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Chitinase Gene Expression in Transgenic Plants: A Molecular Approach to Understanding Plant Defence Responses

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Chitinase gene expression in transgenic plants: a molecular approach to understanding plant defence responses

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SUMMARY

Recent studies suggest that the production of enzymes capable of degrading the cell walls of invading phytopathogenic fungi may be an important component of the defence response of plants. In this chapter, we summarize recent progress on the isolation and characterization of chitinolytic enzymes from higher plants. Emphasis is placed on experiments designed to study the regulation of chitinase gene expression in response to ethylene treatment or pathogen ingress and on determining the role of this enzyme in plant defence. The production of transgenic plants with enhanced resistance to attack by the fungal pathogen *Rhizoctonia solani* is discussed.

INTRODUCTION

Plants posses a wide variety of mechanisms for protecting themselves against infection by pathogenic microorganisms, many of which involve the activation of host defence genes. The activation of these genes produces physical and biochemical changes in the host plants which allow them to become more resistant to microbial attack. Among the physical changes are those that directly affect the properties of the plant cell wall. Examples include: accumulation of cell wall hydroxyproline-rich glycoproteins (Esquerre-Tugaye *et al.* 1979; Showalter *et al.* 1985), lignification and suberization (Vance *et al.* 1980; Espelie *et al.* 1986), callose deposition (Ride 1983; Bonhoff *et al.* 1987), and the accumulation of phenolic compounds (Matta *et al.* 1969; Hunter 1973). Among the major biochemical changes are the biosynthesis and accumulation of phytoalexins, secondary metabolites that are toxic to bacteria and fungi (Darvill & Albersheim 1984; Hahlbrock & Grieseback 1979; Dixon *et al.* 1983), the accumulation of protease inhibitors (Ryan 1973; Peng & Black 1976) and the release of oligosaccharide elicitors of plant origin (Albersheim 1981).

Plants also accumulate a novel class of proteins termed 'pathogenesis-related proteins', or PR-proteins, in response to pathogen attack (Van Loon 1985). Although the exact role of the PR proteins in the defence response of plants is not known, their presence is often correlated with disease resistance (Giazinazzi 1984). Among the PR-proteins that have been identified are several classes that correspond to the hydrolytic enzymes, chitinase and β-1,3 glucanase. These enzymes have received increasing attention as important components of the plant's defence response as they are capable of catalysing the hydrolysis of the two main carbohydrate components of most fungal cell walls, chitin and β-1,3-glucan (Wessels & Sietsma 1981). This chapter summarizes recent studies describing the isolation and characterization of plant chitinolytic enzymes and discusses their role in plant protection.

2. PROPERTIES OF HIGHER PLANT CHITINASES

The enzyme chitinase (poly [1,4-(*N*-acetyl-β-D glucosaminade)] glycanohydrolase, EC 3.2.1.1.4.) catalyses the hydrolysis of chitin, a β-1,4-linked homopolymer of *N*-acetyl-D-glucosamine. Nearly all plant chitinases isolated to date are endochitinases; however, exochitinases have been purified from melon (Roby & Esquerre 1987), carrot (Kurosaki *et al.* 1987) and *Hevea brasiliensis* (Martin 1991). Endo-type chitinases have been found in many different plant species, including both monocotyledonous and dicotyledenous species and occur in widely different tissues. Multiple acidic and basic forms of the protein are known to be present in certain species including bean, tobacco, cucumber and *Arabidopsis thaliana* (Legrand *et al.* 1986; Awade *et al.* 1989). In some cases, the genes encoding these hydrolytic enzymes have been isolated and characterized (Broglie *et al.* 1986; Shinshi *et al.* 1987; Metraux *et al.* 1989; Swegle *et al.* 1989; Samac *et al.* 1990).

Based on a comparison of the predicted amino acid sequences from several plant chitinases, Shinshi *et al.* (1990) have defined three broad classes of plant chitinases. Class I chitinases are basic polypeptides and usually account for most of the chitinase activity

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in plants where both acidic and basic isoforms are found. With the exception of the bean PR4 class I chitinase, which is located extracellularly (Margis-Pinheiro *et al.* 1991), most class I chitinases are vacuolar proteins (Boller & Vogeli 1984; Mauch & Staehelin 1989). All class II chitinases studied to date have been found to be localized in the intracellular space (Legrand *et al.* 1987; Benhamou *et al.* 1990). The vacuolar targeting of class I chitinases is determined by the presence of a carboxy-terminal propeptide (Neuhaus *et al.* 1991).

Class I chitinases are composed of two domains of conserved sequence separated by a variable hinge region (Shinshi *et al.* 1990). These chitinases have an amino-terminal cysteine-rich domain that shows significant sequence homology to two chitin-binding lectins, wheat germ agglutinin and hevein (reviewed by Raikhel *et al.* 1993). The N-terminal domain of these proteins is linked through a small variable glycine- or proline-rich region to the main chitinase domain. Class II chitinases show overall homology to the class I chitinases, except that they lack the amino-terminal cysteine-rich domain, most of the hinge region, and the carboxy-terminal vacuolar targeting signal (Linthorst *et al.* 1990). Examples of class II chitinases are the acidic chitinases from tobacco (Payne *et al.* 1990), petunia (Linthorst *et al.* 1990) and barley (Leah *et al.* 1991). The sequences encoding the cysteine-rich domain of class I chitinases are flanked by imperfect direct repeats of 9–10 base pairs. It has been suggested that these domains arose from a common ancestral gene and were introduced into class I chitinase genes by transpositional events (Shinshi *et al.* 1990). The fact that both class I and class II enzymes exhibit chitinase activity implies that their catalytic site is located within the main structure. Thus, while there is some evidence that the presence of the chitin-binding domain may increase enzymic efficiency (cf. Legrand *et al.* 1987), it does not appear to be required for catalytic activity.

Class III chitinases include the bifunctional lysozyme/chitinases from papaya, *Hevea*, and *Parthenocissus* (Bernasconi *et al.* 1987), and the acidic chitinases from cucumber (Metraux *et al.* 1989) and *Arabidopsis* (Samac *et al.* 1990). Based on comparisons of partial amino acid sequences, these enzymes have considerable sequence homology to each other, but are not similar to class I or class II enzymes and do not contain a cysteine-rich chitin-binding domain.

The antifungal properties of plant chitinases have largely been demonstrated using various *in vitro* bioassays. For example, Schlumbaum *et al.* (1986), using the fungal saprophyte *Trichoderma viride*, showed that purified bean chitinase was inhibitory to fungal growth at concentrations as low as 2 $\mu g\ ml^{-1}$. Similarly, chitinases from barley, maize and wheat (Roberts & Selitrennikoff 1988), thornapple (*Datura stramonium*), tobacco and wheat (Broekaert *et al.* 1988) and from *Arabidopsis thaliana* (Verbug & Huynh 1991) were shown to inhibit the growth of various fungi. The addition of purified bean chitinase to actively growing cultures of the phytopathogenic fungus *R. solani* produced zones of inhibition near the point of application of the protein (Broglie *et al.* 1991). This inhibition of fungal growth was found to increase with increasing concentrations of the lytic enzyme. Previous studies would suggest that the observed inhibition of growth arises from enzyme-catalysed hydrolysis of newly formed chitin and resultant disruption of the growing fungal hyphal tips (Molano *et al.* 1979). Light microscopic examination of *R. solani* hyphae following incubation with bean chitinase showed the presence of both swollen and lysed hyphal tip cells and thus provides support for this explanation (Benhamou *et al.* 1993). Ultrastructural and cytochemical investigation of chitin distribution using wheat germ agglutinin (WGA)/ovomucoid-gold labelling following chitinase treatment revealed noticeable hyphal wall alterations including swelling, plasmalemma retraction, wall disruption and protoplasm leakage (Benhamou *et al.* 1993). These changes were accompanied by a decrease in intensity of gold labelling over the affected areas, indicating an extensive breakdown of chitin. Furthermore, these results demonstrate that chitinase alone is sufficient to cause lysis of *R. solani* hyphae and that the chitin in this fungus is readily accessible to enzymic attack, presumably due to its peripheral location.

3. REGULATION OF CHITINASE GENE EXPRESSION

In healthy tobacco and *Arabidopsis* plants, chitinase expression is both organ-specific and age-dependent. In tobacco, the highest levels of expression are found in roots, older basal leaves and developing flowers (Shinshi *et al.* 1987; Memelink *et al.* 1990; Neale *et al.* 1990), whereas in *Arabidopsis* the highest level of constitutive expression is found in roots (Samac *et al.* 1990). In both cases, exposure to ethylene resulted in increased systemic expression; however, the level of induction was greatest in older plants. Shinshi *et al.* (1987) proposed that the developmental regulation of chitinase in tobacco plants was controlled by auxin and cytokinin gradients within the plant. Similar organ-specific expression patterns have also been observed for class I chitinase in rice (Zhu & Lamb 1991).

In addition to being regulated in an organ-specific manner, chitinase expression is also regulated in a cell-type-specific fashion. In untreated tobacco leaves, the basic isoforms of chitinase are found almost exclusively in epidermal cells (Keefe *et al.* 1990); however, ethylene treatment causes increased expression in both epidermal and non-epidermal cells of the leaf. In contrast, the accumulation of chitinase in ethylene-treated bean leaves is restricted to the epidermis and to parenchyma cells adjacent to vascular strands (Mauch *et al.* 1992).

In bean plants, chitinase is encoded by a multigene family consisting of at least three members. One of these three, the CH5B gene, encodes an abundant mRNA which is known to be subject to induction by ethylene (Broglie *et al.* 1986). To identify the sequences responsible for gene activation by ethylene and elicitors, and to understand the mechanism by

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which these signals are perceived by plant cells, a series of 5′ deletions were introduced into the CH5B gene promoter region. Analysis of deleted chitinase genes in transgenic tobacco plants indicated that DNA sequences located downstream of position −595 were involved in ethylene induction of chitinase gene expression. This study suggested that the promoter of the CH5B gene is, at least in part, composed of a quantitative transcription element, which influences absolute levels of transcription, and an ethylene responsive element (Broglie *et al.* 1989). Consistent with this finding, we have identified a nuclear protein factor by gel mobility shift and DNAse I protection assays that binds to this DNA sequence element (K. E. Broglie, unpublished results). Assay of the chimeric gene deletions using a transient gene expression assay (Roby *et al.* 1991) in the presence of a chitin oligomer showed that the functional profile for this elicitor is qualitatively similar to that for the phytohormone ethylene. Moreover, the timecourses for induction by the two compounds were found to be strikingly similar. These results may directly reflect the known stimulation of ethylene by fungal elicitors. However, the possible existence of an ethylene-independent contribution to chitinase induction by elicitor has not been ruled out.

To study the activation of the bean chitinase 5B promoter by fungal infection, transgenic tobacco plants were produced that contained a chimeric gene consisting of a 1.7 kb fragment of the bean CH5B promoter fused to the reporter gene, β-glucuronidase (GUS). Infection of these transgenic tobacco plants with fungal pathogens gave rise to a significant induction of β-glucuronidase activity that was readily apparent upon histochemical staining of the infected tissue. When the transgenic plants were inoculated on one-half of a leaf with the foliar pathogen, *Botrytis cinerea*, positive staining could be found within and immediately surrounding the necrotic areas (Roby *et al.* 1990). Assays of the infected tissue indicated that although GUS activity was preferentially localized at the site of inoculation, a weaker induction of the chitinase promoter was evident 3–6 mm away from the lesion as well as on the uninoculated half of the leaf. No significant β-glucuronidase activity could be detected in leaves above or below the inoculated leaf. When the infected tissue was fixed, stained with fluorescein isothiocyanate-conjugated wheat germ agglutinin and then subjected to microscopic investigation, fungal mycelia were found to be concentrated within the necrotic lesion. Significantly fewer mycelia were found at the periphery of the lesion in the region which exhibited a blue staining reaction with the chromogenic GUS substrate. These appeared to be confined to the first few cell layers on the surface of the leaf. Beyond this, essentially no fungal structures were apparent. Thus, while the distribution of the fungus was similar to the distribution of GUS enzymic activity, activation of the bean chitinase promoter was clearly found in regions of the leaf which had not been exposed to the fungus as well as in the uninoculated half of the inoculated leaf.

The induction pattern observed for the bean chitinase promoter in the *Botrytis*-infected plants was not restricted to this pathogen. Qualitatively similar results were obtained when the transgenic tobacco plants were infected with two soil-borne pathogens, *Rhizoctonia solani* and *Sclerotium rolfsii*. In both cases, GUS activity was highest in the vicinity of the lesion and declined as the distance from the lesion increased. While inoculation of tobacco plants with *Sclerotium* was achieved by placing an agar plug containing fungus on the surface of the soil near the plant, for *Rhizoctonia* infection, seedlings were transplanted into fungus-infested soil. In the latter case, little if any induction of GUS activity was evident in the roots of the plant, indicating that activation of the chitinase 5B promoter is dependent not only on the presence of the fungus but also on colonization and damage to the plant tissue. A similar, localized induction of GUS enzyme activity has also been observed in transgenic *Arabidopsis thaliana* plants containing a chimeric gene composed of the *Arabidopsis* class III chitinase gene promoter fused to β-glucuronidase and infected with *Rhizoctonia solani* (Samac & Shah 1991).

4. MODIFICATION OF CHITINASE EXPRESSION IN TRANSGENIC PLANTS

Although *in vitro* studies have provided evidence of the antifungal properties of higher plant chitinases, only correlative evidence exists *in planta* to support its role in the defence response. Therefore, to evaluate the role that chitinase plays during pathogen attack, we modified the pattern of bean chitinase expression in transgenic plants. Because the timing of the defence response appears to contribute to the outcome of the interaction between host and pathogen (Bell *et al.* 1986; Joosten & De Wit 1989; Benhamou *et al.* 1990), we have eliminated the temporal factor in chitinase expression. Our results show that constitutive expression of a bean endochitinase in transgenic tobacco and canola plants affords increased protection against disease caused by the fungal pathogen *R. solani*.

A chimeric chitinase gene was constructed by replacing the 5′ regulatory region of the bean chitinase 5B gene with the promoter region of the cauliflower mosaic virus (CaMV) 35*S* transcript. This gene was introduced into *Agrobacterium tumefaciens* by bacterial conjugation and cells carrying the cointegrate plasmids were used to infect leaf discs of *Nicotiana tabacum*. Assays of protein extracts showed increase chitinase activity in all primary transformants containing the 35*S*-chitinase gene.

The 35*S*-chitinase tobacco plants were assayed for fungal resistance using the phytopathogen, *Rhizoctonia solani*. *R. solani* is an endemic, chitinous, soil-borne fungus which infects many plant species, including corn and soybean, and produces severe stem and root rotting symptoms. Planting of seeds in heavily infested fields typically results in poor stands and early season growth of the resulting seedlings.

Damping-off, seedling blight and brown girdling root-rot are examples of important diseases which are attributable to *Rhizoctonia* infection. Homozygous progeny of the transgenic tobacco plants were grown in

the presence of the soil-borne pathogen to determine their susceptibility to infection by this fungus. When 18-day-old tobacco seedlings were transplanted into fungus-infested soil, a decrease in seedling mortality was observed for plants containing the 35*S*-chitinase gene relative to control plants which lacked the 35*S*-chitinase gene. For the four different lines of transformants tested, seedling mortality ranged from 23 to 37% in comparison to 53% for the control. When the surviving plants were compared, a significant difference was evident in the growth and development of control tobacco plants versus plants harbouring the 35*S*-chitinase gene. Control plants were noticeably stunted and their the root systems were poorly developed. In contrast, the 35*S*-chitinase plants were larger, hardier and showed near normal root growth and morphology. The enhanced resistance of 35*S*-chitinase tobacco plants to *R. solani* infection appeared to be correlated with the level of bean chitinase expression: plants containing the higher levels of bean polypeptide survived in greater numbers in the fungus-infested soil. When the 35*S*-chitinase plants were grown in the presence of a pathogen, *Pythium aphanidermatum*, that lacks a chitin-containing cell wall, no difference in survival was detected compared to control plants.

The chimeric 35*S*-chitinase gene has also been introduced into oilseed rape, *Brassica napus* cv. Westar. Consistent with the results observed in transgenic tobacco, canola plants carrying the 35*S*-chitinase gene exhibited increased resistance to infection by the root and stem rot pathogen, *Rhizoctonia solani*. In both systems, seedlings constitutively expressing the bean chitinase polypeptide showed delayed development and progression of disease symptoms. Fifteen days after transplanting into *Rhizoctonia*-inoculated soil, 53% of the transgenic canola seedlings survived while only 24% of the wild-type plants remained (Broglie *et al.* 1991). Similar levels of resistance to *R. solani* have been obtained with transgenic tobacco plants that express either a bacterial chitinase gene (Jach *et al.* 1992) or a ribosome-inactivating protein (RIP) from barley (Logemann *et al.* 1992), both under the control of the CaMV 35*S* promoter.

Analysis of root tissues of infected wild-type canola plants revealed that *R. solani* was capable of extensive colonization of all root tissues including the xylem vessels. Pathogen ingress in these plants was associated with marked host cell wall alterations, such as disruption of the middle lamella matrices. In contrast, the fungal hyphae did not appear to suffer significant damage. In infected transgenic canola plants, however, the pattern of fungal colonization was different than that observed in wild-type plants. Penetration of the host cuticle and invasion of the epidermis was frequently observed, but fungal colonization was usually restricted to the cortex. In all samples examined, severe hyphal alterations ranging from increased vacuolization to cell lysis were also seen. Labeling studies with WGA/ovomucoid revealed that the structural alteration of *R. solani* cell walls in the transgenic plants was accompanied by chitin degradation. Because these features (reduction in fungal biomass, increase in hyphal alterations, fungal lysis, and extensive chitin breakdown) were not seen in the fungus-infected wild-type canola plants, it is likely that constitutive expression of the bean chitinase gene is, at least in part, responsible for the enhanced protection against fungal attack seen in these plants. However, it is uncertain whether constitutively expressed chitinase alone is sufficient to account for the reduction in fungal biomass seen in these transgenic plants and it is possible that other components of the host defence response also contribute to the resistance phenotype. The presence of increased chitinase concentrations from the time of the initiation of infection may allow the host to respond more effectively.

5. CONCLUSION

In recent years considerable progress has been made towards the use of biotechnology to produce disease-resistant plants. As described here, we and others have shown that it is possible to alter the expression of natural defence genes to produce transgenic tobacco and rapeseed plants with increased resistance to the soil-borne pathogen *Rhizoctonia solani*. However, it is unlikely that these changes alone will be sufficient to provide protection against a wide range of fungal pathogens, and it may be necessary to enhance or extend the resistance response by integrating constitutive expression of natural plant defence genes with that of other available anti-fungal polypeptides. Additionally, the identification and isolation of genes involved in race-specific fungal resistance will advance our understanding of the function of these genes in the host defence response and, together with current plant transformation technology, will allow researchers the opportunity to evaluate the potential for the production of high yielding, disease resistant crops.

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