FIELD TESTING AGAINST FOUR FUNGAL PATHOGENS OF TRANSGENIC *BRASSICA NAPUS* PLANTS CONSTITUTIVELY EXPRESSING A CHITINASE GENE

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ABSTRACT

An endochitinase gene placed under the control of the 35S CaMV promoter was introduced into a winter-type oilseed rape (*Brassica napus* var. *oleifera*) inbred line via *Agrobacterium rhizogenes* transformation. Transformed plants, expressing an active 26kD recombinant chitinase were challenged using four different fungal pathogens (*Alternaria brassicae*, *Cylindrosporium concentricum*, *Phoma lingam*, *Sclerotinia sclerotiorum*) in field trials at two different locations. When compared with the non-transgenic parental plants, these plants exhibited an increased resistance to these pathogens.

INTRODUCTION

Oilseed rape is susceptible to many fungal pathogens ; among them, *Cylindrosporium concentricum* , *Alternaria brassicae* , *Phoma lingam*, and *Sclerotinia sclerotiorum* are responsible for severe yield losses. Protection of the plants by chemical treatment or by integration of specific resistance by traditional breeding has not always been effective.

Plant resistance to pathogen attack involves the accumulation in the plant cell of proteins active in defense mechanisms which include strengthening of the cell walls, synthesis of antimicrobial compounds, accumulation of fungal lytic enzymes inhibitors, and induction of lytic enzymes of the fungal cell walls. However, these mechanisms are often too weak or appear too late to be efficient in protecting the plant.

The development of techniques of gene transfer to plants has enabled testing of constitutive over-expression of proteins involved in active defense mechanisms. Chitinases catalyse the hydrolysis of chitin, poly-ß-1,4-N-acetylglucosamine, which is a major component of cell walls of most fungi except the Oomycetes ; their ability to inhibit fungal growth has been shown *in vitro*. The constitutive expression of chitinase genes into transgenic plants has been shown to increase, in greenhouse trials, plant resistance against one pathogen (Broglie *et al*, 1991) ; however, such resistance has not yet been reported for field trials.

We developed transgenic oilseed rape plants constitutively over-expressing a chimeric chitinase gene under the control of the cauliflower mosaic virus 35S promoter. The characterized T_3 progeny (third generation of selfed progeny) was challenged, at two different geographical locations, by artificial inoculation with the four fungal pathogens listed above.

EXPERIMENTAL

Chimeric chitinase gene

A genomic clone was isolated from a tomato DNA library. The alignment of the sequence of this clone with several chitinase sequences showed that the 3'-end of

the coding sequence was lacking. The 1061 bp 5' coding sequence of the tomato clone was completed with a synthetic 78 bp sequence homologous to the 3'-end of a tobacco cDNA. The complete coding sequence was then inserted between the CaMV 35S promoter and the nopaline synthase terminator and cloned into the polylinker of the binary vector pBIN 19. The entire 1860 bp gene sequence is given in patent application EP#WO 92/01792 (Dubois *et al*, 1992).

Rapeseed transformation

The endochitinase gene was introduced into an oilseed rape inbred line RC 624 (Rustica Prograin Génétique) by *Agrobacterium rhizogenes*-mediated transformation. The transformed roots obtained after co-cultivation are analysed for chitinase expression. Those expressing the recombinant chitinase are induced to proliferate into a callus from which plants are regenerated, grown, then selfed after vernalization to give T_1 seeds. Seventy-five transgenic oilseed rape plants expressing the chitinase have thus been generated. T_1 plants expressing the chitinase and lacking the pRiA4 T-DNA were selfed to produce T_2, then T_3 seeds which were used for field experiment. Chitinase specific activity in extracts from young greenhouse-grown transgenic plants (cotyledons and hypocotyls) was 2 to 4.5 times higher than in control plants, with a mean of 3.25. Under field conditions, a similar order of increase was observed in the leaf extracts.

Evaluation of transgenic plants

Plant resistance was evaluated at two locations under different climatic conditions : in the south-west of France (mild and humid winter, warm spring) and in the north of France (cold and dry winter, cool and humid spring).

For *C. concentricum* resistance evaluation, plants were contaminated by spraying a suspension of conidia on the plants once before winter and again after winter when new leaves appear. In the case of *A. brassicae*, field-grown plants were contaminated by an indoor method, because the humid and warm weather conditions expected in the southern location that favour field infections were not encountered during the experiment. A high infection pressure of *P. lingam* was obtained by spreading on the soil at sowing, oilseed rape straws and root collars harboring cankers symptoms. *S. sclerotiorum* inoculation was performed by inserting in the stem a match stick colonized by mycelium of the fungus. For each pathogen tested, 6 randomized block replicates consisting of 4 rows of 100 plants per genotype (T_3 generation) were sown in each location, with the exception of *S. sclerotiorum*, tested in only one site. Inoculation, observation, and disease resistance scoring were performed on the two central rows only to avoid possible interference due to plant competition effects.

RESULTS AND DISCUSSION

Resistance to *C. concentricum* was evaluated at regular intervals until the end of flowering, by measuring the percentage of contaminated plants or, in the case of high disease incidence, the percentage of diseased leaves and of leaf surface colonized by the fungus. In the northern location, where all plants were infected, resistance was observed by a decrease in disease symptoms (for example 49 % mean reduction in the percentage of leaf surface colonization). In the southern location, because of climatic conditions, infection level was lower, and resistance was shown by the decrease of the number of plants affected by the disease (mean reduction of 76 %). A significant correlation exists between the results of the two

sites. The increased disease resistance observed in transgenic plants varied with the infection level observed in the field, a property of quantitative resistance.

As for *A. brassicae*, all but one transgenic family exhibited a significant increase of resistance to this fungus, leading to a reduction in the symptoms severity on pods.

Colonization by *S. sclerotiorum* decreased in several transgenic genotypes. The average stem necrosis length 54 days after inoculation was 6.9 cm for the best transgenic line as compared with 32.6 cm for the non-transgenic control line.

Transgenic plants presented a slight increase of resistance against *P. lingam* (disease index reduction from 23 to 35 %) ; one genotype showed a significant increase of resistance to the basal stem canker in both locations.

Constitutive expression of a tomato endochitinase gene in oilseed rape lines increases field resistance to fungal infection. This higher resistance, demonstrated for the first time in the field, is obtained in oilseed rape against four pathogens responsible for the most severe damages under European culture conditions. The protection observed varies from one disease to another and seems to be complex, involving both a delay in the appearance of symptoms and a reduction in lesion number, size and extent. This protection probably results from both direct effects due to degradation of the chitin in the cell walls of growing hyphae (Benhamou *et al*,1993) and indirect effects due to the release of chitin oligomers which are active elicitors of plant defense mechanisms. Differential plant responses to the pathogens may have different origins : biochemical composition and structure of the fungal cell wall (differing chitin content, localization and accessibility to chitinase), tissue and cellular localization of the chitinase (concordance with the infection start site and progression path of the hyphae), concordance in the chitinase expression kinetics and period of infection, and type of interaction between the plant and the pathogen (duration of the biotrophic phase, etc).

We have shown by chemical and immunocytolocalization studies that the four pathogens used in this experiment possess various chitin content and localization. These observations, combined with the chitinase expression pattern driven by the 35S promoter, can explain the differences in the protective effects against the four pathogens.

Field trials are underway to evaluate the yield of these transgenic plants under fungal infection.

REFERENCES

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