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Identification of Candidate Genes For Use in Molecular Breeding – A Case Study With the Norway Spruce Defensin-like Gene, *Spi 1*

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Abstract

In this study we have investigated whether the defensin-like gene *spi 1*, isolated from Norway spruce, contributes to quantitative disease resistance and is a suitable candidate for utilisation in Norway spruce breeding programmes. The following questions have been raised: (1) Can the putative defense gene, *spi 1*, improve the defense towards microbial pathogens in a model plant species, tobacco? (2) Is it possible to produce transgenic plants of Norway spruce that overexpress *spi 1* and are less susceptible to the pathogenic fungus *Heterobasidion annosum*? Compared to control plants, tobacco plants expressing *spi 1* under an enhanced CaMV 35S promoter permitted less growth of the bacterial pathogen *Erwinia carotovora*. Embryogenic cultures of Norway spruce were transformed with a similar construct. The general phenotype of regenerated transgenic plants was normal, although it was difficult to maintain certain sublines in culture owing to poor initial growth. Among the transformed plants those with the highest content of SPI 1 displayed reduced fungal growth in the sapwood after inoculation with *H. annosum*. In conclusion, the *spi 1* gene increases resistance in both homologous and heterologous systems.

Key words: Molecular breeding, Norway spruce, plant defensin, transformation.

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Introduction

During the last decade biotechnology has been integrated into the breeding programmes of several agricultural and horticultural species. The use of DNA markers for the selection of valuable traits is today an established method (KELLY and MIKLAS, 1998; YOSHIMURA, et al., 1995), and genetic engineering can be applied to several herbaceous species (BIRCH, 1997). However, the use of biotechnology for the breeding of tree species has until now been very limited. This is especially true of the economically important coniferous forest tree species, despite the fact that the benefits for these will be considerable. The breeding cycle of most crop plants takes less than one year, while for tree species it spans several years, for example in Norway spruce one breeding cycle is about 25 years. Anything that can be done to speed up the breeding cycle or improve the genetic gain per cycle will be an important input.

Conifers are outbred and strongly heterozygous, and the traits bred for are quantitative, continuously varying in the population. At least some of the quantitative traits are regulated by major genes, which can be determined by the association of a locus (quantitative trait locus, QTL) with the trait in a segregating population in linkage disequilibrium. In some cases, 'candidate' major genes putatively regulating a quantitative trait have been identified that map close to a known QTL associated with the trait. An example in conifers is the *cad-n1* locus of loblolly pine, which alters the composition of the lignin and maps at or close to a QTL regulating growth (WU et al., 1999). For quantitative disease resistance, candidate gene analysis has been applied successfully to wheat (e.g. FARIS et al., 1999). As a complement to such mapping studies, the importance of specific genes can be tested in transgenic plants that over- or underexpress the gene of interest. This approach

has not yet to our knowledge been tested in conifers. We have now reached the stage where somatic embryogenesis can be integrated into Norway spruce breeding programmes (HÖGBERG, et al., 1998). Furthermore we can routinely produce transgenic plants of Norway spruce (CLAPHAM et al., 2000; WALTERS et al., 1999) which stably express the transgene (BRUKHIN et al., 2000). In this work we show that transgenic plants can be a useful tool for identifying valuable genes in conifers.

Root pathogens cause serious damage to forest trees. One of the major pathogens on Norway spruce is *Heterobasidion annosum*, causing root and butt rot. *H. annosum* is one of the very few fungal pathogens that is able to infect living conifer roots of all ages (HENEEN et al., 1994a and b). About 15% of the Norway spruce trees in Sweden are infected by *H. annosum* (SWEDJEMARK and STENLID, 1995). However there is a significant variation in fungal growth among genotypes (SWEDJEMARK and STENLID, 1997). About 30% of the total variation in fungal growth is due to the genetic variation in susceptibility in the host (SWEDJEMARK et al., 1998).

Antimicrobial peptides of low molecular weight have been identified in plants, animals and various microorganisms (RAO, 1995). The antimicrobial peptides of plants have been categorised into several families according to their presumed mode of activity and their sequence homology (BROEKAERT et al., 1995, 1997). One such family comprises plant defensins. Plant defensins have been found in a number of species, for instance radish, *Arabidopsis*, tomato and sugar beet (BRANDSTÄDTER et al., 1996; KRAGH et al., 1995; PENNINCKX et al., 1996; TERRAS et al., 1995). Plant defensins have been shown to possess antimicrobial activity in *in vitro* experiments (BROEKAERT et al., 1995; CAAVEIRO et al., 1997). Recently, a cDNA, *spi 1* (spruce pathogen induced 1), encoding a plant defensin-like protein was identified from a cDNA library made from Norway spruce seedling-roots infected with *Pythium dimorphum* (SHARMA and LÖNNEBORG, 1996).

Expression analysis of *spi 1* in Norway spruce has shown that within 12 hours of infection with *P. dimorphum* the *spi 1* expression increases slightly, after which it declines to below that of untreated control roots (FOSSDAL, 1999; SHARMA and LÖNNEBORG, 1996). The amount of SPI 1 protein also increases significantly the first 24 hours after infection of Norway spruce seedling roots with *H. annosum*, after which there is a sharp drop in the SPI content (FOSSDAL, 1999). It has previously been shown that fungal pathogens can produce suppressors of plant defense responses (DIXON and LAMB, 1990). The consistent reaction in which both SPI 1 mRNA and protein content culminate within 24 h after infection supports the hypothesis that the infecting pathogens can actively suppress the synthesis of a potentially antifungal protein at the mRNA level (FOSSDAL, 1999). Similarly the expression of the *Arabidopsis* defensin gene *pdf 2.2* seems to be specifically downregulated by *Alternaria brassicola* infection (THOMMA and BROEKAERT, 1998).

In this case study we have investigated whether the defensin-like gene *spi 1* contributes to quantitative disease resistance and is a suitable candidate for incorporation into a breeding programme either by selection or genetic engineering. We have shown that the tools are now available for integrating molecular breeding into breeding programmes of conifers.

Materials and Methods

Plant material

Embryogenic cell lines of Norway spruce (*Picea abies* (L.) KARST.) were initiated from mature zygotic embryos and established as described by (EGERTSDOTTER and VON ARNOLD, 1993). Each cell line represents one genotype. The cell lines were grown as suspension cultures in proliferation medium, i.e. half-strength LP medium (VON ARNOLD, 1987) with 9.0 µM 2,4-D and 4.4 µM BA. The cultures were incubated in darkness at 20 °C and were subcultured weekly.

In vitro grown plants of tobacco (*Nicotiana tabaccum* (L.) cv. Wisconsin 38) were used for transformation. The plants were grown on MURASHIGE-SKOOG medium (ICN) supplemented with 90 mM sucrose and solidified with 2.5 g gelrite per litre medium. The plants were grown at 20°C and constant light (200 µmol m^{-2} s^{-1}).

Fungal and bacterial material

The *Heterobasidion annosum* isolate Rb 175 used in the inoculation experiment is of the S-intersterility-group of *H. annosum* and was obtained in 1985 from a living Norway spruce in southern Sweden (STENLID, 1987). Inocula were prepared by growing the fungus on 5 mm x 5 mm spruce dowels placed on agar medium for four weeks in darkness, at 20 °C.

The gram-negative bacterium *Erwinia carotovora* strain SCC 3193 (PIRHONEN et al., 1988) was grown on L-medium (SAMBROOK et al., 1989) overnight at 28 °C.

Plasmid construction

A 517 bp fragment carrying the entire spi1 coding region plus 45 bp upstream and 210 bp downstream sequence was isolated. The ends of the fragment were filled by Klenow polymerase and a 10 nt *BamHI* linker, 5'-CCGGATCCGG-3', was ligated to both ends. After ligation, the fragment was re-cleaved with *BamHI* and cloned into the binary plant expression vector pPCV702 (KONCZ et al., 1994; WALDEN et al., 1990). This step linked the spi1 fragment downstream to a minimal 460 bp cauliflower mosaic virus (CaMV) 35S promoter fragment: tobacco mosaic virus (TMV) coat protein leader sequence. The strength of the promoter was thereafter increased by linking 4 tandem copies of a 518 bp fragment carrying the CaMV 35S enhancer sequence. The resulting plasmid was denoted p140S:Spi1.km, and was used to transform tobacco plants.

To generate a suitable plasmid for transformation of Norway spruce, the enhanced 35S::Spi1 fragment was excised as a *HindIII* fragment from p140S:Spi1.km and cloned into the *HindIII* site of plasmid pAHC 25 (CHRISTENSEN and QUAIL, 1996), after removal of the *ubi::uidA* sequence in this plasmid. The resulting plasmid, which also carries the *bar* gene (FROMM et al., 1990), giving resistance to the herbicide Basta, was denoted p140S:Spi1.bar and was used for transformation of Norway spruce cells.

Transformation of tobacco and regeneration of plants

In vitro grown tobacco plants were transformed with p140S:Spi1.km using an *Agrobacterium tumefaciens* mediated leaf disc transformation protocol. Fifteen primary transformants were transferred to the greenhouse and seeds were collected after selfing. The seeds were surface-sterilised and sown on MURASHIGE-SKOOG medium (ICN) supplemented with 100 µg ml^{-1} kanamycin, after which resistant seedlings were transferred to MURASHIGE-SKOOG medium without kanamycin and were used in *E. carotovora* inoculation experiments.

Transformation of Norway spruce and regeneration of plants

Embryogenic cells of Norway spruce (genotype 95:88:22) were transformed with p140S:Spi1.bar using a custom-made particle inflow gun as described in CLAPHAM et al. (2000). Transformed cells were selected on proliferation medium containing Basta at 1 mg l^{-1}, corresponding to phosphinothricin at 0.2 mg l^{-1}. Resistant colonies appeared 2 to 6 months after the

bombardment. The colonies were transferred to proliferation medium supplemented with 450 mg l^{-1} of glutamine.

Somatic embryos from the transformed sublines were matured essentially as described by BOZHKOV and VON ARNOLD (1998). Proliferating embryogenic cultures were suspended in liquid medium without growth regulators and the cultures were plated on filter papers placed on BMI-SI medium (KROGSTRUP, 1986) containing 90 mM sucrose, 24 µM ABA, 7.5% PEG and 450 mg l^{-1} glutamine and solidified with 3.5 g l^{-1} gelrite. Mature embryos were partially desiccated under high humidity conditions and the partially desiccated embryos were transferred to germination medium. After germination the somatic embryo plants were transferred to liquid medium (INGESTAD, 1979) in glass tubes. Plants that grew well after one month were grown on in mineral wool. The plants were kept under a regime of 24 hours light (240 µM m^{-2} s^{-1}) and 20 °C.

RNA blot analysis

Total RNA was extracted from a pool of six tobacco seedlings per line, using the method described by LOGEMANN et al. (1987). Total RNA from shoots of individual 8 to 10 months old plants of Norway spruce was extracted according to CHANG et al. (1993).

For RNA blot analysis 15 µg of RNA was separated by gel electrophoresis under denaturing conditions and blotted to Hybond-N^+ nylon membranes. The membranes were probed with a *Spe I*/*Xho I* fragment of *spi 1* with an approximate size of 300 bp, or a 600 bp *Pst I bar* fragment labelled with ^{32}P-dCTP using the oligolabelling-kit (Amersham-Pharmacia, Uppsala, Sweden). Hybridization was performed overnight at 42 °C in 5 X SSC, 4 X Denhardt's solution, 0.1% SDS, 40% formamide, 10% dextran sulphate and 100 µg ml^{-1} denatured salmon sperm DNA. The membranes were washed in 2 X SSC, 0.1% SDS, 2 X 15 minutes at room temperature and in 0.2 X SSC, 0.1% SDS, 2 X 15 minutes at 37 °C and at higher temperatures when necessary.

DNA blot analysis

Total DNA from Norway spruce was isolated by the method of DOYLE et al. (1987) with the modifications described by ROWLAND et al. (1993), but with an extraction buffer containing 100 mM Tris-HCl, 25 mM EDTA, 2 M NaCl, 2% CTAB, 2% PVP and 2% b-mercaptoethanol. 16 µg of genomic DNA was cleaved by *Eco RI* and separated by gel electrophoresis and blotted to a Hybond-N^+ nylon membrane (Amersham). The probe was the 600 bp *bar* gene fragment, as above. Hybridization was performed overnight in 5 X SSPE, 5 X DENHARDT's solution, 10% dextransulphate and 500 µg ml^{-1} denatured salmon sperm DNA at 65 °C. The membranes were washed under high stringency conditions, with the final wash at 65 °C in 0.1 X SSC, 0.1% SDS.

Protein extraction and immunoblot assay

A 12-mer peptide of the predicted mature SPI1 protein was used to generate polyclonal antibodies in a rabbit. The peptide was coupled to Keyhole limpet hemocyanin (KLH) carrier as described by HARLOW and LANE (1988). The antibodies recognised only one protein band from root extracts of Norway spruce. This protein band had an apparent size of 5kD, as expected for SPI1. The details of the procedure and the specificity of the antibodies are described in detail by FOSSDAL (1999).

Shoots from two to three randomly choosen 8 to10 months old Norway spruce plants per subline were snap frozen in liquid nitrogen and stored at –80 °C until use. The samples were boiled in 2X SDS-PAGE buffer with 0.2 M DTT for 10 minutes, undissolved material being removed by centrifugation. Samples were analysed by Tricine SDS-PAGE, as described in SCHÄGGER and VON JAGOW (1987) and SDS-PAGE gels were stained with Coomassie Brilliant Blue R 250 to check equal loading. Following SDS-PAGE the proteins were electroblotted onto a PVDF membrane (BioRad) for 4 hrs at 140 mA in a MINI-Protean blotter (BioRad). For the immunodetection the PVDF membrane was blocked with Blocking buffer (7% [w/v] nonfat dried milk, [pH 7.5] 0.1 M Tris, [pH 7.5] 0.15 M NaCl and 0.5% [w/v] Tween-20) for 30 minutes. The SPI1 protein specific antibodies were diluted 1:2000 and preincubated in Blocking buffer for 2 hours. Goat anti-rabbit alkaline phosphatase linked secondary antibodies (SIGMA) were used to visualize detection. The wash and the colour reaction, using NBT and BCIP as substrates, were performed according to the BioRad immunoblot assay kit protocol.

Inoculation of tobacco plants with Erwinia carotovora

Inoculation of tobacco with *Erwinia carotovora* was done essentially as described by NORMAN-SETTERBLAD and coworkers (NORMAN-SETTERBLAD et al., 2000). Three weeks old *in vitro* grown plants from five transgenic lines, as well as from an empty-vector control line, were inoculated with 15 µl of a 10 times dilution of the bacterial overnight culture (3 x 10^6 CFU μl^{-1}). After the inoculation plants were kept under high humidity conditions to facilitate bacterial growth. Bacterial growth *in planta* was evaluated after 48 h by homogenising the inoculated plants in 10 ml of 10 mM $MgSO_4$ and plating serial dilutions onto L-agar plates. Each sample contained three plants and the number of viable bacteria (colony forming units, CFU) was then counted in six independent samples from each of the tested lines. The variation, represented by the SE, is typically relatively large in the method used, (ANDERSSON et al., 1999).

Infection of Norway spruce somatic embryo plants with Heterobasidion annosum

Somatic embryo plants at an age of 8 to 10 months were inoculated with *H. annosum* as described previously (SWEDJEMARK and STENLID, 1995). A 5-mm circular wound, about 6 cm above the soil surface, was made by removing the phloem, using a sterilised cork-borer, and a *H. annosum*-infected spruce dowel was attached to the wound with parafilm.

After 34 days at 20 °C under constant light (240 µmol m^{-2} s^{-1}) the plants were sampled for analysis of the *H. annosum* extension in the stem. Height and diameter of the stems were measured. Each stem was then consecutively cut into 5 mm thick disks which were immediately placed on moist filterpaper in a Petri-dish. The disks were examined for the presence of conidiophores on the surface ten days after sampling. Fungal growth in the sapwood was expressed in mm.

Results

Expression of spi 1 in tobacco plants

The hypothesis that tobacco plants expressing *spi 1* allow less growth of pathogenic microorganisms was tested. The expression level of *spi 1* in the lines was tested by northern analysis. No expression was found in the vector control, while the expression in the transgenic lines varied (*Figure 1*). Three-week-old *in vitro* grown plants of five p140S:Spi1.km transformed tobacco lines and a vector control line were inoculated with *E. carotovora* (*Figure 1*). The average CFU for the lines presented in *figure 1* are based on six individual samples per line. Each sample contained three plants. The group of transgenic lines permitted significantly less ($p<0.05$) bacterial growth than the control. Furthermore, the lines D-1, D-11 and D-12 had significantly lower CFU values than the vector control

(p<0.05). This experiment indicates that expression of *spi 1* in tobacco improves the defense towards pathogenic micro-organisms.

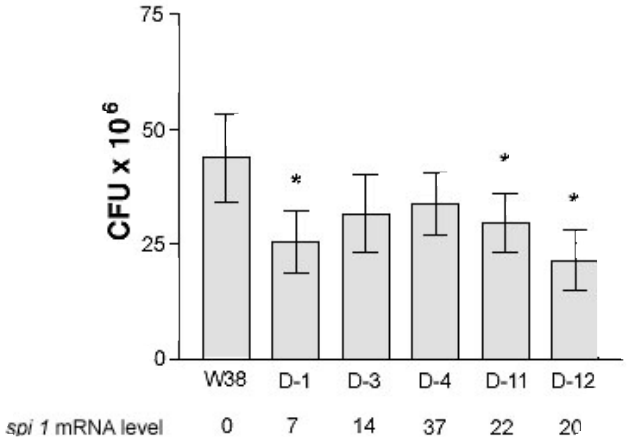

Fig. 1. – Growth of *E. carotovora* on tobacco plants expressing *spi 1.*
Growth of *E. carotovora* on 35S:*spi* 1-transformed tobacco plants was determined 48 h after inoculation. The results are presented as the cumulative mean of six independent samples per line and is given as colony forming units (CFUs). A significant difference between the control and the group of transgenic lines (p<0.05) was found, using the MANN-WHITNEY test. Asterisks indicate that the lines were significantly different from the control (p<0.05). The experiment was repeated with a similar result. To determine if the tobacco lines were expressing *spi 1,* 10 µg of total RNA, extracted from a pool of six plants, was separated on a denaturing gel. The filters were probed with the *spi 1* fragment and studied in a PhosporImager, the expression levels being normalised to the expression of ubiquitin. The expression of *spi 1* is presented as the proportion of the probe (radioactivity) that hybridised to that sample. No expression of *spi 1* was found in the vector control plants.

Overexpression of spi 1 in somatic embryos and regenerated plants of Norway spruce

Embryogenic cell cultures of Norway spruce were transformed with pPCV140S:Spi1.bar. Fourteen embryogenic sublines were recovered on selective medium containing Basta. Six fast growing sublines, i.e. sublines growing at a similar rate as the untransformed control, and expressing the *bar* gene, were analysed further (sublines 4-1.0, 4-1.1, 4-2, 4-3, 4-4 and 4-5). DNA blot hybridization revealed that 1 to 4 copies of the *bar* gene was present in the Norway spruce genome (data not shown). RNA blot hybridization with the *spi 1* probe showed that there was only one clearly overexpressing subline, 4-5, showing up to 2.7 times the expression observed in non-transformed control. Furthermore, the variation in *spi 1* expression was very small between the transformed sublines.

All lines produced normal mature embryos when given a maturation treatment. No significant differences in maturation frequency were observed. From each subline 80 mature embryos were first partly desiccated and thereafter germinated. Germination frequencies varied among the sublines. The lowest germination frequency was observed for the overexpressing subline 4-5 (50%). However, the germination frequency was also low for subline 4-2 (55%), which did not overexpress *spi 1*.

After germination the somatic embryo plants were transferred to liquid medium in glass tubes. Only the plantlets that displayed good growth, and had a white, actively growing root tip after one month of cultivation were potted.

The *spi 1* expression in embryogenic cultures representing different genotypes was relatively low and the variation among different genotypes was small (data not shown). Expression of *spi 1* in somatic embryo plants was up to 30 times higher than in the corresponding embryogenic culture. Furthermore, somatic embryo plants showed a considerable variation in *spi 1* expression both within and among the genotypes. Accordingly, the expression level of *spi 1* in shoots from 8 to 10 months old transgenic somatic embryo plants was higher than in embryogenic cultures (data not shown). There was a variation in the expression of *spi 1*, among plants from the same subline (*Figure 2*). This variaton with a standard deviation of 20% to 30% of the mean, is what is expected for mRNA expression in untransformed or transformed plants. However, the plants derived from the untransformed control always showed lower levels of *spi 1* mRNA than the transgenic sublines. Furthermore, a similar degree of variation in mRNA levels was found in the shoots of *H. annosum*-inoculated plants; but average mRNA levels were consistently higher in subline 4-4 than in the control.

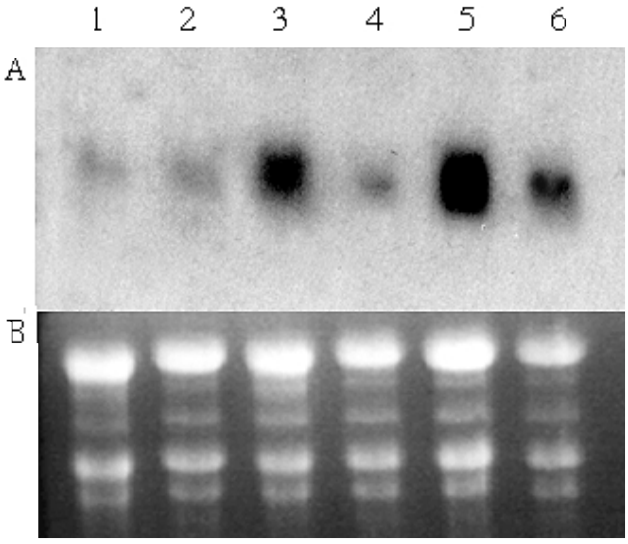

Fig. 2. – RNA blot analysis of the *spi 1* mRNA levels in Norway spruce plants.
Total RNA was extracted from shoots of 8-10 months old control and transformed plants. 15 µg of total RNA was loaded onto a denaturing gel and transferred to a nylon filter after separation. Lanes 1 and 2, untransformed control plants, lanes 3 and 4, plants of subline 4-1.1, lanes 5 and 6, plants of subline 4-4. A. Expression of *spi 1.* The filter was hybridised with the *spi 1* probe, and the autoradiograph was developed after 24h. *B.* Ethidium bromide staining of ribosomal RNA.

The amount of SPI 1 in shoots-samples (from 2 to 3 plants) from 8 to 10 months old plants as determined by Western analysis was higher in plants from five out of the six sublines tested than in the untransformed control. As can be seen in *figure 3*, plants from the sublines 4-1.0, 4-1.1, 4-2, 4-4 and 4-5 displayed an increased level of SPI 1. The levels were so high in these sublines that it was not possible to detect any SPI 1 protein in plants from the control and from subline 4-3 on the same filter, but increasing the developing time showed that both the control and subline 4-3 contained SPI 1.

Infection of plants of Norway spruce with Heterobasidion annosum

Plants at a height of at least 15 cm were infected with *H. annosum* through stem inoculations. Plants regenerated from the control, 4-1.0, 4-1.1, 4-3 and 4-4 were used. *H. annosum* grew significantly less (p<0.001) in the sapwood of plants from subline 4-4 than in the controls (*Table 1*). The growth of *H. annosum* in plants from the other tested sublines did not differ

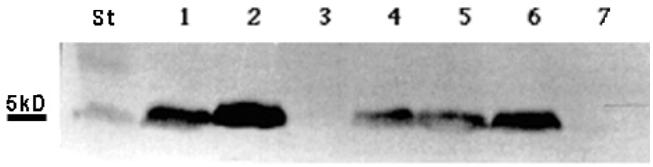

Fig. 3. – Immuno-blot assay of SPI 1 in plants of Norway spruce.
Shoots from 8-10 months old plants were analysed for SPI 1 content. Thirty µg of total protein was loaded in each lane, and denaturing Tricine SDS-PAGE was performed prior to electroblotting and immunodetection using SPI 1-specific antibodies. St, size marker; lane 1, subline 4-5; lane 2, subline 4-4; lane 3, subline 4-3; lane 4, subline 4-2; lane 5, subline 4-1.1; lane 6, subline 4-1.0 and lane 7, untransformed control. The untransformed control plants as well as plants from subline 4-3 do produce SPI 1, but in such low quantities that it can not be detected on the same filter as subline 4-4 and 4-5.

significantly from the control. The SPI 1 content was higher in plants from all sublines, except 4-3, than in the control plants. However, the SPI 1 content was 2 to 3 times higher in plants from subline 4-4 than in plants from the other transgenic sublines (*Figure 3*).

Discussion

Tobacco plants expressing spi 1 permits less growth of Erwinia carotovora

To test if SPI 1 possesses antimicrobial activity, transgenic tobacco plants were generated, and inoculated with *E. carotovora*. Tobacco plants expressing *spi 1* permitted less bacterial growth than control plants did when inoculated with *E. carotovora* (Figure 1). This suggests that *spi 1*, which has high similarity to plant defensins (SHARMA and LÖNNEBORG, 1996), encodes a protein with antimicrobial activity. The results are in accordance with results on tobacco transformed with Rs-AFP 2, a cDNA clone encoding a plant defensin from radish. The expression of Rs-AFP 2 caused smaller lesions to develop on the leaves when infected with *Alternaria longipes* (TERRAS et al., 1995).

Overexpression of spi1 in Norway spruce can decrease susceptibility to Heterobasidion annosum

More than half of all established sublines (8 of 14) putatively transformed with *spi 1* stopped proliferating after 9 to 12 weeks of culture. In contrast, a previous study using reporter genes showed a high yield of sublines after selection on Basta, and once established on proliferation medium essentially all 76 confirmed transgenic sublines continued to proliferate (CLAPHAM et al., 2000). This indicates that the expression of *spi 1* might affect proliferation of embryogenic cell cultures negatively.

It has been shown that defensins are non-toxic to cells of angiosperms (HARRISON et al., 1997; MARCUS et al., 1997). In angiosperms defensins are present in the seeds as well as in the plants. SPI 1 is not present, in detectable amounts using antibodies, in seeds of Norway spruce (FOSSDAL, 1999) and the expression is also very low in embryogenic cultures. We suggest that during these stages elevated levels of the SPI 1 protein have detrimental effects. This is strengthened by the fact that no negative effect of SPI 1 was observed during later stages in Norway spruce. Moreover, other pathogen-induced genes such as chitinase genes (COLLINGE et al., 1993), have been shown to affect embryogenesis. If this is true also for *spi 1*, this could illustrate a problem in using the transgene approach to study resistance genes, namely that the regeneration step can be influenced negatively. This problem could be overcome by using inducible promoters which are not expressed during the critical developmental stages, or a promoter that can be repressed during the tissue culture step. However the enhanced 35S promoter was choosen in this study because the 35S promoter is giving an global expression in most plant species tested, and the enhanced 35S promoter has shown transient expression after particle bombardment of stem and needles of *Larix* ssp. (M. GATINEAU and D. CLAPHAM, unpublished observations).

Previous studies have indicated that SPI 1 is involved in the active host response to invading pathogens (FOSSDAL, 1999). In this work we have shown that transgenic Norway spruce plants with a highly increased content of SPI 1 are less susceptible to *H. annosum*. Previously it has been shown that one transgenic tobacco line expressing the RS-AFP 2 at high levels had increased disease tolerance when challenged with *A. longipes* (TERRAS et al., 1995). Suggesting that production of RS-AFP2 must reach a threshold level in the plants before they can display an increased disease tolerance. Similarily potato plants expressing the defensin alfAFP over the threashold level 1 p.p.m. displayed resistance to *Verticillium dahliae* (GAO et al., 2000). This could be the case for *spi 1*-transformed plants as

Table 1. – Growth of *H. annosum* in transgenic plants.
Two infection experiments were carried out. In the first experiment plants from the sublines 4-1.0 and 4-3 as well as from the untransformed control were infected. In the second experiment plants from the sublines 4-4 and 4-1.1 and from untransformed control were tested. The plants were inoculated with *H. annosum* by making a 5-mm circular wound, to which a *H. annosum*-infected spruce dowel was attached with parafilm. Fungal growth in the sapwood was measured 34 days after the inoculation with *H. annosum*. Each stem was consecutively cut into 5 mm thick disks which were placed on moist filter paper in a Petri-dish. The disks were examined for the presence of conidiophores on the surface ten days after sampling. The fungal growth is presented as a cumulative mean. The data for each experiment were analysed separately, using the MANN-WHITNEY U-test. There was a significant reduction ($p < 0.01$) in fungal growth in plants regenerated from subline 4-4. Asterisks indicates the degree of significance.

Subline	Number of individuals	Fungal growth (mm)	Subline	Number of individuals	Fungal growth (mm)
control	6	78	control	15	92
4-1.0	17	71	4-1.1	18	103
4-3	9	73	4-4	19	72**

well, since plants from subline 4-4 that displayed the highest amount of SPI 1 on Western analysis also permitted less growth of *H. annosum*. In contrast, a moderate increase in SPI 1 level, as in the sublines 4-1.1 and 4-1.0, did not reduce the susceptibility to fungal growth in the sapwood. The method developed for studying variation in fungal growth among genotypes is based on an average from several cuttings per genotype (SWEDJEMARK and STENLID, 1996). Due to the large variation in expression of *spi 1* among plants from the same subline large experimental series have to be run for estimating the threshold level of SPI 1 for decreasing the susceptibility for *H. annosum*.

Transgenic plants can be a useful tool for identifying valuable genes in conifers

Our long-term goal is to make it possible to use molecular breeding in conifers. The basic requirements are fulfilled for Norway spruce: (1) We have a well developed breeding programme (KARLSSON and ROSVALL, 1993). (2) Somatic embryos of Norway spruce can be integrated into the breeding programme (HÖGBERG et al., 1998). (3) Transgenic Norway spruce plants stably expressing the transgene can be produced routinely (BRUKHIN et al., 2000). (4) Several genes of interest for breeding have been isolated from Norway spruce.

All genotypes of Norway spruce tested up to now have been sensitive to *H. annosum* infection. This justified turning to molecular methods. Our result here show that *spi 1* is a candidate gene to include in a molecular breeding programme for Norway spruce aimed at increasing resistance to *H. annosum* Scandinavia's most serious forest tree pathogen. One approach is to screen naturally occuring or conventionally bred genotypes for high levels of *spi 1* expression. Another is to produce transgenic plants overexpressing the gene.

In conclusion, we have shown that a putative defense gene, *spi 1*, increases pathogen resistance in both heterologous and homologous systems. By expressing *spi 1* in tobacco plants it was possible to show that the gene product has an antimicrobial activity. Despite the fact that it was difficult to maintain *spi 1*-transgenic sublines of Norway spruce in culture, we were able to show that plants with a high content of SPI 1 were less susceptible to pathogen infection.

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Stability and Seed Movement for Loblolly Pine in the Western Gulf Region

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Summary

Fifteen test plantings of loblolly pine (*Pinus taeda* L.) were established throughout the Western Gulf Region to assess genotype by environment (G X E) interactions and to establish seed movement guidelines. Open-pollinated families from five selected trees plus a checklot from each of four seed zones were planted at each location. Seed zones tested were southeastern Texas, southern Louisiana, northern Louisiana and southern Arkansas, all in the USA.

Heterogeneity of regressions was significant for height and volume at age five and for volume at age 10. Data suggest that G X E interactions could be managed by stratifying environments. Significant concurrence was detected for volume at ages 5 and 10 indicating a significant portion of G X E interaction was due to changes in family rank. Family differences were significant at ages 5 through 15.

Regression estimates of slope and standard deviation for volume through age 10, indicated northern Louisiana and southeastern Texas families were intermediate in stability while southern Arkansas and southern Louisiana families were equally unstable. Families from southern Arkansas were least responsive to changes in site quality while those from southern Louisiana were most responsive. At age 15, northern Louisiana and southeastern Texas families remained intermediate in stability but southern Arkansas families exhibited increased responsiveness and southern Louisiana families showed decreased responsiveness to site quality. All zones exhibited a linear trend with respect to site quality. Perhaps greater drought tolerance of southern Arkansas families enabled them to continue growing in dense stands where competition for water could be great. The rapid early growth and reduced subsequent growth of southern Louisiana families may highlight a consequence of seed movement without timely regulation of stand density.

Ecovalences and directional responses were large and negative if seed movement exceeded 125 miles north or south of origin. Ecovalences and coefficients of genetic prediction suggested coastal families should not be planted on poor sites. Favorable performances were observed for families planted in regions requiring specific adaptability often along their latitude of origin or areas of higher moisture. Local material showed adaptiveness to local planting conditions.

Key words: Pinus taeda, genotype x environment interaction, plant stability, stability parameters, seed movement.

Introduction

The Western Gulf Region of the southern United States includes the states of Mississippi, Louisiana, Texas, Arkansas and Oklahoma. These states vary widely in weather and soils and resultantly, in site productivity. State and federal agencies and private industry within the Western Gulf Region are planting genetically improved seedlings on diverse sites across large geographical regions. Realization of a consistent and predictable performance from superior seed depends on their use on appropriate sites. An assessment of stability and genotype by environment (G X E) interaction is fundamental to the development of a sound seed movement policy.

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