Chitin and Chitinases
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Chitinases in biological control

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Summary. The public concern over the harmful effects of chemical pesticides on the environment and human health has enhanced the search for safer, environmentally friendly control alternatives. Control of plant pests by the application of biological agents holds great promise as an alternative to the use of chemicals. It is generally recognized that biological control agents are safer and more environmentally sound than is reliance on the use of high volumes of pesticides. Due to the importance of chitinolytic enzymes in insect, nematode, and fungal growth and development, they are receiving attention in regard to their development as biopesticides or chemical defense proteins in transgenic plants and microbial biocontrol agents. In this sense, biological control of some soil-borne fungal diseases has been correlated with chitinase production. Fungi- and bacteria-producing chitinases exhibit antagonism against fungi, and inhibition of fungal growth by plant chitinases has been demonstrated. Insect pathogenic fungi have considerable potential for the biological control of insect pests. Entomopathogenic fungi apparently overcome physical barriers of the host by producing multiple extracellular enzymes including chitinolytic enzymes, which help to penetrate the cuticle and facilitate infection.

In this chapter, the role of chitinases in biological control and their potential use in the improvement of biocontrol agents and crop plants by genetic engineering is analyzed in view of recent findings.

Introduction

In modem agriculture, monoculture is the norm, providing large numbers of generally near-identical plants in one vicinity. Such agricultural practice enables us to continue to provide foodstuffs for the world's ever-increasing population. It is, however, an ecologically unnatural situation, which is inherently unstable and offers considerable opportunity for the invasion of crops by plant pests, weeds and diseases [1]. Pesticides are applied in agricultural systems for the purpose of protecting plants from injury by insects, disease and so on which today still destroy almost 33% of all food crops. The use of pesticides is considered effective if they achieve the desired biological result, and economic if there is a crop yield and a quality response above and beyond the cost of the chemicals and their application. Yet the use of pesticides has also resulted in significant costs to public health and the environment. In general the amount of pesticides released into the environment has risen about 1900% in the 50-year period between 1930 and 1980 [2]. It is clear that this sort of agriculture cannot be sustained if the price for this success is unacceptable destruction of the

environment [3]. However, since there are currently not many alternatives to agricultural practices such as monoculture, even just to maintain current human populations the need remains for scientists to continually seek for new, effective and environmentally friendly ways of controlling pests, weeds and diseases [1]. Biotechnology, in conjunction with conventional breeding programs, could make significant contributions to sustainable agriculture. In this regard, there has been intensive research in agricultural biotechnology aimed at plant protection. This includes, among others, disease-free clones of fruits, vegetables and ornamental crops; plants resistant to insects and microbial pathogens; herbicide-tolerant cultivars; and biopesticides to use as biological control agents [4].

Control of plant pests by the application of biological agents holds great promise as an alternative to the use of chemicals. It is generally recognized that biological control agents are safer and more environmentally sound than is reliance on the use of high volumes of pesticides and other antimicrobial treatments. Besides, there is an equally great or greater need for biological control of pathogens that presently go uncontrolled or are only partially controlled by these "traditional" means [5].

Different meanings have been given to the term "biological control". Representing two extreme views of the concept, we find the following definitions: "Biological control [of plant pathogens] is their control by one or more organisms, accomplished naturally or through manipulation of the environment, host, or antagonist, or by mass introduction of one or more antagonists" [6]. This definition provides us with a broad concept that includes such notions as cultural practices and disease resistance. On the other hand, we have the classical concept: "Biological control is the deliberate use of one organism to control another" [7]. The term "biological control" will be used in this more restricted sense throughout this text.

Classical biocontrol, when effective, is an outstanding method of pest control not only because it eliminates the use of powerful, environmentally dangerous pesticides, but also because if the introduced biocontrol agent becomes properly established, it is long lasting and further investments in control are not necessary. In this way, it differs from the use of pesticides, which require repeated application. This has led to a renewed interest in the discovery, development and refinement of biological control agents. Such efforts have followed classical plant pathology screening strategies but have also begun to utilize the methodologies made available through molecular biology. We can now look at micoorganisms with inhibitory activity against pathogens as potential sources of genes for disease resistance.

Physiological role of chitinases

Chitinases have been detected in a great variety of organisms, including those that contain chitin, such as insects, crustaceans, yeasts and fungi, and

also organisms that do not contain chitin, such as bacteria, higher plants and vertebrates. In arthropods, chitinases are involved in molting and digestion. Insects periodically sheed their old cuticles and resynthesize new ones. This process is mediated by the elaboration of chitinases in the molting fluid that accumulates between the old cuticle and the epidermis. The products of hydrolysis are recycled for the synthesis of the new cuticle. Often larvae will ingest the old cuticle. Apparently, chitinases found in the gut have a digestive function in addition to their role in breaking down chitin present in the gut lining [11].

The model of fungal cell wall growth proposed by Bartnicki-Garcia [12] envisages the role played by lytic enzymes in maintaining a balance between wall synthesis and wall lysis during hyphal apical growth, providing plasticity to the apex and permitting insertion of nascent chitin into the wall. Evidence for the association of chitinases and chitin synthases comes from parallel behavior of the two activities during spore germination in ***Mucor mucedo*** [13], during exponential growth in ***Mucor rouxii*** [14] and ***Candida albicans*** [15], and from the finding of a chitinase activity in the same cell fraction as chitin synthase in M. ***mucedo*** [16, 17]. Sahai et al. [18] showed that chitinase is present during spore swelling, germination, sporangium formation and response to mechanical injury in ***Choanephora cucurbitarum*** and four other Zygomycetes fungi. Failure to localize chitinase at the hyphal tips suggests its possible lack of involvement in apical growth.

The process of autolysis of mature fruiting bodies of ***Coprinus lagopus*** is accomplished by the action of chitinases which are formed shortly before spore release begins [19]. Demonstration of lysosomal chitinases was based on sedimentation studies. Chitinase activity was localized intracellularly in vacuoles together with other lytic enzymes. Chitinases had no apparent function in intracellular digestion since they were synthesized shortly before autolysis in gills [19]. This enzyme is passively released into the wall when metabolic activity stops in senescing cells. It has been described that some autolytic enzymes including chitinases are bound to subapical walls of *Neurospora crassa* and *Aspergillus nidulans* [20, 21]. These data led to the suggestion that chitinases are associated with hyphal branching rather than autolytic wall turnover. Thus, fungal chitinases have been implicated in apical growth, spore swelling and germination, liberation of spores, cell separation and budding.

Considerable interest in the physical, chemical, kinetic and biocidal properties of chitinases has been stimulated by their possible involvement as defense agents against chitinous pathogenic or pestiferous organisms such as fungi and insects. Resistance to organisms can be imparted by the degradation of vital structures such as the peritrophic membrane or cuticle of insects, the cell wall of fungal pathogens or by liberation of compounds that subsequently elicit other defense responses [22].

Chitinases in insect control

Insect pathogenic fungi have considerable potential for the biological control of insect pests of plants. The majority of these fungi occur in the Deuteromycotina and Zygomycotina. Many attempts have been made to exploit the Deuteromycotina fungi Metarhizium anisopliae, Bauveria spp., Nomurae rileyi, Aschersonia *aleyrodis* and *Verticillium* lecanii, as well as some Entomophtorales for insect control. In this sense, the peritrophic membrane and exoskeleton of insects act as physicochemical barriers to environmental hazards and predators. However, entomopathogenic fungi apparently overcome these kinds of barriers by producing multiple extracellular enzymes, including chitinolytic and proteolytic enzymes that help to penetrate the cuticle and facilitate infection [23–26]. Some insect venoms also contain chitinolytic enzymes that might serve to facilitate the entry of venomous components into prey [27]. Similarly, the nematode *Brugia* malayi utilizes a chitinase to break down a protective chitinous extracellular sheath and/or the peritrophic membrane to gain entry into the mosquito host [28, 29]. Baculoviruses also contain genes for chitinases, but their precise role(s) in host infection is unclear [30]. Nevertheless, hydrolytic enzymes used by insects, fungi and other organisms for molting or barrier penetration are potentially useful in pest management because their physiological action is to destroy vital structures such as the exoskeleton or peritrophic membrane of insects.

A Manduca sexta chitinase has been shown to increase the killing rate of a recombinant baculovirus [31]. In that study a recombinant nonoccluded baculovirus, *Autographa californica* nuclear polyhedrosis virus (AcMNPV) carrying the M. sexta chitinase complementary DNA (cDNA) under the control of the polyhedrin gene promoter, expressed the chitinase. This enzyme was secreted into the medium when insect cell lines were infected with the virus. When the recombinant virus was injected in M. sexta larvae, chitinase was found in the hemolymph, where it does not normally occur. The recombinant baculovirus expressing the chitinase killed larvae of fall armyworms (S. *frugiperda*) in approximately three quarters of the time required for the wild-type virus to kill the larvae [31].

Chitinases have also been used in mixing experiments to increase the potency of entomopathogenic microorganisms. Bacterial chitinolytic enzymes have been used to enhance the activity of microbial insecticides including Bacillus thuringiensis and a baculovirus. Larvae of spruce budworm, Choristoneura *fumiferana*, died more rapidly when exposed to a chitinase-Bacillus mixture than when exposed to the enzyme or bacterium alone [32–34]. In another study, mortality of gypsy moth (Lymantria dispar) larvae was enhanced when chitinase was combined with B. *thuringiensis* compared with treatment with the bacteria alone, and this effect was correlated with enzyme levels. The larvicidal activity of a nuclear polyhedrosis virus toward gypsy moth larvae was increased fivefold when it

was coadministered with bacterial chitinase [35]. In that case, chitinases were supposed to cause perforations in the gut peritrophic membrane, facilitating entry of the pathogens into the hemocoel of susceptible insects [36].

Chitinases appear to be involved in the penetration of host cuticle by entomopathogenic fungi [25, 26, 37]. In this regard, chitinases and β-N-acetylglucosaminidases are secreted when the entomopathogens *M.* anisopliae, B. bassiana and *Verticillium* Iecanii are grown on insect cuticles [24]. Virulent isolates of N. rileyi exhibit substantially higher levels of chitinase activity than avirulent strains at the time of cuticle penetration [23]. Chitinase gene expression in entomopathogenic fungi is believed to be controlled by a repressor-inducer system in which chitin or the oligomeric degradation products serve as inducers [24]. However, bacterial chitinases were ineffective in assays in which insects were fed a diet containing the enzymes. No mortality of the nymphal stages of the rice brown plant hopper, *Nilaparvata lugens*, occurred when 0.09% w/v Streptomyces *griseus* chitinase was added to an artificial diet [38]. Similarly, Serratia and Streptomyces chitinases at 1–2% levels in the diet of the merchant grain beetle, *Oryzaephilus* mercator, caused no mortality.

Chitinases in the control of phytopathogenic fungi

Even with intensive fungicide use, the destruction of crop plants by fungal pathogens is a serious problem worldwide that annually leads to losses of about 15% [3]. Hence, any development aimed to diminish this problem will be useful, especially if at the same time it helps to decrease the strong fungicide application.

Biological control of some soil-borne fungal diseases has been correlated with chitinase production [39]; bacteria-producing chitinases and/or glucanases exhibit antagonism in *vitro* against fungi [40, 41]; inhibition of fungal growth by plant chitinases and dissolution of fungal cell walls by a streptomycete chitinase and β-(1,3)-glucanase have been demonstrated [42, 43]. The importance of chitinase activity was further demonstrated by the loss of biocontrol efficacy in *Serratia* marcescens mutants in which the *chi*A gene had been inactivated [44].

Molecular techniques have also facilitated the introduction of beneficial traits into rhizosphere competent and model organisms to produce potential biocontrol agents. A recombinant Escherichia coli expressing the *chiA* gene from S. marcescens was effective in reducing disease incidence caused by Sclerotium *rolfsii* and Rhizoctonia *solani* [45, 46]. In other studies, chitinase genes from S. marcescens have been expressed in Pseudomonas sp. and the plant symbiont Rhizobium meliloti. The modified Pseudomonas strain was shown to control the pathogens *F. oxysporum f.* sp. *redolens* and Gauemannomyces *graminis* var. tritici [47, 48]. The anti-

fungal activity of the transgenic Rhizobium during symbiosis on alfalfa roots was verified by lysis of R. solani hyphal tips treated with cell-free nodule extracts [49].

The use of mycoparasites is a promising alternative for disease control by biological means. Mycoparasitism is defined as a direct attack on a fungal thallus, followed by nutrient utilization by the parasite [50]. According to Barnett and Binder [51] mycoparasites can be divided into biotrophic and necrotrophic. Necrotrophic mycoparasites are those that kill the host cells before, or just after, invasion and use the nutrients released. These mycoparasites tend to be more aggressive and destructive than biotrophs, have a broad host range extending to wide taxonomic groups, and are relatively unspecialized in their mode of parasitism. The antagonistic activity of necrotrophs is due to the production of antibiotics, toxins or hydrolytic enzymes in such proportions as to cause death and destruction of their host [52]. Instead, in biotrophic parasitism the development of the parasite is favored by a living rather than a dead host structure [50]. Biotrophic mycoparasites tend to have a more restricted host range and in many cases produce specialized structures (haustoria) to absorb nutrients from their host [52].

There are a number of examples of fungi that parasitize plant pathogens. Of these only a few have been studied to any extent with the aim of biological control. Trichoderma species and *Gliocladium virens* have probably been studied more extensively. Other mycoparasites reported to have some potential for biocontrol are Ampelomyces quisqualis, *Coniothyrium* minitans, *Laetisaria arvalis*, Pythium *nunn*, Talaromyces *flavus* and Sporidesmium sclerotivorum [5, 50, 53].

The potential for the use of Trichoderma species as biocontrol agents was suggested 67 years ago by Weindling [54], who was the first to demonstrate the parasitic activity of members of this genus toward pathogens such as Rhizoctonia solani [54, 55]. Several species of Trichoderma spp. have been tested as biocontrol agents; among them Trichoderma harzianum has proved to be more effective [56], and it has been shown to attack a range of economically important soil-borne plant-pathogenic fungi. The parasitic process by Trichoderma apparently includes (i) chemotropic growth, (ii) recognition of the host by the parasite, (iii) secretion of extracellular enzymes, (iv) hyphae penetration and (v) lysis of the host or their combination. Penetration of the host mycelium takes place aparently by partial degradation of its cell wall [57, 58]. Microscopic observations [59, 60] lead to suggest that Trichoderma spp. produced and secreted mycolytic enzymes responsible for the partial degradation of the host's cell wall. Results supporting this hypothesis have shown that indeed Trichoderma produces extracellularly a complex set of β-(1,3)-glucanases, chitinases, lipases and proteases when grown on cell walls of R. solani [61, 62].

The level of hydrolytic enzymes produced differs for each host parasite interaction analyzed. This phenomenon correlates with the ability of each

Trichoderma isolate to control a specific pathogen. However, the specificity of ***Trichoderma*** cannot be simply explained by a difference in enzyme activity, since the nonantagonistic ***Trichoderma*** isolates produce lower but significant levels of lytic enzymes [63]. This observation supports the idea that recognition is an important factor in the mycoparasitic activity of ***Trichoderma.*** The effect of the cell wall-degrading enzymes on the host has been observed using different microscopy techniques. Interaction sites have been stained by fluoresceinisothiocyanate-conjugated lectins or calcofluor. The appearance of fluorescence indicated the presence of localized cell wall lysis at points of interaction between the antagonist and its host. Electron microscopy analysis has shown that during the interaction of ***Trichoderma*** spp. with either ***S. rolfsii*** or ***R. solani*** the parasite hyphae contacted their host and enzymatically digested their cell walls [57].

The purification and characterization of three chitinases from I: ***harzianum*** was reported by De la Cruz et al. [64]. They reported the isozymes to be 37, 33 and 42 kDa, respectively. Only the purified 42-kDa chitinase hydrolyzed ***Botrytis cinerea*** purified cell walls ***in vitro***, but this effect was heightened in the presence of either of the other two isoenzyrnes [64]. However, the chitinolytic system of ***T. harzianum*** was recently found to be more complex [65], consisting of six distinct enzymes. The system is apparently composed of two β-(1,4)-N-acetylglucosaminidases of 102 and 73 kDa, respectively, and four endochitinases of 52, 42, 33 and 31 kDa, respectively. All the chitinolytic enzymes were induced and secreted during growth of ***Trichoderma*** on chitin as the sole carbon source.

The complexity and diversity of the chitinolytic system of I: ***harzianum*** involves the complementary modes of action of six enzymes, all of which might be required for maximum efficiency against a broad spectrum of chitin-containing plant pathogenic fungi. Probably the most interesting individual enzyme of the system is the 42-kDa endochitinase because of its ability to hydrolyze ***B. cinerea*** cell walls ***in vitro***. Since the report of the purification of this enzyme the corresponding gene has been cloned [66]. Expression of the gene (***ech42***) is strongly induced during fungus-fungus interaction. Its expression is apparently repressed by glucose and may be affected by other environmental factors such as light and nutritional stress and may even be developmentally regulated [66]. A second endochitinase and a β-(1,4)-N-acetylglucosaminidase encoding genes from ***Trichoderma*** have been cloned [67, 68].

Recently we have analyzed the role of the ***T. harzianum*** endochitinase Ech42 in mycoparasitism by genetic manipulation of its coding gene ***ech42*** [69]. Several transgenic I: ***harzianum*** strains carrying multiple copies of ***ech42*** as well as the corresponding gene disruptants were generated. The level of extracellular endochitinase activity when I: ***harzianum*** was grown under inducing conditions increased up to 42-fold in multicopy strains as compared with the nontransformed strain, whereas gene disruptants

showed practically no activity. In greenhouse experiments, no differences in the efficacy of the gene disruptants to control ***Rhizoctonia solani*** or ***Sclerotium rolfsii*** were observed, as compared with the nontransformed control strains. However, multicopy transformants allowed about 10% lower disease incidence. Furthermore, 30% higher degradation of the chitin content in the R. ***solani*** cell walls was observed during interaction with the overexpressing ***Trichoderma*** than with the wild type, when quantified by transmission electron microscopy.

In an attempt to increase its effectiveness, *T. harzianum* was transformed with plasmid pSL3chiAII containing a bacterial chitinase gene from S. ***marcescens*** under the control of the CaMV35S promoter. Two transformants showed increased constitutive chitinase activity and expressed a protein of the expected size (58 kDa). When evaluated in dual cultures against the phytopathogenic fungus S. ***rolfsii***, both showed higher antagonistic activity, as compared with the nontransformed control [70].

Other necrotrophic mycoparasites also secrete chitinases. An extracellular chitinase produced by ***Myrothecium verrucaria*** inhibits germination and germ tube elongation of the groundnut rust fungus ***Puccinia arachidis.*** Similarly, ***Acremonium obclavatum*** produces and secretes a chitinase ***in vitro*** which inhibits germination of uredospores of the peanut rust [71].

Penetration of fungal hosts by the biotrophic mycoparasite ***Piptocephalis virginiana*** [72] occurs by both mechanical and enzymatic mechanisms. Light and scanning electron microscopy studies have shown inpushing of the susceptible host cell wall and enzymatic erosion of the resistant host cell wall by the advancing infection hyphae [73]. Interestingly, culture filtrates of I! ***virginiana*** contained only negligible levels of chitinases and chitosanases, thus indicating strict regulatory control of these lytic enzymes, which is characteristic of a biotrophic mycoparasite [74]. Manocha [72] has proposed that metabolic shifts favoring chitinase occur in the susceptible host, ***Choanophora cucurbitarum*** and chitin synthase in the resistant host, ***Phoscolomyces articulosus*** when attacked by I! ***virginiana.*** Increased levels of chitinase activity induced in ***C. cucurbitarum*** may culminate in enhanced plasticity of the host cell wall and a limited incorporation of a chitin precursor at the penetration site due to degradation of nascent chitin by chitinase. By contrast, higher levels of chitin synthase may be present in I! ***articulosus*** because of deposition of chitin and papilla formation at the penetration sites [72].

Chitinases as defense and transgenes in plants

The role of plant chitinases in disease resistance is well documented [75]. Numerous plant chitinase genes or cDNAs have been cloned. In a successful case, transgenic tobacco plants were generated which constitutively expressed a bean endochitinase gene under the control of the cauliflower

mosaic virus 35S promoter. The transgenic tobacco plants were less susceptible to infection by Rhizoctonia solani, and either the disease development was delayed or they were not affected at all [76]. In other very interesting work, the possible functional interactions between two different hydrolytic enzymes, the rice RCH10 basic chitinase and the alfalfa AGLU1 acidic glucanase, by constitutive coexpression in transgenic tobacco was analyzed. Hybrid plants were generated by crossing transgenic parental lines exhibiting strong constitutive expression of CaMV35S enhancer1 RCH10 and CaMV 35S double promoter/AGLU1 gene fusions, respectively. Evaluation of disease development in these hybrids, heterozygous for each transgene, and homozygous selfed progeny, showed that combination of the two transgenes gave substantially greater protection against the fungal pathogen Cercospora nicotianae than either gene. These data led to the suggestion that combinatorial expression of antifungal genes could be an effective approach to engineering enhanced crop protection against fungal disease [77].

There are many other examples of the introduction of chitinase genes into plants under the control of constitutive promoters, resulting in enhancement of resistance of the host plant to fungal pathogens [78–80]. However, not all cases have been successful. When a tobacco chitinase gene was expressed in high levels in Nicotiana sylvestris, the transgenic plants were still as susceptible to C. nicotianae infection as wild-type plants [81]. Unfortunately, the role of various chitinases in mediating plant resistance to insects is less well understood.

Although the successful use of plant chitinases for controlling fungi is well documented, no reports of successful use of a plant chitinase in controlling insect pests are available. In fact, in spite of the substantial levels of chitinases found in cereal grains (10–100 μg/g), they are susceptible to insect attack, suggesting that stored-product insects have evolved to overcome plant chitinases. Furthermore, recently Kramer et al. [11] found that transgenic rice plants expressing relatively high levels of a rice chitinase have no detrimental effects on the growth of the fall armyworm Spodoptera *frugiperda*.

In other work, a cDNA encoding the major molting fluid chitinase of Manduca sexta was characterized. The M. sexta chitinase gene is not expressed during larval feeding behavior; it is switched on only during a narrow time frame just prior to larval-larval and larval-pupal molting. The activity of this gene is apparently tightly regulated by hormones, both positively and negatively [82]. Based on the tight developmental and hormonal regulation of the chitinase expression, the authors suggested that plants constitutively expressing it might be resistant to insects that feed on them because exposure to this enzyme might damage the gut lining. The same group generated chimeric gene constructs carrying the M. sexta chitinase under the control of single or double CaMV 35S promoter which were introduced into tobacco and tomato plants. Leaves from transformed

and control plants were excised and fed to first instar larvae of the tobacco budworm. After 3 weeks, the total mass of surviving larvae on control plants was 966 mg, whereas that on the chitinase transformed leaves was only 177 mg, a reduction of more than 80% [83].

To determine whether the Manduca chitinase from transgenic tobacco and several chitinases from other sources were directly toxic to insects, a beetle feeding study was conducted using purified enzymes. Recombinant Manduca chitinase from transgenic tobacco and chitinase from Serratia, *Streptomyces* and Hordem species were incorporated into a diet at a 1–2% level and fed to neonate beetle larvae. Whereas growth and mortality of larvae consuming the bacterial and plant chitinase-supplemented diets were the same as those of larvae consuming the untreated diet, all larvae consuming the insect chitinase-supplemented diet died a few days after egg hatch [83]. These data led to the suggestion that insect chitinases are potential host plant resistance factors in transgenic plants and might be more potent than chitinases from other sources.

Concluding remarks

All together, the data summarized in this chapter allow us to envisage chitinases as an important factor in the development of improved agents and novel strategies for biological control. Further work on cloning and characterization of chitinases will provide us the tools and understanding needed to make better use of these genes. The potential of chitinases is likely to be enhanced by combining them with other bioactive peptides and lytic enzymes, such as glucanases, as is found in natural systems [22, 84]. Thus, special emphasis should be made of the use of combinatorial strategies. The enormous potential of genetic engineering will allow us to combine the natural responses of plants with transgenes of microbial and/or insect chitinases, other bioactive peptides and improved microbial biocontrol agents.

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