

Bacterial disease resistance of transgenic hybrid poplar expressing the synthetic antimicrobial peptide D4E1

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Summary Important losses in poplar productivity occur because of susceptibility to microbial pathogens. To enhance disease resistance in susceptible genotypes, the gene coding for D4E1, a synthetic antimicrobial peptide consisting of 17 amino acid residues, was introduced into poplar (*Populus tremula* L. × *Populus alba* L.) via *Agrobacterium*-mediated transformation. Four kanamycin-resistant transformants were selected based on significant accumulation of the *D4E1* transcript and confirmed by reverse transcription-polymerase chain reaction and RNA dot-blot analysis. These transgenic poplar lines were tested for resistance to *Agrobacterium tumefaciens*, *Xanthomonas populi* pv. *populi* and *Hypoxyton mammatum* (Wahl.) Miller. One transgenic poplar line, Tr23, bearing the highest transcript accumulation for the *D4E1* gene, showed a significant reduction in symptoms caused by *A. tumefaciens* and *X. populi*. However, none of the transgenic poplar lines showed a significant difference in disease response to the fungal pathogen *H. mammatum*.

Keywords: *Agrobacterium tumefaciens*, *Hypoxyton mammatum*, *Xanthomonas populi*.

Introduction

The economic importance of poplars and their hybrids is related to their high growth capacity, their adaptability to varied climates and soils, and the various uses of their wood (Ahuja 1987, Stettler et al. 1996). Nevertheless, there remain many obstacles to their exploitation, in particular with respect to pathogen sensitivity (reviewed in Newcombe 1996). Among fungal pathogens, the most harmful include species of *Melampsora* (Widin and Schipper 1981, Newcombe et al. 1994), *Septoria* (Ostry et al. 1989) and *Hypoxyton* (Anderson 1964). Injuries are also caused by bacterial pathogens such as *Xanthomonas* spp. (Nesme et al. 1994) and *Agrobacterium* spp. (Ebinuma et al. 1997). Recent progress in both recombinant DNA technologies and plant genetic transformation should facilitate production of transgenic plants with enhanced resistance to pathogens.

After a pathogen attack, plants exhibit various defense mechanisms based on induction of specific genes (see Soms-

sich and Hahlbrock 1998, Dixon 2001 for reviews). One of these reactions is based on the specific expression of genes encoding small peptides with antimicrobial activities. Recently, this non-specific defense and protection mechanism has been studied in detail. Many antimicrobial genes have been isolated and attempts are being made to engineer pathogen resistance in plants by introducing these genes. Antimicrobial peptides have been isolated from several plant and animal species and characterized (Rao 1995, Broekaert et al. 1997, Hancock and Lehrer 1998). Antimicrobial peptides can be classified into several groups. One of these groups comprises short linear peptides (20–40 amino acids) that form amphipathic α -helices in solution. The cecropin and magainin families are part of this group (Duclohier 1994, Matsuzaki et al. 1995). Another group consists of cysteine-rich peptides, which have a complex disulfide bond, and includes the defensins (Hoffmann and Hetru 1992). A third group is that of synthetic antimicrobial peptides, which have been modified by substitution, chain extension or deletion of amino acids to increase their α -helicity and cationic nature (Bessalle et al. 1992, Everett 1994, Maloy and Kari 1995).

Antimicrobial peptides appear to exert their antimicrobial effect at the level of the plasma membrane of the target microorganism (Broekaert et al. 1997). Cecropins and magainins associate with lipid membranes to form transient ion channels, leading to permeability changes and consequent cell lysis (Duclohier 1994, Matsuzaki et al. 1995). In vitro experiments have demonstrated that synthetic antimicrobial peptides inhibit the germination of a broad spectrum of fungal conidia (De Lucca et al. 1998). Potent activity against bacteria was also observed (Powell et al. 1995, Zhong et al. 1995). Synthetic antimicrobial peptide genes have been expressed in transgenic plants to elucidate the effects of these molecules in vivo. Increased disease resistance was reported in transgenic tobacco expressing a modified cecropin B peptide (Jaynes et al. 1993). However, other reports showed no significant increase in disease resistance in transgenic tobacco expressing a synthetic cecropin peptide (Hightower et al. 1994). More recently, broad-spectrum resistance to phytopathogens has been successfully engineered in potato with a gene encoding a synthetic antimicrobial peptide (Osusky et al. 2000).

In vitro assays with the synthetic peptide D4E1 (FKLRK IKVRLRAKIKL) demonstrated a large inhibition of spore germination of various fungal pathogens (De Lucca et al. 1998, Jacobi et al. 2000). Expression of D4E1 in tobacco plants confirmed this antimicrobial effect: symptoms on transgenic tobacco leaves were reduced after inoculation with *Colletotrichum destructivum* (Cary et al. 2000). However, there is little information on expression of synthetic antimicrobial peptides in forest trees. In this work, we show the stable integration and expression of the synthetic antimicrobial gene *D4E1* in transgenic poplar (*Populus tremula* L. × *Populus alba* L.) and present data on its in vivo activity against plant bacterial and fungal pathogens.

Materials and methods

Plasmid construction

The plasmid ubi7-D4E1 contains the ubiquitin 7 promoter (Garbarino et al. 1995) fused to the coding region of the gene for the synthetic peptide D4E1 (Cary et al. 2000), followed by the polyadenylation signal of the gene coding for nopaline synthase. The 2.4-kb *KpnI*-*PstI* fragment was subcloned into the multiple cloning site of the binary vector pBINPLUS *KpnI*-*Sse8387I* (van Engelen et al. 1995). This binary vector has a nopaline synthase (*nos*) promoter driving the *nrpII* gene with a *nos* terminator. The *nrpII* gene was used as a plant-selectable marker conferring kanamycin resistance. This plasmid was introduced into *Agrobacterium tumefaciens* strain AGL1 (Lazo et al. 1991) by electrotransformation (Dower et al. 1988).

Plant materials and transformation

The study was conducted with *Populus tremula* × *P. alba* clone 717 IB 4 (provided by L. Jouanin, INRA, France). Aseptic poplar plantlets were maintained in vitro on hormone-free half-strength MS medium (Murashige and Skoog 1962) and cultured in vitro in sterile Magenta boxes under controlled environmental conditions (day/night temperature of 25/22 °C and 16-h photoperiod at 100 µmol m⁻² s⁻¹). Stem sections (1 cm in length) obtained from in-vitro-grown plantlets were precultured, then co-cultured with *A. tumefaciens* and transferred to a selective regeneration medium using the conditions described by Lepié et al. (1992), except that the preconditioning period was extended to 3 days. Plantlets thus derived were subjected to polymerase chain reaction (PCR), reverse transcription-polymerase chain reaction (RT-PCR) and RNA dot-blot analyses (see next section). Transgenic plants, when confirmed, were propagated in vitro from stem cuttings. Plantlets were then transplanted to soil medium PromixTM (Premier Tech, Rivière-du-Loup, QC, Canada) and grown in the greenhouse.

PCR analysis of genomic DNA

Poplar genomic DNA was extracted from in-vitro-grown plants of transformed and untransformed control lines with Qiagen Genomic-tips (Qiagen, Mississauga, ON, Canada) ac-

cording to the Qiagen protocol for plants. The PCR analysis was conducted on DNA samples from individual transgenic lines, non-transgenic lines and a negative control that contained no DNA. Each PCR reaction mixture (50 µl) contained 1.5 mM MgCl₂, 100 µM of each dNTP, 1 µM of each primer, 1× of the supplied buffer, 2.5 units of Taq DNA polymerase (Life Technologies, Burlington, ON, Canada) and 100 ng of template DNA. Thermocycler (Thermolyne, Amplitron[®], Barnstead, Conroe, TX) parameters were: 10 min preheat at 95 °C, followed by 25 cycles of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min. The first PCR amplification was conducted with the primers 5'-CTGGCCACGACGGGCGTT CCTTG-3' and 5'-GAATCGGGAGCGGCGATACCGTAAA-3', corresponding to the *nrpII* coding region, and amplified a 545-bp DNA fragment. Another combination of primers was used to verify that the ubi7-D4E1-*nos* expression cassette was present in the plant genome of transgenic clones. The ubiquitin promoter primer 5'-CTAATCAGGGAGTGTG-3' and the *D4E1*-specific primer 5'-TAGCTCTCAATCTAACC-3' were used to amplify a *D4E1* gene-specific fragment of 349 bp. The PCR products were analyzed by electrophoresis on a 1% agarose gel stained with ethidium bromide.

RNA extraction and *D4E1* transcript accumulation analysis

Because of the small size of the expected *D4E1* transcript, standard northern analysis was not an efficient way to detect these transcripts. Consequently, RT-PCR amplification and dot-blot analysis were performed. Total RNA was isolated from leaves (1 g) by the phenol-chloroform LiCl method according to Chang et al. (1993). First-strand cDNA synthesis from total RNA (250 ng) and amplification of the target cDNA was done with the SuperScriptTM first-strand synthesis system from Invitrogen (Burlington, ON, Canada).

For RT-PCR analysis, a sense oligonucleotide primer matching the 5'-end of the *D4E1* gene (5'-GGTGGAAAGTT CTGATACAATCGAC-3') and an antisense primer matching the 3'-end of the *D4E1* gene (5'-GCTCTCAATCTAACCCTTA ATCTTAGCTCTC-3') were used. First-strand cDNA representing the *D4E1* transcript was amplified using the following thermocycler parameters: 94 °C for 2 min; 25 cycles of 94 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min; and 72 °C for 5 min. The PCR products were separated by electrophoresis in a 10% polyacrylamide gel. All cDNAs from the reverse transcription were also amplified with two primers, a flanking 5'-primer (5'-GCGGAGTTCAGTTCTGGTGCTGAACG-3') and a 3'-primer (5'-GGTCTGAAGTCCACAAGGTGAGATT GT-3') corresponding to a poplar *14-3-3* gene (GenBank accession number AF121195), to confirm that there was no DNA contaminating the RNA samples. Because of the presence of an intron in the coding region of this *14-3-3* gene, the expected PCR product from the mRNA is 307 bp, whereas amplification from genomic DNA would result in a fragment of 494 bp. The presence of a larger DNA fragment was never observed among the RT-PCR amplification products, and the absence of reverse transcriptase in the first-strand cDNA synthesis reaction mix resulted in the absence of DNA amplification (data not shown).

For dot-blot analysis, RNA (10 µg) was transferred onto a nylon membrane (Zeta-Probe) with a dot-blot apparatus (Convertible™ Filtration Manifold System, Life Technologies) and cross-linked by UV irradiation. Overnight pre-hybridization at 65 °C and a 24-h hybridization at 60 °C were carried out in hybridization buffer (Sugita et al. 1987): 0.9 M NaCl, 50 mM NaH₂PO₄, pH 7.4, 5 mM EDTA, 0.1% SDS, 100 µg ml⁻¹ denatured salmon sperm DNA and 1× Denhardt's solution (1% bovine serum albumin, 1% Ficoll 400 and 1% polyvinylpyrrolidone). The *D4E1* oligonucleotide probe (CAACTTAATCTTAGCTCTCAATCTAACCTTAATCTTA GCTCTCAA) was labeled with γ³²P-ATP according to the 5'-end-labeling procedure described by Sugita et al. (1987). The membrane was washed four times (5 min) with 6× SSC at 60 °C, and washed again once (60 min) at 65 °C. Finally, it was exposed to pre-flashed X-OMAT™ AR Scientific Imaging film (Eastman Kodak, Rochester, NY) at -80 °C for 18 h.

Pathogen strains and disease-resistance assays

To evaluate *D4E1* antimicrobial activity in transgenic poplar lines, we inoculated plants with two bacterial strains and one fungal strain.

Agrobacterium tumefaciens Wild-type C58 and B10 (supplied by L. Jouanin) are nopaline *Agrobacterium* strains; i.e., they induce tumors containing nopaline. Glycerinated stocks were stored at -70 °C. Bacteria were cultured as required on YEP medium (10 g l⁻¹ yeast extract, 10 g l⁻¹ bactopeptone, 5 g l⁻¹ NaCl and 15 g l⁻¹ agar) for 2 days at 28 °C. Four-month-old transgenic and control plants were used. Inoculation was performed according to the method of Cervera et al. (1998). Three inoculations per plant were performed at the same time. Plant stems were wounded three times with a sterile scalpel at 5–7 cm spacing, with the first wound 5 cm from the soil. Five µl of *A. tumefaciens* suspension in water (OD₅₉₅ = 0.3) was added to the wound with a micropipette. The wounds were then covered with plastic wrap for 2 weeks to prevent desiccation. Control plants were also wounded and 5 µl of sterile water was applied to each wound. The greenhouse growth conditions were a day/night temperature of 21/17 °C and a 16-h photoperiod. The experimental assay was a completely randomized design with two bacterial strains, five clones (four transformants and one control) and four plants per clone per treatment.

Tumor formation was visually monitored; the diameters of the tumors were measured in three positions along the stem of each inoculated plant, on each monitoring date (6, 12 and 18 weeks). Tumor diameter was transformed to its cubic root before analysis to achieve normality and variance homogeneity. After 18 weeks, when tumor growth was significantly low, tumors were excised and weighed.

Xanthomonas populi* pv. *populi An isolate of *X. populi* (provided by M. Ménard, INRA, Angers, France) was maintained on LPG medium (5 g l⁻¹ yeast extract, 5 g l⁻¹ bactopeptone, 5 g l⁻¹ glucose and 15 g l⁻¹ agar) for 10 to 15 days at 18 °C. Bacterial suspension, adjusted to 10⁸ to 10⁹ cells ml⁻¹, was used as inoculum. For each plant (eight leaves stage), one drop (30 µl) of this suspension was placed on the petiole base of the sixth re-

moved leaf. Negative controls consisted of plants inoculated with sterilized water. Growth conditions were: day/night temperature of 21/17 °C; day/night relative humidity of 80/100%; and a 16-h photoperiod. The experimental design was a completely randomized block design with five clones (four transformants and one control) and five replicates. Disease development was evaluated 2 months after inoculation by measuring both the longitudinal canker extensions and the lateral canker extensions, based on the girdling index of Ridé and Ridé (1978).

***Hypoxyylon mammatum* (Wahl.) Miller** One strain (121-96) of *Entoleuca mammata* (*H. mammatum*), a causal agent of the hypoxyylon canker (provided by M.J. Mottet, Direction de la recherche forestière, Sainte-Foy, QC, Canada) was tested on 3-month-old plants in a greenhouse trial. Fungus was maintained on ME (2% malt extract) agar medium. Inoculation was performed with a 1-week-old mycelium culture according to the method of Mottet (1992): two 2-mm diameter bark wounds were made to the stem with a leather punch; one 14 cm and the other 21 cm above ground. The upper wound received a similar sized piece of ME agar that contained mycelium fungi. Strips of mycelium were placed with the mycelium side down along the wound. The control wound (14 cm above ground) received a piece of sterile agar. To protect the inoculum and prevent tissue desiccation, inoculated wounds were wrapped with poly-ether foam and then with Parafilm™ (Structure Probe, West Chester, PA). The impact of disease on transgenic and control poplar plants was evaluated by measuring canker length 3 months after inoculation. Results were evaluated by analysis of variance with a completely randomized block design with five replications.

Results

Analysis of transgenic plants

Transgenic poplar plants were obtained through *Agrobacterium* transformation and selection for kanamycin resistance. Integration of the *npfII* gene into the poplar genome was confirmed by PCR analysis. All transgenic lines tested produced an amplification product specific for the *npfII* gene (Figure 1, panel A and data not shown). No visible amplification product was obtained from the negative control (no template DNA) or from untransformed poplar. Positive amplification of the *ubi7-D4E1-nos* expression cassette was obtained with DNA extracted from transgenic clones. The *D4E1* gene-specific fragment of 349 bp was present only in transgenic trees, and no amplification was observed for the non-transformed control or the control sample lacking DNA (Figure 1, panel B).

Transgenic poplar lines that were healthy and growing vigorously were selected for further molecular analysis. The expression of *D4E1* was tested at the RNA level by RT-PCR and RNA dot-blot analysis, and expression was confirmed in all transgenic lines (Figure 2). In RNA dot-blot analysis, the amount of *D4E1* transcript varied significantly between plants, and no RNA product was detected from the control plants (Figure 2, panel A). To confirm the expression of the



Figure 1. Ethidium-bromide stained PCR products from amplification of poplar genomic DNA to confirm the presence of the *apfI* and *D4E1* genes. The PCR products were obtained from genomic DNA from four *D4E1*-transgenic poplar lines (Tr15, Tr16, Tr21 and Tr23), untransformed poplar (NTrans), control without template DNA (control) and from the plasmid bearing the *D4E1* construct (plasmid). (A) Amplification of a 545-bp region from the *apfI* gene. (B) Amplification of a 349-bp region spanning from within the ubiquitin promoter to the 3'-end of the *D4E1* gene.

D4E1 gene in various transgenic lines, we performed RT-PCR using RNA from these lines. The primer used in the PCR amplification gave the expected products of 226 bp. We note that we used primers to amplify a large fragment (larger than the expected 60 bp corresponding to the 17 amino acids of the *D4E1* peptide) in order to facilitate molecular analysis. The

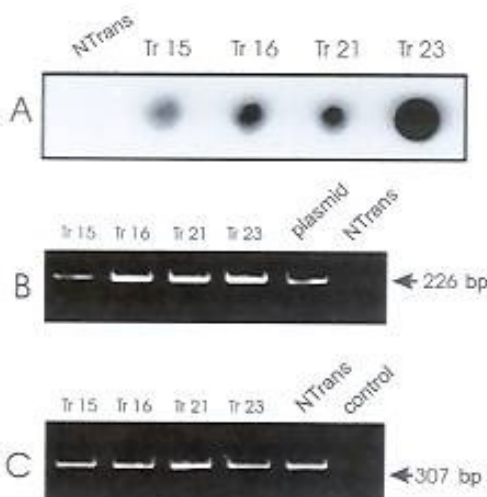


Figure 2. Expression profile of the *D4E1* gene. (A) Dot-blot analysis of total RNA from control and transgenic poplar lines. Hybridization was carried out with a *D4E1* probe to RNA from control plants (NTrans) and from four transgenic poplar lines (Tr15, Tr16, Tr21 and Tr23). (B) Ethidium-bromide stained RT-PCR products amplified with *D4E1* specific primers from transgenic poplar cDNA, untransformed poplar cDNA (NTrans) and with plasmid bearing the *D4E1* construct as a positive control (plasmid). (C) Ethidium-bromide stained RT-PCR products amplified with *14-3-3* specific primers.

sense and antisense primers were specific to the engineered peptide signal located at the 5'-end of the *D4E1* gene and to the 3' non-coding region of the *D4E1* transcript, respectively. The first-strand cDNA of the transgenic poplar samples as well as the positive control using the plasmid bearing the *D4E1* gene gave a positive signal of the expected size (Figure 2, panel B). The untransformed poplar sample produced no signal. Primers were designed to anneal to a poplar *14-3-3* gene to monitor potential DNA contamination of the mRNA samples. All samples, except the control (no DNA), gave one product of 307 bp, indicating the absence of DNA contamination. Therefore, the PCR products obtained with the respective primer sets resulted from the amplification of their particular gene transcripts. Growth and development of transgenic poplar lines were similar to those of control plants, and no abnormal phenotypes were observed (data not shown).

Agrobacterium bioassay

In all cases, no significant differences were observed among upper, intermediate and lower inoculation sites. *Agrobacterium* strain B10 was ineffective on the 717-1-B4 poplar hybrid, and no tumors were observed 18 weeks after inoculation with this strain. However, stem segments of control and transgenic poplars inoculated with *Agrobacterium* strain C58 showed 100% tumor formation frequency. No control plants (wounded but not inoculated) showed symptoms of infection. There were no significant differences in tumor mass or diameter between the inoculated control and the transgenic lines Tr15, Tr16 and Tr21. Transgenic line Tr23 displayed smaller tumors compared with the other transgenic lines and controls, and the reduced tumor size was evident 12 weeks after inoculation (Figure 3). The mass of tumors produced by the Tr23 transgenic line was 65% less than in the control lines (Figure 4). All tumors had single lobes and were light green to brown (Figure 5).

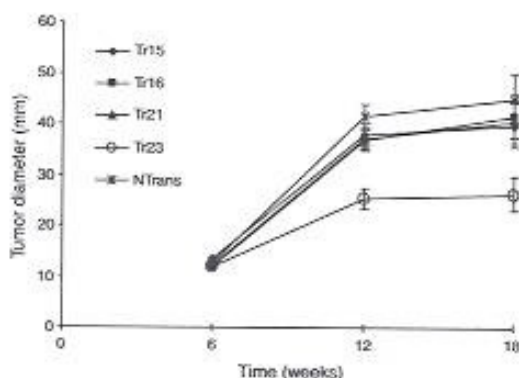


Figure 3. Tumor growth on poplar after inoculation with *Agrobacterium tumefaciens* (C58): mean tumor diameter (mm) of control and transgenic poplar lines over four replicate trees and three positions. Observations were made at 6, 12 and 18 weeks.

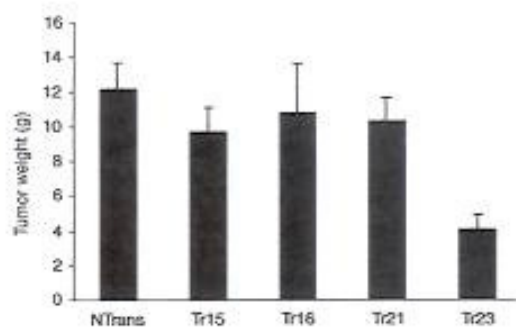


Figure 4. Tumor mass of control (NTrans) and four D4E1-transgenic poplar lines (Tr15, Tr16, Tr21 and Tr23) over four replicate trees and three inoculate positions. Tumor mass was evaluated 18 weeks after inoculation with *A. tumefaciens*.

Xanthomonas bioassay

No symptoms were observed in control plants (non-inoculated), whereas all inoculated plants showed canker formation. In both control and transgenic lines Tr15, Tr16 and Tr21, longitudinal cracked lesions were observed at the inoculation site; the bark surface became necrotic and swollen. Canker lengths varied from 5.4 to 12.2 mm and no significant differences were observed between lines. In contrast, transgenic line Tr23 had only small lesions and canker severity was reduced to 57% compared with control plants (Figure 6). Infection sites were lightly necrotic and no swelling was reported (Figure 7). Transgenic line Tr23 also differed in lateral canker extension, presenting a mean Girdling Index (GI) of 1 compared with a GI of 3 for all controls and other transgenic lines (Figure 7).

Hypoxyton bioassay

Three months after inoculation, the morphology of the wound-inoculated site of all transgenic and control plants was similar (data not shown) and no significant differences in canker elongation were observed (Figure 8). Mean canker lengths ranged

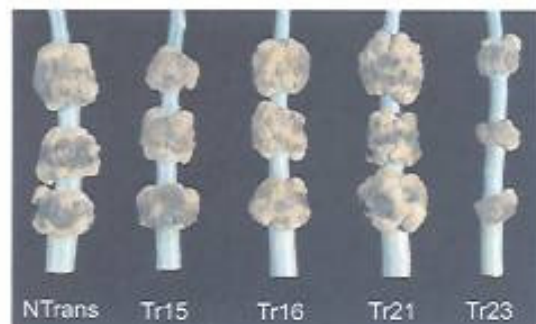


Figure 5. Tumor formation induced by *A. tumefaciens* (CS8) on control (NTrans) and four D4E1-transgenic poplar lines (Tr15, Tr16, Tr21 and Tr23). Photograph was taken 18 weeks after inoculation.

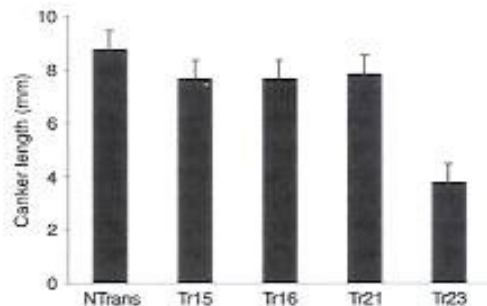


Figure 6. Variations in canker length on one untransformed line (NTrans) and four D4E1-transgenic poplar lines (Tr15, Tr16, Tr21 and Tr23) 2 months after inoculation with *Xanthomonas populi*.

from 6.1 to 11.2 mm. Stems of inoculated plants seemed to be unable to collapse the inoculated wound. However, 100% callus production (wound closure) was observed in all control (wounded and non-inoculated) plants.

Discussion

Poplar species have great potential for intensive plantation forestry. However, considerable losses as a result of bacterial and fungal pathogens are frequently encountered. Genetic engineering provides an opportunity to produce transgenic plants with enhanced disease resistance (see Klopferstein et al. 1997 and citations therein). Several studies have reported on the use of genes encoding natural antimicrobial peptides, and synthetic analogs of these genes or totally synthetic antimicrobial peptides have also been used (Rao 1995). Compared with natural antimicrobial peptides, the synthetic peptides often provide a broad spectrum of antimicrobial activity, are less sensitive to plant proteases and have reduced phytotoxic effects (Jaynes et al. 1993, Powell et al. 1995, Cavallarin et al.

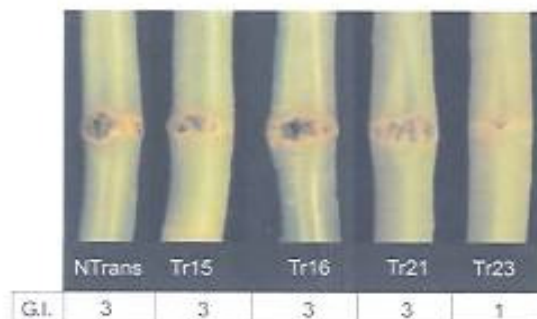


Figure 7. Disease symptoms caused by *Xanthomonas populi* on stems of one untransformed poplar line (NTrans) and four D4E1-transgenic poplar lines (Tr15, Tr16, Tr21 and Tr23). Lateral canker extension, expressed as girdling index (GI) 2 months after inoculation with *X. populi*, is also presented for one untransformed line (NTrans) and four D4E1-transgenic poplar lines (Tr15, Tr16, Tr21 and Tr23).

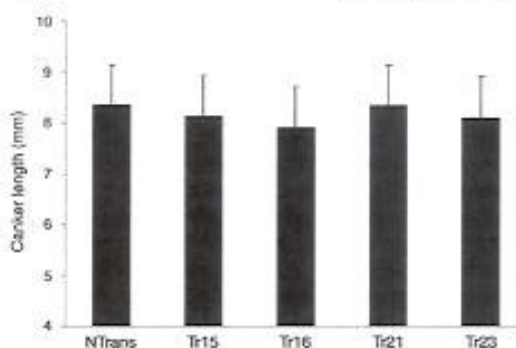


Figure 8. Variations in canker length in one untransformed line (NTrans) and four D4E1-transgenic poplar lines (Tr15, Tr16, Tr21 and Tr23) 3 months after inoculation with *Hypoxyylon mammatum*.

1998). In vitro tests have shown the synthetic peptide D4E1 to be effective against several agronomic and forest pathogens (De Lucca et al. 1998, Cary et al. 2000, Jacobi et al. 2000). For example, De Lucca et al. (1998) reported that D4E1 inhibited germination of conidia of *Aspergillus* spp. and *Fusarium oxysporum*, and Jacobi et al. (2000) showed that the D4E1 peptide inhibited spore germination of various forest pathogens. In an in vivo study, Cary et al. (2000) observed that D4E1 reduced disease symptoms in transgenic tobacco after inoculation by a fungal strain of *Colletotrichum destructivum*.

Based on the broad-spectrum antimicrobial activity of D4E1 in vitro, combined with its resistance to fungal and plant protease degradation (De Lucca et al. 1998), we used the D4E1 peptide to develop transgenic poplar. Our results indicate that D4E1 has the potential to control bacterial disease of poplar. The PCR analysis indicated that all transgenic plants tested contained intact copies of the D4E1 gene expression cassette. We observed significant reduction in symptoms caused by *A. tumefaciens* (C58 strain) and *X. populi* in the transgenic poplar line Tr23 compared with the other clones tested. Reduced symptoms in response to these bacteria correlated with the presence of large amounts of D4E1 transcript in the transgenic line. It is possible that large accumulations of the D4E1 transcripts are necessary to provide sufficient accumulation of the peptide in transgenic poplar. In transgenic tobacco plants, the antibacterial effect of a synthetic peptide, the cecropin analog MB39, was shown to confer increased resistance to *Pseudomonas syringae* (Huang et al. 1997).

We found no dramatic differences in disease response to *H. mammatum* between the control and the transgenic lines. Possible explanations for this negative result include: (1) expression of the D4E1 peptide in transgenic lines was insufficient to elicit antifungal action; (2) fungal membranes interacted with the D4E1 peptide less than do bacterial membranes; (3) the D4E1 peptide may be more susceptible to degradation by fungal protease than by bacterial protease. In an in vitro study, De Lucca et al. (1998) reported some degradation of D4E1 by *Aspergillus flavus* protease, but this was insufficient

to counteract the inhibitory action of D4E1 on conidium germination. There is no information about *H. mammatum* proteases, and the interaction of this synthetic peptide with biological membranes is not well understood.

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