Bacterio-opsin gene overexpression fails to elevate fungal disease resistance in transgenic poplar (*Populus*)

Rozi Mohamed, Richard Meilan, Michael E. Ostry, Charles H. Michler, and Steven H. Strauss

Abstract: Overexpression of the bacterio-opsin (*bO*) gene in tobacco had previously been shown to induce hypersensitive-response-like lesions, increase viral and bacterial disease resistance, and stimulate pathogenesis-related gene expression. To see if this gene enhanced resistance to fungal pathogens of poplar, we generated a total of 35 transgenic lines in two clones of *Populus trichocarpa* Torr. & A. Gray × *Populus deltoides* Bartr. ex Marsh. and one clone of *P. trichocarpa* × *Populus nigra* L. and challenged them with the fungal pathogens *Melampsora occidentalis* H. Jack (leaf rust), *Venturia populina* (Vuill.) Fabric. (leaf and shoot blight), *Septoria musiva* Peck, and *Septoria populicola* Peck (leaf spot and stem canker) in greenhouse, field, or laboratory inoculations. Northern analysis showed that the *bO* gene was expressed in the transgenic poplars; however, no increase in expression of phenylalanine ammonia-lyase (*PAL1*) or two wound-inducible poplar chitinase genes (*WIN6* and *WIN8*) were observed, even in one line that showed very high *bO* expression, intensive lesion development, and retarded growth. Poplars required a high threshold of *bO* expression for lesion development, and susceptibility to all of the pathogens tested was unaffected by *bO* overexpression.

Résumé : Des études antérieures ont démontré que la surexpression du gène de la bactério-opsine (*bO*) chez le tabac induisait des lésions semblables à celles associées à la réponse hypersensible, augmentait la résistance aux maladies virales et bactériennes et stimulait l'expression de gènes liés à la pathogénèse. Afin de vérifier si ce gène augmente la résistance à l'endroit d'agents pathogènes du peuplier, nous avons produit un total de 35 lignées transgéniques chez deux clones de *Populus trichocarpa* Torr. & A. Gray × *Populus deltoides* Bartr. ex Marsh. et chez un clone de *P. trichocarpa* × *Populus nigra* L. que nous avons inoculées avec les champignons pathogènes *Melampsora occidentalis* H. Jack (rouille foliaire), *Venturia populina* (Vuill.) Fabric. (brûlure des feuilles et des pousses) ainsi que *Septoria musiva* Peck et *Septoria populicola* Peck (tache foliaire et chancre caulicole) en serre, au champ et au laboratoire. L'analyse Northern a confirmé l'expression du gène *bO* chez les peupliers transgéniques; on n'a cependant pas observé d'augmentation de l'expression de la phénylalanine ammonia-lyase (*PAL1*) ni de deux gènes de chitinase inductibles par blessure (*WIN6* et *WIN8*) et ce, même chez la lignée chez laquelle on observe une expression de *bO* très élevée, le développement intense de lésions et une croissance ralentie. Les peupliers requièrent un seuil d'expression de *bO* élevé pour que les lésions se développent, tandis que la surexpression de *bO* n'a changé la susceptibilité à aucun des agents pathogènes étudiés.

[Traduit par la Rédaction]

Introduction

Plants have developed complex defensive mechanisms as a result of their long history of coevolution with pathogens. In the course of infection, a pathogen has to successfully overcome the pre-existing physical and chemical barriers in plants and cope with systems programmed to recognize pathogens and induce defense mechanisms. A primary induced mechanism of resistance by plants is the hypersensitive response (HR). It is characterized by the formation of necrotic lesions resulting from localized cell death at the site of infection. The HR restricts further growth and spread of the pathogen into neighboring healthy cells (Dixon and Harrison 1992) and appears to be a programmed suicidal act of plant cells (Greenberg 1997). Concurrent with the onset of the HR is the activation of overall defense responses of plants (Gilchrist 1998; Greenberg 1997; Hammond-Kosack and Jones 1996). These include reinforcement of the plant cell wall, generation of an oxidative burst, accumulation of phytoalexins, and activation of defense-related genes.

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R. Mohamed, R. Meilan, and S.H. Strauss.[1] Department of Forest Science, Richardson Hall, Oregon State University, Corvallis, OR 97331-5752, U.S.A.
M.E. Ostry. USDA Forest Service, North Central Research Station, St. Paul, MN 55108, U.S.A.
C.H. Michler. Hardwood Tree Improvement and Regeneration Center, USDA Forest Service, North Central Research Station, Department of Forestry and Natural Resources, Purdue University, West Lafayette, IN 47907-1159, U.S.A.

[1]Corresponding author. e-mail: steve.strauss@orst.edu

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The bacterio-opsin (*bO*) gene encodes a protein that functions as a light-driven proton pump (Krebs and Khorana 1993). Transgenic tobacco (*Nicotiana tabacum* L.) plants expressing the *bO* gene from *Halobacterium halobium* (Petter) Elazari-Volcani produce an HR-lesion phenotype, and in the absence of pathogen show elevated expression of several defense-related genes associated with HR, including chitinase, glucanase, *PAL*, an antiviral compound, and salicylic acid (SA) (Mittler et al. 1995). The transgenic plants also had heightened protection against tobacco mosaic virus (TMV), tobacco necrotic virus (TNV), and a bacterial pathogen (*Pseudomonas syringae* pv *tabaci* (Wolf & Foster)) when challenged in laboratory assays.

Similar results were obtained by Abad et al. (1997), who observed upregulation of pathogenesis-related (PR) genes and SA in transgenic potato (*Solanum tuberosum* L.) expressing *bO*. However, protection against pathogens was not very successful when the *bO* potatoes were challenged with several pathogens in growth chamber trials and laboratory assays. The *bO* gene did enhance resistance toward one race of a fungal pathogen (*Phytophthora infestans* (Mont.) de Bary mating type A1) but failed to impart resistance toward another race of the same pathogen (mating type A2). It also failed to impart resistance to a bacterial pathogen (*Erwinia carotovora* (Jones) Bergey et al.) and actually increased susceptibility to a viral pathogen (potato virus X, PVX) (Abad et al. 1997). Contrary to expectations that promotion of the HR via the *bO* gene could provide broad-spectrum resistance, it instead appeared that *bO*-induced resistance had high specificity (Abad et al. 1997).

To test whether the *bO* gene would stimulate the HR response and elevate disease resistance in poplar, we produced transgenic plants using clones that are susceptible to different types of fungal pathogens and tested them via greenhouse and field inoculations. Each of these pathogens have caused major disease epidemics in poplar plantations (Newcombe 1996). We report that overexpression of *bO* only rarely led to the generation of lesion-mimic phenotypes in poplar and failed to affect expression of defense-related genes or resistance to disease.

Materials and methods

Gene construct and bacterial strains

The vector pEL301 (Mittler et al. 1995) was provided by Dr. Eric Lam (Rutgers University). The T-DNA of this binary vector contains a synthetic *bO* gene (Nassal et al. 1987) with a chlorophyll *a*/*b* transit peptide (*Cab-t*) and an Ω translation-enhancing sequence. Expression of the whole cassette (Ω-*Cab-t-bO*) was controlled by the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase terminator (*nos 3′*). The plasmid EL301 was created by insertion of this transcriptional unit into pBI100 (Clontech, California), which has a kanamycin resistance gene (*NPTII*) for in vitro selection. It was then transformed into competent *Escherichia coli* (Migula) Cast. & Chal. DH5α (Gibco BRL) for multiplication before being inserted into *Agrobacterium tumefaciens* (Smith & Townsend) Conn strain C58 for poplar transformation.

Plant materials and transformation

The plasmid EL301 was used to transform three hybrid poplar clones: 189-434 and 195-529 (*Populus trichocarpa* Torr. & Gray × *Populus deltoides* Bartr. ex Marsh.), and 311-93 (*P. trichocarpa* × *Populus nigra* L.) following an established *Agrobacterium*-mediated transformation protocol (Han et al. 2000). Positive transformants were selected based on their ability to root in kanamycin-containing medium (25 mg/L) and were propagated in vitro. After 3–4 weeks in tissue culture, plantlets were transferred to 5 × 5 cm pots containing fertilized soil in a greenhouse.

Polymerase chain reaction (PCR) analysis of transgenic plants

Genomic DNA samples from individual transformants grown in tissue culture were extracted from leaf tissue using a small-scale sodium dodecyl sulfate (SDS) based DNA preparation protocol (<http://www.fsl.orst.edu/tgerc/dnaext.htm>). PCR analysis was carried out using the following primers: forward, 5′-ATGCAAGCTCAAATTACTGGAC-3′; reverse, 5′-AAGATAGCTCGAGAACGCAG-3′. These primers amplify a DNA fragment of approximately 700 base pairs (bp) from the *bO* gene. PCR conditions were as follows: 94°C for 1 min 30 s, 53°C for 1 min 30 s, and 72°C for 2 min (a total of 40 cycles).

Field trial and spontaneous lesion assessment

Greenhouse-grown plants that were 5–6 months of age were transplanted into two different field sites near Corvallis, Oreg. (44°34′N, 123°16′W), in summer 1997. Three ramets of each independent transgenic line (separate insertional events) and their non-transgenic controls were planted in a completely randomized design at each site. The two sites, Peavy Arboretum (PA) and Marchel Tract (MT), differed in several aspects. The PA site is in a hilly area, has a hard clay soil, and Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco var. *menziesii*) trees are abundant in the vicinity. The MT site has a sandy soil, is in the floodplain of a large river (Willamette), and is near to extensive wild and planted poplars. Trees were planted in a 14.6 × 23.2 m plot with a spacing of 1.8 m between rows and 1.2 m between trees within a row (18 trees per row, 7 rows). Height and diameter were measured 2 weeks after planting, and again in fall 1998. Tree volume index (VI) was used to assess yield and was calculated as height × diameter^2 (cm^3). Data underwent natural logarithm transformation for statistical analysis. Net growth was defined as the difference between the ln VI at the beginning and the ln VI at the end of a growing season. Growth data were analyzed via SAS (SAS Institute Inc. 1990) using general linear models (GLM) and analysis of variance (ANOVA) procedures. Full analyses included site, clone, line (clone), and site × clone and site × line (clone) interactions, where clone and line (clone) were considered fixed effects, and site and error were random effects.

Spontaneous lesions (necrotic tissues) were scored on field-grown trees in early spring 1998 prior to fungal inoculation. The first fully opened leaf on the main stem was identified as leaf No. 1, and the six leaves below it as leaf nos. 2–7. Lesions severity was rated by counting the number of lesions that developed on leaves 2–7 and categorized as follows: (0) no lesions, (1) 1–5 lesions, (2) 6–10 lesions, and (3) >10 lesions (or too many to count). Because lesions were irregular in shape and size, the overall percentage of necrotic tissue on each leaf was also rated as follows: (1) 1–25%, (2) 26–50%, (3) 51–75%, and (4) 76–100%; the necrosis was given weights of 5, 15, 23, and 33.3, respectively (similar to scoring *Venturia* symptoms, described below (Newcombe and Oosten 1997)). The area of leaves 2–7 was also estimated; the product of the severity and necrosis indices yielded an estimate of lesion area per leaf. Lesion density (in relative units) was determined for every indexed leaf by dividing lesion area by leaf area. Combination of lesion densities from leaves 2–7 gave the total lesion density (TLD) for each ramet.

Fungal inoculum

Transgenic plants were screened for resistance to four different fungal pathogens. *Melampsora occidentalis* H. Jack rust urediniospores were collected from diseased poplar leaves in early fall 1997 from a plantation near Monmouth, Oreg. (35 km north of Corvallis). Conidia of *Venturia populina* (Vuill.) Fabric. leaf and shoot blight fungus were collected in mid-spring 1998 from newly infected leaves of hybrid poplar in a different plantation, also near Monmouth, Oreg. *Melampsora* and *Venturia* were used directly after collection for inoculation in the greenhouse and field, respectively. *Septoria musiva* Peck and *Septoria populicola* Peck cultures were obtained from leaves of hybrid poplar clone NE 299, near Milaca, Minn., and from black cottonwood (*P. trichocarpa*) leaves near Toutle, Wash., respectively. Cultures were maintained on V-8 juice agar at 20°C under continuous light (Ostry et al. 1988).

Plant inoculation and disease symptom assessments

For rust inoculation a urediniospore suspension at a concentration of 3×10^4 spores/mL in 0.05% Tween 20 was prepared (Newcombe et al. 1996). We selected a total of 19 transgenic lines drawn approximately evenly from the three poplar clones and also included their non-transgenic parental clones as controls. Experiments utilized two 3- to 4-month-old ramets from each transgenic and control line in a completely randomized design (in a tray) and were repeated twice over a period of 3 months. Plants were inoculated individually by brushing each leaf with the spore suspension. After inoculation the tray was transferred into a plastic tent ($1.8 \times 1.5 \times 1.1$ m) on the greenhouse bench and sealed to keep leaves moist. After 18 h, trees were taken out and placed on the bench at a constant greenhouse temperature of about 20°C. Disease severity was assessed 14 days later following Newcombe et al. (1996). The most rusted leaf on a tree was rated as 1 (light), 5 (moderate), or 25 (heavy). Occurrence of rust was given a value of 1, 2, 3, or 4, corresponding to the overall proportion of rusted leaves on each tree. Multiplying the rating and occurrence values yielded a scale ranging from 0 (highly resistant) to 100 (highly susceptible) (Newcombe et al. 1996; Schreiner 1959).

For *Venturia* inoculation, a conidial suspension of *Pollacia elegans* Serv., the anamorph of *V. populina* was prepared at a concentration of 5×10^4 conidia/mL by brushing the black, velvety lesions from freshly infected leaves into distilled water (Newcombe and Oosten 1997). Nine-month-old trees grown at the two field sites were sprayed with the suspension, which was allowed to dry for 2 h. To keep leaves moist, an individual plastic tent was put over each tree using a clear plastic bag (approximately 61×91 cm) mounted on a conical bamboo frame. After 48 h, the tent was removed, and disease severity was assessed after 2 weeks. Leaf and shoot blight symptoms were rated following Newcombe and Oosten (1997), where blight severity was scored as 1, 2, or 3, corresponding to their emergence on the leaves, petioles, or stems. The percentage of blighted tissues on a tree was estimated in four classes (1–25, 26–50, 51–75, and 76–100%) and assigned weights of 5, 15, 23, and 33.3, respectively, (Newcombe and Oosten 1997). Multiplication of symptom scores and severity weights resulted in a scale ranging from 0 (highly resistant) to 100 (highly susceptible).

Screening for resistance to *S. musiva* and *S. populicola* was carried out using an in vitro leaf assay and a greenhouse stem assay at the USDA Forest Service laboratories, North Central Research Station, in St. Paul, Minn. Trees were grown in the greenhouse until the age of 2–5 months, and three ramets were utilized for each transgenic and control line. Fully expanded leaves numbered 3, 4, and 5 (counting down from the shoot apex) were collected from a ramet for the leaf assay. Inoculation procedures for the leaf assay were as described by Ostry et al. (1988). A total of 12 leaf discs (18 mm diameter) per ramet were made from the specified leaves; six discs were used for inoculation with each pathogen. The leaf discs were placed in holes made in 2% agar in Petri dishes, with the abaxial surface facing up. A suspension prepared from 7- to 10-day-old cultures was made using distilled water to give a concentration of 1×10^6 conidia/mL. Leaf discs were inoculated with 100 μL of the conidial suspension and incubated at 20–25°C under continuous light for 32 days. Inoculation experiments were repeated two or three times over a period of 4 months. Disease development was measured beginning on the fourth day following inoculation using a dot grid, and the percentage of green leaf tissue was determined at the end of the 32nd day. A high percentage of green tissue indicates high resistance. Lines having >50% mean healthy leaf tissue (HLT) were considered to be resistant (Ostry et al. 1988).

Stem assays for *Septoria* canker utilized three ramets of 2- to 3-month-old transgenic and control trees. Three petioles approximately 15 cm apart were removed from each tree to expose inoculation sites. Agar sections (9 mm^2) bearing spores were taken from 7- to 10-day-old cultures of *S. musiva* and *S. populicola* and were applied to two wounded, petiole attachment sites for every tree. A section taken from pathogen-free agar was applied to the third petiole attachment site to serve as an inoculation control. Wounds were sealed with Parafilm® for 2 weeks and canker formation was measured as area affected (cm^2) 12 weeks after inoculation. Larger canker size indicates higher susceptibility. This experiment was conducted only once.

Northern analysis and hybridization probes

The shoot apex and the first expanding leaves were collected from 3- to 4-month-old plants for total RNA extractions according to Hughes and Galau (1988). RNA samples (20 μg) were run in 0.8% formaldehyde agarose gels and blotted onto Zeta-Probe nylon membranes (BioRad). Northern blot analysis was carried out according to Sambrook et al. (1989). DNA probes were radioactively labeled (^{32}P) using a nick translation labeling kit (Pharmacia Biotech). The poplar phenylalanine ammonia-lyase (*PAL*) cDNA (pPAL7) was provided by Dr. Carl Douglas, University of British Columbia, Vancouver (Subramaniam et al. 1993). Poplar wound-inducible chitinase genes, *WIN6* and *WIN8* (pWIN6 and pWIN8FL), were provided by Dr. John Davis, University of Florida, Gainesville (Parsons et al. 1989). Poplar nuclear 18S rDNA in pPD5 was used to estimate RNA loading (D'Ovidio et al. 1991). Except for the pEL301 (as described above), all of the genes were subcloned in pBluescript (Stratagene) and maintained in *E. coli* DH5α (Gibco BRL). RNA gel blots were hybridized sequentially with *bO* (a 761-bp *Hin*dIII–*Sst*I fragment from pEL301), *PAL1* (a 994-bp *Sac*I–*Xho*I fragment from pPAL7 cDNA), *WIN6* (a 725-bp *Eco*RI fragment from pWIN6), *WIN8* (a 770-bp *Eco*RI fragment from pWIN8FL), and 18S rDNA (a 1.1-kb *Sac*I–*Xba*I fragment from pPD5). Washing and stripping probes from blots was done as recommended by the manufacturer (BioRad). Hybridization was performed at 43°C in 50% formamide – 0.12 M Na_2HPO_4 – 0.25 M NaCl and 7% SDS at pH 7.2. Three sequential washes were done at 43°C in 2× SSC – 0.1% SDS, followed by washes at 55°C in 0.5× SSC – 0.1% SDS and at 55°C in 0.1× SSC – 0.1% SDS. Probes were stripped in 0.1× SSC/0.5% SDS at 95°C. Radioactive membranes were scanned using a Phosphorimager (Molecular Dynamics), band intensity quantified using ImageQuaNT (version 4.2), intensities adjusted based on rRNA levels, and relative expression levels calculated as a ratio with the level observed in the line with lowest expression. On average, *bO* expression was 9.6% of 18S rRNA expression.

Induction of chitinase genes through mechanical wounding

Two ramets each from transgenic lines 6, 41, and 12 in clone 195-529 and its non-transgenic control (C2) were grown in a greenhouse for 5–6 months. One ramet was wounded prior to RNA

extraction, and the other ramet was left unwounded. Leaf nos. 5–8 (where leaf No. 1 is a newly expanding leaf at shoot apex) were chosen for wounding. Using a small pair of pliers, half of each leaf (divided by the midvein) was wounded with a total of 15–20 "bites" at 0, 4, 17, 20, and 24 h. Thirty-six hours following the first wounding treatment, the undamaged half of the wounded leaf (D) was harvested. For controls, half of each specified leaf was collected from undamaged trees (UD). RNA extraction and northern analysis procedures used were as described above. The RNA blot was probed sequentially with *WIN6*, *WIN8*, *PAL1*, and 18S rDNA. For each gene, relative expression levels were calculated as a ratio with the level observed in the UD control line (C2) after adjustment based on rDNA hybridization.

Results

Lesion formation and tree growth

We produced 12, 16, and 7 independent transgenic lines from clones 189-434, 195-529, and 311-93, respectively, that were resistant to kanamycin, verified as containing *bO* via PCR (data not shown) and that showed detectable *bO* expression via northern analysis (shown below). Lesions started to develop in the greenhouse 4–8 weeks after transfer into soil and continued in the field (Fig. 1). Lesion intensity varied markedly among lines, with one line (No. 6) showing very intense lesion development and most others showing few or no lesions (Fig. 2). We focused on line 6 and another line from clone 195-529 that had a moderate level of *bO* expression and some lesion development in the greenhouse (No. 41; Fig. 2) for several intensive studies of gene expression and lesion development.

To reduce the confounding effect of insect attack and other biological factors that tend to increase lesion formation in the field as the growing season progressed, we assessed lesion frequency in the field at both locations (MT and PA) early in spring 1998, as new leaves developed. In the high *bO*-expressing line 6, lesions were visible as early as leaf No. 2, lesion density gradually increased as leaves aged, and then lesion number remained constant until leaves began to senesce. Lesions varied in shape and size and were usually brown in color. They appeared at margins of the leaves or scattered across the surface (Fig. 1). Older leaves exhibited chlorosis and eventually senesced. Lesion formation was striking in all ramets of line 6; its mean TLD was at least fourfold higher than in all of the other transgenic and control lines and ninefold higher than in the control line for clone 195-529 (C2) (Fig. 2). None of the other lines were significantly different from each other or from their control lines (Dunnett's test, $\alpha = 0.05$).

Analysis of net growth after a year in the field showed that the trees planted at MT grew significantly faster than those at PA ($p < 0.01$); mean increment in volume index at MT was almost 180-fold greater than at PA. There was no statistically significant difference in growth among the clones ($p < 0.17$), nor was evidence of site × clone statistical interaction ($p < 0.32$). Line 6 was retarded in growth, compared with its control (C2) from clone 195-529. Based on logarithmic net growth, C2 grew 10.3 times faster than line 6 ($p < 0.01$, Dunnett's test). Visual inspection also showed line 6 was by far the smallest among all the transgenic lines; its absolute net growth was 1500 ± 1071 cm^3; (mean ± 1 standard error, SE), whereas the growth of C2 was 117 400 ± 70 300 cm^3, about 78-fold higher. No other transgenic lines were significantly different in growth rate compared with their control plants. There was a weak but statistically significant line × site interaction ($p < 0.05$).

Northern analysis

Total RNA was extracted from shoots of plants that had grown for 3–4 months after transplanting from tissue culture media into soil in the greenhouse. Nearly all of the 38 transgenic lines under study were confirmed to be expressing *bO* mRNA. A band of the expected size (761 bp) was observed for transgenic lines but not for control plants (Fig. 3). Two lines selected as being kanamycin resistant and that contained *bO* gene, as verified through PCR, were shown to lack the *bO* mRNA based on Northern analysis. After standardizing for variation in 18S rDNA, the levels of *bO* expression varied approximately 33-fold among transgenic lines (10-fold among 16 lines in clone 195-529, 13-fold among seven lines in 311-93, and 33-fold among 12 lines in 189-434). Despite substantial levels of *bO* expression in many lines, lesion development that was statistically above that of controls was only observed in line 6.

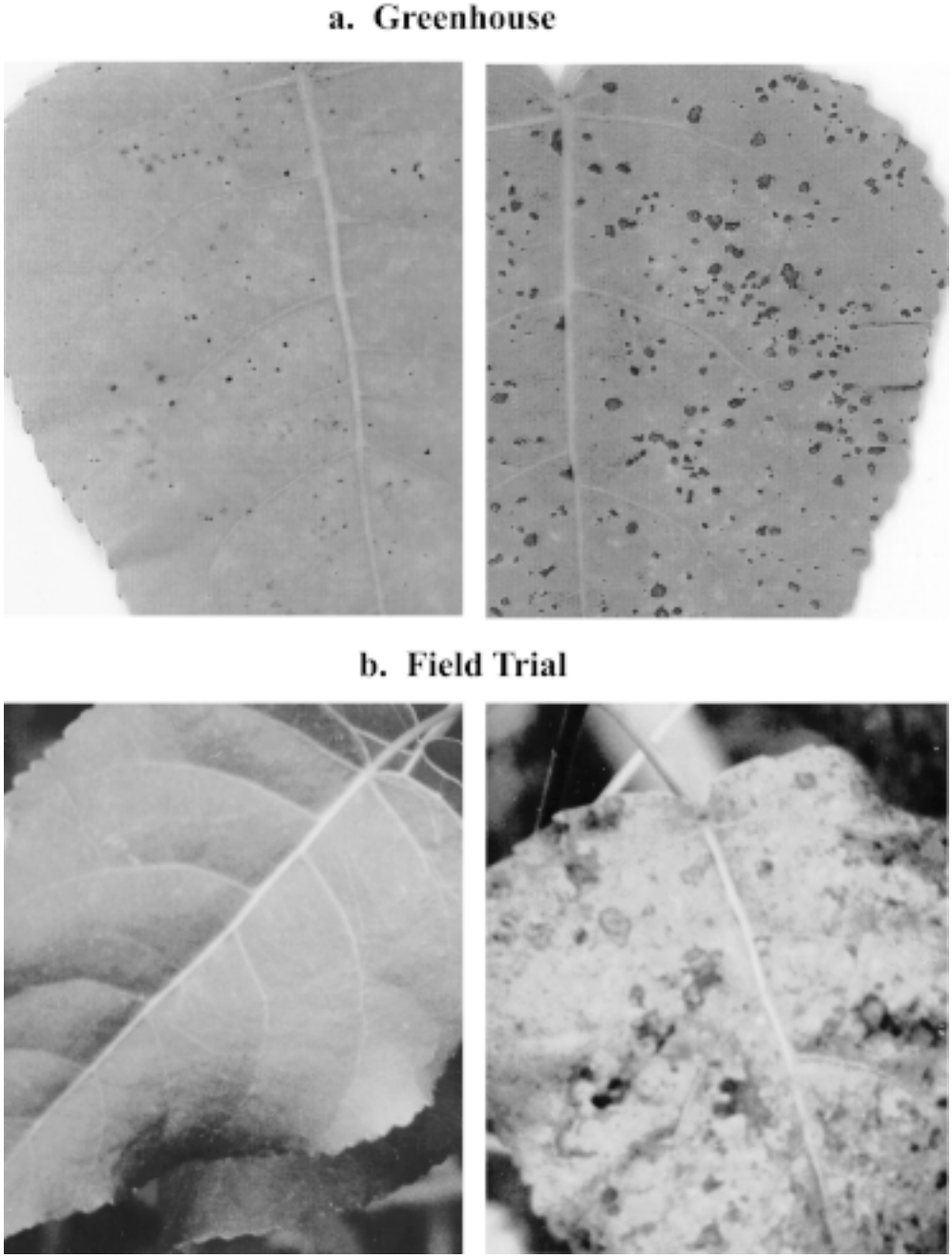
To determine whether the transgene triggers expression of defense-related genes, we probed the RNA blots with *bO*, *PAL1*, and two different wound-inducible chitinase genes (*WIN6* and *WIN8*). The *bO* transgene did not induce increased *PAL1* expression compared with controls (e.g., Fig. 3). In fact, when analyzed over all lines it appeared to do the opposite, although the difference did not reach statistical significance ($p < 0.08$); the relative expression level of *PAL1* for *bO* transgenics was 8.8 ± 1.3 and that of the controls was 14.6 ± 2.0. In two separate Northern blot experiments containing different subsets of transgenic lines and control lines, we also found that *PAL1* and *bO* levels showed no evidence of statistical association (experiment 1: $r = 0.00$, $p > 0.99$, $n = 20$ lines; experiment 2: $r = -0.13$, $p < 0.53$, $n = 25$ lines).

Because the level of expression of the chitinases studied was nearly undetectable in the total RNA samples prepared from shoot tissues of greenhouse-grown plants, we induced expression via mechanical damage on leaves 5–8. For this study, we used four lines from clone 195-529 with variable lesion formation. We selected transgenic lines 12 and 41 to represent low level of lesion formation, line 6 for high lesion formation, and the nontransformed line (C2) as a control. We studied the expression of *WIN6*, *WIN8*, and *PAL1* from leaves samples before and after induction. The three genes had different levels of induced expression ($p < 0.01$; Fig. 4). Wounding upregulated expression greatly (mean of 2.3-fold in leaves for *WIN6*, 19.5-fold for *WIN8*, and 4.4-fold for *PAL1*). However, the transgenics did not differ from controls in expression levels for *PAL1* or any of the *WIN* genes, whether considered before or after induction. Even for the high *bO*-expressing line 6, where lesions were frequent on the older leaves (Fig. 2), there were no significant differences from the control line in *PAL1* or *WIN* gene expression before or after induction (Fig. 4).

Ineffective resistance to *Venturia* leaf and shoot blight

Disease symptoms were 1.4-fold greater on the trees at the MT site compared with those at the PA site ($p < 0.00$).

Fig. 1. Expression of spontaneous lesions on leaves from trees grown in the greenhouse (*a*) and field (*b*). The left panel in Fig. 1*a* shows leaves from the moderate *bO*-expressing line 41. The left panel in Fig. 1*b* is its non-transgenic counterpart (clone 195-529). The right panels in Figs. 1*a* and 1*b* are the high *bO*-expressing line 6; the lowermost leaf has begun to senesce early as a result of intense lesion formation.



The three clones also differed in their degree of susceptibility ($p < 0.05$). Clone 195-529 was the most susceptible to the race of *Venturia* tested among the three clones studied (Fig. 5). In clone 195-529, *Venturia* symptoms appeared as early as 10 days post-inoculation and progressed very rapidly. At early stages of infection, dark necrosis appeared on young leaves, especially at the margins, resulting in curling. Necrosis could also be seen as black patches on older leaves. Eventually, infected leaves became severely necrotic and died, with necrosis spreading into petioles and terminal shoots, leading to the "sheperd's crook" symptom characteristic of *Venturia* infection in poplar. In contrast, the disease was confined mostly to the leaves in clones 189-434 and 311-93.

Transgenic trees exhibited nearly the same blight severity as control plants in all clones (Dunnett's test, $\alpha = 0.05$). Clone 195-529 was most susceptible, with its severity (33.0 ± 1.8) approximately five- to seven-fold greater than the other two clones (clone 189-434, 4.7 ± 0.2; clone 311-93, 5.9 ± 0.4). The line ranking in blight severity was slightly

Fig. 2. Mean lesion density and *bO* expression of some transgenic and non-transgenic (non-trans) lines. Lesion density was scored in the field prior to fungal inoculation. Data are mean lesion density (TLD) from a total of six ramets for each line grown at the two field sites. Error bars are SE. The amount of *bO* expression was quantified from Northern analysis after standardization based on rDNA expression.

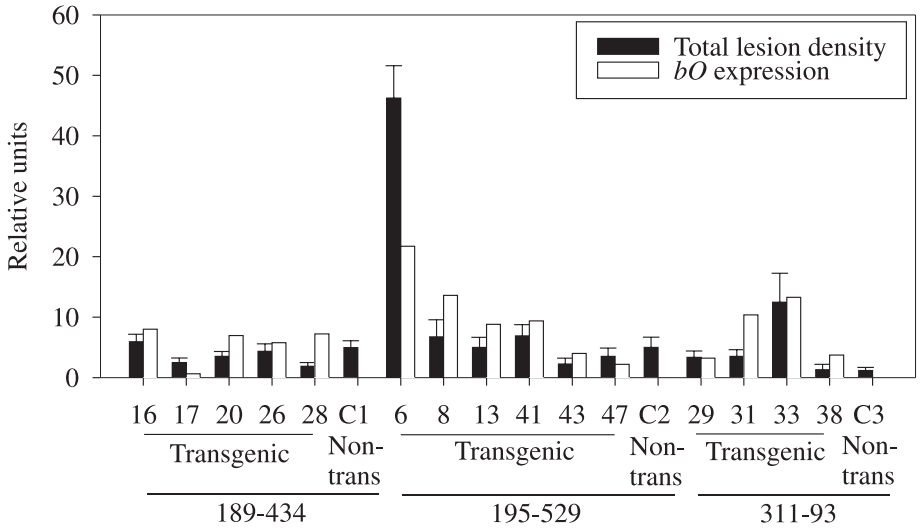
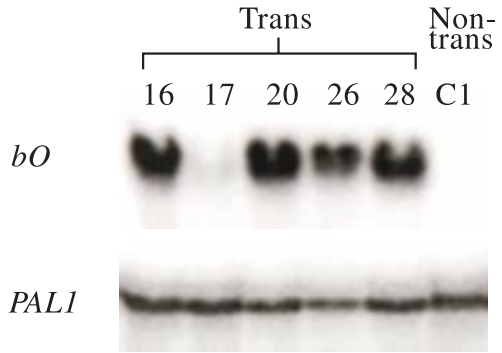


Fig. 3. Northern blot analysis of a sample of transgenic (trans) lines expressing *bO* and *PAL1* genes. C1 is the non-transgenic (non-trans) counterpart for these transgenic lines from clone 189-434.



different at both sites ($p < 0.05$), where line (clone) by site interaction was small but statistically significant.

Although the *bO* gene was moderately expressed in line 41 and highly expressed in line 6 of clone 195-529, and line 6 showed a strong propensity for lesion formation, neither line showed significantly greater resistance to *Venturia* than the other transgenic lines or the control lines (Figs. 2 and 5).

Ineffective resistance to *Melampsora* rust

We selected a number of greenhouse-grown *bO* transgenic lines from the three clones and challenged them with *Melampsora* rust through artificial inoculation. Fungal spores appeared as early as 7 days after inoculation in some plants, in the form of orange pustules containing urediniospores on both sides of the leaves. The three clones varied in their degree of resistance to rust infection ($p < 0.04$). Clones 195-529 and 311-93 were highly resistant to rust with mean rust severity scores below 10 (the cut-off value for resistance is usually considered to be <25; Schreiner 1959). No new orange pustules developed or spread on the leaf surface in these two clones 10 days after inoculation. However, clone 189-434 was very susceptible to rust; its mean rust severity was 36 ± 7 for the transgenics and 38 ± 15 for the control plants. Transgenic lines within a clone were not significantly different in resistance against rust ($p < 0.64$). The moderate (line 41) and high (line 6) *bO*-expressing lines appeared to be 2.5- and 4.5-fold more resistant to rust than the control

Fig. 4. Expression of several defense-related genes before and after induction via mechanical wounding of leaves. RNA samples were collected from leaves of undamaged (UD) and damaged (D) trees. Data are from two moderate *bO*-expressing lines (12 and 41), the high *bO*-expressing line 6, and the non-transgenic line (C2) from clone 195-529. The levels of gene expression were quantified from Northern analysis after standardization based on rDNA expression. No significant differences in expression level for *PAL1* and *WIN* genes were detected in any of the transgenic lines when compared with its control, C2.

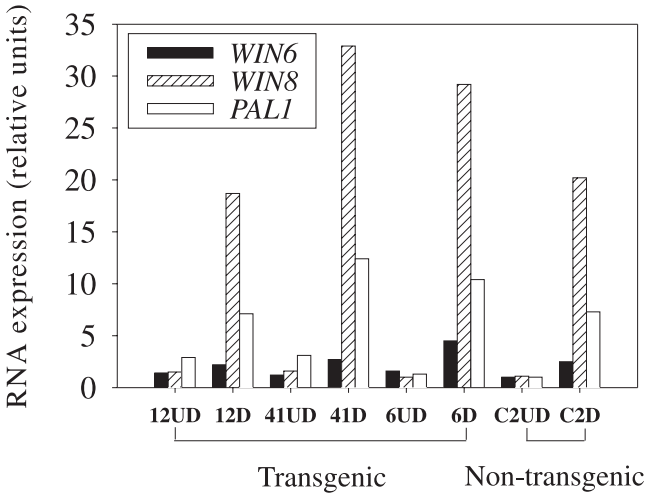
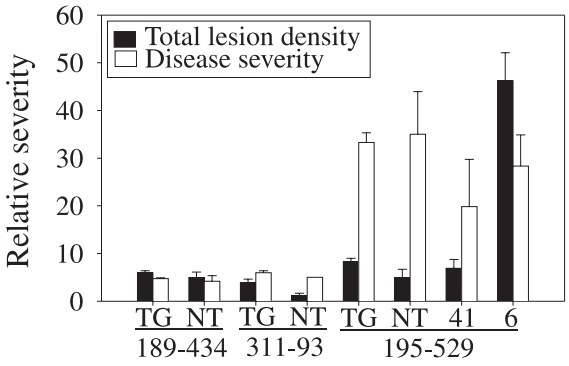


Fig. 5. Spontaneous lesion development prior to inoculation, and disease development following artificial inoculation with fungal spores causing *Venturia* leaf and shoot blight. Data are means over two field test sites; non-transgenic (NT) bars are based on six ramets and transgenic (TG) bars are based on the mean of six ramets per line and 8–18 lines per clone. Data from the moderate (line 41) and high (line 6) *bO*-expressing lines from clone 195-529 are shown separately. Error bars are SE. There is no error bar for disease severity for the control of clone 311-93, because all six ramets had the same score.



line from clone 195-529. However, the modest absolute difference in rust severity between these two lines, as well as between all the transgenic lines and the control lines, were not statistically significant (Dunnett's test, $\alpha = 0.05$). In clone 189-434, both the transgenic and control plants were highly susceptible to rust infection, and all transgenics performed nearly identically to the control plants in all clones (Dunnett's test, $\alpha = 0.05$).

Ineffective resistance to *Septoria* leaf spot and stem canker

Results from the in vitro leaf assay showed that transgenic and control plants from all three clones were susceptible (≤50% HLT) to both *Septoria* species; however, clonal differences in susceptibility were apparent. All clones were more susceptible to *S. musiva* than to *S. populicola*. Clone 195-529 was the least susceptible to *S. musiva*, and clones 189-434 and 311-93 displayed about the same level of susceptibility. Clone 189-434 was the most susceptible clone to *S. populicola*. There was no difference between transgenic and control lines in their susceptibility to either fungus. Stem assays on plants growing in the greenhouse resulted in similar findings. Clonal differences were significant, with clone 189-434 the most susceptible to *S. musiva*, and clone 311-93 the most susceptible to *S. populicola*. The two fungi caused similarly sized cankers in clones 189-434 and 195-529. There were no differences in canker susceptibility between the transgenic and control plants in any of the three clones towards both fungi (data not shown).

Discussion

We found that overexpression of the *bO* gene caused formation of spontaneous HR-like lesions in at least two lines in the greenhouse (Nos. 6 and 41) and in a single line in the field (No. 6). Line 6 consistently exhibited severe lesions both in the greenhouse and in the field, and its growth was severely retarded compared with the other transgenic and control lines. Its extremely high level of *bO* expression appears to have exceeded a threshold required for extensive lesion development in field-grown poplar (Fig. 2). The lesion phenotype displayed had never before been observed in any other of the more than 2000 independent lines of transgenic poplars produced in our laboratory, making it very unlikely to be the result of a somaclonal mutation rather than because of its high level of *bO* expression. Lesion development and growth of the other transgenic lines in the field were not significantly different from that of the control lines, despite considerable levels of *bO* expression in most of them.

Lesion phenotypes of the *bO*-poplar leaves were similar to *bO*-expressing tobacco and potato; however, lesions were also observed on stems of the latter two species (Abad et al. 1997; Mittler et al. 1995), whereas none were observed in poplar. Lesions also formed on flowers in the *bO* potato but were not detected on tubers or roots. Although growth of the *bO*-expressing tobacco plants was normal, some stunting was reported in the *bO*-expressing potato (Abad et al. 1997). Furthermore, *bO*-tobacco plants transformed with a *bO* gene construct containing the stronger figwort mosaic virus (FMV) promoter, instead of the CaMV 35S promoter, were also stunted (M.S. Abad, personal communication). It appears that very high levels of *bO* expression can cause substantial growth reductions.

In contrast to results in transgenic tobacco, we demonstrated that *PAL1* expression was not elevated in the *bO* poplars, even in the high *bO*-expressing line. In addition, the expression of two wound-inducible chitinases, *WIN6* and *WIN8*, was very low in *bO* poplars and did not differ from control plants either before or after wounding. Both WIN6 and WIN8 amino acid sequences show strong similarities to class IA chitinases (Pfam HMM data base: <http://pfam.wustl.edu/hmmsearch.shtml>), which are known to have antifungal activities (Bol et al. 1996; Collinge et al. 1993). In constrast, in *bO*-tobacco plants both *PAL* and chitinase mRNAs were more abundant than in controls, both in older leaves with lesions and in younger leaves that had few or no lesions (Mittler et al. 1995).

The effects of the *bO* gene on disease resistance can vary widely. Abad et al. (1997) challenged *bO*-expressing potato plants with several pathogens and found that resistance level and symptoms varied widely with fungal strain. In our study, we tested resistance of *bO* poplars to four different fungal pathogens. There was no observable difference detected in resistance between the *bO* and control plants; all pairs were equally susceptible. As expected, however, there was some variation in susceptibility to the pathogens among poplar clones. When challenged with *Melampsora* rust, transgenic lines from clone 189-434, a clone that was very susceptible to rust, did not produce any fewer rust pustules than its control. When *bO* poplars of the *Venturia*-susceptible clone 195-529 were challenged with *Venturia*, it caused necrosis on leaves, petioles, and stems similar to that in controls, and similar to that observed in *bO* potato infected with *Phytophthora infestans* mating type A2 (Abad et al. 1997). Additional negative results were obtained when *bO* poplars were assayed for resistance against two *Septoria* species that cause leaf spot and stem canker.

Although overexpression of the *bO* gene was evident from RNA analysis and formation of the spontaneous HR lesions, prelesion induction of biochemical markers (*WIN6*, *WIN8*, and *PAL1*) involved in induced resistance was not detected. This was true even in line 6, which showed extensive lesion formation as leaves aged. Thus, the prelesion induction of defense genes mediated by bO protein reported in tobacco and potato appeared to be absent in poplar. Although we cannot rule out that this negative result may be a consequence of not monitoring a sufficient number of genes, or sufficient time points and tissues, we believe that it may instead reflect a different reaction to *bO* in *Populus*, or in trees generally, when compared with herbaceous species. Perhaps the size and longevity of trees, which cause them to serve as hosts to a myriad of insects and microbial species during their lifetimes, requires them to "demand" a higher induction threshold before they devote major cellular resources to increase pathogen defense and risk massive HR-associated tissue necrosis. If so, trees may require different transgenic approaches to genetic engineering of disease resistance than short-lived plant species. Studies of additional plant species, defense genes, and inductive treatments will be necessary to test this hypothesis.

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