,167,183,199, 203). The biological significance of these chitinases have yet to be elucidated, and they may have as yet undetermined functions in plant development. In carrot, for example, chitinase was shown to enhance somatic embryo development (39).

In general, chitinases are induced by numerous unrelated factors: infection by viruses (4,12,16,19,64,93,96,122,123, 128,139,141,183,190,194), viroids (20), pathogenic fungi (5,28,37,46,61,76, 81,82,105,112,116,123,124,139,144,145, 153,154,156,164,169,180,194,185), mycorrhizal fungi (40,162,171), endophytic fungi (148), and bacteria (22,35,73,116, 123,193); application of ethylene (13,14,

19,20,25,28,52,69,70,78,112,114,115,120, 135,146,152,186), chitosan (79,84,111, 202), salicylic acid (69,111,142,194,186, 196), acetylsalicylic acid (77), salt solutions (68,146), heavy metals (4,71,128), fungal cell wall components and oligosaccharides (22,61,62,79,86–88,152,155,203), and pectic polysaccharides (22); exposure to UV light (19,62) and ozone (43,163); insect (27) or nematode (18,148) feeding; and mechanical wounding (61,69,140,186,202, 203). There are reports of a reduction in chitinase expression by various plant hormones (167), heat shock (21,186), and mycorrhizal development in roots (92). Chitinase induction in plants is therefore generally nonspecific and enhanced by both biotic and abiotic stresses, and is only one component of the plant response to various pathogens and stresses (12,17,99).

Following induction, chitinases may accumulate locally at the sites of challenge or systemically in other tissues (16,68,123, 139,140,155,184,194). In addition, chitinases may be extracellular or vacuolar (16, 100,118). In some plants, several closely related isoforms of chitinase can be induced (62,82,112,186). Thus, chitinases are encoded by members of a small gene family (28,38,61,62,64,81,93,95, 96,128,135,140,144,186,204).

Characteristics of Plant Chitinases

Many chitinases, like many other pathogenesis-related (PR) proteins (17,99,117) are acid extractable, have low molecular weights, are resistant to proteases, and are secreted extracellularly (12,99). Plant chitinases generally range in molecular weight from 25 to 36 kD and may be either acidic or basic. Based on amino acid sequences, chitinases can be grouped into at least four classes, may of which can occur in the same plant species. Class I includes the majority of chitinases described to date, e.g., in *Arabidopsis* (159), bean (25,109,151), barley (178), chickpea (192), pea (185), poplar (38,140), potato (51,89), rice (65,135,204), sugarbeet (124), and tobacco (100,168, 175). Class I chitinases have an N-terminal cysteine-rich lectin or "hevein" (chitin-binding) domain and a highly conserved catalytic domain (70–88% homology). Class I chitinases are generally basic and vacuolar. A C-terminal extension of seven amino acids is involved in targeting of the protein to the vacuole (6,118,126,133), and a model for intracellular transport has been recently described (175,176).

Class II chitinases, e.g., in tobacco (141), petunia (100), and barley (95), are similar in sequence to Class I (60–64% homology) but lack the cysteine-rich domain. Class II chitinases are generally acidic and targeted to the apoplast. Class III chitinases have a different amino acid sequence in the catalytic domain from Classes I and II and lack a cysteine-rich domain. Class III chitinases include the acidic extracellular chitinases from *Arabidopsis* (159), adzuki bean (69,70), chickpea (192), cucumber (122), sugarbeet (48,134), and tobacco (93), and the basic chitinases of *Parthenocissus* (10), rubber (74,110), and tobacco (93).

Other described chitinases do not belong to the above three classes and may represent new classes. Acidic extracellular chitinases with a cysteine-rich domain occur in bean (109), poplar (38), and yam (1,2) and may be a subclass of Class I. A basic chitinase from rapeseed (140) and sugarbeet (124) was found to have little homology with any of the other classes and could constitute class IV (124,144,145). Because of the homology in amino acid sequences within the above classes, many (but not all) of the chitinases show serological relatedness, and several have strikingly similar biochemical and physicochemical characteristics (20,23,96). The chitinases from monocots appear to have diverged from those in dicotyledonous plants (63,66). For several plant chitinases, complementary DNA clones and genomic clones have been isolated, and the amino acid sequences have been deduced, as in *Arabidopsis* (159), adzuki bean (69), barley (95), bean (61,108,109), corn (66), cucumber (122), garlic (186), peanut (62), potato (51), rapeseed (58,144), rice (65,135,204), sugarbeet (124), and tobacco (28,50,64,93,

100,141,167,168). The promoter regions of the chitinase genes in bean and *Arabidopsis* have been characterized (25,160).

Chitinases show inhibitory activity to fungal spore germination and mycelial growth in disc plate diffusion or microtiter plate assays with partially purified or purified proteins. This finding has led to the long standing hypothesis that they must have a defense role against pathogen invasion in plants. The most frequently used test organisms have been species of *Trichoderma* (23,33,66,95,113,150,179,184), *Fusarium* (66,95,113,165,179), and *Alternaria* (66). However, because the proportions of polysaccharides such as chitins and glucans, and other components such as lipids and proteins, can vary considerably in fungal cell walls (157), their susceptibility to lysis by chitinase alone would be expected to differ, as has been observed for several fungi (23,66). In Oomycete fungi, chitin is almost absent and is replaced by cellulose (157), making plant pathogens in this group a less likely target for chitinases.

The different isoforms of plant chitinases may also differ in substrate-binding characteristics and specific activities (66, 70,96,106,192), important factors that can be overlooked when measuring total tissue chitinase activity in antifungal activity tests. Different chitinases from a given host species can also differ in specific and antifungal activity (66,96,106,165,192). For example, acidic (class II or III) chitinase from tobacco or chickpea (119,165,192) displayed less antifungal activity when tested in vitro than basic (class I) chitinase (119, 165,192). Therefore, all chitinases do not have equal antifungal activity, a fact that could impact the outcome of genetic engineering with chitinase-encoding genes.

Roles of Chitinases in Disease Resistance

Because chitinases are induced by agents that simultaneously enhance other defense reactions and pathogenesis-related proteins in the same plant tissues (12,17,99), elucidation of the specific roles of these enzymes in resistance is difficult. Important considerations are the rapidity of chitinase induction, the concentrations in tissues, and localization in cells relative to growth of the incoming pathogen. Numerous studies have compared chitinase induction in tissues that are resistant (incompatible) or susceptible (compatible) to a fungal pathogen with regard to rate of induction and final concentrations in tissues. The results from these studies are not clear. In some plant species, resistant tissues accumulated chitinases more rapidly and in some instances to higher final concentrations than susceptible tissues (9,37,61,68, 76,144,145,185,191,199). Because in many of these cases, the resistant response initially was a hypersensitive reaction, with very rapid localized cell death (12,57,61, 164,190,193), the injury and stress response of the cells could have rapidly induced chitinase production in adjoining cells or tissues.

In other plant species, however, there was no difference between chitinase accumulation in susceptible and resistant tissues, or paradoxically the susceptible tissues accumulated higher levels of the enzymes (5,82,164,192). The latter response can be explained by greater fungal biomass accumulation in the diseased tissues than in healthy tissues, and greater stress on the diseased plant, two factors that can induce greater levels of chitinases. Thus, in the latter host–pathogen interactions, chitinases may have either no role or a secondary role in retarding pathogen development following infection. Chitinases may also indirectly trigger defense reactions within the plant, because fungal cell wall fragments released by enzymatic digestion can act as elicitors of the biosynthetic pathways that lead to the accumulation of phenolic compounds and lignins in the cell (13,49,87,147).

The rapidity of chitinase induction in plant tissues varies considerably depending on the specific host–pathogen interaction. The use of cell suspension cultures has greatly facilitated experimental approaches to study the effects of elicitors on

transcription and subsequent protein accumulation. These cultures permit synchronous induction and provide high quantities of mRNA. However, the results from suspension cultures should be extrapolated carefully to intact differentiated tissues. In bean, chitinase mRNA was detectable within 5–20 minutes after elicitation, with a maximum at 2 hours (61); in other species, mRNA or protein levels were maximal between 4 and 16 hours after elicitation (43,62,68,69,73,155,163). These findings point to transcriptional activation of gene expression, which in some plant species resulted in a differential pattern of gene expression depending on the type of stimulus. For example, specific mRNAs or isoforms of the chitinases were induced only by certain pathogens or specific stimuli (28,35,62,108,139,144). Although the signal transduction pathway is unknown, potential receptors for chitinase gene induction could be general and modulated by ethylene response (14,69) or salicylic acid (107,194) or be elicited by specific cues (90,91).

In a majority of the plant species examined, chitinase activity was enhanced after 1 to 28 days following induction by abiotic and biotic factors (16,20,46,81,82,93,96, 105,116,123,139,142,145,153,154,156, 184,185,190). This time frame indicates that the gradual accumulation of chitinases in diseased tissues may be involved only in slowing down pathogen growth and perhaps reducing growth and sporulation at later stages of disease development; in these cases, chitinases are not specifically involved in the early events of host–pathogen interactions. The delayed accumulation of chitinases in potato did not account for the race–cultivar specificity of *Phytophthora infestans* following the hypersensitive reaction (164).

The tissues in which chitinases accumulate can also influence their potential role in the defense response. Extracellular chitinases would intuitively be expected to have an initial role in limiting pathogen growth upon entry of hyphae into the host, with vacuolar chitinases having a secondary or delayed effect following cell lysis (115). In bean and tobacco leaves treated with ethylene, chitinase accumulated within specific cell types (78,114). The pathogen behavior and host–pathogen interaction at the cellular level may also influence the effectiveness of chitinases. Fungal pathogens may be obligate biotrophs or necrotrophs and can grow intercellularly or through cells. Obligate biotrophs or intercellular pathogens may never encounter the vacuolar forms of chitinase, whereas necrotrophs or intracellular hyphae would encounter both extracellular and intracellular forms of chitinase. As already discussed, various isoforms may differ in localization and in antifungal activity. Direct injection of chitinase into epidermal cells was shown to inhibit development of intracellular fungal haustoria (182). The accessibility of chitin in the fungal cell walls to chitinase action is another important consideration, given that chitin and β-1,3-glucans are generally found in the innermost layers of the cell wall and thus may be protected, except at the hyphal tips (8,13,195). Removal of fungal cell wall proteins and soluble carbohydrates by heat treatment increased binding of chitinase to the wall (171). Further cytochemical and immunocytochemical studies (6,7, 9,30,114,115,171,198) are needed to elucidate the chitinase–fungal cell wall interactions at the cellular level in planta.

Genetic Engineering of Plants with Chitinase Genes

The currently available techniques in transformation of many plant species have permitted experiments to answer the following intriguing question: would the overexpression of a cloned chitinase gene, behind a constitutive or inducible promoter, lead to enhanced resistance or tolerance against fungal infection in the transgenic tissues? Another approach that could be utilized to elucidate the roles of constitutive chitinases in plants is through antisense or sense transformation (60, 132). There are several recent examples

of the successful introduction of heterologous chitinase genes into plants. The first such success was the introduction of an exochitinase gene from *Serratia marcescens* into tobacco (41,42,75,103,104,125,181). Subsequently, genes or promoter sequences of plant origin encoding endochitinases have been introduced into various plant species. In transgenic tobacco, the promoter regions of a bean and rice chitinase gene were shown to be regulated by ethylene (25) and fungal elicitors (203), respectively. In transgenic *Arabidopsis* and tomato, the promoter region of an *Arabidopsis* chitinase gene was regulated developmentally in various tissues and by pathogen infection (160). In both transgenic hosts, expression of a reporter gene under control of the chitinase gene promoter was enhanced around necrotic lesions caused by fungal infection (160). Similar results were also obtained with a bean chitinase promoter in transgenic tobacco following infection by various pathogens (151). These studies show that chitinase gene promoter activity can be induced by pathogen attack, ethylene, and elicitors (25,151,160).

Genes expressing chitinases under control of the cauliflower mosaic virus 35S promoter have been introduced into several plant species. Results from inoculation studies of these transgenic plants with various filamentous fungal pathogens have differed. In tobacco transformed with the exochitinase gene from *Serratia* (41) or the vacuolar chitinase from bean (26), the disease severity and rate of development of *Rhizoctonia solani* (a necrotrophic pathogen) were reduced (26,41). Different levels of chitinase activity occurred in leaves, stems, and roots of transgenic plants (26, 60,131). Rapeseed plants transformed with a bean chitinase gene also had reduced disease development due to *R. solani* (26). In contrast, transgenic tobacco plants with enhanced constitutive levels of vacuolar chitinase were not more tolerant to infection by *Cercospora nicotianae* than the untransformed plants (131). Perhaps the intracellular localization of the chitinase in these transgenic plants precluded early involvement in defense against this pathogen, which initially grows intercellularly. Recent reports of the reaction of transformed tobacco (134) and tomato (63) plants containing acidic chitinase genes from sugarbeet and corn, respectively, to inoculation with various fungi indicated that there was no increase in resistance to infection. The greater antifungal activity of basic chitinases compared to acidic chitinases discussed previously could in part explain this lack of resistance. Manipulation of the carboxy-terminal region involved in vacuolar targeting can result in secretion of these basic chitinases into the extracellar space (118,165). These plants would be worthy of further study to determine if inhibition of pathogen growth was subsequently enhanced. The events that take place at the cellular level in these transgenic plants following inoculation should also be studied using ultrastructural and immunocytochemical techniques.

Considerable evidence exists for the coordinated regulation and expression of other pathogenesis-related proteins in tissues that also express chitinases (12,14,54, 99,189,194). In particular, accumulation of β-1,3-glucanases closely parallels accumulation of chitinases in diseased tissues or abiotically stressed tissues (9,13,14,112, 189). Furthermore, β-1,3-glucanases and chitinases act synergistically to inhibit fungal growth in vitro (36,113,165). Other potential synergisms occur with lectins (24). Therefore, the engineering of chitinase-encoding genes into plants in conjunction with other genes encoding antifungal proteins should provide enhanced protection against fungal pathogens (31,36,59,91). Indeed, when a basic chitinase and a glucanase were simultaneously introduced into tomato, the level of protection was significantly higher than in plants expressing either one alone (119). This approach could lead to control of a broad range of fungal pathogens. Yet another strategy would be to introduce specific inhibitors of chitin synthesis into plants, such as polyox-

ins, to reduce development of pathogens that contain chitin (32,147,173).

Current Research in Our Laboratory

We are currently characterizing chitinase isozyme banding patterns in both cucumber and carrot following induction by biotic and abiotic factors. We have observed the appearance of new chitinase isoforms in cucumber cotyledons following wounding, pathogen inoculation, and application of salicylic acid; these isoforms differ from the constitutively produced isoforms (201,202). The induction of these isoforms in other plant tissues, in different cultivars, by different pathogens, and in tissue-cultured cells of cucumber and carrot are being studied (201,202). In additional research, we are introducing chitinase-encoding genes into both crops via *Agrobacterium*-mediated transformation (143,161) and evaluating the response of these transgenic plants to inoculation with various leaf- and root-infecting fungi. The results from this work should provide additional insights into the roles of chitinases in plant defense in these two important horticultural crops.

Application of Chitinases to Nematode Control

In some cases, the addition of chitinaceous amendments, e.g., crab shell, to soil has reduced the severity of diseases caused by plant-parasitic nematodes (53,172,177). This reduction was partly attributed to the enhancement of populations of soil microorganisms with chitinolytic activity (e.g., various fungi, bacteria, and actinomycetes), which could have reduced nematode activity and survival (53,172,177).

Chitin is a major structural component of nematode egg shells. This chitin layer can vary in thickness and is located between the outer vitelline layer and the inner lipid layer and may occur in association with proteins (11). As such, the chitin may not be readily exposed to the effects of chitinases, similar to the case with fungal pathogens. Furthermore, eggs that are laid within a gelatinous matrix may be protected against enzymatic activity. Studies are needed to determine the effects of partially or totally purified chitinases of plant and microbial origin on nematode egg shell integrity and larval emergence and growth. In a recent study, chitinases increased hatch rates of *Meloidogyne* eggs (121); however, premature hatching led to mortality of juveniles and, in some cases, the eggs died. If chitinases do indeed have this type of activity against nematodes, then screening of currently available transgenic plants expressing chitinases for increased resistance to nematode development may be worthy of further investigation. Intuitively, endoparasitic pathogens (e.g., *Meloidogyne, Pratylenchus*) would be excellent candidates for evaluation because their eggs are laid on or in the host tissues, allowing maximum exposure to the chitinases. However, as with fungal pathogens, the same concerns of cellular localization, in vivo activity, and isoform of chitinase would have to be addressed in experiments with nematodes. As the results with fungal pathogens seem to indicate, chitinases alone may not significantly affect nematode development within the transgenic tissues. Therefore, the introduction of genes that encode proteins with proven nematicidal properties should also be considered in genetic engineering of plants for resistance to nematodes.

Conclusion

The widespread occurrence of chitinases in plants and their induction by a broad range of biotic and abiotic factors suggests that these enzymes must serve some general function related to plant stress or defense, or have as yet undetermined roles in development or senescence. Because different isoforms of the enzyme are known in plants, however, these isoforms may have different specific activities and roles and may be elicited by different agents through as yet undetermined signal transduction pathways. The introduction of genes encoding chitinases into trans-

genic plants will provide opportunities to specifically elucidate roles of these enzymes in defense. However, the outcome may be affected by the specific activity, concentration, and targeting of the enzyme within the cell, and the characteristics of the fungal–host cell interaction. The effects of plant chitinases on nematode development in vitro and in vivo are worthy of investigation.

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Note Added in Proof

The following articles on chitinase–fungal interactions have been published while this article was in press.

Benhamou, N., K. Broglie, I. Chet, and R. Broglie. 1993. Cytology of infection of 35S-bean chitinase transgenic canola plants by *Rhizoctonia solani:* Cytochemical aspects of chitin breakdown in vivo. Plant Journal 4:295–305.

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