THE DEVELOPMENT OF TRANSGENIC PLANTS FOR THE CONTROL OF PLANT DISEASES

L S Melchers and M H Stuiver
Mogen International, Leiden, the Netherlands

The development of transgenic plants for the control of plant diseases

L S Melchers, M H Stuiver
MOGEN International N.V., P.O. Box 628, 2300 AP Leiden, The Netherlands

ABSTRACT

Plant disease control is entering an exciting period with the development of transgenic plants showing improved resistance against pathogenic viruses, bacteria, fungi and insects. This paper will review the first successful attempts for engineering fungal resistance in crops and highlights two promising strategies. These advances form the basis of new integrated disease management strategies combining modern fungicides and transgenic approaches to provide effective disease control in agricultural crops.

INTRODUCTION

Protection against attack by detrimental micro-organisms and pests represents a major challenge for crop production in agriculture. Fungal diseases have been one of the main causes of crop losses ever since mankind started to cultivate plants. Presently, the control of epidemic spread of fungal diseases is mainly based on three strategies: (a) various husbandry techniques, such as crop rotation and avoiding the spread of infested soil and pathogen-contaminated plant materials, (b) breeding for resistant cultivars of crops, and (c) the application of agrochemicals. Although conventional plant breeding has made a significant impact in improving resistance of many crops to important diseases, the time-consuming processes of making crosses and back-crosses and the selection of the desired resistant progeny make it difficult to react adequately to the evolution of new virulent fungal races. Moreover, for many major fungal diseases these plant breeding techniques will not provide a solution because there are simply no natural sources of resistance available to the breeder.

Therefore, farmers often use fungicides in modern agriculture for crop protection. However, the use of these compounds is limited by their high costs and potentially harmful impact on the environment. In addition, extended application can reduce their efficiency due to the evolution of tolerant or resistant pathogens. The growing concern about the environment, together with a strong motivation to lower production costs, encourages the development of cultivars of crops that require less chemicals. The advent of plant transformation and advanced molecular techniques in plant breeding provides powerful tools for genetically improving crops for enhanced resistance to fungal diseases. The distinct advantage of transgenic technology is that it enables the plant breeder to cross species barriers, allowing genes from non-related plants and other organisms to be introduced into crop plants.

In this review, we discuss the current status of engineering resistance to fungal pathogens in crops and highlight two highly promising approaches for generating broad spectrum fungal-resistant crops. First, a strategy based on the expression of genes that encode proteins that have either a direct or indirect inhibitory effect on the growth of fungi (i.e., antifungal protein

strategy). Second, a strategy aiming for increased fungal resistance in plants by engineering controlled hypersensitive death based on the specific interaction of avirulence (Avr) gene and resistance (R) gene products (i.e. Avr/R two-component strategy) (De Wit 1992).

ANTIFUNGAL PROTEINS

Plants have several inducible defence mechanisms that act to limit pathogen infection, including increased lignification and cell wall cross-linking, production of small antibiotic molecules (i.e., phytoalexins), host cell death at the site of infection (i.e., the hypersensitive response) (Bowles 1990 and ref. therein), and the production of reactive oxygen species (Mehdy 1994). Many plants also develop an increased resistance against subsequent pathogen infection in uninfected tissues. This systemic acquired resistance (SAR) can be effective against viruses, bacteria, and fungi and is accompanied by the expression of a large set of genes termed pathogenesis related (PR) genes (van Loon 1987, Ward *et al.*, 1991).

These PR-genes most likely play an important role in the defence response of plants against fungal infections. The "antifungal protein" strategy is based on the constitutive expression of genes encoding proteins with fungitoxic or fungistatic capacity in transgenic plants to enhance resistance to fungal pathogens. There have been several reports of transgenic plants showing increased resistance obtained with the above approach (Table 1).

The hydrolytic enzymes, chitinase and ß-1,3-glucanase, capable of degrading the major cell wall constituents (chitin and ß-1,3-glucan) of most filamentous fungi, have been extensively studied in plants. Expression of a plant or bacterial chitinase gene in transgenic tobacco was shown to enhance the resistance of plants to *Rhizoctonia solani*, an endemic, chitinous, soilborne fungus that infects numerous plant species (Broglie *et al.*, 1991; Jach *et al.*, 1995).

Interestingly, the tobacco plants showed no enhanced tolerance to *Cercospora nicotianae* (Neuhaus *et al.*, 1991) indicating that results obtained *in vitro* are difficult to extrapolate to an *in planta* situation and that tolerance to a range of pathogens may require more than the simple over-expression of a single gene.

Researchers at Zeneca MOGEN were the first to demonstrate that constitutive co-expression of tobacco chitinase and ß-1,3-glucanase genes in tomato plants conferred higher levels of resistance to a fungal pathogen (*Fusarium oxysporum*) then either gene alone, indicating a synergistic interaction between the two enzymes *in planta* (Van den Elzen *et al.*, 1993; Jongedijk *et al.*, 1995). The effectiveness of this approach was further demonstrated in tobacco, where the simultaneous expression of a rice chitinase and alfalfa glucanase gene in tobacco showed a substantially greater protection against the fungal pathogen *Cercospora nicotianae*, than either transgene alone (Zhu *et al.*, 1994). Similar effects were found after co-expression of class II chitinase, class II glucanase and a type-I ribosome inactivating (RIP) genes from barley in transgenic tobacco (Jach *et al.*, 1995). Certain combinations (chitinase/glucanase and chitinase/RIP) provided 'significantly enhanced protection' against *Rhizoctonia solani* (Table 1). The group of J. Ryals at Novartis, has made a significant effort in the evaluation of these and other PR-proteins for their potential to provoke disease resistance in transgenic tobacco plants. Transgenic tobacco plants constitutively expressing PR1a, a protein with unknown

biochemical function, showed tolerance to infection by two Oomycete pathogens, *Peronospora tabacina* and *Phytophthora parasitica* (Alexander *et al.*, 1993) although again, this resistance did not extend to other pathogens tested. An interesting observation in this work was that the apparent disease resistance of a transgenic line did not correlate with the level of expression of the transgene. This is an observation that has been made on several occasions in other reported work.

Table 1 Increased resistance in transgenic plants

Plant	Gene(s)	Pathogen	Reference
Tobacco	PR-1a	*Peronospora tabacina, Phytophthora parasitica, Pythium*	Alexander *et al.* 1993
	SAR 8.2	*Peronospora tabacina, Phytophthora parasitica*	"
	SAR 8.2 + PR-1a	*Peronospora tabacina*	"
	Class III chitinase	*Phytophthora parasitica*	"
	Chi-I	*Rhizoctonia solani*	"
	Bean chitinase (CH5B)	*Rhizoctonia solani*	Broglie *et al.* 1991
	Barley RIP	*Rhizoctonia solani*	Logemann *et al.* 1992
	S. marcescens Chi-A	*Rhizoctonia solani*	"
	Barley Chi + Glu	*Rhizoctonia solani*	Jach *et al.* 1995
	Barley Chi + RIP	*Rhizoctonia solani*	"
	Rice Chi + alfalfa Glu	*Cercospora nicotianae*	Zhu *et al.* 1994
	Radish Rs-AFP	*Alternaria longipes*	Terras *et al.* 1995
Carrot	Tobacco Chi-I + Glu-I	*Alternaria dauci*	Melchers, unpubl.
	Tobacco AP24	*Alternaria radicina*	"
		Cercospora carotae	"
		Erysiphe heracleï	"
Tomato	Tobacco Chi-I + Glu-I	*Fusarium oxysporum*	Jongedijk *et al.* 1995
B. napus	Bean chitinase	*Rhizoctonia solani*	Broglie *et al.* 1991
	Tom./Tob. Chitinase	*Cylindrosporium conc.*	Grison *et al.* 1996
		Sclerotinia sclerotiorum	"
		Phoma lingam	"
Potato	AP24	*Phytophthora infestans*	Liu *et al.* 1994
	Glux-ox	*Phytophthora infestans*	Wu *et al.* 1995
		Verticillium dahliae	"
	Aly AFP	*Verticillium*	Liang *et al.* 1998
Rice	Rice Chi	*Rhizoctonia solani*	Lin *et al.* 1995
Wheat	Aly AFP	*Fusarium sp.*	Liang *et al.* 1998

There are several other examples of reported enhanced tolerance being conferred by the overexpression of a single gene. Overexpression of tobacco osmotin, a PR-5 protein, in potato significantly delayed the expression of lesions caused by *Phytophthora infestans* (Liu *et al.*, 1994; Zhu *et al.*, 1996). Interestingly, recent studies with transgenic monocot crops, including

rice and wheat, have yielded the first promising results in development of *Rhizoctonia solani* (Lin *et al.*, 1995) and *Fusarium* sp. (Liang *et al.*, 1998) resistance respectively, in these economically most important crops.

In addition to PR-proteins, a broad family of small antifungal peptides, termed plant defensins, has been discovered in plants. Several of these peptides have been isolated from seeds of a variety of plants, and demonstrated to possess antifungal activity *in vitro* against a broad spectrum of fungal pathogens. Constitutive expression of Rs-AFP2 (a small cysteine-rich plant defensin from radish), in tobacco conferred a high level of resistance to the foliar pathogen *Alternaria longipes* in plants producing high levels of Rs-AFP2 (Terras *et al.*, 1995).

Other examples of plant antifungal peptides include thionins from mono- and dicotyledonous plants, hevein, a lectin from *Urtica dioica* and lipid transfer proteins from different plant species (Broekaert *et al.*, 1997 and ref. therein). Recently, the first reports on the efficacy of these peptides at controlling fungal diseases were published. Eppel and co-workers (1997) demonstrated increased protection of *Arabidopsis* to *Fusarium oxysporum* by the overexpression of an endogenous thionin. Molina & Garcia-Olmedo reported that expression of a barley non-specific lipid transfer protein in *Arabidopsis* and tobacco conferred enhanced resistance to the bacterial pathogen *Pseudomonas syringae*. Such proteins have also been shown to demonstrate antifungal effects *in vitro*.

Finally, there are a number of proteins which do not fall into any of the classes described above. For example, the H_2O_2-producing enzyme oxalate-oxidase has been shown to accumulate in barley attacked by powdery mildew, *Erysiphe graminis* (Zhang *et al.*, 1995). Interestingly, Wu *et al.* demonstrated that constitutive expression in potato of another H_2O_2-generating enzyme, glucose oxidase, provided disease resistance against a range of plant pathogens, including *Erwinia carotovora*, *Phytophthora infestans* and *Verticillium* wilt disease (Wu *et al.*, 1995).

Most of the above promising reports are solely based on observations of increased fungal resistance in transgenic plants tested in phytotrons or greenhouse facilities. The technical challenge is now to translate such results to a robust effect in the field. A comprehensive study on field evaluation of transgenic carrot plants (*Daucus carota*) containing the tobacco class I chitinase and ß-1,3-glucanase genes demonstrated a high level of resistance against major pathogens of carrots (Melchers & Stuiver, unpublished). These include *Alternaria dauci, Alternaria radicina, Cercospora carotae* and *Erysiphe heraclei* (powdery mildew). A majority of the transgenic carrot lines which had resistance against one pathogen exhibited significant resistance against all four pathogens.

The demonstration of broad spectrum fungal resistance in transgenic carrot plants indicates proof of concept of the "antifungal gene" strategy. Another study on field-testing of transgenic canola constitutively expressing a chimeric chitinase gene resulted in enhanced resistance to *Cylindrosporium concentricum*, and to a lesser degree, *Phoma lingam* and *Sclerotinia sclerotiorum* following artificial inoculation (Grison *et al.*, 1996).

Avr/R two-component strategy

The hypersensitive response (HR) is one of the most powerful mechanisms that plants possess to resist pathogen attack. The genetically controlled induction of the HR is triggered in plant-pathogen interactions only if the plant contains a disease resistance protein (R) that recognises the corresponding avirulence (Avr) protein from the pathogen.

Figure 1. Transgenic carrot plants resistant against the fungus *Alternaria dauci* (left) and non-transgenic carrot plants severely affected by the same fungus (right)

In the absence of a functional resistance gene or avirulence gene product, no recognition occurs and the interaction between plant and pathogen results in disease. Resistance genes involved in race-specific interactions often provide full disease resistance and are well known from conventional breeding programmes. Over the past years, many disease-resistance genes have

been cloned and have been shown to present five different classes of genes. The specificity of HR-associated disease resistance limits its applicability in molecular breeding approaches. Therefore, it would be desirable to engineer broad-spectrum disease resistance based on the HR.

An interesting so called Avr/R strategy proposed by De Wit (1992) involves the transfer of an avirulence gene (i.e. *Cladosporium fulvum avr9* gene) into a plant containing the corresponding resistance gene (i.e. tomato *Cf9* gene) and its subsequent expression under the direction of a promoter that is rapidly and locally inducible by a wide range of fungal pathogens. Pathogen-induced expression of the *avr9* gene results in an interaction, either direct or indirect, between the small elicitor protein (Avr9) and the *Cf9* gene product, which will provoke a resistance reaction manifested by a hypersensitive response. A localized HR will be induced which prevents further spread of any invading pathogen that can be inhibited by an HR, followed by a general defense response.

Since, constitutive production of the two components of this necrosis-inducing system will result in cell death, the expression of either the elicitor or resistance gene has to be regulated very strictly. This strategy for developing fungal resistance in plants can be based on various Avr-R gene combinations identified to date, and is upon success superior to other known strategies because of its low specificity and universal applicability. The success of the Avr / R gene concept will depend on the correct timing and expression levels of the resistance gene (e.g.*Cf9*) and corresponding avirulence gene (e.g. *avr9*). Results from our lab and others have demonstrated proof of concept for this strategy in various crops including tobacco, tomato and potato. Transgenic Cf9-tomato lines containing the *avr9* gene under control of a pathogen-inducible promoter (i.e. PRP1, Martini *et al.*, 1993) showed increased resistance to *Oidium lycopersici* (powdery mildew), *Cladosporium fulvum* and the viral pathogen TSWV (Stuiver & Melchers, unpublished). Leaves of these resistant tomato lines, droplet-inoculated with *Phytophthora infestans*, exhibited clearly restricted growth of the late blight lesions compared to the infected control transgenic lines. Keller and co-workers reported a non-specific resistance upon pathogen-induced expression of a fungal elicitor (cryptogein) in tobacco containing the corresponding resistance gene (Keller *et al.*, 1998). This engineered resistance was effective against several diseases (including *Phytophthora parasitica* var. *nicotianae*, *Erysiphe* and tobacco mosaic virus). An alternative strategy exploring the *avr9*/*Cf9* system is based on the finding that transgenic tomato plants containing an active transposable element in *Cf9* and constitutively expressing *avr9* show some characteristics of SAR. These plants displayed a developmentally regulated cell death (i.e. HR) in plant sectors in which excision of the transposable element resulted in restoration of *Cf9* gene function and subsequently enhanced disease resistance to tomato powdery mildew and *Phytophthora infestans* (Hammond-Kosack *et al.*, 1994). Together these data demonstrate the feasibility of strategies that exploit the Avr/R system for engineering disease resistance in solanaceous plants. Studies to achieve broad spectrum resistance with the Avr-R strategy in other species, including monocotyledonous crops, are currently in progress. Next to the *avr9*-*Cf9* system a large number of genes of other two component systems have been cloned and are now available for plant biotechnologists to engineer resistance. Further understanding of the molecular mechanism responsible for the Avr-R gene mediated resistance is critical for establishing durable resistance in genetically engineered crops.

Future prospects

The field of plant disease control is in an exciting period with major breakthroughs in the elucidation of the molecular basis of disease resistance to a wide range of plant pathogens. Increased understanding of both plant defence mechanisms and pathogen action initiated world-wide the development of transgenic strategies for improving the resistance of plants towards pathogens. In contrast to the great success in production of commercially useful insect- and virus resistant crops, the production of fungus-resistant crops with commercially useful levels of resistance has not been achieved to date. However, an increasing number of reports on broad spectrum fungal resistance in different transgenic crops indicates that commercial introductions of fungus-resistant crops are to be expected within the next 4-8 years.

There is a considerable challenge for scientists in industry, academia and government institutions to provide solutions for the growing global demand for increased food-production and to achieve simultaneously the consumers needs for high quality food. Fungicides are expected to continue to form the main option for growers to achieve high levels of disease control and improving crop yields in the near future. The continuous effort in agrochemical research has developed a range of modern fungicides with new modes of action providing broad spectrum control in economically important crops (see Powell and Bright, this volume). It is clear now that the introduction of a single transgene is not sufficient to achieve a durable and broad spectrum resistance. One can predict that a combination of transgenic strategies will be needed to reduce the requirement for agrochemicals to control crop diseases. Future disease resistance research will focus on the successful integration of transgenic strategies into breeding programmes to develop durable resistance based on improved resistance of new commercial varieties in combination with a selective use of low doses of fungicides. In the longer term it is envisaged that farmers will achieve optimal disease control in individual crops by balancing the use of both transgenes and fungicides through integrated crop management.

ACKNOWLEDGEMENTS

We would like to thank Bart van Wezenbeek for critical reading of the manuscript and Melanie van Spronsen for her assistance in preparation of the manuscript.

REFERENCES

Alexander D; Goodman R M; Gut-Rella M; Glascock C; Weymann K; Friedrich L; Maddox D; Ahl-Goy P; Luntz T; Ward E; Ryals J (1993). Increased tolerance to two oomycete pathogens in transgenic tobacco expressing pathogenesis-related protein 1a. *Proceedings of the National Academy of Science USA* **90**, 7327-7331.

Bohlman G L (1994). The role of thionins in plant protection. *Critical Review in Plant Science* **13**, 1-16.

Bowles D J (1990). Defence-related proteins in higher plants. *Annual Review of Biochemistry* **59**, 873-907.

Broekaert W F; Cammue B P A; De Bolle M F C; Thevissen K; de Samblanx G W; Osborn R W (1997). Antimicrobial peptides from plants. *Critical Review in Plant Science* **16**, 297-323.

Broglie K; Chet I; Holliday M; Cressman R; Biddle P; Knowlton S; Mauvis C J; Broglie R (1991). Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. *Science* **254**, 1194-1197.

Cornelissen B J C; Melchers L S (1993). Strategies for control of fungal diseases with transgenic plants. *Plant Physiology* **101**, 709-712.

Cammue B P A; Thevissen K; Hendriks M; Eggermont K; Goderis I; Proost P; Damme J; Osborn R; Guerbette F; Kader J-C; Broekaert W F (1995). A potent antimicrobial protein from onion seeds showing sequence homology to plant lipid transfer proteins. *Plant Physiology*, **109**, 445-455.

De Wit P J G M (1992). Molecular characterisation of gene-for-gene systems in plant-fungus interactions and the application of avirulence genes in control of plant pathogens. *Annual Review of Phytopathology*. **30**, 391-418.

Epple P; Apel K; Bohlman H (1997). Overexpression of an endogenous thionin enhances resistance of Arabidopsis against *Fusarium oxysporum*. *The Plant Cell* **9**, 509-520.

Grison R; Grezes-Besset B; Schneider M; Lucante N; Olsen L; Leguay J-J; Toppan A (1996). Field tolerance to fungal pathogens of *Brassica napus* constitutively expressing a chimeric chitinase gene. *Nature Biotechnology* **14**, 643-646.

Hammond-Kosack K E; Harrison K; Jones J D G (1994). Developmentally regulated cell death on expression of the fungal avirulence gene *Avr9* in tomato seedlings carrying the disease resistance gene *Cf-9*. *Proceedings of the National Academy of Science USA* **91**, 10444-10449.

Jach G; Gornhardt B; Mundy J; Logemann J; Pinsdorf E; Leah R; Schell J; Maas C (1995). Enhanced quantitative resistance against fungal disease by combinatorial expression of different barley antifungal proteins in transgenic tobacco. *The Plant Journal*, **8**, 97-109.

Jongedijk E; Tigelaar H; van Roekel J S C; Bres-Vloemans S A ; Dekker I; van den Elzen P J M; Cornelissen B J C; Melchers L S (1995). Synergistic activity of chitinases and b-1,3-glucanases enhances fungal resistance in transgenic tomato plants. *Euphytica* **85**, 173-180.

Liang J; Wu Y; Rosenberger C; Hakimi S; Castro S; Berg, J (1998). AFP genes confer disease resistance to transgenic potato and wheat plants. *5th International Workshop on Pathogenesis-related proteins in plants; signalling pathways and biological activities.* Aussois, France.

Lin W; Anuratha C S; Datta K; Potrykus I; Muthukrishnan S; Datta S K (1995). Genetic engineering of rice for resistance to sheath blight *Bio/Technology* **3**, 686-691.

Liu D; Raghothama K G; Hasegawa P M; Bressan R A (1994). Osmotin over-expression in potato delays development of disease symptoms *Proceedings of the National Academy of Science USA* **91**, 1888-1892.

Logemann J; Jach G; Tommerup H; Mundy J; Schell J (1002). Expression of a barley ribosome inactivating protein leads to increased fungal protection in transgenic tobacco plants *Bio/Technology* **10**, 305-308.

Martini N; Egen M; Rüntz I; Strittmatter G (1993). Promoter sequences of a potato PR gene mediate transcriptional activation selectively upon fungal infection. *Molecular General Genetics* **236**, 179-186.

Mehdy M C (1994). Active oxygen species in plant defence against pathogens *Plant Physiology* **105**, 467-472.

Molina A; Garcia-Olmedo F (1997). Enhanced tolerance to bacterial pathogens caused by the transgenic expression of barley lipid transfer protein LTP2. *The Plant Journal*, **12**, 669-675.

Neuhaus J M; Ahl-Goy P; Hinz U; Flores S; Meins F (1991). High-level expression of a tobacco chitinase gene in *Nicotiana sylvestris*; susceptibility of transgenic plants to *Cercospora nicotianae* infection. *Plant Molecular Biology* **16**, 141-151.

Powell K A; Bright S W J (1998). Commercial considerations in the development of transgenic crops and agrochemicals for crop protection. *Brighton Conference*, Brighton, U.K.

Terras F R G; Eggermont K; Kovaleva V; Raikhel N V; Osborn R W; Kester A; Rees S B; Torrekens S; van Leuven F; Vanderleyden J; Cammue B P A; Broekaert W F (1995). Small cysteine-rich antifungal proteins from radish (*Rhaphanus sativus* L): Their role in host defence. *The Plant Cell*, **7**, 573-588.

Van den Elzen P J M; Jongedijk E; Melchers L S; Cornelissen B J C (1993). Virus and fungal resistance: from laboratory to field. *Phil. Trans. Royal Society of. London B*, **342**, 271-278.

Van Loon L C; Gerritsen Y A M; Ritter C E (1987). Identification, purification and characterisation of pathogenesis-related proteins from virus-infected Samsun NN tobacco leaves. *Plant Molecular Biology* **9**, 593-609.

Ward E R; Uknes S J; Williams S C; Dincher S S; Wiederhold D L; Alexander D C; Ahl-Goy P; Metraux J.-P; Ryals J A (1991). Coordinate gene activity in response to agents that induce systemic acquired resistance. *The Plant Cell* **3**, 1085-1094.

Wu G; Shortt B J; Lawrence E B; Levine E B; Fitzsimmons C; Shah D M (1995). Disease resistance conferred by expression of a gene encoding H_2O_2-generating glucose oxidase in transgenic potato plants. *The Plant Cell* **7**, 1357-1368.

Zhang Z; Collinge D B; Thordal-Christensen H (1995). Germin-like oxalate oxidase, a H_2O_2 producing enzyme, accumulates in barley attacked by the powdery mildew fungus. *The Plant Journal* **8**, 139-145.

Zhu B; Chen T H H; Li P H (1996). Analysis of late-blight disease resistance and freezing tolerance in transgenic potato plants expressing sense and antisense genes for an osmotin-like protein. *Planta* **198**, 70-77.

Zhu Q; Maher E A; Masoud S; Dixon R A; Lamb C J (1994). Enhanced protection against fungal attack by constitutive co-expression of chitinase and glucanase genes in transgenic tobacco. *Bio/Technology* **12**, 807-812.