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Update on Biotechnology

Strategies for Control of Fungal Diseases with Transgenic Plants

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Fungal diseases have been one of the principal causes of crop losses ever since humans started to cultivate plants. To date, the epidemic spread of fungal diseases is controlled by (a) various crop husbandry techniques, such as crop rotation and avoiding the spread of infested soil and pathogen-carrying plant materials, (b) breeding of fungus-resistant cultivars of crops, and (c) the application of agrochemicals. Although breeders have succeeded in producing cultivars resistant to fungal diseases, the time-consuming processes of making crosses and back-crosses and the selection of progenies for the presence of resistance traits make it difficult to react adequately to the evolution of new virulent fungal races. Therefore, farmers often have to use chemicals. However, agrochemicals are costly, and eventually they become less efficient due to the evolution of the pathogen. In addition, both their production and their persistence in the soil after use are potentially harmful to the environment.

The growing concern about the environment, together with a strong motivation to lower production costs, encourages the development of cultivars of crops that require few chemicals. Newly developed technologies in plant breeding such as restriction fragment length polymorphism techniques and gene transfer methods can be used to develop these cultivars. In contrast to conventional breeding, this latter technology allows the transfer of traits from one species into the genomes of plants of other species, with the preservation of the intrinsic properties of the acceptor plant.

Currently, much energy is being applied to 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. Furthermore, there is an extensive search for genes that encode enzymes involved in the synthesis of compounds toxic to fungi and for genes that encode proteins with a direct inhibitory effect on the growth of fungi. Moreover, possibilities to exploit genes encoding inhibitors of fungal enzymes to obtain resistance are being investigated.

RACE-CULTIVAR-SPECIFIC RESISTANCE TRAITS

The type of resistance trait most frequently used in breeding programs protects plants against one or a few races of a pathogen species only. Often, this so-called race-cultivar-specific resistance is manifested in a hypersensitive response that is characterized by fast, localized necrosis at the site of infection. As a result, the pathogen is contained usually in the region immediately surrounding the infection site, and spreading to noninfected parts of the plant is prevented. With regard to genetics, the race-cultivar-specific resistance reaction is explained by the gene-for-gene hypothesis, which states that the product of a dominant resistance gene in the plant interacts specifically with a pathogen race-specific elicitor, the product of a genetically dominant "avirulence" (*avr*) gene, resulting in the elicitation of a hypersensitive reaction (for review, see de Wit, 1992).

Resistance genes involved in race-cultivar-specific interactions are well known from conventional breeding programs. To isolate such resistance genes, various approaches are being pursued, including map-based cloning and transposon tagging (for review, see Bennetzen and Jones, 1992). The latter approach recently has resulted in the isolation of the first specific fungal resistance gene, the genetically defined *Hm1* locus in maize conferring resistance to race 1 of the fungus *Helminthosporium carbonum* (Johal and Briggs, 1992). Biochemically, resistance to this fungus is characterized by the presence in resistant maize genotypes of an NADPH-dependent reductase capable of reducing a small cyclic tetrapeptide called HC toxin (Meeley et al., 1992). This pathogen-produced toxin mediates the specific pathogenicity of *H. carbonum* race 1 on maize. The primary structure of the *Hm1* gene shows homology with the primary structure of other plant reductase genes, especially in the region conserved in NADPH- and NADH-dependent reductases and dehydrogenases and, therefore, most likely encodes the HC toxin-detoxifying enzyme responsible for resistance (Johal and Briggs, 1992).

After fungal resistance genes have been isolated they can be transferred to other cultivars and possibly to other species to provide resistance to specific races of fungal pathogens. However, the resulting transgenic plants will remain susceptible to other races of the pathogen. Furthermore, the large-scale growth of the newly bred fungus-resistant cultivars is likely to lead to the evolution of new virulent races, and the cultivation of crops as monocultures will promote rapid epi-

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Abbreviation: RIP, ribosome-inactivating protein.

demic outbreaks. Thus, the use of resistance genes involved in race-cultivar-specific interactions in the breeding for fungal resistance is limited by the specificity of the traits and by the relative ease with which they are overcome by new races of fungal pathogens. However, as will be discussed below, the pathogen-inducible simultaneous expression in transgenic plants of a specific resistance gene, together with the corresponding avirulence gene, has the potential to result in broad-range, nonspecific pathogen resistance (de Wit, 1992).

TWO-COMPONENT SYSTEM FOR NONSPECIFIC RESISTANCE

Races of the fungus *Cladosporium fulvum* with avirulence gene *avr9* elicit a hypersensitive response in tomato lines containing resistance gene *Cf9* (for review, see de Wit, 1992). Induction of the hypersensitive response is provoked by a small peptide of 28 amino acids, which is the mature product of the fungal *avr9* gene. When injected into leaves the purified protein elicits the same reaction as races of *C. fulvum* containing the *avr9* gene. Recently de Wit (1992) postulated a model for obtaining resistance against a broad range of pathogens in plants containing the *Cf9* gene. The model proposes the transfer of the fungal *avr9* gene into *Cf9* tomato and its subsequent expression under the direction of a promoter that is rapidly and locally inducible by a wide range of pathogens. Expression of the *avr9* gene will encourage an interaction between the small elicitor protein and the *Cf9* gene product, which will result in a resistance reaction manifested in a hypersensitive response. Because constitutive production of the two components of this necrosis-inducing system will result in cell death, the expression of the *avr9* gene should be regulated very strictly.

Upon isolation of the *Cf9* resistance gene, the regulation of its expression could be modified so that both components of the system are synthesized only in cells attacked by pathogens. Furthermore, the *avr9-Cf9* two-component system could be introduced as a gene cassette into plants that lack the *Cf9* gene. In plants in which the *avr9-Cf9* system does not function, a cassette could be introduced that contains another combination of a pathogen avirulence gene and the corresponding plant resistance gene.

If the concept of obtaining resistance, as proposed by de Wit, is achieved through practice, a strategy for pathogen resistance will have been developed that is superior to other known strategies because of its low specificity and its universal applicability. The success of the concept will depend on the availability of suitable promoters to direct the expression of resistance genes and corresponding avirulence genes.

COMPOUNDS TOXIC TO FUNGI

Under certain conditions, both microorganisms and plants produce low mol wt, antimicrobial substances. In plants, such compounds known as phytoalexins are often synthesized locally and accumulate after exposure to pathogens and/or stresses. In many cases, a correlation has been found between the concentration of phytoalexins and resistance to specific pathogens. For example, in grapevine the presence of the phytoalexin resveratrol (3,4′,5-trihydroxystilbene) is associated with resistance to the fungus *Botrytis cinerea*. Such correlations suggest that the production of phytoalexins in plants can result in resistance to certain pathogens. Recently, Hain and coworkers (1990) transferred a gene encoding stilbene synthase into tobacco. In groundnut, stilbene synthase is the key enzyme in the synthesis of resveratrol. Although the substrates required for the synthesis of resveratrol are present in tobacco as well as in most other plant species, stilbene synthase is not. The constitutive expression of the groundnut stilbene synthase gene in transgenic tobacco plants results in the synthesis of resveratrol (Hain et al., 1990), and the transgenic plants appear to be more resistant to infection by *B. cinerea* than control plants (R. Hain, personal communication). As is the case with the resistance genes involved in race-cultivar-specific interactions, generally the resistance that will be obtained by the production of toxic substances is specific and limited to those races of pathogens sensitive to the toxin.

ANTIFUNGAL PROTEINS

Proteins with the ability to inhibit the growth of fungi in vitro are abundantly present in the plant kingdom. Whether they are involved in the defense against fungal infections in vivo is not known. Nevertheless, the constitutive or pathogen-inducible expression of the corresponding genes in transgenic plants may render these plants fungus resistant. This idea is supported by examples of transgenic tobacco plants that show enhanced resistance against the fungus *Rhizoctonia solani*, which is brought about by the constitutive expression of genes encoding proteins shown to have in vitro antifungal activity (Broglie et al., 1991; Logemann et al., 1992).

Chitinases and β-1,3-Glucanases

The antifungal proteins most frequently described are probably chitinases and β-1,3-glucanases. These enzymes catalyze the hydrolysis of chitin and β-1,3-glucan, respectively, both major components of the cell walls of many fungi (Wessels and Sietsma, 1981). Through the breakdown of these components, the two hydrolases are thought to inhibit fungal growth. Four classes (I–IV) of plant endochitinases have been described (Mikkelsen et al., 1992), of which at least three are present in tobacco (Melchers et al., 1993a, and refs. therein). Three major classes (I–III) of β-1,3-endoglucanases are also found in this plant species (Ward et al., 1991). The class I hydrolases are localized in the plant vacuoles and are potent inhibitors of growth in vitro of many fungi. The combination of a class I chitinase and a class I β-1,3-glucanase, both in quantities insufficient to show an effect by themselves, results in a very strong antifungal activity, indicating that these hydrolases act synergistically (Leah et al., 1991; Melchers et al., 1993a). In contrast, class II hydrolases, which are very homologous to the class I hydrolases but which are localized extracellularly, are not antifungal, either alone or in combination with other proteins (Melchers et al., 1993a). It is not yet known whether hydrolases from classes III and IV exhibit fungal growth-inhibiting activities in vitro.

The tobacco genes encoding class I hydrolases are constitutively expressed in the roots and lower leaves. Still, tobacco

plants are susceptible to the fungal root pathogen *R. solani*. Upon introduction of a cauliflower mosaic virus 35S promoter-driven bean class I endochitinase gene in tobacco, the transgenic plants constitutively expressing the gene showed an enhanced resistance to *R. solani* (Broglie et al., 1991). Apparently, a certain basal level of an antifungal endochitinase is not sufficient to protect plants against fungi, whereas elevated levels may lead to enhanced resistance. In contrast, Neuhaus and coworkers (1991) found that the constitutive expression in *Nicotiana sylvestris* of a tobacco class I chitinase gene under the control of the cauliflower mosaic virus 35S promoter did not alter the susceptibility of the transgenic plants to the fungal leaf pathogen *Cercospora nicotianae*, despite the fact that *Cercospora* species are sensitive to chitinases in vitro. This could indicate that results obtained in vitro are difficult to extrapolate in vivo or that the in vitro antifungal proteins are not localized at the preferred site of action in the transgenic plants.

Most fungal infections are initiated in the spaces between cells and subsequently penetrate into the cells. Some fungi, including *Cercospora* species, grow predominantly or even exclusively in the intercellular spaces. Therefore, to protect plants against such fungi, antifungal compounds may need to be present in the intercellular spaces rather than inside the cells. The class I hydrolases showing antifungal activity in vitro are naturally localized in the vacuoles of the plant cell, and the transgenic plants constitutively expressing the class I chitinase gene produce the antifungal protein inside the cell and not in the intercellular spaces (Neuhaus et al., 1991). To target vacuolar antifungal proteins out of the cell, both a class I chitinase and a class I β-1,3-glucanase gene have been modified (Melchers et al., 1993b, and refs. therein). In transgenic plants expressing either of the two modified genes, the two hydrolases were found extracellularly. Both targeted proteins retained their fungal growth-inhibiting activity and together they still showed synergy in the inhibition of fungal growth in vitro. The transgenic plants have not yet been tested for resistance against *C. nicotianae*.

RIPs

Plant RIPs inhibit protein synthesis in target cells by a specific modification of 28S rRNA. As a consequence, elongation factor 2 binds less efficiently and the elongation step in protein synthesis is inhibited (for review see Stirpe et al., 1992). RIPs do not affect ribosomes of plants in which they are produced and show various degrees of specificity toward ribosomes of other plants. Fungal ribosomes can be targets of RIPs as well. In in vitro assays a barley RIP has a lower antifungal activity than chitinases or β-1,3-glucanases from barley. However, a strong synergy is observed when barley RIP is mixed with either of these two hydrolases (Leah et al., 1991). Recently, a barley RIP gene under the control of a wound-inducible promoter was introduced into tobacco. R_1 progeny showed an increased resistance to *R. solani* (Logemann et al., 1992).

Other Antifungal Proteins

In their search for proteins able to interfere with the growth of fungi lacking chitin in their cell walls, Woloshuk and coworkers (1991) identified in tobacco a salt-stress-inducible, vacuolar protein with an inhibitory effect on the growth in vitro of *Phytophthora infestans*. It was suggested that this protein, described as osmotin in the literature, inhibits the growth of *Phytophthora* by interfering with the fungal membrane, hence disturbing cellular function. As with the class I hydrolases, the protein could be targeted extracellularly by modification of the corresponding gene (Melchers et al., 1993b).

In addition to the plant antifungal proteins mentioned above, there is a whole array of small, basic, Cys-rich proteins exhibiting in vitro fungal growth-inhibiting activity. These include (a) recently identified seed proteins from *Raphanus sativus* (Terras et al., 1992), *Amaranthus caudatus*, and *Mirabilis jalapa*; (b) hevein, a lectin from *Urtica dioica*; and (c) thionins, which are antimicrobial peptides occurring in seeds and leaves of both mono- and dicotyledonous plants (Broekaert et al., 1992, and refs. therein). Some of these proteins, notably hevein, lectin, and the peptides from *A. caudatus*, bind specifically to chitin and share striking homologies in their primary structures with the N-terminal domain of class I chitinases. As yet, nothing is known about the mode of action of these antifungal proteins.

Although most proteins shown to inhibit the growth in vitro of saprophytic and phytopathogenic fungi have been identified in plant materials thus far, antifungal peptides do occur in microorganisms as well. For example, the fungus *Aspergillus giganteus* produces an extracellular protein of 51 amino acids with an inhibitory effect on the growth of other fungi (Nakaya et al., 1990, and refs. therein). One can envision that the constitutive production of antifungal proteins of microbial origin in transgenic plants may render these plants resistant to fungi as well.

INHIBITORS OF FUNGAL ENZYMES

To facilitate fungal colonization of plant tissue, pathogens produce endopolygalacturonases that hydrolyze plant cell wall homogalacturonans, thereby generating oligogalacturonides. Generally, these degradation products are too small to act as elicitors of defense reactions in the plant. The in vitro inhibition of the hydrolysis of polygalacturonic acid by a plant cell wall-associated, proteinaceous inhibitor results in prolonged existence of oligogalacturonides large enough to act as elicitors. This suggests that the inhibitor plays a role in the resistance of plants to fungal pathogens. It has been hypothesized that high-level, constitutive production of the inhibitor in transgenic plants may render these plants fungus resistant (Toubart et al., 1992, and refs. therein). The presence of an inhibitor would hamper fungal colonization of plant tissue as well as increase the half-life of in vivo generated elicitor-active molecules. Recently, the gene encoding the polygalacturonase-inhibiting protein of bean has been cloned and characterized (Toubart et al., 1992) and now can be used to test the effectiveness of the proposed strategy to obtain fungal resistance in plants.

CONCLUSIONS

Since the advent of plant genetic engineering the question has been asked regarding its possible contribution to the

control of fungal diseases. Now, the first reports of transgenic plants show enhanced resistance against specific fungi, although such crops have not reached the market yet. In addition, new concepts have been developed to obtain broad-range pathogen resistance, and these concepts will be worked out further in the next few years. Undoubtedly, increasing knowledge of the molecular basis of both pathogenicity and resistance will lead to the development of new strategies in the future.

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