

Enhancing Resistance to *Sclerotinia minor* in Peanut by Expressing a Barley Oxalate Oxidase Gene¹

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Sclerotinia minor Jagger is the causal agent of Sclerotinia blight, a highly destructive disease of peanut (*Arachis hypogaea*). Based on evidence that oxalic acid is involved in the pathogenicity of many Sclerotinia species, our objectives were to recover transgenic peanut plants expressing an oxalic acid-degrading oxalate oxidase and to evaluate them for increased resistance to *S. minor*. Transformed plants were regenerated from embryogenic cultures of three Virginia peanut cultivars (Wilson, Perry, and NC-7). A colorimetric enzyme assay was used to screen for oxalate oxidase activity in leaf tissue. Candidate plants with a range of expression levels were chosen for further analysis. Integration of the transgene was confirmed by Southern-blot analysis, and gene expression was demonstrated in transformants by northern-blot analysis. A sensitive fluorescent enzyme assay was used to quantify expression levels for comparison to the colorimetric protocol. A detached leaflet assay tested whether transgene expression could limit lesion size resulting from direct application of oxalic acid. Lesion size was significantly reduced in transgenic plants compared to nontransformed controls (65%–89% reduction at high oxalic acid concentrations). A second bioassay examined lesion size after inoculation of leaflets with *S. minor* mycelia. Lesion size was reduced by 75% to 97% in transformed plants, providing evidence that oxalate oxidase can confer enhanced resistance to Sclerotinia blight in peanut.

Sclerotinia blight of peanut, caused by the necrotrophic fungus *Sclerotinia minor*, is one of the most devastating diseases of peanut (*Arachis hypogaea*) in Virginia, northeastern North Carolina, Oklahoma, and Texas. The fungicide fluzinam provides some protection (Smith et al., 1992), but its benefit to peanut producers is offset by the expense of the multiple applications necessary to achieve modest control. Yield losses due to Sclerotinia blight can be significant. For example, prior to the availability of fluzinam, losses to Sclerotinia blight averaged 10% in Virginia, with pod loss exceeding 50% in severely affected fields (Porter and Melouk, 1997).

Oxalic acid is considered a pathogenicity factor in *Sclerotinia sclerotiorum* and many other fungal pathogens (Maxwell and Lumsden, 1970; Godoy et al., 1990). A wide variety of other fungi also secrete oxalic acid, including *Poria placenta* (Ritschkoff et al., 1995), *Septoria musiva* (Liang et al., 2001), *Sclerotium rolfsii* (Bateman and Beer, 1965), *Endothia (Cryphonectria) parasitica* (Havir and Anagnostakis, 1985; Bennett and Hindal, 1989), *Penicillium oxalicum* (Ikotun, 1984),

Cristulariella pyramidalis (Kurian and Stelzig, 1979), *Sclerotium cepivorum* (Stone and Armentrout, 1985), *Mycena citricolor* (Rao and Tewari, 1987), and *S. minor* (Hollowell et al., 2001). Direct application of oxalic acid to stem or leaf tissue causes marked tissue injury and wilting, similar to plant responses to fungal infection by *S. rolfsii* (Bateman and Beer, 1965) and *S. sclerotiorum* (Noyes and Hancock, 1981). The most compelling evidence for the involvement of oxalic acid in disease initiation was the demonstration that mutant isolates of *S. sclerotiorum*, deficient in oxalic acid production, were not pathogenic on bean (*Phaseolus vulgaris*), but revertants became pathogenic once they regained the ability to produce oxalic acid (Godoy et al., 1990). Screening for resistance to oxalic acid has also been studied as an indirect test of physiological resistance to white mold in common bean (Kolkman and Kelly, 2000).

Oxalic acid may aid in infection through a number of proposed routes, including acidification to facilitate cell wall-degrading enzyme activity, through pH-mediated tissue damage, or via sequestration of Ca²⁺ ions (for review, see Dutton and Evans, 1996). The optimal pH for many cell wall-degrading enzymes lies in the acidic range (Lumsden, 1979). Degradative synergy between oxalic acid and polygalacturonase has been shown for Sclerotinia species and other fungal pathogens (Bateman and Beer, 1965; Kurian and Stelzig, 1979). Rollins (2003) demonstrated that both oxalic acid production and endopolygalacturonase activity are regulated by pH in *S. sclerotiorum*. The low pH resulting from oxalic acid production may weaken plants to improve access to fungal infection.

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Oxalic acid is also a strong chelator of divalent cations, and sequestration of calcium may weaken cell walls (Bateman and Beer, 1965). Oxalic acid can directly suppress the oxidative burst associated with the detection of pathogens by the plant (Cessna et al., 2000). Recently, oxalic acid has been reported to disturb guard cell function during infection by *S. sclerotiorum* by inducing stomatal opening and inhibiting abscisic acid-induced stomatal closure (Guimarães and Stotz, 2004).

Oxalate oxidase belongs to the germin family of proteins and catalyzes the degradation of oxalic acid to produce carbon dioxide and hydrogen peroxide (H_2O_2 ; Chiriboga, 1966; Lane et al., 1993). Oxalate oxidase is expressed during germination, where it associates with cell wall components such as glucuronogalactoarabinoxylans (Lane, 2000). Plant oxalate oxidase activity increases the pH at the site of infection following contact with oxalate-secreting pathogens (Rollins, 2003). Oxalate oxidase has received considerable attention for its possible utility in plant defense. It has been proposed that through the production of H_2O_2 , oxalate oxidase or oxalate oxidase-like proteins may cause cross-linking of plant cell wall proteins in papillae at the site of infection (Wei et al., 1998) or play a role in the plant hypersensitive response (Lane, 1994; Zhou et al., 1998), previously shown to be orchestrated by H_2O_2 from the oxidative burst (Levine et al., 1994). Wheat (*Triticum aestivum*) oxalate oxidase activity increased following biotic (e.g. fungal pathogens) and abiotic (e.g. salt or heavy-metal ions) stress (Hurkman et al., 1994; Dessaigne et al., 1997; Berna and Bernier, 1999). Barley (*Hordeum vulgare*) oxalate oxidase is also induced in leaves following challenge with the fungus *Erysiphe graminis* (Dumas et al., 1995; Zhang et al., 1995).

Reports of oxalate oxidase activity in response to pathogen attack have been restricted to cereals (Pundir, 1991; Lane et al., 1993); however, transgenic technology has been used to successfully transfer oxalate oxidase genes from cereals to other plants. Transgenic oilseed rape (*Brassica napus*) expressing a barley oxalate oxidase can break down exogenously applied oxalic acid (Thompson et al., 1995). Soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), and sunflower (*Helianthus annuus*) transformed with a wheat oxalate oxidase gene demonstrated increased resistance to *S. sclerotiorum* (Zaghmout et al., 1997; Donaldson et al., 2001; Hu et al., 2003). Poplar (*Populus* spp.) expressing the same gene was resistant to *S. musiva* (Liang et al., 2001). Although not examined in the soybean or poplar studies, it was demonstrated in transformed sunflower that oxalate oxidase expression resulted in the induction of plant defense proteins (Hu et al., 2003). There are also reports of increased resistance to insect predation in corn (*Zea mays*) expressing a wheat oxalate oxidase (Ramputh et al., 2002). Expression of another oxalate-degrading enzyme, oxalate decarboxylase, isolated from *Collybia velutipes*, also resulted in resistance of transgenic tomato (*Lycopersicon esculentum*) and tobacco to *S. sclerotiorum* (Kesarwani et al., 2000).

Further evidence for the utility of H_2O_2 -generating enzymes in protective plant responses was provided by an examination of Glc oxidase expression in transgenic rice (Kachroo et al., 2003). Expression of Glc oxidase and release of H_2O_2 led to enhanced resistance to the bacterial pathogen *Xanthomonas oryzae* and the fungus *Magnaporthe grisea*. Multiple strategies for engineering oilseed crops for resistance to *S. sclerotiorum*, including introduction of H_2O_2 -producing enzymes, have recently been reviewed (Lu, 2003). Together, these reports suggest that introduction of an oxalate oxidase to degrade oxalic acid and generate H_2O_2 can be used in peanut to enhance resistance to oxalic acid-producing fungi, including *S. minor*.

RESULTS

Assay of Transformants for Oxalate Oxidase Activity

Following establishment of embryogenic tissue culture lines for several Virginia peanut cultivars, embryos were transformed by particle bombardment with a plasmid vector containing the barley oxalate oxidase coding sequence. Selection and regeneration resulted in greater than 200 viable plants from three different cultivars. A simple colorimetric assay was used to screen hygromycin-resistant transformants for oxalate oxidase activity, and 40% to 60% of regenerated T_0 plants showed elevated activity levels, depending on cultivar (data not shown). Eight transformed plants were chosen for a closer examination of enzyme activity (Fig. 1). Oxalate oxidase activity levels were higher in seven of the transformants than in all three nontransformed controls (Wilson, Perry, and NC-7). One additional regenerated plant, W1, was included as a nonexpressing control transformant.

Figure 1 shows a comparison of results from two different protocols for detection of oxalate oxidase activity. Both the colorimetric assay and the commercially available fluorescent kit relied on the detection of H_2O_2 produced by the activity of oxalate oxidase on the substrate, oxalic acid. The difference between the two protocols was the greater sensitivity and lower background of the fluorescent assay, which required 5-fold less reaction volume for a comparable response. Both methods are easy to perform in a microtiter format, which allowed for high throughput analysis of transformed material. The assay results were highly repeatable with comparable levels of expression in different leaves of the same plant. One line, W3, gave variable oxalate oxidase activity results at different sampling dates, with some leaflets showing moderate activity and some with only background levels, indicating possible chimerism (data not shown).

Southern and Northern Analysis

Genomic DNA samples from transformed and control peanut plants were analyzed by Southern-blot hybridization to demonstrate the integration of the

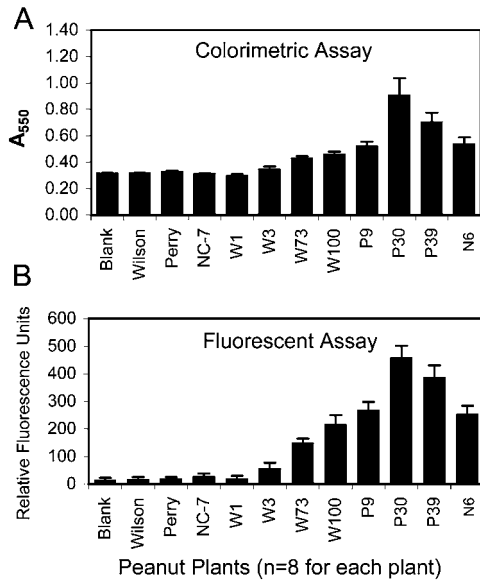


Figure 1. Comparison of two oxalate oxidase activity assays using transgenic and control peanut plants. Transgenic plants were regenerated from embryogenic peanut cultures bombarded with an oxalate oxidase gene under the control of the cauliflower mosaic virus 35S promoter and selected in the presence of hygromycin. Transformants were identified by screening for transgene expression using a leaf disc assay for oxalate oxidase activity. Quantitative measure of enzyme activity was determined for eight transformants and three untransformed controls using two different detection methods. A, The colorimetric detection protocol used 100 μ L of the enzyme reaction, and results were measured spectrophotometrically at A_{550} . B, The Amplex Red detection kit used 20 μ L of the enzyme reaction, and results were measured with a fluorescence plate reader with an excitation filter of 530 nm and emission filter of 590 nm. (The same reaction was assayed by both methods for each of the eight leaf discs per plant.) Untransformed controls were Perry, Wilson, and NC-7. P9, P30, and P39 were regenerated from bombardment of Perry embryogenic cultures. W1, W3, W73, and W100 were regenerated from bombardment of Wilson embryogenic cultures. N6 is derived from NC-7 embryos, although some caution in making this assignment was described in "Materials and Methods." "Blank" indicates a reaction performed without plant material (buffer alone). Means and \pm SE are presented for eight replicates for each plant.

oxalate oxidase transgene into the peanut genome. The number of insertion sites varied among transformants, as illustrated in Figure 2. The variable intensity of bands also suggested the possible insertion of multiple copies of the coding sequence in several lines, as is frequently observed for microprojectile bombardment. Hybridization patterns differed for the three Perry transformants, P9, P30, and P39, indicating that they were all independent transformation events. Hybridization patterns for two of the Wilson transformants (W3 and W100) were similar, raising the possibility that they may have originated from the same bombardment event. However, the difference in oxalate oxidase expression and other parameters tested below suggested that W3 and W100 originated from different events and that the patterns appeared similar due to the inability to resolve small size differences for large

fragments on the gel. A similar consideration applies to transformant N6 obtained from bombardment of NC-7 cultures. The similarity of its hybridization pattern to P39 called into question whether it could have been inadvertently mislabeled during transfer to the greenhouse. We have retained the N6 designator while recognizing this caveat and will confirm its origin if progeny from this plant are used for any future studies.

Oxalate oxidase RNA expression was examined by northern-blot analysis to confirm the enzyme activity results. RNA from young leaflets was hybridized with the oxalate oxidase probe (Fig. 3). The seven oxalate oxidase-positive transformants showed the presence of a single 700-nucleotide RNA band that was absent from nontransformed controls. RNA expression levels were variable among lines. Line W3 showed very low levels of oxalate oxidase RNA, while P30 had the highest level of expression, consistent with enzyme activity results.

Resistance to Oxalic Acid

To determine whether transgenic peanut plants expressing oxalate oxidase showed increased resistance to

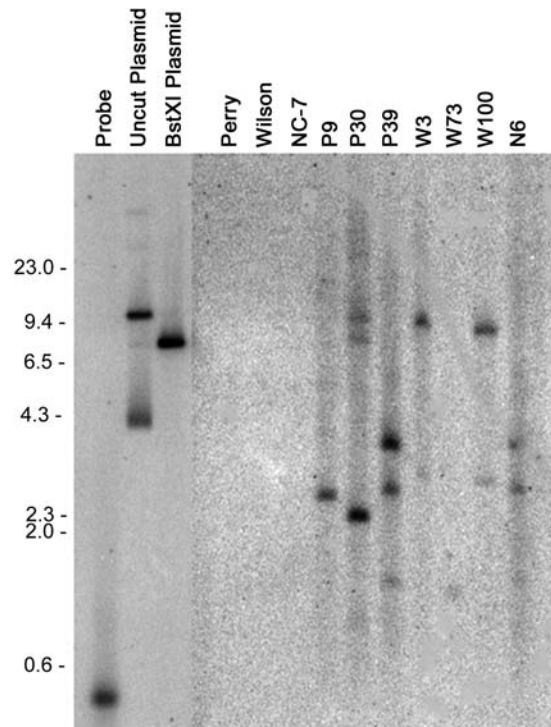


Figure 2. Southern-blot analysis of peanut genomic DNA with the oxalate oxidase probe. *Bst*XI-digested DNA was probed with a 32 P-labeled fragment amplified by PCR from the barley oxalate oxidase cDNA. Size markers were *Hind*III-digested lambda DNA (sizes to the left of the blot are in base pairs). The first three lanes show hybridization of the probe to the PCR product used as the probe (470 bp), to undigested pOxOx plasmid, and to *Bst*XI-digested pOxOx plasmid. Untransformed controls are cultivars Perry, Wilson, and NC-7. P9, P30, and P39 were Perry transformants. W3, W73, and W100 were Wilson transformants, and N6 is believed to be a NC-7 transformant.

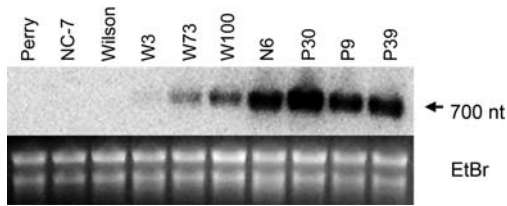


Figure 3. Northern-blot analysis of peanut leaflet RNA with the oxalate oxidase probe. A, Total RNA from transformants and nontransformed controls was probed with the same 470-bp probe as described for the Southern blot in Figure 2. Expression in transgenic peanuts is observed by the presence of a 700-nucleotide mRNA band. B, Ethidium bromide (EtBr) staining was performed to demonstrate sample loading.

the damaging effects of oxalic acid, varying concentrations of oxalic acid were applied to the surface of detached leaflets. Resistance was measured as a reduction in lesion size on transgenic plant leaflets compared to lesion size on nontransformed controls. Transformed peanut plants showed decreased damage from exogenously applied oxalic acid over a wide range of oxalic acid concentrations, as illustrated in Figures 4 and 5. The assays showed that expression of oxalate oxidase in

transgenic peanut was able to neutralize the effects of oxalic acid at pathophysiological concentrations (Fig. 4A) and at concentrations up to 20-fold higher than normally found in infected tissue (Fig. 4, B–D). At high (200 mM) oxalic acid concentrations, lesions were reduced by 65% to 89% compared to the corresponding nontransformed controls (Fig. 5A). Figure 5B shows the appearance of leaflets from several transformants following application of oxalic acid, illustrating the reduced lesion size. Liang et al. (2001) found that lesion area resulting from exposure to 200 mM oxalic acid on leaf discs from oxalate oxidase-expressing poplar transformants was reduced by 18% compared to nontransformed controls. The apparent difference in oxalic acid resistance in our transgenic peanut lines compared to the transgenic poplar may have resulted from differences in parameters such as plant species, expression levels, and/or assay conditions.

Resistance to *S. minor*

To test for enhanced resistance to *S. minor*, thin agar plugs from the actively growing edge of a fungal culture were used to inoculate isolated leaflets from

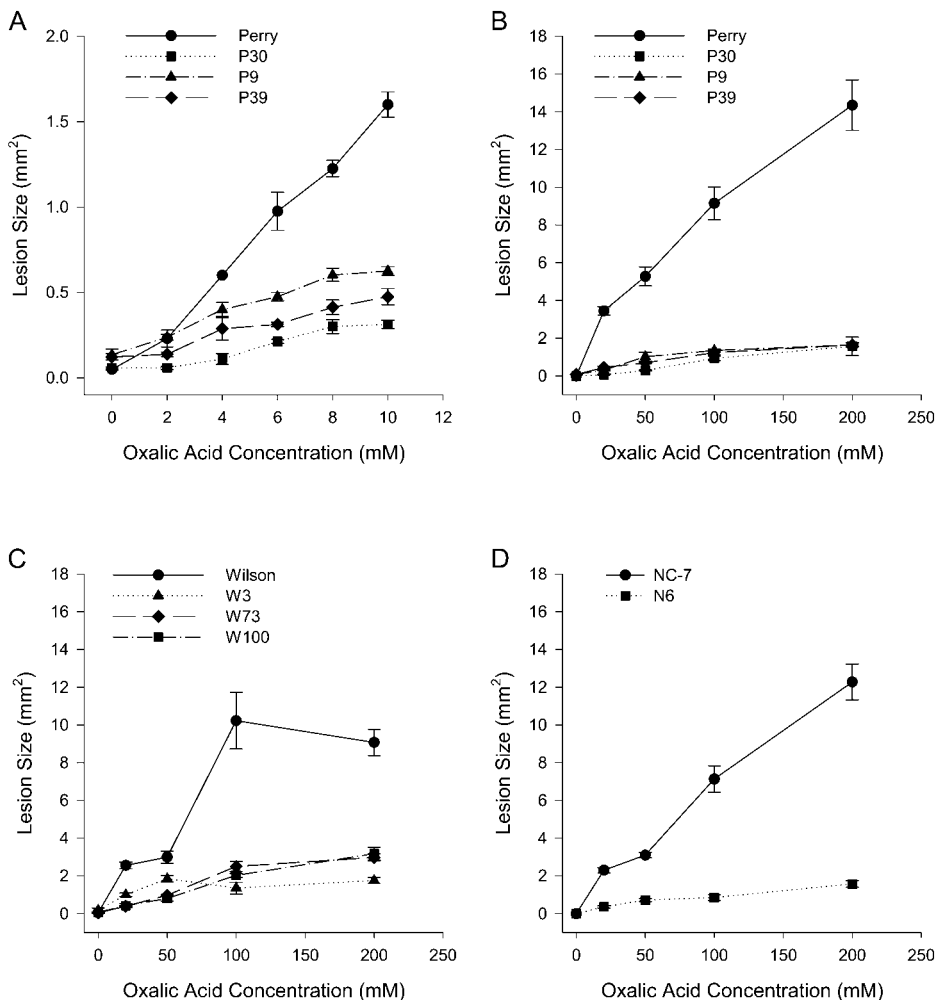


Figure 4. Resistance of transgenic plants to application of oxalic acid. Different concentrations of oxalic acid were applied to detached leaflets from transformants and nontransformed controls. Oxalic acid in A was applied at 0, 2, 4, 6, 8, and 10 mM. Oxalic acid in B to D was applied at 0, 20, 50, 100, and 200 mM. A, Nontransformed Perry and three transformants (P9, P30, and P39) at low oxalic acid concentrations. B, Same samples as in A except for high range of oxalic acid concentrations. C, Nontransformed Wilson and three transformants (W3, W73, and W100). D, Nontransformed NC-7 and transformed N6. Means and SE are shown for four replicates.

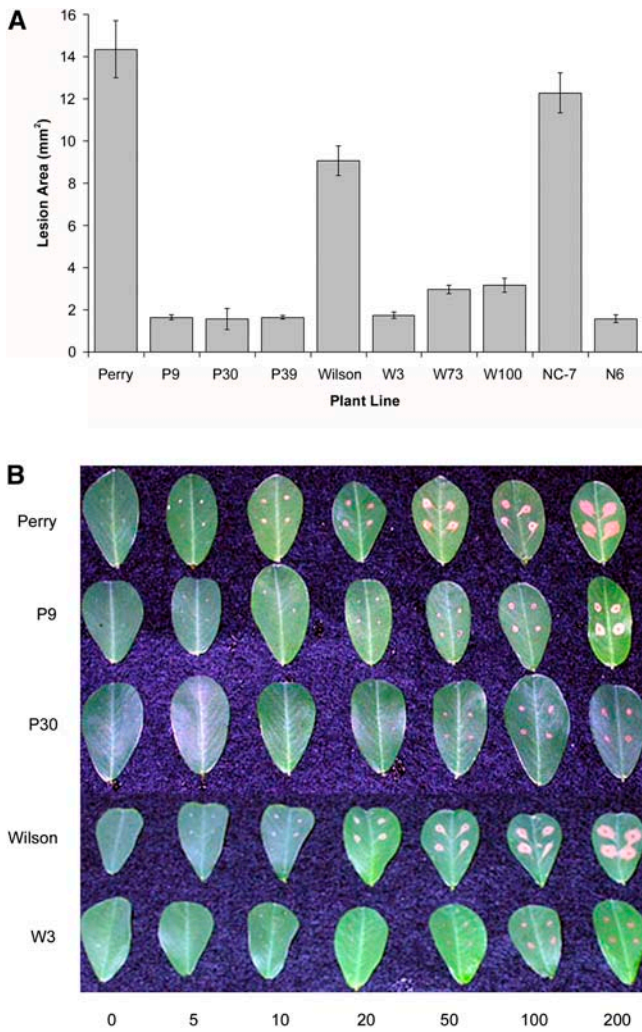


Figure 5. Resistance to oxalic acid in control and transformed peanut. A, Bar graph showing lesion size (in mm²) for transformed lines and nontransformed controls following application of 200 mM oxalic acid to peanut lines. B, Photograph of selected peanut lines showing lesion size and appearance on detached leaflets after application of increasing amounts of oxalic acid. Means and SE are shown for four replicates.

both transformed and nontransformed peanut plants. All the transgenic lines showed increased resistance to *S. minor* as measured by a reduction in lesion area (Fig. 6A). The type of lesion also varied between transgenic and control lines. Controls showed a large, continuous, tan-colored lesion, whereas the transgenic lines typically exhibited numerous smaller lesions resembling a hypersensitive response (Fig. 6B). The average reduction compared to the respective nontransformed control cultivars ranged from 75% for P39 to 97% for W100. The resistance results in peanut correlated well with previously published reports in other plants. Lesion size resulting from inoculation with the oxalate-secreting pathogen *S. musiva* was reduced by 63% in poplar expressing a wheat oxalate oxidase gene (Liang et al., 2001). Using oxalate decarboxylase to degrade oxalic acid, Kesarwani et al. (2000) showed

decreased lesion size of approximately 89% in transgenic tomato inoculated with *S. sclerotiorum*.

Fertility of Transgenic Lines

Primary transgenic plants often show reduced fertility and lower yields than nontransformed plants. This can be due to epigenetic effects related to long tissue culture regimens or to the direct effect of the transgene itself, either because of position effects or the toxic nature of some transgene products. For example, Kachroo et al. (2003) showed that ectopic, wound-inducible expression of a Glc oxidase gene in transgenic rice resulted in lesion mimicry. Our T₀ peanut transformants did not grow as well as non-regenerated control plants and exhibited variable fertility. T₁ progeny plants showed improved growth, and no lesion mimicry has been observed. Line P30 had the highest average enzyme activity, but, although it flowered and produced several pods, all seeds were aborted at an early stage. Lines P9, P39, W73, and N6 produced seed yields similar to the nonexpressing

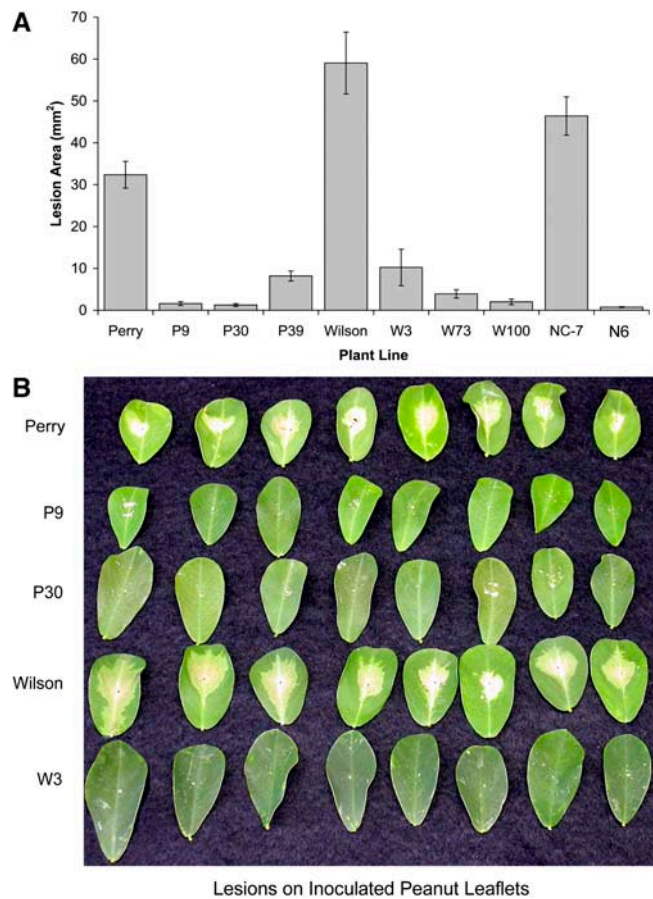


Figure 6. Resistance to *S. minor* in oxalate oxidase-expressing peanut lines compared to controls. A, Lesion size (in mm²) on detached leaflets in response to inoculation with *S. minor*. B, Comparison of selected peanut lines in response to inoculation with *S. minor* showing lesion size and appearance on detached leaflets. Means and SE are shown for 16 replicates.

transformant from line W1 (21–54 seeds). Line W100 produced only five viable seeds. Line W3 showed the lowest levels of transgene expression and most variable enzyme activity but also failed to produce any viable seed. From a comparison of RNA expression levels and seed recovery, we concluded that the lack of seed production in several transformants could not be attributed to the expression of oxalate oxidase alone.

DISCUSSION

These studies demonstrated that the expression of a barley oxalate oxidase in transgenic peanut increased resistance to exogenously applied oxalic acid over a wide range of concentrations (up to 20 times pathophysiological levels). Transgenic peanut plants also exhibited enhanced resistance to *S. minor* as measured by reduced lesion size in detached leaflet assays. Peanut leaf inoculations were previously shown to serve as a reliable indicator of host resistance to *S. minor* (Hollowell and Shew, 2003).

In addition to meeting the goal of enhancing resistance to *S. minor*, the project results also demonstrated the potential utility of oxalic acid as a screen for fungal disease resistance. Previous studies used wilting during immersion of cut bean seedlings in 20 mM oxalic acid as a screen for white mold resistance (Kolkman and Kelly, 2000). Our studies quantified the response to oxalic acid over a much wider range of concentrations. In addition, the leaflet assay is much more rapid and simple to perform.

Due to the absence of obvious deleterious effects of the oxalate oxidase transgene in most peanut transformants, we have used it as a sensitive marker for following transformation and regeneration, and as a reporter gene to evaluate expression in transformants. It is inexpensive and easy to assay, allowing screening of many progeny plants for transgene segregation patterns during generation of sufficient peanut seeds for field studies. The utility of oxalate oxidase as a reporter gene and its advantages over luciferase, β -glucuronidase, and green fluorescent protein have been recently described for other dicots (soybean) and monocots (maize) alike (Simmonds et al., 2004).

As encouraging as these results are in support of oxalate oxidase as a useful resistance gene, a limitation to the experiments is that the leaflet assays were performed under highly controlled conditions, including constant temperature, humidity, and use of a single fungal isolate. This will not be the case in field conditions. *S. minor* is an obligate necrotrophic fungus and requires dead or decaying tissue for successful establishment of infection. Variable growth and environmental conditions will come into play in a field production situation. Oxalic acid is considered a pathogenicity factor for *Sclerotinia* spp. (Godoy et al., 1990) but may be only one of a number of factors that contribute to successful infection by fungal pathogens.

The oxalate oxidase gene may also play a role in cell wall defense unrelated to its enzyme activity. For example, Schweizer et al. (1999) showed that the expression of a mutant wheat oxalate oxidase sequence lacking enzyme activity conferred increased resistance to penetration by *S. sclerotiorum* (20%–67% of control levels). Other studies have suggested that additional factors, such as hydrolytic enzymes, are likely to be important in establishing infection and progression of disease caused by *S. minor*. Based on the observation that coexpression of glucanase and chitinase genes in transgenic tobacco enhanced protection against fungal attack (Zhu et al., 1994), these genes have also been introduced into peanut (Chenault et al., 2002). Expression of multiple resistance genes may offer a better approach for providing greater and long-lasting fungal resistance.

Although there are probably multiple factors involved in the establishment of fungal infection, this study demonstrates the importance of oxalic acid as a pathogenicity factor in infection of peanut by *S. minor*, and that counteracting the effects of oxalic acid through oxalate oxidase expression can enhance resistance to injury and establishment of fungal infection. Further studies are in progress to assess disease resistance and peanut yields for a number of oxalate oxidase-expressing peanut transformants under field conditions.

MATERIALS AND METHODS

Tissue Culture and Regeneration

Embryos from mature seeds of Virginia-type peanut (*Arachis hypogaea*) cultivars (NC-7, Wilson, and Perry) were surface sterilized by soaking the seeds in 70% ethanol for 5 min, twice in 1% dichloroisocyanuric acid for 5 min, and finally rinsing the seeds with sterile distilled water. Following sterilization, the embryo was excised and the radicle end removed. Excised embryos were washed in 1% dichloroisocyanuric acid for 30 s and rinsed with three volumes of sterile distilled water. All reagents for tissue culture and subsequent assays were obtained from Sigma (St. Louis) unless otherwise specified.

Embryogenic callus was induced by culturing the mature embryos on MP3 medium, which contained Murashige and Skoog (MS; Murashige and Skoog, 1962) salts and vitamins (Caisson Laboratories, Sugar City, ID), 3% (w/v) Suc, 1 g L⁻¹ Gln, 0.8% (w/v) agar, and 3 mg L⁻¹ picloram. Embryogenic callus was maintained on MP3 medium at 28°C, in the dark, with a subculture period of 3 to 4 weeks.

Maintenance, bombardment, and regeneration of peanut embryogenic cultures were conducted as described previously (Livingstone and Birch, 1999). For regeneration, differentiated embryos with a clearly distinguishable “apex” and “root” bipolarity were transferred to MS medium, which contained MS salts and vitamins, 2% (w/v) Suc, and 0.1% activated charcoal, and allowed to grow for 1 month. The embryos were transferred to a thin layer of MS medium and allowed to desiccate for approximately 1 month before final transfer to fresh MS medium. Any embryos that failed to turn green or show signs of growth and differentiation were discarded. Both regeneration steps were performed at 28°C in the light (80 $\mu\text{mol m}^{-2} \text{s}^{-1}$) with a 16-h photoperiod.

Vector Construction

RNA was purified from germinating barley seedlings (RNeasy kit; Qiagen, Valencia, CA) for amplification of the oxalate oxidase coding region by reverse transcription-PCR. Oligonucleotide primers were designed from the

published sequence for barley oxalate oxidase (accession no. Y14203; Zhou et al., 1998). Upstream (5' GCAAGGTACCATGGGTTACTCTAAAAACC-TAG) and downstream (5' GCAAGTCGACTGGGGCTCATGGAAGTTAAG) primers included *KpnI* and *SalI* sites, respectively, for cloning into the transformation vector. First-strand synthesis was carried out using Super-script reverse transcriptase according to manufacturer's specifications (Invitrogen, Carlsbad, CA), and amplification was performed using *Taq* PCR master mix (Qiagen). PCR conditions were denaturation for 30 s at 94°C, annealing for 1 min at 44°C and 50°C for the first and second cycles, respectively, followed by 56°C for the remaining 38 cycles, and extension for 1 min at 72°C. After cloning the 710-bp PCR product into the *SmaI* site of an intermediate vector pTZ19R and verification of the sequence for oxalate oxidase, cDNA was excised as a *KpnI-SalI* fragment and used to replace the coding sequence for a fungal *phyA* gene in a previously described plasmid vector (Li et al., 1997) under control of the dual-enhanced cauliflower mosaic virus 35S promoter. The final oxalate oxidase construct (pOxOx) included a selectable marker for hygromycin resistance.

Plant Transformation and Growth of Regenerated Plants

Embryogenic callus was bombarded with the pOxOx plasmid DNA that had been coated onto 1- μ m-diameter tungsten microparticles. Particles were accelerated using a PDS-1000/He Particle Delivery System and an 1,100-psi rupture disc according to manufacturer's recommendations (Bio-Rad, Hercules, CA). Callus was incubated on MP3 medium supplemented with 0.4 M D-mannitol for bombardment. After 2 h of recovery, callus was transferred to MS medium for 2 to 3 d and then plated onto MP3 medium supplemented with filter-sterilized hygromycin B at a concentration of 40 mg L⁻¹ for selection. After 3 to 6 months, transformed somatic embryos were regenerated on antibiotic-free medium as described previously (Livingstone and Birch, 1999). Once plants were transferred from Magenta boxes to the greenhouse, growth conditions included daylight plus supplemental light (sodium halide and high-intensity discharge lighting) for 12 h a day. Plants were treated with Osmocote fertilizer (Scotts, Marysville, OH) and Nitro-Fix (TCI, Pekin, IL) according to manufacturers' recommendations.

DNA and RNA Extraction

DNA was extracted by a modification of the hexadecyltrimethylammonium bromide method described by Murray and Thompson (1980). Unexpanded peanut leaflets (1–1.5 g) were ground in a mortar and pestle with liquid nitrogen and transferred to 10 mL of extraction buffer containing 2% hexadecyltrimethylammonium bromide, 2% polyvinylpyrrolidone (PVP 40; Sigma), 50 mM MES, and 1.4 M NaCl, pH 5.5, which was preheated to 65°C. A volume of 100 μ L of 0.2 M β -mercaptoethanol was added to each tube immediately before adding the frozen tissue. The mixture was incubated at 65°C for 10 min with several gentle inversions to ensure mixing, and debris was pelleted at 12,000 rpm in a prewarmed (>15°C) Sorvall SA-600 rotor (Kendro, Asheville, NC) for 10 min. The supernatant was poured through two layers of Miracloth (Calbiochem, La Jolla, CA) into a clean tube containing 10 μ L of 10 mg mL⁻¹ RNase A and incubated for 30 min at 37°C. Following RNase digestion, the sample was transferred to a clean tube, and an equal volume of chloroform was added. The tube was gently agitated for 5 min before centrifugation at 12,000 rpm for 10 min. The supernatant was retained and the DNA precipitated with 0.1 volumes of 7.5 M ammonium acetate and 2 volumes of ice-cold ethanol. After 30 min at -20°C, the DNA was spooled into a fresh tube and allowed to stand at room temperature to evaporate the remaining alcohol. The sample was resuspended in 3 mL of TE (10 mM Tris, pH 8, 1 mM EDTA). RNA was extracted from 100 to 150 mg of fresh, tightly folded peanut leaflets using an RNeasy kit (Qiagen).

Southern and Northern Hybridization

To confirm integration of the transgene in peanut transformants, genomic DNA was digested with *BstXI*. *BstXI* cuts the pOxOx plasmid once in the oxalate oxidase coding sequence, adjacent to the region amplified as the hybridization probe. DNA fragments were separated by agarose gel electrophoresis and transferred to positively charged nylon membranes (Nytran supercharge; Schleicher and Schuell BioScience, Keene, NH). The membrane was hybridized with a ³²P-labeled oxalate oxidase probe. The probe sequence was a 470-bp fragment amplified by PCR using puReTaqReady-To-Go PCR beads (Amersham Biosciences, Piscataway, NJ) with forward primer

5'CCCTCTACAGGACTTCTGCG3' and reverse primer 5'CTGGTGTGTAAGGAACACAA3'. The PCR product was purified using a QIAquick PCR purification kit (Qiagen) and labeled using a Prime-It RmT Random Primer labeling kit (Stratagene, La Jolla, CA) and radiolabeled ³²P-dCTP (Perkin Elmer, Boston). Hybridization and subsequent washes were performed at 65°C using conditions described by Sambrook et al. (1989) and analyzed on a Storm 850 phosphorimager (Molecular Dynamics, Piscataway, NJ).

RNA was separated by formaldehyde agarose gel electrophoresis and transferred to positively charged nylon membranes as described previously (Sambrook et al., 1989). Membranes were probed with the same oxalate oxidase sequence as described above for Southern blots.

Oxalate Oxidase Assays

To determine oxalate oxidase activity in transgenic peanut plants, leaf discs (5 mm diameter) were placed in 1.5-mL microfuge tubes and assayed using a modification of the procedure by Sugiura et al. (1979). Next, 200 μ L of assay buffer (18 mg oxalic acid in 100 mL of 2.5 mM succinic acid, pH 4) were added to tubes and reactions incubated for 15 min at 37°C. After incubation, developing solution (135 μ L) was added to the tubes and the reactions allowed to continue at room temperature for 30 min. Developing solution consisted of 6 mg of aminoantipyrene dissolved in 30 μ L of *N,N*-dimethylaniline, which was added to 100 mL of 0.1 M sodium phosphate buffer, pH 7.0, containing 57 μ L of a 140 mg mL⁻¹ solution of horseradish peroxidase. The absorbance was measured at a wavelength of 550 nm. For assay in a microtiter plate format, volumes were reduced to one-half the microfuge assay reaction size.

An alternative assay for release of H₂O₂ as a measure of oxalate oxidase activity was performed with an Amplex Red kit (Molecular Probes, Eugene, OR). Amplex Red in the presence of H₂O₂ and horseradish peroxidase forms the fluorescent compound resorufin. For the fluorescent assay, leaf discs were incubated in microtiter wells with the same assay buffer as described above. After incubation of leaf discs with oxalic acid substrate, a 20- μ L aliquot of each sample was transferred with a multichannel pipettor to a fresh plate and brought to a volume of 50 μ L with kit reaction buffer. To start the detection reaction, 50 μ L of the Amplex Red/horseradish peroxidase reagent was added and incubated for 30 min in the dark. Fluorescence was detected in a plate reader (Bio-Tek Instruments, Winooski, VT), using a 530-nm excitation filter and 590-nm emission filter. A standard curve allowed calculation of the total H₂O₂ released. The products of the Amplex Red reaction can also be measured spectrophotometrically at 550 nm due to the high extinction coefficient.

Oxalic Acid Bioassays

Leaflet assays were conducted to assess the ability of oxalate oxidase expression to prevent damage in response to application of oxalic acid to plant tissue. Detached peanut leaflets were arranged on an inverted weigh boat in 15-cm petri dishes containing dampened paper towels. Eight leaflets were used for each plant line, one for each concentration of oxalic acid. Each leaflet was wounded in four locations on the abaxial surface with an 18-gauge needle, and 15 μ L of oxalic acid was applied to each wound. For oxalic acid concentrations in the 0 to 200 mM range, the leaflets were incubated for 6 h at room temperature. For concentrations in the 0 to 10 mM range, leaflets were incubated for 48 h at room temperature. To quantify lesion size, leaflets were washed and viewed with a Zeiss Stemi SV 11 dissecting microscope (Carl Zeiss, Jena, Germany). Images were recorded with an attached Scion video camera and imaging software (Scion, Frederick, MD). The lesion area was traced with the computer mouse on the image displayed on the computer monitor. The delimited area was calculated in square pixels by the software package and converted to mm².

Fungal Bioassays

Fungal assays were performed using detached leaflets inoculated with an agar plug of *S. minor* mycelia. *S. minor* cultures were grown on thin potato dextrose agar plates and 5-mm plugs taken from the actively growing edge. Leaflets were wounded with an 18-gauge needle, and plugs were placed on the adaxial surface, near the midvein. Eight leaflets were inoculated for each plant line tested using a minimal quantity of agar in each plug. The plates were incubated for 48 h at 19°C, and lesion area was measured as in the oxalic acid assays above.

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