Sclerotinia Blight Resistance in Virginia-Type Peanut Transformed with a Barley Oxalate Oxidase Gene

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ABSTRACT

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Transgenic peanut lines expressing oxalate oxidase, a novel enzyme to peanut, were evaluated for resistance to *Sclerotinia* blight in naturally infested fields over a 5-year period. Area under the disease progress curve (AUDPC) for transgenic lines in single rows planted with seed from single-plant selections averaged 78, 83, and 90% lower than nontransgenic parents in 2004, 2005, and 2006, respectively. In addition, AUDPC in 14 transgenic lines planted with bulked seed in two-row plots averaged 81% lower compared with nontransgenic parents in 2005 and 86% lower

in 16 transgenic lines in 2006. Six transgenic lines yielded 488 to 1,260 kg/ha greater than nontransgenic parents in 2005, and 10 lines yielded 537 to 2,490 kg/ha greater in 2006. Fluazinam (0.58 kg a.i./ha) fungicide sprays in 2008 and 2009 reduced AUDPC in transgenic and nontransgenic lines but AUDPC was lowest in transgenic lines. Without fluazinam, yields of transgenic lines averaged 1,133 to 1,578 kg/ha greater than nontransgenic lines in 2008 and 1,670 to 2,755 kg/ha greater in 2009. These results demonstrated that the insertion of barley oxalate oxidase in peanut conveyed a high level of resistance to *Sclerotinia* blight, and negated the need for costly fungicide sprays.

Additional keywords: *Arachis hypogaea*, disease resistance, oxalic acid, *Sclerotinia minor*.

Sclerotinia blight, caused by the soilborne fungus *Sclerotinia minor* Jagger, is a major disease of peanut (*Arachis hypogaea* L.) in the United States. The disease was first found in Virginia and northeastern North Carolina (2,21) and subsequently in parts of Texas (30), Oklahoma (26), and New Mexico (25). During favorable conditions for *Sclerotinia* blight (19), yield losses can reach 57% (24). Production of oxalic acid by *S. minor*, *S. sclerotiorum*, and *Sclerotium rolfsii* is thought to be a pathogenicity factor that predisposes plants to infection and disease (1,7,8). Oxalate oxidase is an enzyme that degrades oxalic acid to produce carbon dioxide and hydrogen peroxide and prevents the predisposition of cells to infection by oxalic acid-producing fungi (29). Barley oxalate oxidase shows a high degree of homology to wheat germin, a protein in wheat that is expressed during germination (12). Similar proteins with oxalate oxidase activity are naturally found in cereals such as corn, wheat, barley, rye, oat, sorghum, and rice (4,6,22,29). Oxalate oxidase is upregulated in wheat and barley in response to pathogen attack by powdery mildew (10,31).

The oxalate oxidase gene is thought to enable plant cells to destroy oxalic acid and slow or prevent disease development. The release of hydrogen peroxide catalyzed by the oxalate oxidase protein may also trigger cross linking of plant cell wall proteins in papillae at the site of infection (28) and plant hypersensitive responses (11,32) that would prevent fungal infection. Oxalate oxidase gene has been successfully inserted into Virginia-type peanut and shown in growth-chamber studies to enhance resistance to *Sclerotinia* blight through degradation of oxalic acid (17).

Field indices of available moisture in the form of rainfall or humidity >95%, air or soil temperatures <29°C, vine growth, and density of foliar canopy are used for predicting outbreaks of *Sclerotinia* blight (13,19) and the need for fungicide sprays. Although this approach has improved the efficiency of fungicide applications in disease control, it has not reduced the need for fungicide sprays. Furthermore, commercial cultivars of Virginia- and runner-type peanut exhibit some differences in susceptibility to *Sclerotinia* blight (18,20) but none have allowed a reduction in the need for fungicide sprays.

The high cost of crop production and reduced value of peanut since the 2002 Farm Bill have forced many growers in the Virginia-North Carolina region to stop growing peanut. Losses of yield to *Sclerotinia* blight and the need for two or three fungicide sprays of fluazinam (Omega 500; ISK BioSciences) costing \$208 to 312/ha were among the important factors accounting for production in Virginia falling from 23,473 ha in 2002 to 6,880 ha in 2006. The importance and impact of *Sclerotinia* blight on peanut production in the Virginia-Carolina region served as the rationale for employing a biotechnology approach to improving disease resistance.

The current study characterized disease resistance and yield of transgenic lines of Virginia-type peanut enhanced with a barley oxalate oxidase gene. The primary goal was to evaluate the effectiveness of this novel enzyme in peanut in providing resistance under natural disease conditions in the field and define yield advantages of transgenic lines compared with their corresponding nontransgenic parents.

MATERIALS AND METHODS

Origin of transgenic lines. Transgenic peanut lines were generated from three Virginia-type cultivars (NC 7, Perry, and

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Wilson) by introduction of a cloned barley oxalate oxidase gene. The barley oxalate oxidase cDNA was amplified from total barley RNA using upstream primer 5'GCAAGGTACCATGGGTTA CTCTAAAACCTAG and downstream primer 5'GCAAGTC GACTGGGGCTCATGGAAGTTAAG designed from the reported sequence (accession no. Y14203) (32). The resulting product was inserted as a *KpnI-SalI* fragment (sites in primers underlined above) under transcriptional control of the dual-enhanced *Cauliflower mosaic virus* promoter into a transformation vector (14) to yield pOxOx. Embryogenic cultures were initiated and maintained according to the method of Livingstone and Birch (16). Microprojectile bombardment with the gene gun was used for transformation (Bio-Rad, Hercules, CA). Plantlets were regenerated as described by Livingstone et al. (17) following selection on hygromycin for transformed callus. After transfer of plantlets to soil, growth conditions included daylight plus supplemental light (sodium halide and high-intensity discharge lighting) for 12 h a day. Seed were produced and harvested from transgenic plants (T_0 generation) in the greenhouse in 2003 and T_1 seed of superior lines were planted in the greenhouse for seed increase during winter 2003–04. After confirming the presence of the oxalate oxidase gene and expression of enzyme activity in detached leaflet assays, T_2 seed were harvested from transgenic plants with acceptable agronomic traits for field trials.

Field trials. Each year, the Animal and Plant Health Inspection Service (APHIS) approved testing of the T_2 to T_9 generations of transgenic lines at the Virginia Tech Tidewater Agricultural Research and Extension Center (TAREC) in Suffolk, VA in 2004 to 2009. Disease intensity from Sclerotinia blight at TAREC was generally moderate to high each year, except for 2007, when above-normal temperatures and below-normal rainfall limited disease development to low levels in all plots. In addition, APHIS granted approval for field trials of the T_5 and T_6 generations in 2007 and 2008 at the Upper Coastal Plain Research Station (UCPRS) in Rocky Mount, NC. Unfortunately, the trials at UCPRS were inconclusive in demonstrating disease resistance or susceptibility to Sclerotinia blight in transgenic and nontransgenic parents due to low and nonuniform disease intensity. Therefore, the results for trials at TAREC in 2007 and at UCPRS in 2007 and 2008 are not presented.

Field trials at TAREC were Kenansville loamy sand with a history of cotton-peanut rotation prior to 2004 and a cotton-corn-peanut rotation in 2005 to 2009. Metam sodium 42% (Vapam HL; AMVAC Chem. Corp., Los Angeles) at 35.8 kg a.i./ha was applied either once or twice at 20.3 cm under rows and at least 2 weeks prior to planting each year for control of Cylindrocladium black rot (CBR) and nematodes. Disks and a bed shaper produced smooth beds measuring 61 cm wide and 10.2 cm high over treated rows. Standard practices for production of Virginia-type peanut were followed during each growing season.

Rainfall at TAREC in May to October totaled 109.4 cm (39.1 cm above normal) in 2004, 65.7 cm (4.5 cm below normal) in 2005, 92.5 cm (21.9 cm above normal) in 2006, 44.6 cm (25.9 cm below normal) in 2007, 49.9 cm (20.6 cm below normal) in 2008, and 65.2 cm (4.8 cm below normal) in 2009. Overhead irrigation was applied at TAREC during periods of drought stress for a total of 10.7 cm in 2005, 5.7 cm in 2006, 14.6 cm in 2007, 7.6 cm in 2008, and 7.6 cm in 2009.

Transgenic seed from single-plant selections and certified seed of nontransgenic parent cultivars (NC 7, Wilson, and Perry) were hand planted 15.2 cm apart (to allow for single-plant selections) in single-row plots of 15, 20, or 25 seeds/row according to seed availability in 2004, 50 plants/row in 2005, and 40 plants/row in 2006. T_2 seed from 56 single-plant selections were pregerminated in paper towels saturated with water and ethephon at 0.173 g a.i./liter (Cerone Brand-4 Plant Regulator; Bayer CropScience, Research Triangle Park, NC) prior to planting in the field on 9 June 2004. The treatment was required to break seed dormancy be-

cause seed were harvested from the greenhouse just prior to planting in the field. Thereafter, the treatment was not needed because seed were harvested from field plantings the previous year and placed in cold storage. T_3 seed from 20 single-plant selections were planted on 1 June 2005, and T_4 seed from 22 single-plant selections were planted on 3 May 2006, along with their nontransgenic parents. In all 3 years, rows were spaced 0.9 m apart. Each year, single-plant and bulk selections of plants from superior lines were made based on oxalate oxidase expression, disease resistance, and yield. Seed harvested from these plants were used to plant field trials in subsequent years. Plants were dug on 27 October 2004, 1 November 2005, and 19 October 2006 and harvested on 1 and 2 November 2004, 9 November 2005, and 2 November 2006. Yield was determined after drying and adjusting the weight of whole pods to 7% moisture.

Bulked T_3 to T_7 seed were harvested from field plots in 2004 to 2008 and planted in replicated field trials in 2005 to 2009 for comparing disease resistance and yield in transgenic lines and nontransgenic parent cultivars. Transgenic lines and their parents were replicated in four randomized complete blocks in plots composed of two rows spaced 0.9 m apart and 7.6 m long in 2005 and 9.1 m long annually in 2006 to 2009. Trials at TAREC with bulked seed were planted on 31 May 2005, 2 May 2006, 14 May 2007, 8 May 2008, and 12 May 2009 at a rate of 9.8 seed/m of row with KMC planters (Kelly Manufacturing Company, Tifton, GA). These trials were harvested on 1 November 2005, 31 October 2006, 2 November 2007, 16 October 2008, and 26 October 2009.

Response to fungicide treatment. Field trials comparing the response of transgenic lines and their nontransgenic parents to fungicide treatments for control of Sclerotinia blight were conducted at TAREC in 2007, 2008, and 2009. The experimental design was a split-plot with fungicide treatment in main plots and subplots of transgenic lines and their nontransgenic parents. The commercial standard treatment with fluazinam at 0.58 kg a.i./ha (Omega 500; Syngenta Crop Protection, Research Triangle Park, Raleigh, NC) was applied by a tractor-mounted sprayer according to weather-based Sclerotinia blight advisories (13) that were issued daily during each growing season. The sprayer was equipped with three disc-core combination (D_323) nozzles per row and delivered a spray volume of 140 liters/ha. Chlorothalonil at 1.26 kg a.i./ha (Bravo Weather Stik; Syngenta Crop Protection) was applied for control of early leaf spot and other foliar diseases according to daily leaf-spot advisories in all trials. No other fungicides were applied.

Enzyme activity assay. Oxalate oxidase enzyme activity in transgenic plants was determined using a colorimetric method (10,17) to detect hydrogen peroxide released from degradation of oxalic acid. Leaf discs (0.5 cm in diameter) were placed in individual wells of a microtiter plate with 200 μ l of assay buffer containing 180 μ g of oxalic acid (Fisher Chemicals, Fairlawn, NJ) in 2.5 mM succinic acid solution (Fisher Chemicals) at pH 3.5 and incubated for 25 min at 37°C. After incubation, 100 μ l of the solution from each reaction was transferred to a new 96-well plate and 70 μ l of developing solution (6 mg of aminoantipyrene [Sigma-Aldrich, St. Louis] plus 30 μ l of N,N-dimethylaniline [ACROS Organics, Morris Plains, NJ] dissolved in 100 ml of 0.1 M sodium phosphate buffer at pH 7.0 with 57 μ l of a solution of horseradish peroxidase EC 1.11.1.7 at 140 mg ml⁻¹ [Sigma-Aldrich]) was added to each well. A purple color developed to indicate the presence of the hydrogen peroxide product. The reaction was allowed to continue for 25 min at room temperature, at which time absorbance was measured at a wavelength of 562 nm by a microplate reader EL311 (Bio-Tek Instruments, Inc., Winooski, VT) in trials in 2004 to 2008. In 2009, absorbance was measured at 550 nm by a microplate reader EL800 (Bio-Tek Instruments). All plants were sampled from each of the hand-planted trials and 10 plants were flagged arbitrarily and sampled in each two-row plot of replicated trials in 2006 to 2009.

Oxalic acid bioassay. Leaflets were taken from the fully-expanded leaf near the tip of the mainstem on each plant in hand-planted trials in 2005 and 2006. The detached leaflets were arranged in humid boxes containing wet paper towels. Each leaflet was wounded in four locations with an 18-gauge needle on the abaxial surface. A 15- μ l droplet of 100 mM oxalic acid (Fisher Chemicals) was applied to each wound. Leaflets were incubated for 6 h at room temperature (25°C) and leaves were rinsed and photographed under a stereo microscope (Nikon SM2645, Tokyo). The number of pixels per lesion was quantified from the digital image using Adobe Photoshop 7.0 (Adobe Systems Incorporated, San Jose, CA) and converted to area in square millimeters.

Disease assessment. Disease severity in 2004, 2005, and 2006 was scored on a plant-by-plant basis in small plots on a scale from 0 to 3, where 0 = none, 1 = $\leq$ 10% of limbs with disease, 2 = 11 to 50% of limbs with disease, and 3 = 51 to 100% of limbs with disease. In addition, three to five counts of disease incidence were obtained in field trials with large plots in 2005 to 2009 at 2- or 3-week intervals, with the first at initial disease onset and the final just prior to harvest. Incidence of Sclerotinia blight was determined by counting the number of infection centers with signs of *Sclerotinia minor* or symptoms of Sclerotinia blight in each two-row plot or a total of 15.2 m of row in 2005 and 18.3 m of row in 2006, 2007, 2008, and 2009. An infection center was a point of active growth by *S. minor* and included 15.2 cm on either side of that point. Area under the disease progress curve (AUDPC) was calculated to compare disease intensity in parent cultivars and transgenic lines (23). Occurrences of other diseases in field plots were recorded based on symptoms and signs in field trials.

Data analysis. Data from single-plant selections were combined for each original transformation event (T_0) and tested for statistical significance from nontransgenic parents by *t* test. A randomized complete block design was used in trials planted to bulked seed in two-row plots in 2005 and 2006. Analysis of variance (ANOVA) (version 9.1; SAS Institute Inc., Cary, NC) and Fisher's protected least significant difference (LSD) were used to compare transgenic lines and their nontransgenic parents. In 2008 and 2009, the experimental design was a split-plot with

and without fluazinam fungicide treatment in main plots and subplots of transgenic lines and their nontransgenic parents in four randomized complete blocks. Significant interactions between fungicide treatments (treated versus untreated) and lines (transgenic and nontransgenic) were detected in both years. Therefore, the data analysis used ANOVA and Fisher's protected LSD at $P \leq 0.05$ to detect differences in lines within main plots (fluazinam-treated and nontreated).

RESULTS

Trials with seed from single-plant selections. In 2004, T_2 seed from 56 T_1 single-plant selections representing 13 original transformation events (T_0) were planted along with nontransgenic parent cultivars in a field naturally infested with *S. minor*. Oxalate oxidase expression in 21 of the 56 transgenic lines from single-plant selections in the T_2 generation showed oxalate oxidase gene expression ≥ 0.065 absorbance, which was adopted as the threshold for transformed plants in colorimetric assays for hydrogen peroxide production (data for individual lines not shown). When grouped according to the original transformation event (T_0), 9 of the 13 original transformation events showed oxalate oxidase expression that averaged ≥ 0.065 absorbance and 7 were significantly different from the corresponding nontransgenic parents (Table 1). Three lines of transformed Perry appeared to be segregating for the oxalate oxidase transgene in the T_2 generation because some plants in each row showed no oxalate oxidase expression. Sclerotinia blight severity was reduced in all 13 transgenic lines compared with their nontransgenic parents. In 2004, transgenic lines had an average of 77, 75, and 80% lower disease severity compared with their corresponding nontransgenic parents NC 7, Perry, or Wilson, respectively.

In 2005, T_3 seed from 20 T_2 single-plant selections representing 10 original T_0 transformation events were planted. Of the 20 transgenic lines in the T_3 generation, 19 showed strong oxalate oxidase expression (≥ 0.065) according to the colorimetric assay (data not shown). When grouped according to their T_0 parent, 9 of the 10 lines showed significant oxalate oxidase expression (Table 1). All 10 transformation events had reduced severity of Sclero-

TABLE 1. Oxalate oxidase expression (Oxox), Sclerotinia blight severity, and oxalic acid (OA) sensitivity in transgenic lines from individual plant selections hand-planted in field trials in 2004, 2005, and 2006^a

Line (T_0)	T_2 (2004)			T_3 (2005)				T_4 (2006)			
	N	Oxox	Sev	N	Oxox	Sev	OA lesion	N	Oxox	Sev	OA lesion
NC7 (nontransgenic)	3	-0.008	1.74	1	-0.006	1.02	6.70	1	0.018	1.44	8.94
N70	3	0.096*	0.64****	2	0.366****	0.07****	3.00****	2	0.451****	0.05****	3.64****
N99	3	0.225****	0.16****	2	0.375****	0.01****	2.57****	2	0.486****	0.08****	2.89****
Perry (nontransgenic)	9	0.015	0.98	1	-0.008	0.84	6.28	1	0.021	0.97	7.81
P99	4	0.093	0.41****	3	0.323****	0.01****	1.64****	6	0.613****	0.05****	2.21****
P39	2	0.086	0.03****	1	0.304****	0.02****	1.72****	2	0.700****	0.02****	2.06****
P53	9	0.089	0.31****	3	0.172****	0.22****	3.21****	4	0.183****	0.34****	3.65****
Wilson (nontransgenic)	9	-0.007	1.54	1	-0.005	1.02	7.06	1	0.018	2.19	8.86
W14	3	0.053*	0.24****	2	0.163****	0.00****	2.66****	2	0.260****	0.14****	4.23****
W171	9	0.074****	0.35****	2	0.311****	0.28****	3.59****	2	0.162****	0.42****	3.78****
W59	5	0.077****	0.32****	3	0.160****	0.31****	3.98****	2	0.181****	0.18****	5.26****
W73	9	0.074****	0.23****	1	0.266****	0.50****	3.45****	...	—	—	—
W83	4	0.102****	0.34****	1	0.062	0.22****	3.32****	...	—	—	—
W147	1	0.032	0.00****	...	—	—	—	...	—	—	—
W24	1	0.043	0.00****	...	—	—	—	...	—	—	—
W51	3	0.003	1.05****	...	—	—	—	...	—	—	—

^a Significant differences in nontransgenic parent and its transgenic lines were determined by a *t* test, where *, **, ***, and **** denote significance at $P = 0.05$, 0.01, 0.001, and 0.0001, respectively; — indicates lines not tested in 2005 or 2006. N = number of single-plant selections from each original transformation and rows of nontransgenic parent evaluated in the field, with data pooled according to nontransformed parent or original transformation (T_0 = parent or original transformed plant with oxalate oxidase gene, T_2 = second generation, T_3 = third generation, and T_4 = fourth generation). Average Oxox determined from leaflet samples taken in September of each year using a colorimetric detection method. Sev = disease severity rated on a scale of 0 to 3 for each plant, where 0 = no disease, 1 = $\leq$ 10% limbs with disease, 2 = 11 to 50% of limbs with disease, and 3 = 51 to 100% of limbs with disease on 13 October 2004, 26 October 2005, and 12 October 2006. OA lesion = detached leaflets were wounded with 18-gauge needle on abaxial side and a 15 μ l droplet of 100 mM OA was applied to each wound. The number of pixels within each lesion was converted to square millimeters.

TABLE 2. Oxalate oxidase expression (Oxox), Sclerotinia blight severity, and incidence for nontransgenic parent and 19 T₃ transgenic lines in 2005^y

Line (T ₀ -T ₁ -T ₂) ^w	Oxox ^x	Sclerotinia blight severity ^y			Sclerotinia blight incidence ^z		
	29 September	4 October	27 October	9 September	4 October	27 October	
NC7 (nontransgenic)	-0.008 c	1.13 a	2.35 a	9.3 a	33.0 a	35.8 a	
N99-4-B	0.405 a	0.25 b	0.55 b	0.8 b	7.5 b	8.9 b	
N70-6-B	0.081 c	0.30 b	0.58 b	0.5 b	6.3 b	7.3 b	
N70-8-B	0.237 b	0.13 b	0.65 b	0.3 b	4.5 b	9.8 b	
LSD	0.135	0.35	0.43	5.6	15.0	12.7	
Perry (nontransgenic)	-0.011 f	0.60 a	1.25 a	2.8 a	20.0 a	25.8 a	
P99-4-B	0.153 de	0.58 a	1.10 a	2.8 a	17.8 a	18.8 a	
P99-1-B	0.091 ef	0.43 a	1.03 a	0.5 b	13.5 a	19.8 a	
P53-4-B	0.300 a-c	0.15 b	0.35 bc	0.5 b	4.5 b	7.0 b	
P53-30-B	0.212 b-d	0.05 b	0.28 bc	0.3 b	5.0 b	4.8 b	
P39-8-B	0.230 b-d	0.13 b	0.48 b	0.3 b	3.8 b	6.8 b	
P53-27-B	0.213 b-d	0.18 b	0.50 b	0.0 b	3.3 b	6.0 b	
P39-7-B	0.320 ab	0.03 b	0.35 bc	0.8 b	2.3 b	6.8 b	
P53-26-B	0.195 c-e	0.05 b	0.13 c	0.0 b	1.3 b	2.3 b	
P53-28-B	0.151 de	0.05 b	0.10 c	0.0 b	0.5 b	2.5 b	
P99-2-B	0.387 a	0.05 b	0.13 c	0.0 b	1.0 b	1.0 b	
LSD	0.109	0.23	0.35	1.6	7.6	7.4	
Wilson (nontransgenic)	-0.008 d	0.83 bc	2.45 a	7.0 a	25.3 ab	31.3 a	
W51-9-B	-0.009 d	1.30 a	2.48 a	3.0 b	30.3 a	33.3 a	
W59-10-B	0.018 cd	0.90 b	2.15 a	3.0 b	21.0 b	29.8 a	
W83-7-B	0.014 cd	0.55 cd	2.25 a	2.0 b	19.5 b	26.3 a	
W59-11-B	0.153 b	0.43 de	1.33 b	2.3 b	9.8 c	14.0 b	
W171-17-B	0.235 a	0.13 e	0.55 c	0.3 b	5.0 c	6.8 bc	
W73-27-B	0.087 bc	0.10 e	0.20 c	0.3 b	2.5 c	3.8 c	
LSD	0.078	0.34	0.42	2.9	7.6	7.8	

^v Means followed by the same letter in a parent group and column are not significantly different according to Fisher's protected least significant difference (LSD) ($P = 0.05$).

^w Name of transformed parent, and single-plant selection in each subsequent generation; B = bulked seed generation.

^x Oxox determined by assay of leaflets from 10 plants/plot using a colorimetric detection method.

^y Disease severity on a scale of 0 to 3 for each plant, where 0 = no disease, 1 = $\leq 10\%$ of limbs with disease, 2 = 11 to 50% of limbs with disease, and 3 = 51 to 100% of limbs with disease.

^z Counts of infection centers in two 7.6-m rows/plot. An infection center was a point of active growth by *Sclerotinia minor* and included 15.2 cm on either side of that point.

Fig. 1. Area under the disease progress curve (AUDPC) for *Sclerotinia blight* and yield of T₃ and T₄ transgenic lines and their nontransgenic parents in field trials in 2005 and 2006. **A**, AUDPC for T₃ lines in 2005. **B**, Yield (kg/ha) of T₃ lines planted in 2005. **C**, AUDPC for T₄ lines in 2006. **D**, Yield of T₄ lines in 2006. Within each cultivar group and year, bars with the same letter(s) are not significantly different according to Fisher's protected least significant difference ($P = 0.05$).

tinia blight and reduced lesion size when leaflets were wounded and treated with 100 mM of oxalic acid. The T₃ lines had an average of 96, 90, and 74% lower disease severity compared with their corresponding nontransgenic parents NC 7, Perry, or Wilson, respectively.

In 2006, T₄ seed from 22 T₃ single-plant selections representing eight original transformation events were planted. All 22 T₄ lines had strong oxalate oxidase gene expression (≥ 0.065) (data not shown). When grouped according to the eight original transformation events, all of the T₄ generation lines showed significant oxalate oxidase expression, reduced severity of Sclerotinia blight, and reduced lesion size on leaflets treated with oxalic acid (Table 1). These T₄ lines had an average of 96, 86, and 89% less disease than their corresponding nontransgenic parents NC 7, Perry, or Wilson, respectively.

Trials with bulked seed. In 2005, assays of 10 arbitrarily selected plants per plot showed oxalate oxidase expression in 16 of 19 transgenic lines in the T₃ generation (≥ 0.065), while only 14 were significantly different from their corresponding parents (Table 2). Strong gene expression was observed in two transgenic lines of NC, nine transgenic lines of Perry, and three transgenic lines of Wilson. Nomenclature defines transformation event, single-plant selection, or bulk selection (B) in T₀-T₁-T₂ generations, respectively. Sclerotinia blight severity was assessed for each of the 10 individual plants tested for gene expression in each plot. These ratings were significantly lower in transgenic lines compared with their nontransgenic parents, except for lines P99-1-B, P99-4-B, W51-9-B, W59-10-B, and W83-7-B, on 4 and 27 October. These lines had few to no plants with oxalate oxidase gene expression.

Fig. 2. Comparison of genetically modified Virginia-type peanut lines (N70-8-B, P53-28-B, and W73-27-B) with resistance to Sclerotinia blight and their corresponding susceptible nontransgenic parents (NC 7, Perry, and Wilson).

Whole-plot evaluations of bulked seed lines in 2005 showed that all T₃ transgenic lines had significantly lower incidence of Sclerotinia blight on 27 October (Table 2) and reduced AUDPC compared with their corresponding nontransgenic parents, except lines P99-4-B, P99-1-B, W51-9-B, W59-10-B, and W83-7-B (Fig. 1). The resistant transgenic lines had an average of 81, 86, and 77% less disease than their corresponding nontransgenic parents NC 7, Perry, or Wilson, respectively. This was visually evident in the field when comparing transgenic and nontransgenic plots (Fig. 2). All transgenic lines yielded equal to or greater than their corresponding nontransgenic parents (Fig. 1). Although the average yield of transgenic lines N99-4-B, N70-6-B, and N70-8-B was 1,200 kg/ha greater than nontransgenic parent NC 7, they were not significantly different, presumably due to variability in disease and possibly some segregation for the transgene in the bulked population. Transgenic lines P53-4-B, P39-8-B, P53-27-B, P39-7-B, W171-17-B, and W73-27-B had significantly higher yields of 489 to 1,261 kg/ha greater than their corresponding nontransgenic parents (Fig. 1).

In 2006, data from 10 arbitrarily selected plants per plot confirmed oxalate oxidase gene expression (≥ 0.065) in all 16 T₄ transgenic lines tested (Table 3). Sclerotinia blight severity was assessed for each of the 10 individual plants tested in each plot for gene expression. Disease severity was significantly less in all T₄ transgenic lines compared with their nontransgenic parents on 20 September and 9 October.

Whole-plot evaluations with bulked seed in 2006 found that all T₄ transgenic lines had significantly less disease incidence on 20 September and 9 October than their nontransgenic parents (Table 3). They also showed reduced AUDPC (Fig. 1). The resistant transgenic lines had an average of 88, 95, and 74% less disease than their corresponding parents NC 7, Perry, or Wilson, respectively. Transgenic lines yielded equal to or greater than their corresponding nontransgenic parent and 10 transgenic lines (N70-8-24-B, N70-6-B-B, N70-8-B-B, N99-29-10-B, P99-2-8-B, P99-4-14-B, W14-10-2-B, W171-17-15-B, W171-17-B-B, and W73-27-B-B) had significantly higher yields of 537 to 2,490 kg/ha greater (Fig. 1).

Response to fluazinam fungicide. Treatments with fluazinam at 0.58 kg a.i./ha in 2007, 2008, and 2009 were designed to compare the response of transgenic lines and their nontransgenic parents to fungicide treatments 0.58 kg a.i./ha for control of Sclerotinia blight. Although dry weather stress in 2007 resulted in low disease intensity (19), differences in disease incidence and AUDPC between transgenic lines and their corresponding parent cultivars were significant but yields were not significantly different (data not shown).

The split-plot analysis of data for 2008 at TAREC showed significant differences ($P \leq 0.05$) in lines (transformed and non-transformed), fungicide, and the interaction of fungicide–lines for disease incidence, AUDPC and yield. Incidence of Sclerotinia blight and AUDPC in untreated main plots was significantly lower in transgenic lines compared with nontransgenic lines in 2008 (Table 4). Fungicide treatments with fluazinam at 0.58 kg a.i./ha were applied twice (30 July and 2 September) and reduced AUDPC by 82, 94, and 80% in nontransgenic NC 7, Perry, and Wilson (data not shown). Transgenic lines alone reduced AUDPC by 80, 93, and 94% compared with nontransgenic parents NC 7, Perry, or Wilson, respectively (Table 4). Yields of transgenic lines without fluazinam were significantly higher in N70-8-24-B-B, P39-7-9-B-B, W14-10-2-B-B, and W73-27-B-B compared with their nontransgenic parent cultivars in 2008. No significant differences in yields of nontransgenic parent cultivars and their corresponding transgenic lines were detected in plots treated with fluazinam.

As in 2008, the split-plot analysis of data for 2009 was significant ($P \leq 0.05$) for fungicide, cultivar, and the interaction of fungicide–cultivar. Transgenic lines showed significantly lower

disease incidence and higher yields compared with their nontransgenic parents in main plots without fluazinam treatment (Table 5). AUDPC was reduced 98, 97, and 92% by transgenic lines without fluazinam treatment compared to their corresponding parent cultivar NC 7, Wilson, or Perry, respectively. Two applications of fluazinam (20 August and 14 September) reduced AUDPC by 57, 46, and 62% in nontransgenic cultivars NC 7, Wilson, and Perry, respectively (data not shown). Yields without the fluazinam treatment for all transgenic lines were significantly greater than their corresponding nontransgenic parent cultivars. The yields of nontransgenic cultivars and their corresponding transgenic lines were not significantly different when treated with fluazinam.

Incidence of other diseases. Occurrences of diseases other than Sclerotinia blight in field trials were low as a result of using

recommended practices. Based on observations of disease incidence, Sclerotinia blight was the primary cause of disease and accounted for differences in yield. Other diseases were widely scattered and similar across transgenic lines and their nontransgenic parent in each trial and included tomato spotted wilt (*Tomato spotted wilt virus*), CBR (caused by *Cylindrocladium parasiticum*), and southern stem rot (caused by *Sclerotium rolfsii*). Initial laboratory studies have shown that infection by *S. rolfsii* is also limited by the oxalate oxidase gene in these peanut lines (D. E. P. Telenko, *unpublished data*) but, due to regulations by the United States Department of Agriculture, the transformed lines can only be planted in a limited area. Therefore, we are unable at this time to evaluate the effectiveness of this gene toward southern stem rot. Foliar diseases such as early and late leaf spot (caused

TABLE 3. Oxalate oxidase expression (Oxox), Sclerotinia blight severity, and incidence for nontransgenic parents and 16 T₄ transgenic lines in 2006^v

Line (T ₀ -T ₁ -T ₂ -T ₃) ^w	Oxox ^x	Sclerotinia blight severity ^y			Sclerotinia blight incidence ^z		
	11 September	20 September	9 October	23 August	20 September	9 October	
NC7 (nontransgenic)	0.026 d	0.90 a	1.85 a	5.50 a	23.8 a	35.5 a	
N70-6-B-B	0.291 c	0.33 b	0.73 b	0.25 b	6.0 b	8.8 b	
N70-8-B-B	0.512 b	0.18 bc	0.38 bc	0.00 b	2.8 b	7.5 bc	
N99-29-10-B	0.834 a	0.00 c	0.13 c	0.25 b	1.3 b	3.5 bc	
N70-8-24-B	0.764 a	0.03 c	0.05 c	0.00 b	1.5 b	2.0 c	
Perry (nontransgenic)	0.020 d	0.45 a	0.90 a	1.50	9.8 a	18.8 a	
P39-8-B-B	0.334 b	0.03 b	0.08 b	0.00	0.8 b	4.5 b	
P53-28-B-B	0.194 c	0.03 b	0.13 b	0.00	1.0 b	2.8 b	
P99-1-10-B	0.561 a	0.03 b	0.05 b	0.25	0.3 b	1.5 b	
P99-2-8-B	0.579 a	0.00 b	0.03 b	0.00	0.0 b	1.5 b	
P53-30-21-B	0.195 c	0.00 b	0.00 b	0.00	0.0 b	0.8 b	
P39-7-9-B	0.590 a	0.00 b	0.03 b	0.00	0.0 b	0.5 b	
P99-4-14-B	0.528 a	0.00 b	0.00 b	0.00	0.0 b	0.3 b	
Wilson (nontransgenic)	0.019 d	1.03 a	2.03 a	2.75 a	31.5 a	48.0 a	
W171-17-15-B	0.237 bc	0.40 b	1.00 b	0.50 b	13.5 b	23.5 b	
W171-17-B-B	0.222 bc	0.25 bcd	0.65 bc	0.50 b	10.5 b	18.0 c	
W59-8-2-B	0.299 b	0.33 bc	0.35 c	0.75 b	9.5 b	11.8 d	
W73-27-B-B	0.183 c	0.03 d	0.25 c	0.00 b	3.0 c	9.5 d	
W14-10-2-B	0.441 a	0.08 cd	0.38 c	0.25 b	2.0 c	9.0 d	

^v Means followed by the same letter in a parent group and column are not significantly different according to Fisher's protected least significant difference (LSD) ($P = 0.05$).

^w Name of transformed parent, and single-plant selection in each subsequent generation; B = bulked seed generation.

^x Oxox determined by assay of leaflets from 10 plants/plot using a colorimetric detection method.

^y Disease severity on a scale of 0 to 3 for each plant, where 0 = no disease, 1 = $\leq 10\%$ of limbs with disease, 2 = 11 to 50% of limbs with disease, and 3 = 51 to 100% of limbs with disease.

^z Counts of infection centers in two 9.1-m rows/plot. An infection center was a point of active growth by *Sclerotinia minor* and included 15.2 cm on either side of that point.

TABLE 4. Oxalate oxidase expression (Oxox), Sclerotinia blight incidence, area under the disease progress curve (AUDPC), and yield for nontransgenic parents and six T₆ transgenic lines in Virginia in 2008^v

Line (T ₀ -T ₁ -T ₂ -T ₃ -T ₄ -T ₅) ^w	Oxox ^x		Sclerotinia blight ^y					AUDPC	Yield (kg/ha) ^z
	3 July	4 September	18 August	3 September	19 September	7 October			
NC7 (nontransgenic)	0.009 c	0.012 c	1.3 a	7.3 a	11.5 a	16.8 a	491 a	4,739 b	
N70-8-24-B-B-B	0.434 a	0.563 a	0.0 b	0.3 b	0.3 b	1.0 b	17 c	6,412 a	
N70-6-B-B-B-B	0.157 b	0.425 b	0.0 b	4.5 b	4.5 b	5.5 b	180 b	5,331 ab	
LSD	0.067	0.120	1.0	2.3	4.5	5.7	159	1,138	
Perry (nontransgenic)	0.011 c	0.009 c	1.0 a	6.8 a	13.8 a	22.3 a	560 a	4,304 b	
P39-7-9-B-B-B	0.322 a	0.458 a	0.0 b	0.3 b	1.8 b	2.8 b	59 b	6,326 a	
P53-30-21-B-B-B	0.201 b	0.300 b	0.0 b	0.3 b	0.3 b	0.8 b	15 b	5,189 ab	
LSD	0.090	0.087	0.8	1.0	3.4	4.6	77	1,479	
Wilson (nontransgenic)	0.014 c	0.013 c	2.3	7.8 a	12.8 a	18.0 a	545 a	4,332 b	
W14-10-2-B-B-B	0.328 a	0.478 a	0.0	0.0 b	0.8 b	2.0 b	31 b	5,813 a	
W73-27-B-B-B	0.210 b	0.273 b	0.0	0.3 b	2.0 b	2.0 b	39 b	6,059 a	
LSD	0.058	0.1	n.s.	6.2	6.8	8.1	321	687	

^v Means in each parent group and column followed by the same letter are not significantly different according to Fisher's protected least significant difference (LSD) ($P = 0.05$); n.s. = not significant.

^w Name of transformed parent, and single-plant selection in each subsequent generation; B = bulked seed generation.

^x Oxox determined by assay of leaflets from 10 plants/plot using a colorimetric detection method.

^y Counts of infection centers in two 9.1-m rows/plot. An infection center was a point of active growth by *Sclerotinia minor* and included 15.2 cm on either side of that point.

^z Yields are weight of peanut with 7% moisture.

TABLE 5. Oxalate oxidase expression (Oxox), Sclerotinia blight incidence, area under the disease progress curve (AUDPC), and yield for nontransgenic parents and advanced generations of six transgenic lines in Virginia in 2009^y

Line (T ₀ -T ₁ -T ₂ -T ₃ -T ₄ -T ₅ -T ₆₋₈) ^w	Oxox ^x	Sclerotinia blight ^y				Yield (kg/A) ^z
	4 June	24 August	2 September	21 October	AUDPC	
NC7 (nontransgenic)	0.018 b	0.8	5.5 a	30.3 a	905 a	3,493 b
N70-8-24-5-6-2-B-B	0.189 a	0.0	0.0 b	1.3 b	31 b	6,086 a
N70-6-B-B-48-5-9-B-B	0.236 a	0.0	0.0 b	0.0 b	0 b	6,409 a
LSD	0.059	n.s.	2.4	11.2	338	1,251
Wilson (nontransgenic)	0.015 c	0.3	2.5	20.8 a	582 a	4,116 b
W14-10-2-27-11-B-B	0.273 a	0.0	0.0	0.8 b	18 b	6,102 a
W73-25-17-34-8-2-4-B-B	0.159 b	0.0	0.3	2.8 b	75 b	5,737 a
LSD	0.057	n.s.	n.s.	8.5	258	643
Perry (nontransgenic)	0.021 b	0.5	2.8 a	24.5 a	682 a	4,554 b
P39-7-9-43-10-10-B-B	0.391 a	0.0	0.0 b	0.3 b	6 b	6,064 a
P39-7-9-1-40-B-B	0.357 a	0.0	0.0 b	1.5 b	37 b	6,383 a
LSD	0.063	n.s.	2.2	2.4	86	659

^v Means in each parent group and column followed by the same letter are not significantly different according to Fisher's protected least significant difference (LSD) ($P = 0.05$); n.s. = not significant.

^w Name of transformed parent, and single-plant selection in each subsequent generation; B = bulked seed generation.

^x Oxox determined by assay of leaflets from 10 plants/plot using a colorimetric detection method.

^y Counts of infection centers in two 9.1-m rows/plot. An infection center was a point of active growth by *Sclerotinia minor* and included 15.2 cm on either side of that point.

^z Yields are weight of peanut with 7% moisture.

by *Cercospora arachidicola* and *Cercosporidium personatum*), and web blotch (caused by *Phoma arachidicola*) were controlled by four or five foliar sprays of chlorothalonil at 1.26 kg a.i./ha and exhibited low incidence (data not shown).

DISCUSSION

Early generations (T₂, T₃, and T₄) of transgenic peanut lines from single-plant selections were evaluated for gene expression, resistance to Sclerotinia blight, and yield. Results verified the functionality of the oxalate oxidase gene in providing resistance to Sclerotinia blight in the field and identified superior lines with strong gene expression and disease resistance. All transgenic lines had phenotypic characteristics that were identical to their nontransgenic parents. In 2004, 21 of the 56 single-plant selections in the T₂ generation of transgenic lines exhibited disease resistance when compared with their nontransgenic parent cultivars, and all advanced selections of T₃ and T₄ lines had reduced lesion size in bioassays of leaflets with oxalic acid as well as reduced severity of Sclerotinia blight in field trials in 2005 and 2006 (Table 1). These studies demonstrated the effectiveness of the oxalate oxidase gene in reducing levels of infection and disease caused by *S. minor*, presumably through the ability of the enzyme to degrade oxalic acid.

In all, 19 transgenic lines of the T₃ generation in 2005 (Table 2) and 16 transgenic lines of the T₄ generation in 2006 (Table 3) showed high oxalate oxidase expression and reduced AUDPC by 81 and 86%, respectively, when compared with their nontransgenic parents. This confirmed the value of the oxalate oxidase gene in providing resistance to Sclerotinia blight. These studies corroborate reports that a wheat oxalate oxidase gene was effective in increasing resistance to *S. sclerotiorum* in transgenic soybean (3), oilseed rape (5), tomato (27), and sunflower (9) and to *Septoria musiva* in transgenic poplar (15). Disease resistance in transgenic peanut lines was evident at the end of the season, because the nontransgenic plots could be easily identified by more severe damage caused by Sclerotinia blight (Fig. 2).

Transgenic lines in the T₆ and advanced generations showed good to excellent expression of oxalate oxidase in 2008 and 2009. As in previous years, Sclerotinia blight appeared first in nontransgenic parent cultivars and incidence was significantly lower in transgenic lines until harvest. AUDPC was significantly lower in transgenic lines compared with their nontransgenic parent cultivars, and yields were increased significantly in four of six lines

tested in 2008 and all six lines in 2009. Replicated blocks of nontransgenic parent cultivars and transgenic lines treated with two applications of fluazinam at 0.58 kg a.i./ha for control of Sclerotinia blight showed similar disease incidence and yield in both nontransgenic and transgenic lines throughout the growing season (data not shown). Overall, the transgenic cultivars without fungicide treatments for control of Sclerotinia blight performed equally as well as nontransgenic parent cultivars with two sprays of fluazinam; therefore, by planting the transgenic lines, there was no benefit of fungicide treatments for control of Sclerotinia blight and the high fungicide input cost.

The results of these trials have demonstrated that the reduction in disease by the oxalate oxidase gene was significantly correlated with yield ($r = -0.784$, $P \leq 0.001$ in 2005; $r = -0.750$, $P \leq 0.0001$ in 2006; $r = -0.641$, $P \leq 0.0001$ in 2008; and $r = -0.886$, $P \leq 0.0001$ in 2009). All transgenic lines yielded better than their nontransgenic parent cultivars, with yield increases of 489 to 2,490 kg/ha in 2004, 2005, and 2006 trials and 592 to 2,593 kg/ha in 2008 and 2009. The increase in yield was attributed to season-long resistance to Sclerotinia blight, which was associated with expression of oxalate oxidase. All plant parts, including roots, limbs, leaves, flowers, pegs, and pods, are susceptible to infection by *Sclerotinia minor*, which can lead to losses of yield (24). We believe that the oxalate oxidase gene provides resistance to Sclerotinia blight in Virginia-type peanut by degradation of oxalic acid, which results in a reduction in disease incidence and a concomitant increase of yield.

Through use of biotechnology, transgenic peanut lines containing the novel enzyme oxalate oxidase offer an effective means for peanut producers to overcome a high risk of Sclerotinia blight in the field and increase profitability through elimination of costly fungicide sprays. This study has demonstrated that the addition of the oxalate oxidase gene, not formerly present in Virginia-type peanut, has the potential to eliminate fungicide costs for control of Sclerotinia blight, increase profits, and promote a more environmentally sound strategy for disease management.

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