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Field performance of chitinase transgenic silver birches (*Betula pendula*): resistance to fungal diseases

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Abstract A field trial of 15 transgenic birch lines expressing a sugar beet chitinase IV gene and the corresponding controls was established in southern Finland to study the effects of the level of sugar beet chitinase IV expression on birch resistance to fungal diseases. The symptoms caused by natural infections of two fungal pathogens, *Pyrenopeziza betulicola* (leaf spot disease) and *Melampsoridium betulinum* (birch rust), were analysed in the field during a period of 3 years. The lines that had shown a high level of sugar beet chitinase IV mRNA accumulation in the greenhouse also showed high sugar beet chitinase IV expression after 3 years in the field. The level of sugar beet chitinase IV expression did not significantly improve the resistance of transgenic birches to leaf spot disease. Instead, some transgenic lines were significantly more susceptible to leaf spot than the controls. The level of sugar beet chitinase IV expression did have an improving effect on most parameters of birch rust; the groups of lines showing high or intermediate transgene expression were more resistant to birch rust than those showing low expression. This result indicates that the tested transformation may provide a tool for increasing the resistance of silver birch to birch rust.

Introduction

In recent years biotechnology and plantation forestry have been seen as potential solutions for meeting the globally increasing demands for pulp, paper and timber products. Biotechnology has many benefits compared to conventional tree breeding by providing the potential to transfer specific traits into selected genotypes without the need for time-consuming breeding programmes. Genetic transformation provides the tools for shortening the long juvenile phases and overcoming the difficulties caused by complex reproductive systems and inbreeding. In addition to manipulations in the molecular control of flowering (Weigel and Nilsson 1995; Nilsson and Weigel 1997; Brunner et al. 2000; Rottmann et al. 2000; Peña et al. 2001) most efforts in molecular tree improvement have been directed to modifications in wood properties, especially the quantity and quality of lignin (Hu et al. 1999; Lapierre et al. 1999; Franke et al. 2000; Pilate et al. 2002; Li et al. 2003). To a lesser extent, biotechnological methods have been tested in attempts to increase the insect and disease resistance of commercially important tree species (Schuler et al. 1998; Jacobi et al. 2000; Delledonne et al. 2001; Liang et al. 2001). Promising results against viral diseases have been obtained by introducing viral coat proteins into trees (Tennant et al. 1994; Scorza et al. 2001). The possibilities to improve resistance against bacterial and fungal diseases have been tested by introducing genes encoding antimicrobial recombinant proteins of plant or non-plant origin into trees, but these approaches are in the early stages of development (Peña and Séguin 2001).

Chitinases are examples of antifungal cell-wall degrading enzymes that have been successfully used in genetic engineering in various economically important plant species in order to improve plants' resistance against fungal diseases (Broglie et al. 1991; Toyoda et al. 1991; Asao et al. 1997; Lorito et al. 1998; Tabei et al. 1998). Chitinases are produced as a general stress response, and various differentially regulated isoforms possess distinct roles in plant defence (Collinge et al. 1993). Chitinases catalyse the hydrolysis of chitin, a β-1,4-linked polymer of *N*-acetyl-D-glucosamine that is the major component of the cell wall of most filamentous fungi (Bartnicki-Garcia 1968). Hydrolysis of the chitin found in the fungal hypha by chitinases kills the fungus without causing damage to the cells of the plant. In addition to acting in plant defence reactions, chitinases may play a role in functions related to plant growth such as cell division, differentiation and

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development (Collinge et al. 1993; Sahai and Manocha 1993; Patil and Widholm 1997).

The expression of a basic chitinase IV gene from sugar beet in transgenic silver birch (*Betula pendula* Roth) improved the resistance of birch against the leaf spot fungus *Pyrenopeziza betulicola* (Fuckel) in a greenhouse experiment (Pappinen et al. 2002). *Pyrenopeziza betulicola* has recently been identified as the major causative agent of leaf spot disease in silver and pubescent (*Betula pubescens* Ehrh.) birch in Finland (Paavolainen et al. 2000). The pathogen causes the premature yellowing and shedding of leaves, which may reduce photosynthesis and cause problems in frost hardening. Another main fungal pathogen on silver birch is the leaf rust fungus *Melampsoridium betulinum* (Kleb.) that infects and colonises the green leaves of the host plant in late summer. This pathogen is considered to be harmful mainly in nurseries where premature shedding and frost damage are common in rust-infected birch seedlings (Kurkela 1994; Poteri 1998). Birch rust infection has also been reported to decrease the growth of the seedlings during the next spring (Lilja 1973). In nurseries, fungal diseases are usually controlled by fungicides.

An essential prerequisite for the use of genetically modified (GM) trees is the development of more thorough methods and procedures for evaluating any potential environmental risks included in the release of these trees. Because of the complex nature of forest ecosystems and the longevity of forest trees, risk assessments should be very sensitive to temporal and spatial factors. In many cases, the unavailability of applicable ecological and biological data has created a problem in the risk assessment of GM organisms (Koivisto et al. 2001). Secondly, the longevity of forest trees also creates challenges for studying the stability of transgene expression in different environmental conditions over long periods of time. Although the number of studies on the molecular mechanisms and other factors influencing the stability of transgene expression in trees is gradually increasing (Fladung 1999; Cervera et al. 2000; Kumar and Fladung 2000, 2001, 2002), there are still relatively few studies on transgene expression under natural conditions (but see, for example, Kumar and Fladung 2001). The critical point for stable transgene expression often is the transfer of transgenic lines from in vitro conditions to the greenhouse or field (Kumar and Fladung 2001), which emphasises the importance of field trials.

Extensive field trials with transgenic trees, including poplars, aspen, silver birch, pines, spruce, eucalyptus and fruit trees like apple, plum, cherry and orange, are currently being carried out in many countries throughout the European Union and America (Peña and Séguin 2001; McCord and Gartland 2003). In the European Union, 43 field tests of transgenic fruit and forest trees had been reported by 2003, and the transgenes include resistance genes (viruses, insects, fungi, herbicides), lignin biosynthesis genes, marker genes and *rol* genes (McCord and Gartland 2003). Since most of the field trials have been established during the last few years, not many reports have been published to date. In the investigation reported here, we studied the effects of the level of sugar beet chitinase IV gene expression in transgenic silver birches on the resistance against two fungal diseases—leaf spot disease and birch rust—in a 3-year field trial. The field trial was designed to produce information that can be utilised in assessing the risks of GM silver birches as well as in evaluating the possibilities of using sugar beet chitinase to improve birch resistance against fungal diseases.

Materials and methods

Plant material

Agrobacterium strain LBA4404 carrying the Ti-plasmid pAL4404 was used in gene transfer (Hoekema et al. 1983). The binary plasmid (pBKL4 K4, courtesy of Dr. Mikkelsen, Danisco) contained a sugar beet chitinase IV gene with the enhanced (fourfold) cauliflower mosaic virus (CaMV) 35S promoter. Transformation was performed as described in Keinonen-Mettälä et al. (1998). Leaf explants from an elite Finnish birch clone (JR 1/4) in tissue culture were used as the recipient plant material in gene transformations. Pre-culturing the plant tissue, co-culturing with *Agrobacterium* and selection of transgenic tissue have been described in more detail in Pappinen et al. (2002). Fifteen transgenic lines were selected for further analyses and transferred after in vitro propagation to the greenhouse. In autumn 2000, after 1.5 years in the greenhouse, 15 seedlings from each chitinase transgenic line and the corresponding non-transgenic control clone were planted in the field. The field trial was established in the Viikki area of Helsinki as a randomised block design consisting of 15 different blocks, each containing one replicate of each type of the seedling, and was carried out with the permission of the Board of Gene Technology, Ministry of Social and Health Affairs, (notification no. 2/MB/00). Regulations on the safe handling of GM material were applied (Directive 2001/18 EC). The field trial was monitored for three growing periods from September 2001 to September 2003. The field trial was harvested in October 2003.

Molecular analysis

Expression of the sugar beet chitinase IV gene in different transgenic lines was confirmed by Northern blot analysis by studying the level of sugar beet chitinase IV transcript accumulation. Leaf samples were collected from each chitinase transgenic line and the corresponding non-transgenic control clone from one selected field trial block in August 2003. Total RNA was extracted from each sample as described by Chang et al. (1993). The RNA was blotted and hybridised as described in Church and Gilbert (1984) (see more details in Pappinen et al. 2002). Northern analysis was repeated twice.

Disease scoring in the field

The data presented in this paper were collected from the field trial between September 2001 and September 2003, which covered the whole duration of the field trial excluding the year in which the trial was established. After natural infections, the symptoms of two fungal diseases—birch rust caused by *Melampsoridium betulinum* and leaf spot disease caused by *Pyrenopeziza betulicola*—were analysed from the leaves of the seedlings. The following disease parameters were measured: the number of leaf spots or rust postules on the surface of the leaves; percentage of the leaf area covered by leaf spots or rust postules; general disease score that was given to each seedling on the basis of the general appearance of the fungal

disease. The disease parameters were analysed from three leaves per seedling. Leaf spot and birch rust were analysed from different leaves. For leaf spot, three branches from different heights were selected from each seedling and the disease symptoms analysed from the lowest leaf of the lowest side branch. For birch rust, three branches from varying heights were selected and the disease symptoms analysed from the fifth highest leaf of that branch. The selected positions of the analysed leaves represented well the general occurrence of the fungal diseases studied.

The number of disease spots was individually counted from the selected leaves of each seedling in July 2002 and in August 2003. In 2001, individual disease spots could not be separated and counted due to the severe symptoms of both diseases. Percentage area covered by the fungal diseases and general disease scores were estimated at the end of the growing season in September 2001, 2002 and 2003. The percentage of leaf area covered by the fungal diseases was estimated visually using a scale of 1 to 5 (1=no disease spots; 2=1–5%; 3=6–25%; 4=26–75%; 5=76–100% of the leaf area covered by disease spots). A general disease score was given to each seedling based on the severity of the diseases. The following classification was used for both fungal diseases: (1) no disease; (2) a few disease spots on some leaves (disease spots cover less than 50% of the leaf area); (3) a few disease spots on 50% of the leaves; (4) very few disease spots but on almost all leaves (disease spots cover 1–10% of the leaf surface); (5) some disease spots on almost all leaves (disease spots cover 10–50% of the leaf surface); (6) some or fairly many disease spots on almost all of the leaves (disease spots cover 50% or more of the leaf surface); (7) all leaves heavily infected (disease spots cover 80–100% of the leaf surface).

Data analysis

The differences among the lines with respect to the number of leaf spots or rust postules, percentage leaf area covered by the disease and the general disease score were studied by one-way ANOVAs using seedling height as a covariate. Dunnet's test was used to test the differences between the transgenic lines and the non-transgenic control clone. In order to study the effects of the level of sugar beet chitinase IV gene expression on the disease parameters, we classified the transgenic lines into three groups based on their level of chitinase IV mRNA accumulation: high expression, intermediate expression and low expression. One-way ANOVA was used to study the effects of the level of transgene expression on the disease parameters. If necessary, square-root transformation was applied to normalise the data. Parametric tests were used for all of the parameters studied except for the mean number of rust postules that was analysed by the non-parametric Kruskal-Wallis test. For the mean number of rust postules, Mann-Whitney *U*-tests were used for pairwise comparisons between the transgenic lines and the non-transgenic control clone. Data collected from different years were analysed separately. The SPSS 10.0 statistical package for Windows was used for all the statistics.

Results

Sugar beet chitinase IV gene expression in the field

Northern blot analyses of the leaf samples collected from the field trial in August 2003 showed a sugar beet chitinase IV transcript accumulation in all of the lines studied, indicating that they were stable transgenics. The transgenic lines could be classified into three groups according to the level of sugar beet chitinase IV mRNA accumulation. Lines 4, 8, 10, 12, 14 and 15 showed high expression, lines 2, 3, 5 and 9 intermediate and lines 1, 6 and 13 low expression (Fig. 1).

Leaf spot disease

After the first growing season in the field, 14 transgenic lines showing varying levels of sugar beet chitinase IV transcript accumulation differed significantly from the non-transgenic control clone with respect to the general disease score, but none of the former showed improved resistance to leaf spot disease (data not shown). In 2002, significant differences between the transgenics and the control were only detected for mean number of leaf spots, with four lines being significantly more susceptible to leaf spot disease than the control clone (Fig. 2). In 2003, four lines for percentage leaf area covered by leaf spots and nine lines for the general disease score showed significantly more severe symptoms of leaf spot disease than the control (Fig. 3).

One-way ANOVA of the effects of the level of sugar beet chitinase IV transcript accumulation on the studied parameters of leaf spot disease revealed significant effects of the transgene expression on the number of disease spots in 2002 and on the percentage leaf area and general disease score in 2003 (Table 1). In 2002, the number of leaf spots was significantly lower for the group of lines showing high expression than for the groups of intermediate or low expression, but the opposite trend was detected for percentage leaf area and general disease score in 2003.

Birch rust

The symptoms of birch rust were more severe in the first 2 years of monitoring the field trial than in the last year

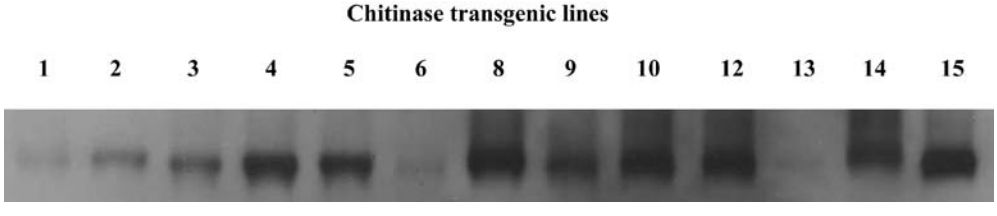

Fig. 1 Northern analysis of the leaf samples collected from the field in August 2003 (lines 7 and 11 were not included in the analysis). The samples contained 15 μg RNA

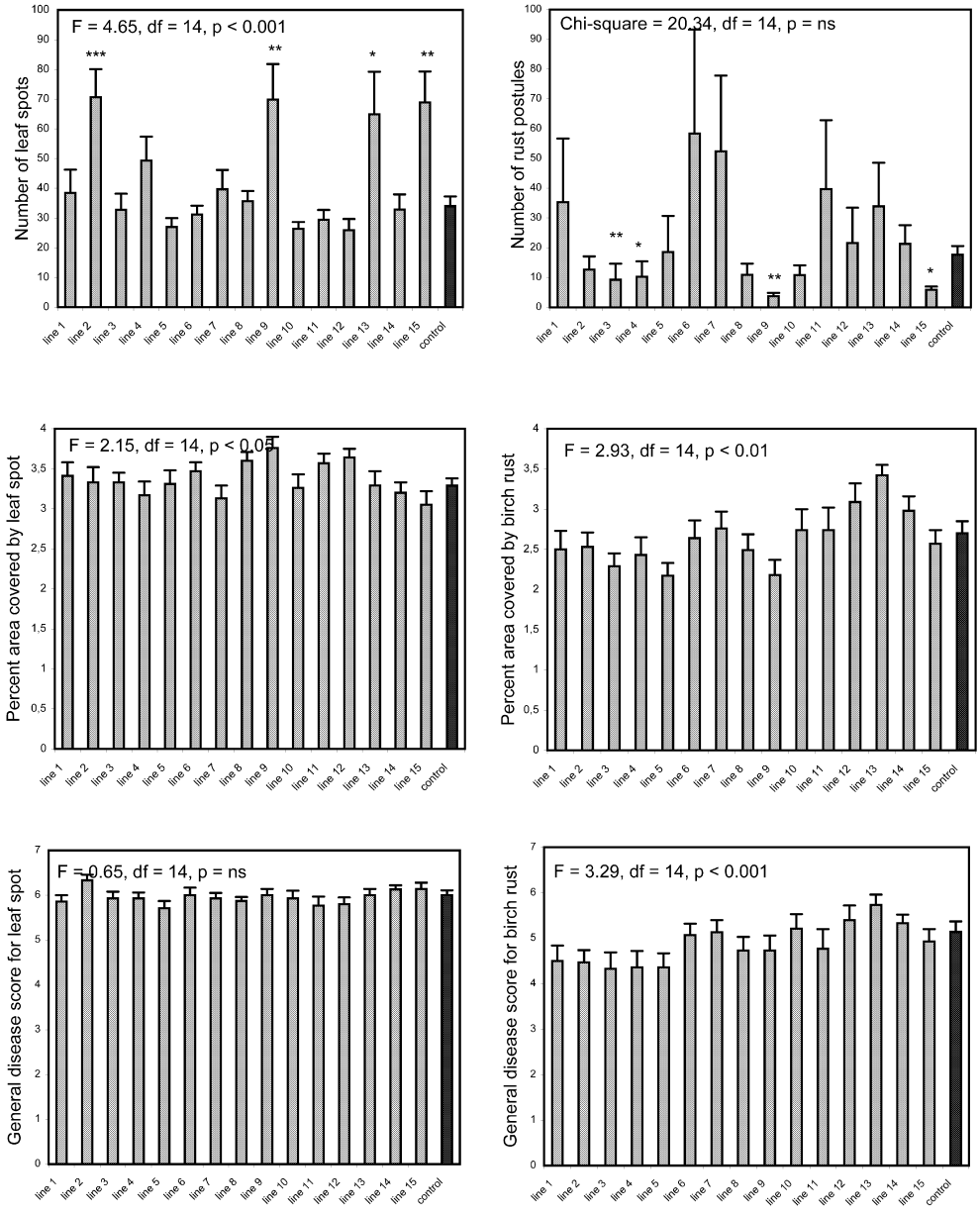

Fig. 2 Variation among the transgenic lines with respect to three parameters of leaf spot disease and birch rust in 2002. *Stars above columns* indicate significant differences between the transgenic line and the non-transgenic control clone. **P*<0.05; **0.01>*P*>0.001; ****P*<0.001

Table 1 One-way ANOVA of the effects of the level of sugar beet chitinase IV transcript accumulation on three parameters of leaf spot disease and the means of the groups of transgenic lines showing varying levels of transgene expression

Year	Variable	Expression groups			One-way ANOVA	
		High expression	Intermediate expression	Low expression	*F*	*P*
2001	Number of disease spots	–	–	–		
	Percentage leaf area	3.39	3.49	3.49	0.01	0.992
	General disease value	4.79	5.00	5.02	0.99	0.372
2002	Number of disease spots	39.03	49.85	43.94	3.75	0.025
	Percentage leaf area	3.32	3.42	3.37	0.36	0.699
	General disease value	5.96	6.02	5.93	0.52	0.596
2003	Number of disease spots	28.36	25.96	24.07	1.94	0.146
	Percentage leaf area	2.60	2.56	2.40	3.10	0.047
	General disease value	5.93	5.72	5.63	3.48	0.033

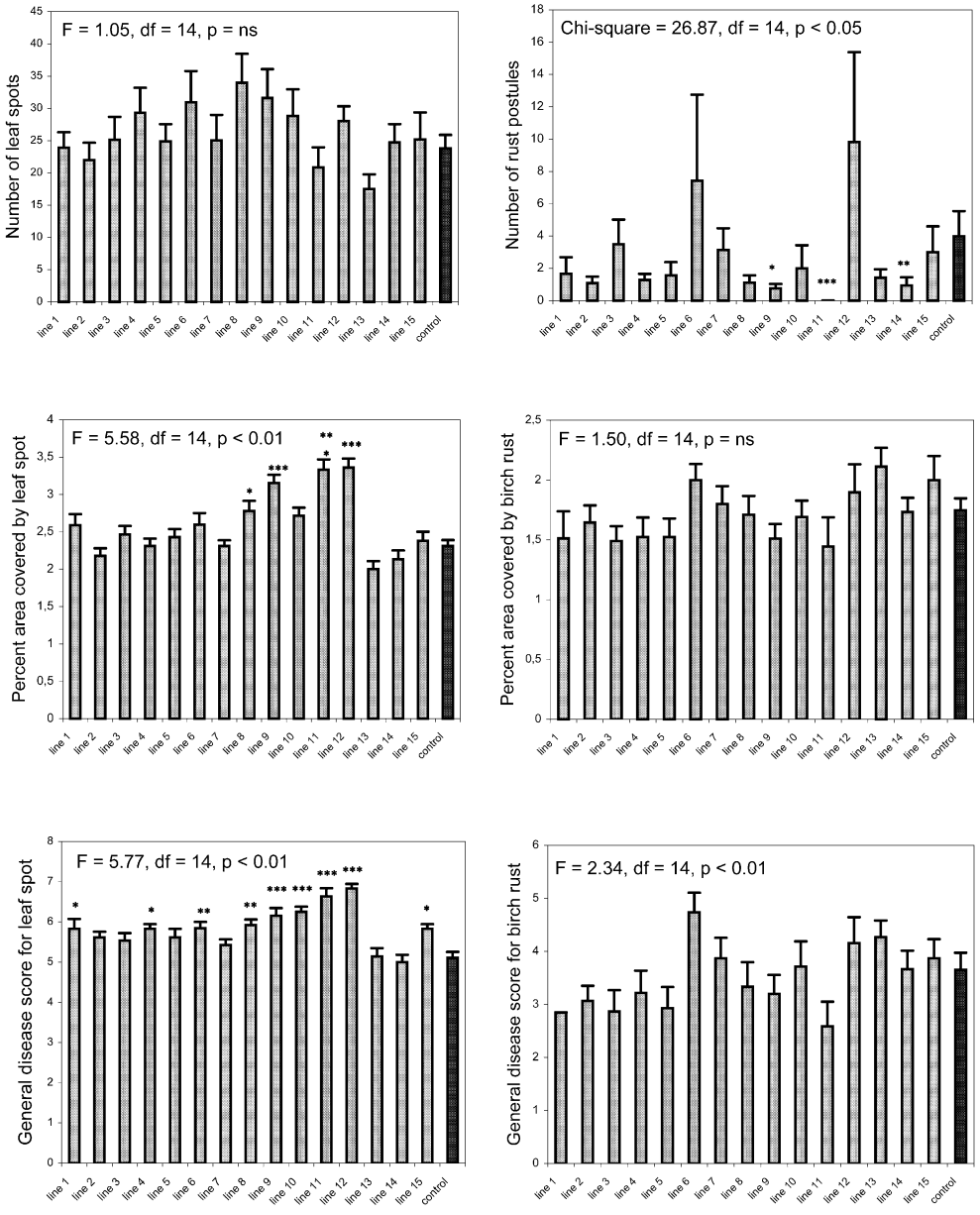

Fig. 3 Variation among the transgenic lines in three parameters of leaf spot disease and birch rust in 2003. *Stars above columns* indicate significant differences between the transgenic line and the non-transgenic control clone. **P*<0.05; **0.01>*P*>0.001; ****P*<0.001

when natural fungal infections were unusually slight. In 2001, significant differences among the transgenic lines were detected for percentage leaf area covered by birch rust and for the general disease score, but only one transgenic line (line 10) differed significantly from the control clone, showing more severe symptoms of birch rust than the latter (data not shown). In 2002 and 2003, significant differences between the transgenic lines and the control were detected only for one parameter—the number of rust postules (Figs. 2, 3). Four (2002) and three (2003) transgenic lines showed improved resistance to birch rust. Interestingly, some of the lines (lines 9 and 15) showing improved resistance to birch rust in 2002 showed concurrently decreased resistance to leaf spot disease when compared to the non-transgenic control clone (Fig. 2). However, no significant negative correlations between the parameters of leaf spot disease and birch rust were found.

The level of sugar beet chitinase IV transcript accumulation had a significant influence on all of the parameters of birch rust studied in 2002 and on percentage leaf area covered by rust and general disease score in 2003. The general trend was that the group of trees showing low sugar beet chitinase IV expression had more severe symptoms of birch rust than the groups with high or intermediate expression (Table 2). This pattern was especially clear in the number of rust postules in 2002. Of all of the measured parameters, the group showing intermediate sugar beet chitinase IV expression showed the best performance in terms of resistance to birch rust. On the basis of this data it seems that sugar beet chitinase IV may enhance the resistance of silver birch against birch rust.

Table 2 One-way ANOVA of the effects of the level of sugar beet chitinase IV transcript accumulation on three parameters of birch rust and the means of the groups of transgenic lines showing varying levels of transgene expression. Variation among the groups in the number of rust postules was analysed by the non-parametric Kruskal-Wallis test

Year	Variable	Expression groups			One-way ANOVA	
		High expression	Intermediate expression	Low expression	*F*	*P*
2001	Number of rust postules	–	–	–		
	Percentage leaf area	3.23	2.75	2.93	1.77	0.173
	General disease value	3.88	3.37	3.49	1.45	0.236
2002	Number of rust postules	13.45	11.21	43.52	χ^2=7.61	0.022
	Percentage leaf area	2.72	2.28	2.85	7.50	0.001
	General disease value	5.00	4.44	5.09	4.67	0.010
2003	Number of rust postules	3.00	1.67	3.46	χ^2=0.80	0.670
	Percentage leaf area	1.77	1.53	1.87	3.88	0.022
	General disease value	3.68	2.98	3.93	5.87	0.003

Discussion

We report here stable sugar beet chitinase IV gene expression expressed as mRNA transcript accumulation in transgenic silver birches and the effects of the transgene expression on birch resistance against two fungal diseases during a 3-year field trial. Expression of the chitinase IV gene was studied by Northern analyses of the leaves of the transgenic lines grown in the greenhouse before they were transferred to the field (Pappinen et al. 2002) and before harvesting the field trial. The same four lines that showed a high level of sugar beet chitinase IV transcript accumulation in the greenhouse also showed high sugar beet chitinase IV expression after 3 years in the field. The main difference between transgene expression in the greenhouse and that in the field was that some lines showing no sugar beet chitinase IV expression in the greenhouse did show varying levels of chitinase IV transcript accumulation in the field. There are many factors that can cause fluctuations in transgene expression. In the transformation process, foreign DNA is randomly integrated into the plant genome, and the integration site, configuration and copy number of the integrated transgene along with metabolic and environmental factors may all influence the stability of transgene expression (Fladung 1999; Levée et al. 1999; Cervera et al. 2000; Kumar and Fladung 2001). In chitinase transgenic birches, the copy numbers of the sugar beet chitinase IV gene were studied before the seedlings were transferred to the field and found to vary between two and four (Pappinen et al. 2002). Contrary to some other studies (Cervera et al. 2000; Kumar and Fladung 2001), no association between copy number and the stability of the transgene expression was detected in this study. Based on the greenhouse and field analyses, the expression of sugar beet chitinase IV gene in transgenic silver birches was fairly stable, on the transcriptional level during the whole 3-year field trial.

The main objective of this study was to evaluate the effects of the expression of sugar beet chitinase IV gene in transgenic silver birches on their resistance against fungal diseases. Several studies have demonstrated that overexpression of chitinase genes in transgenic plants can increase their resistance against fungal pathogens (Broglie et al. 1991; Toyoda et al. 1991; Asao et al. 1997; Lorito et al. 1998; Tabei et al. 1998). In the experiment carried out in a greenhouse, chitinase transgenic birch lines with high levels of sugar beet chitinase IV mRNA showed a high degree of resistance to *P. betulicola* (Pappinen et al. 2002). During the 3-year field trial, the level of sugar beet chitinase IV expression had varying effects on the resistance against leaf spot disease that were year- and parameter-dependent. However, the general trend among the individual transgenic lines was that they were more susceptible to leaf spot than the non-transgenic control clone. This trend was consistent across the whole 3-year period, indicating that the pattern of resistance did not fluctuate under varying weather and natural disease conditions.

There are several potential causes that would explain the contradictory results in the resistance of transgenic birches to leaf spot in the greenhouse and in the field trial. In the greenhouse experiment, a high inoculum of *P. betulicola* isolate 97171/1 (Paavolainen et al. 2000) was used to infect the seedlings, while various genotypes of the same pathogen may have infected the transgenic birches in the field. Genetically distinct individuals have been detected in different spots within one leaf, indicating that multiple infection by *P. betulicola* naturally occurs on birch leaves (Paavolainen et al. 2001). The expression of an excess of the chitinase gene under a strong, constitutive promoter may also divert energy and resources from the cellular functions involved in growth and natural defence. Fitness costs associated with foreign protein expression in a host cell are known to occur in transgenic organisms (Glick 1995; Purrington and Bergelson 1997). The effects of changes in resource allocation may not occur in the greenhouse under constant nutrient and water supply, but the situation may differ in the field where plants are exposed to more variable and harsh conditions.

In addition, the reduced resistance to leaf spot in the field may also have been caused by a variety of other biological interactions such as stress caused by insectivorous herbivores or disturbances in mycorrhizal associations. The transgenic production of chitinases may harm symbiotic mycorrhizal fungi that represent a crucial link between the root system and soil, thereby enhancing plant

nutrition and water acquisition, as well as resistance against parasites and other stress factors (Smith and Read 1997). In nature, birch roots are colonised by different ectomycorrhizal fungi. High expression of an introduced chitinase gene could potentially hinder mycorrhiza formation in transgenic plants by the degradation of the hyphal cell walls by the heterologous chitinase. Purified chitinase applied to the hyphal tip of the arbuscular mycorrhizal fungus *Glomus mosseae* produced an inhibition of hyphal extension, lysis of the apex and alterations in the growth pattern of the fungus (Vierheilig et al. 2001). Studies on the associations between chitinase transgenic birches and various ectomycorrhizal fungi both in the field and in vitro are in progress.

In contrast to leaf spot disease, the level of sugar beet chitinase IV expression in transgenic birches had a significant influence on the resistance to birch rust: the groups of lines showing high or intermediate accumulation of the chitinase IV transcript were more resistant to birch rust than those showing a low accumulation. Among the individual lines, those differing significantly from the non-transgenic control clones were always more resistant to birch rust than the control. This was especially clear in the number of rust postules, a parameter that can be considered to be the most accurate measure of the severity of disease infection. In broad-leaved trees, year-to-year variation in rust epidemics is usually considerably large (Kurkela 1973; Widin and Schipper 1980; Hamelin et al. 1993). This was also observed during our field trial as the rust infection in 2003 was much less severe than those in the preceding two years. There are many environmental and host-plant associated factors that can influence rust infection, including moisture and temperature conditions at the beginning of spore germination (Beckett et al. 1990), physiological condition of the host (Mendgen 1981) and the functioning and density of stomata (Wetzstein 1986; see Poteri 1998). In the present study, no other background variables in addition to plant size were measured. In most cases, plant size correlated negatively with disease resistance.

In chitinase transgenic plants, variation in the degree of resistance to different fungal diseases has been explained by differences in the biochemical composition and structure of the fungal cell wall, tissue and cellular localisation of the recombinant chitinase, concordance in chitinase expression kinetics and the period of infection and the type of interaction between the plant and the pathogen (Grison et al. 1996). In an in vitro assay, sugar beet chitinase IV inhibited the growth of the root-rot fungus *Heterobasidion annosum*, but no inhibiting effect on the growth of the scleroderris canker fungus, *Gremmeniella abietina*, was detected (Susi et al. 1995). In a field study, transgenic *Brassica napus* plants constitutively expressing a chimeric chitinase gene showed different degrees of protection against three fungal pathogens that differed in the localisation and the amount of chitin as well as in the infection start site and the progression path of the hyphae (Grison et al. 1996). In the case of the leaf spot and rust fungi analysed in this study, the localisation and the amount of chitin in the fungal cell walls is not known. The differential degree of resistance of chitinase transgenic birches against leaf spot and birch rust may partly be due to the totally different nature of these two fungi. Rust fungi are biotrophs that derive their nutrition from living plant cells and prefer to grow in vital host plants. The leaf spot fungus, in contrast, is a necrotrophic fungus living and proliferating in dead plant tissue (Kurkela 1994).

To date, there are only a few reports of studies employing recombinant chitinases to increase fungal disease resistance in trees. In this study we showed that sugar beet chitinase IV expression was fairly stable in transgenic silver birches over the 3-year field trial and that the transformation tested may provide a tool for increasing the resistance of silver birch to birch rust. However, the introductions of single chitinase genes under a strong constitutive promoter are not likely to provide an equal response against various fungal pathogens in transgenic birches. This study also emphasises the importance of testing biotechnological applications under field conditions where the test organisms are exposed to a variety of biotic and abiotic interactions.

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