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Heterologous chitinase gene expression to improve plant defense against phytopathogenic fungi

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Agricultural crops worldwide suffer from a vast array of fungal diseases which cause severe yield losses. Upon interaction with a pathogen, plants initiate a complex network of defense mechanisms, among which is a dramatic increase in chitinase activity. Chitinases are capable of hydrolyzing chitin-containing fungal cell walls and are therefore thought to play a major role in the plant's response. One of the strategies to increase plant tolerance to fungal pathogens is the constitutive overexpression of proteins involved in plant-defense mechanisms. The level of protection observed in transgenic plants harboring heterologous chitinase genes varies, depending on the particular combination of enzyme, plant and pathogen tested. Nevertheless, most of these transgenic plants exhibit increased tolerance to fungal diseases relative to their non-transgenic counterparts. The combined expression of chitinases with other plant-defense proteins such as glucanases and ribosome-inactivating proteins further enhances the plant's resistance to fungal attack.

Keywords: plant defense; chitinase; heterologous genes

Introduction

Plant protection is a major challenge to agriculture worldwide, with fungi being one of the main causes of significant yield losses. The control of fungal disease in modern agriculture is mainly achieved by the extensive use of chemical fungicides. However, growing concern about the environment and the high cost of chemicals have encouraged farmers and researchers to look for substitutes, such as the use of biocontrol agents and fungus-resistant crop cultivars. Although intensive activity towards the development of means of biological control is currently taking place, commercial products are few. The genetic approach of breeding to produce crops which are resistant to fungal diseases has proven successful; however, this time-consuming process is expensive, and makes it difficult to react adequately to the evolution of new virulent fungal races. Newly developed technology for the identification, isolation and transfer of specific genes, currently in use for plant breeding, has enabled the insertion of traits for resistance without interfering with the intrinsic properties of the acceptor plant. Therefore, much effort is being put into identifying and isolating genes that, upon transfer, may render target plants resistant to fungi. Some of these efforts are being focused on resistance genes, known from conventional breeding programs. Genes encoding toxic compounds and enzymes involved in direct inhibitory effects on fungi are another direction for intensive research.

Chitin, an unbranched homopolymer of 1, 4-β-linked *N*-acetyl-d-glucosamine, is a major cell-wall component of most phytopathogenic fungi [56] which does not occur in plants, vertebrates or prokaryotes. Besides chitin, the skeleton of filamentous fungal cell walls contains 1,3-β-glucan, proteins and lipids [20]. Chitinases (poly[1,4(*N*-acetyl-glucosaminide] glycanohydrolase, EC 3.2.1.14) are abundant proteins, found in a wide variety of seed-producing plants. Although the physiological function of chitinases has yet to be clarified, there is strong correlative evidence that they are defense proteins with antifungal activity [46]. Chitinases, along with proteases and 1,3-β-glucanases, degrade fungal cell walls, inhibit fungal growth at the hyphal tips [1,21,35,47], and have been shown to associate with hyphal walls *in planta* [2,4]. Nearly all plant chitinases isolated to date are endochitinases, ie they hydrolyze chitin, a polymer of *N*-acetyl-glucosamine from within the polymer rather than at its terminus. Several reported plant chitinases are exochitinolytic [14].

Chitinases fall into three broad classes, as proposed by Shinshi *et al* [50]. Class I chitinases are basic and contain a cysteine-rich N-terminal domain with putative chitin-binding properties. They are usually localized in the vacuole and are potent growth inhibitors *in vitro* of many fungi [24,34]. Class II chitinases consist of a monomeric catalytic domain with strong homology to the catalytic domain of class I chitinases, but lacking the cysteine-rich domain. Class II chitinases are generally acidic and extracellular, and can be detected in the apoplastic fluid or culture medium of protoplasts [5,12] They are not thought to be antifungal, either alone or in combination with other proteins [36]. A possible role for these chitinases in plant defense is to act as signaling molecules, releasing elicitors from invading fungal hyphae and acting as a first line of defense [34]. Class III chitinases are extracellular hydrolases whose conserved catalytic-domain amino-acid sequence differs from the conserved sequence of class I or II chitinases. Most of the class III chitinases are classified

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as such on the basis of homology to previously described lysozymes with chitinase activity [14]. A newly proposed group of class IV chitinases [11], includes several chitinases with structural similarities, but sequence differences relative to class I. Class IV chitinases lack the C-terminal extension, and are therefore assumed to be accumulated extracellularly [37]. Thus, these chitinases may fulfill an antifungal role similar to that of class I within the apoplast.

The role of chitinases in plant defense

The interaction between a pathogen and its host plant initiates a complex network of defense mechanisms, including: the synthesis of polymers forming physical barriers, such as lignin and cellulose; the synthesis of antimicrobial metabolites (phytoalexins); and the synthesis of pathogenesis-related (PR) proteins, chitinases among them [2,16]. It is therefore complicated to elucidate the specific roles of chitinases in plant defense, despite the fact that the inducibility of chitinases and chitinase genes as a result of pathogen attack is very well documented [42]. One way to resolve this problem is to compare induction rates and final concentrations of chitinases in tissues that are resistant (incompatible) or susceptible (compatible) to the fungal pathogen. The results of such studies reveal a complicated picture, in which the role of chitinases depends upon the specific combination of pathogen, plant and chitinase in question. For example, in the interaction between *Cladosporium fulvum* and tomato, resistance against the fungus correlates with early transcription-induction of genes encoding apoplastic chitinase and 1,3-β-glucanase and the accumulation of these proteins in inoculated tomato leaves. For vacuolar, basic isoforms of chitinase and 1,3-β-glucanase, however, early gene transcript accumulation was observed in both incompatible and compatible interactions. Moreover, studies on the tissue-specific expression of genes encoding these hydrolytic enzymes revealed only temporal differences in gene-transcript accumulation for each isoform studied. Expression of the acidic chitinase gene was observed primarily near leaf vascular tissue. Expression of the basic chitinase was less confined to a particular tissue. No preferential accumulation of gene transcripts in the tissue near penetrating hyphae was observed in compatible or incompatible interactions. However, injection of purified race-specific elicitors induced primarily differential expression of acidic chitinase, which was observed most abundantly in resistant genotypes, and correlates well with the differences in gene expression previously observed in time-course experiments on compatible and incompatible *C. fulvum*–tomato interactions [57]. The time for chitinase induction is also dependent on the specific pathogen-host interaction, and varies from minutes to 15–20 h [42]. After induction, the time course of chitinase activity is in the range of several days [14]. This time frame suggests that the role of chitinases in plant defense is mainly to reduce pathogen growth and sporulation at later disease stages, rather than to be involved in the early events of the host-pathogen interaction. However, as mentioned before, different chitinase classes may play different roles in plant defense. The dramatic increase in chitinase activity as a result of PR induction [10,14,27], together with evidence of chitinase antifungal activity *in vitro* [17,28,40,45,47], strongly support the correlative observation that chitinases are key enzymes in antifungal plant defense. The use of fungi and bacteria as biological control agents is based mainly on their antagonistic activity towards phytopathogenic fungi. This activity involves the secretion of extracellular lytic enzymes, such as 1,3-β-glucanases, chitinases and proteases, which degrade the main components of the fungal cell walls. The mechanism of hydrolytic enzymes in the control of plant pathogenic fungi has been intensively studied, and chitinases were found to be directly involved in inhibition of spore germination and germ-tube elongation, as well as in degradation of fungus hyphal tips [8,9,17]. These results provide further evidence of chitinases' role in plant defense.

Heterologous chitinase gene expression

Chitinases hydrolyze chitin, a polysaccharide that is foreign to plants but is one of the main cell-wall components of fungi. Thus, chitinase genes are attractive candidates for expression studies geared towards the production of resistant cultivars, mainly of important crop plants. Moreover, the currently available techniques for the transformation of many plant species have enabled experiments which could answer the intriguing questions regarding the precise role of different chitinases in plant defense.

As a first step in evaluating the feasibility of such an approach, transformation studies were conducted. Jones *et al* [22] used the photosynthetic gene promoters of ribulose bisphosphate carboxylase (*rbcS*) and the chlorophyll a/b-binding protein (*cab*) from petunia to express the bacterial chitinase gene (*ChiA*) from *Serratia marcescens* in tobacco. Under the *rbcS* promoter, ChiA protein accumulated to about 0.25% of the total leaf protein, and the transformed plant exhibited significantly higher chitinase enzymatic activity relative to controls. The successful introduction of the *ChiA* gene from *S. marcescens* into tobacco has also been shown by others, using additional promoters, such as that for the nopaline synthase (*nos*) gene from *Agrobacterium tumefaciens* and that of the cauliflower mosaic virus (CaMV) 35S-RNA gene [31,32,38]. The 35S CaMV was also successfully used to drive expression of the *ChiA* gene in the mycoparasitic fungus *Trichoderma harzianum*, to enhance its biological control activity [18]. However, the high expression of the 35S promoter in lower eukaryotes and prokaryotes might be a disadvantage in the production of transgenic plants since 'false transformants', resulting from promoter activity in endophytic prokaryotes or fungi, might occur. Recently, Mass *et al* [33] developed a system in which insertion of an intron (198-bp intron 2 of the potato *STLS 1* gene) into a selectable marker gene (*NPT II*) driven by the 35S promoter completely abolished gene expression in prokaryotes without affecting the expression in monocotyledonous and dicotyledonous plants. This intron-interrupted system can be used with different genes of interest for efficient 35S CaMV-driven plant expression.

Subsequently, genes of plant origin were introduced into various plant species. In transgenic tobacco, the promoter regions of a bean chitinase gene were shown to be regulated by ethylene [7]. Regulation by fungal elicitors was found

for rice chitinase [58], and fungal spores enhanced the peanut *Chi2;1* gene [25], expressed in transgenic tobacco. Recently, Raharjo *et al* [43] introduced three chitinase genes (an acidic chitinase from petunia, and basic chitinases from tobacco and bean) into pickling cucumber. Driven by the 35S CaMV promoter, all three chitinases were expressed in cucumber leaves, and showed varying but enhanced levels of activity relative to non-transformed controls.

The first attempt to evaluate the role of transformed chitinases in plant defense was made by Suslow *et al* [52]. They developed various populations of *ChiA*-expressing plants using mesophyll-specific or constitutive plant promoters fused to the bacterial gene. In the transformed plants, the bacterial chitinase protein approached 0.2% of the plant's total soluble protein. At this protein level, the bacterial enzyme increased the endogenous chitinase activity by 30–40% over the best-comparable homozygous population. The ChiA-expressing tobacco leaves were assayed for resistance to the phytopathogen *Alternaria longipes*. Necrotic-lesion development and chlorosis were significantly reduced when transformed control plants were at peak susceptibility. However, further maturation of the ChiA-transformed plants eliminated these differences. In a later work independent lines of transgenic tobacco plants which expressed high levels of the *S. marcescens* ChiA protein intracellularly or extracellularly were found to exhibit tolerance to the fungal pathogen *Rhizoctonia solani* in the field [19].

Broglie *et al* [6] were the first to assess the role of transformed plant chitinases in resistance to fungal pathogens. They modified the timing of the natural host defense mechanisms from temporal to constitutive expression by transforming the bean *CH5B* chitinase gene into tobacco plants under the 35S CaMV constitutive promoter. The size of the bean protein in the transgenic tobacco plants was indistinguishable from that of the native protein, indicating that the precursor protein had been correctly processed in the heterologous tobacco system. The transformed plants showed increased chitinase enzyme activity, up to four-fold in the roots and 44-fold in the leaves relative to control plants, in a constitutive manner. To determine the susceptibility of the 35S-chitinase transgenic tobacco to fungal attack, homozygous progeny were grown in the presence of the soilborne phytopathogenic fungus *R. solani*. Transgenic tobacco expressing high levels of chitinase grew faster, lost at least three-fold less root weight (15 *vs* 46%), and had a lower seedling mortality rate (37 *vs* 53%) relative to control seedlings. The 35S-*CH5B* construct was also transformed into canola plants, which were grown in a soil infested with *R. solani*. The extent of the infection was lessened and was contained mainly within the root cortex. *R. solani* hyphae on the transgenic plants appeared physically damaged and suffered increased vacuolization and cell lysis as compared to the metabolically active fungi found on control plants [3].

The same approach was taken by Lin *et al* [29], who transformed a rice chitinase under the control of the 35S CaMV promoter into rice plants. Constitutive expression of chitinase in cereal plants could potentially improve resistance to fungal attack in two ways: besides the ability to attack fungal cell walls directly, chitinase releases oligo-*N*-acetylglucosamines which function as elicitors for the activation of defense-related responses in rice cells [44]. Progeny from the chitinase-positive plants were tested for their resistance to the sheath blight pathogen *R. solani*, and the degree of resistance displayed by these transgenic plants correlated with the level of chitinase expression. Although lesions appeared on both control and transgenic plants, the number and size of the lesions were smaller, and confined to the lower half of the sheath in the transgenic plants, whereas in control plants lesions spread to the upper half of the plant and covered a larger area.

Grison *et al* [15] took the resistance evaluation of chitinase-expressing transgenic plants one step further, by challenging such plants with three different fungal pathogens (*Cylindrosporium concentricum*, *Phoma lingam* and *Sclerotinia sclerotiorum*) in field trials at two different geographical sites. Oilseed rape (*Brassica napus*) was transformed with a tomato chitinase gene under the control of the 35S CaMV promoter. The transgenic genotypes showed different degrees of protection against the three fungal pathogens at the different field sites, but in all cases, ranging from roughly 23% to 79%, symptom reduction was exhibited.

The phenomenon of chitinase's variable antifungal effect is, however, problematic. The defense mechanism depends on both the chitinase type and the fungus tested. In *in vitro* assays, for example, a class I chitinase from *Arabidopsis* was effective against *Trichoderma reesie*, but not against commercially important pathogens such as *Fusarium oxysporum*, *Alternaria solani*, *Sclerotium rolfsii* or *Phytophthora megasperma* [14,53]. The chitinase from *S. marcescens* was, on the other hand, very effective against *S. rolfsii* [49]. Also problematic is the observation that not all transformed plants expressing high levels of chitinase exhibit the expected increase in resistance to fungal pathogens. Neuhaus *et al* [39] introduced a gene for a class I tobacco chitinase regulated by the 35S CaMV RNA-expression signal into *Nicotiana sylvestris*. The gene was expressed to give mature, enzymatically active chitinase targeted to the intracellular compartment of leaves. Most transformants accumulated high levels of chitinase—up to 120-fold that in control plants. However, some transformants exhibited chitinase levels lower than in non-transformed plants, suggesting that the transgene inhibited expression of the homologous gene, as was also observed for *T. harzianum* transformed with the *S. marcescens chiA* gene [18]. Neuhaus *et al* [39] challenged the highly expressing chitinase transformants with the fungus *Cercospora nicotianae*, a major pathogen of tobacco. They used an inoculum density that was quite high relative to field conditions, which induced a class I chitinase in infected leaves of non-transformed plants. Nevertheless, disease symptoms in the chitinase transformants were only slightly reduced, indicating that tobacco class I chitinase is not the limiting factor in the defense reaction to this pathogen. Transgenic tobacco plants bearing the gene for the SE2 class III chitinase from sugar beet were also not appreciably protected from infection by *C. nicotianae* [41]. These observations do not, however, rule out a role for chitinases in the defense reaction. In many cases chitinase is only an effective fungicide *in vitro*, when applied in combination with 1,3-β-glucanase

[35,47]. Zhu *et al* [59] took this approach to enhance protection against fungal attack by constitutive co-expression of chitinase and glucanase genes. They introduced the gene encoding the RCH10 rice basic chitinase under a 35S CaMV enhancer and the AGLU1 alfalfa acidic 1,3-β-glucanase under a 35S caMV double promoter into separate parental lines. Hybrid plants were generated by crossing the transgenic parental lines exhibiting strong constitutive expression of either gene. The generation of such hybrid lines enables a direct evaluation of the protective interaction between the transgenes by comparing the protection in the hybrid plants with that afforded by each transgene alone at the same respective loci in the parental lines. This approach also overcomes the problem of variation in the level of transgene expression among independent transformants containing the same construct. As in previous studies [39,41], some protection against *C. nicotianae* was observed in the parental line strongly expressing either transgene alone. However, markedly higher protection was observed in hybrid plants expressing both chitinase and 1,3-β-glucanase transgenes. The protective effects involved a delay in the appearance of the first visible lesions and subsequent reduction in both the number and size of the lesions. Jongedijk *et al* [23] introduced two chitinase and two 1,3-β-glucanase genes (representatives of class I and class II chitinase/glucanase from tobacco) into tomato plants. Again, tomato plants expressing both hydrolytic enzymes, ie chitinases and glucanases, exhibited higher resistance to *F. oxysporum* f sp *lycopersici*, than transgenic plants expressing any one of these genes alone which were not protected against fungal infection. Their results also demonstrated that resistance is achieved by the simultaneous expression of only class I chitinase and 1,3-β-glucanase, as had been suggested for such synergistic activity against *F. oxysporum in vitro* [35,48].

Plants respond to pathogen attack by activating an array of defense mechanisms, and the strategy of combined gene expression may therefore not be limited to hydrolytic enzymes. Genes encoding ribosome-inactivating protein (RIPs) are also candidates as defense transgenes. RIPs possess 28S rRNA *N*-glycosidase activity which, depending upon their specificity, leads to the inactivation of nonspecific/foreign ribosomes [13,51]. Synergistically enhanced antifungal activity of barley endosperm RIP combined with barley class I chitinase or class II 1,3-β-glucanase was observed *in vitro* by Leah *et al* [28], and expression of this RIP gene in tobacco plants resulted in increased stability against *R. solani*, without influencing plant growth [30]. The synergistic effect was validated *in vivo* by Guido *et al* [21], who compared the tolerance of transgenic tobacco plants expressing cDNA encoding basic class II chitinase (CHI), basic class II 1,3-β-glucanase (GLU), or a type I RIP from barley, all under the control of the 35S CaMV promoter, with the tolerance of isogenic tobacco plants harboring various combinations of these genes. Transgenic seedlings were transplanted into soil infected with *R. solani*, and disease severity was ranked on a scale from 0 to 4, 0 representing no disease symptoms and 4 representing macerated/rotted or dead plants. Although plant lines expressing the barley transgene individually exhibited relatively high levels of protection against this fungus (disease reduction of 35–53%), significantly enhanced protection was found for plants expressing a combination of these defense genes. Not only did the combination of the hydrolytic enzymes chitinase and 1,3-β-glucanase result in the expected increased protection, but the combination of chitinase and RIP increased protection as well. Preliminary infection assays with other phytopathogenic fungi such as *Alternaria alternata* and *Botrytis cinerea* also revealed significantly enhanced protection of glucanase/chitinase/RIP transgenic tobacco lines against fungal attack [21]. These results indicate that the combined expression of different antifungal proteins can lead to improved protection against a broad range of phytopathogenic fungi.

Plant improvement via genetic engineering may be useful when the manipulation does not interfere with the intrinsic valuable traits of the plant. Plant roots are colonized not only by pathogens, but also by beneficial symbiotic fungi. Most herbaceous plants are hosts for vesicular-arbuscular mycorrhizal fungi that enhance the uptake of mineral nutrients in exchange for assimilates provided by the plant [26]. The cell walls of fungi involved in this symbiosis contain chitin and 1,3-β-glucan, and constitutive expression of chitinases and 1,3-β-glucanases may therefore interfere with their colonization. Interestingly, *N. sylvestris* plants constitutively expressing different forms of tobacco chitinases, and *N. tabacum* constitutively expressing different forms of chitinases and 1,3-β-glucanases were colonized by the mycorrhizal symbiont to the same degree, following the same time course, as control plants lacking the transgenes [54,55]. Of particular interest is the observation that plants expressing two enzymes simultaneously were colonized with the vesicular-arbuscular mycorrhizal fungi to the same degree as the control plants. However, plants expressing high levels of the acidic class II 1,3-β-glucanase, an enzyme with very little antifungal potential, were colonized more slowly and to a lesser extent than control plants. These results suggest that transgenic plants should be assessed not only with respect to disease resistance but also with respect to their symbiotic abilities. Despite this limitation, this work further encourages the approach of modifying plant lines towards increased resistance by constitutive expression of chitinases, together with other PR proteins.

Conclusion

Chitinases have been shown to be an integral component of the plant's response to fungal attack, in concert with other defense-related proteins. In most cases, plants which have been transformed with chitinase genes show improved resistance to phytopathogenic fungi. Although a great deal of knowledge has been gathered pertaining to the different classes and functions of chitinases and PR proteins, the relative activities of these proteins against specific pathogens have only been partially elucidated. It is therefore necessary to evaluate each combination of plant–pathogen-introduced gene for enhanced resistance and plant viability and productivity. Additional knowledge of the signaling pathways for chitinase induction, together with an elucidation of the responsive elements in their genes, may lead to a better and more uniform system for heterologous

expression. Future research, together with the current results of enhanced resistance, may prove transgenic plants expressing chitinases and PR proteins to be the best means of plant protection.

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