Hydrolase Activity in Transgenic Peanut

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ABSTRACT

Fungal diseases of peanut are responsible for increased production costs and yield losses of up to 50% for peanut producers in the U.S. Few cultivars with disease resistance have been developed through traditional breeding practices. There is an urgent need for developing cultivated peanut (*Arachis hypogaea* L.) cultivars that are resistant to the broad spectrum of fungal pathogens that pose a recurring threat to peanut health. Hydrolases such as chitinase and β-1-3-glucanase are known to degrade the cell walls of many fungi that attack plants, making them likely candidates for over-expression through genetic engineering to produce disease-resistant crops. Somatic embryos of the peanut cultivar Okrun were transformed with a chitinase gene from rice, and/or a β-1-3-glucanase from alfalfa via microprojectile bombardment. Regenerated Okrun lines were tested for the presence of the transgenes by polymerase chain reaction (PCR) and Southern blot and for transgene expression by colorimetric assays. Transgenic lines exhibited hydrolase activities 0-37% above levels observed in nontransformed Okrun plants.

Key Words: *Arachis hypogaea*, chitinase, glucanase, transformation.

Cultivated peanut (*Arachis hypogaea* L.) is an economically important crop throughout the world. Peanut is susceptible to many types of pathogens, with most damage being caused by fungi (21). Soilborne fungi cause diseases that adversely affect peanut health and productivity throughout the growing areas of the U.S. Diseases such as pod rot (*Rhizoctonia solani* Kühn, *Pythium myriotylum* Drechs), crown rot (*Aspergillus niger* Tiegh), and southern blight (*Sclerotium rolfsii* Sacc) occur in all U.S. peanut-producing areas, while others such as Sclerotinia blight (*Sclerotinia minor* Jagger) are limited to certain geographic regions. Depending upon severity of field infestation, yield losses due to soilborne fungi may be as high as 50% (21). Traditional breeding and screening practices have resulted in few cultivars resistant to fungal diseases that are suitable for commercial use. Expensive fungicide applications throughout the growing season are often required for effective disease management. However, chemical pesticides have been the subject of substantial criticism, mainly due to their adverse effects on the environment. These facts along with the reduction in the peanut price support program have resulted in the need for effective alternative methods of disease management needed that will provide disease resistance without the use of pesticide application.

Most fungi contain chitin, a homopolymer of β-1,4-linked N-acetyl-glucosamine, as a major component of their cell walls (28, 30). All organisms that contain chitin also produce chitinases, which are hydrolases that degrade the polymer by breaking its β-1,4 linkages, presumably for morphogenesis of cell walls and exoskeletons (10). Although plants do not produce chitins, many plants have been shown to produce chitinases as a defense response to chitin-containing pathogens (3, 4, 24). Recently, another hydrolase, β-1,3-glucanase, has been suggested to be part of certain plant defense systems against fungal infection (19, 20, 25). Also, purified plant enzymes have been shown to hydrolyze fungal cell walls, inhibit the growth of fungal pathogens, and inhibit the induction of the chitinase promoter associated with the plant defense response (3, 11). The objective of this study was to introduce two hydrolase genes, a glucanase from alfalfa (*Medicago sativa* L.), and a chitinase from rice (*Oryza sativa* L.) into peanut somatic embryos via microprojectile bombardment and to assay their expression in transgenic plants.

Materials and Methods

Plant Tissue Culture. Somatic embryogenic cultures of peanut were generated from the cultivar Okrun following procedures similar to those published previously (29). Individual embryos were excised from Okrun seed, surface sterilized, and placed on petri plates (100 × 15 mm) of PIC medium [MS salts (22), B5 vitamin, 30% sucrose, 1.5 mg/L picloram, 0.1 g/L myoinositol, 1.5 g/L phytagel, 6 g/L agar, pH 5.8]. Embryogenic cultures were incubated at 24 C, transferred at 4-wk intervals for up to 8 mo, and bombarded 2 wk after subculture.

Plasmid Constructs. The plasmid pBZ56 containing a rice chitinase gene (31) was obtained courtesy of Dr. Rick Dixon, Samuel Roberts Noble Foundation, Ardmore, OK. The gene cassette (1.3 Kbp) was excised from pBZ56 by *Bam*HI digestion, blunt-ended, and subcloned (2) into a plant expression cassette from plasmid pRTL2 (6) containing a dual-enhanced CaMV 35S promoter. The resulting expression cassette was isolated by *Hind*III digestion and ligated with plasmid pTRA141 containing a gene for hygromycin resistance (*hph*) which has been used previously as a selectable marker for screening primary transformants of peanut (29). The resulting plasmid is referred to as pAB2.5.

The plasmid pMU2X containing an alfalfa glucanase gene AGLU1 (31) also was obtained from Dr. Dixon. The glucanase gene (1.38 Kbp) was excised from pMU2X by *Eco*RI digestion, blunt-ended (2), and then inserted into

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pRTL2 (6). The resulting gene cassette was excised by HindIII digestion and ligated with pTRA141 as discussed above. The resulting plasmid is referred to as pAB8.

Microprojectile Bombardment and Regeneration of Transgenic Plants. Five bombardment experiments were carried out using DNA from pAB2.5, pAB8, or both constructs together. Each experiment consisted of 7-10 plates, each containing eight to 10 embryonic clusters 2-3 cm in dia. A PDS 1000/helium-driven biolistic device (BioRad, Hercules, CA) was used to bombard the tissues at 1800 psi under 27" Hg vacuum. DNA (5 µg) was delivered for each shot and each plate was shot twice. After bombardment, embryonic cultures were maintained for 2 wk on PIC plates and then transferred to liquid PIC media with the addition of 0.3 M glutamine and 10 mg/mL hygromycin where they were maintained at 28 C in darkness for 12 wk, with transfers every 2 wk. Then viable embryonic tissue was transferred to shoot regeneration (SR) plates [MS basal medium (22), 30% sucrose, 3 g/L phytagel, 4 mg/L BA, 1 mg/L NAA, pH 5.8, 10 mg/mL hygromycin] and maintained in light with a 16-hr photoperiod at 28 C with transfers every 4 wk for 4-6 mo. Developing shoots were excised, placed on rooting medium (MS basal medium, 1 mg/L NAA, 3 g/L phytagel), and maintained in light with a 16-hr photoperiod at 28 C. When root systems were well developed, plants were transferred to maturation medium (coarse perlite, coarse vermiculite, and peat moss, 1:1:1) and grown to maturity under greenhouse conditions. Individual plant lines were numbered in order of placement into greenhouse. The experiment from which a plant line was regenerated is denoted as a numerical superscript to the plant line number.

PCR Analysis of Putative Transformants. DNA was isolated from leaves of putative transformants by harvesting 2 g of fresh leaf tissue and grinding in liquid nitrogen. DNA was extracted from frozen tissue by adding extraction mixture [equal parts of extraction buffer (0.1 M glycine, pH 9.0, 50 mM NaCl, 10 mM EDTA, 2% SDS, 1% sodium lauryl sarcosine) and phenol:chloroform:isoamyl alcohol, 25:24:1]. After centrifugation at 12,000Xg for 15 min at 4 C, DNA was precipitated by the addition of an equal volume of 2-propanol to the aqueous phase. DNA was spooled, washed in 70% ethanol, air-dried at 25 C, and resuspended in 2-mL sterile TE buffer. Preliminary screening of primary transformants was done by PCR for the presence of the *hph* gene. A 400-bp region of the *hph* gene was amplified using primers F6 5' CGCAAGAATCGGTCAATACACTAC 3' and B11 5' TCCATACAAGAAAACCACGG 3'. Amplification of an 1100-bp region of the β-1,3-glucanase gene was generated using primers 3349 5' TCCCGGATCCTAACATGTCACTC 3' and 3450 5' GATTCCGTGGCATATGCCTTCTTTC 3'. A 750-bp region of the chitinase gene was amplified using primers CFOR 5' CACCATGAGAGCGCTCGCTG 3' and CREV 5' CCGCGTTGCACGACGGCTTG 3'. Multiplex PCR was performed using primers for both the glucanase and the chitinase genes when the presence of both genes was possible. Amplification for all PCR products was carried out in a PTC-100 thermal cycler (MJ Research, Watertown, MA) under the following conditions: 1 min at 95 C, 1 min at 55 C, and 1 min at 72 C, for 40 cycles. PCR products were visualized via electrophoresis on a 1% agarose gel in 1 X Tris-acetate-EDTA (TAE) buffer and subsequent staining with ethidium bromide (2). The marker used for PCR analysis was a 1-Kbp ladder (Gibco-BRL, Grand Island, NY).

Transgene Expression by Colorimetric Enzyme Assay. Second generation (R_2) transgenic plants were assayed for transgene activity by colorimetric assay (18). Enzyme extract was prepared by homogenizing 0.5 g fresh leaf tissue from 10-wk-old plants in 5 mL of ice cold extraction buffer (100 mM sodium acetate, pH 5.0, 2 mM dithiothreitol). After centrifugation at 27,000Xg for 15 min at 4 C, the supernatant was collected and assayed for activity. Reaction mixtures were composed of 1 mL of enzyme extract, 0.5 mL of reaction buffer (100 mM sodium acetate, pH 5.0), and 1 mL of substrate. The substrate for chitinase was CM-Chitin-RBV (2 mg/mL) and for glucanase, the substrate was CM-Curdlan-RBB (4 mg/mL) (Loewe Biochemica, Germany). Reactions were carried out at 37 C for 2 hr with 0.4-mL samples taken at 0, 30, 60, 90, and 120 min. Reactions were stopped with the addition of 100 µL of 2 M HCl and cooled on ice for 30 min prior to centrifugation at 17,000Xg for 10 min at RT. The absorption of the supernatant for each sample was determined and recorded (Abs_{600nm} for glucanase reactions, Abs_{550nm} for chitinase reactions). Three independent samples were taken from each plant line. For each sample taken, reactions were performed in triplicate and the supernatant absorption readings were averaged for each time point. Statistical analysis on absorbance readings was performed using Dunnett's test (26). Estimated least squared mean, slope, and curve area values were calculated from absorbance readings for each enzyme assay performed on transgenic lines. Probability levels reported for each value reflect comparison to the nontransgenic Okrun control.

Results

Thirty-seven hygromycin-resistant plant lines were regenerated from five bombardment experiments (Table 1), of which 32 were positive for the hph gene when assayed by PCR. All plant lines included in this study originated from

Table 1. Number of transgenic peanut lines positive for hygromycin resistance (*Hgy*^{r}) and *hph* PCR.

Experiment	Plasmid[a]	*Hgy*^{r}	PCR[b]
E2 (10 plates)	C	29	26
E12 (8 plates)	C	21	
E13 (8 plates)	C + G	3	3
E14 (8 plates)	G	2	1
E15 (7 plates)	G	1	1
Total		37	32

[a]C = pAB2.5 containing a chitinase gene; G = pAB8 containing a glucanase gene.

[b]PCR analysis using primers for the *hph* gene.

independent embryonic cultures and thus were considered to have arisen from independent transformation events. Figure 1 shows the PCR analysis of 16 representative transgenic peanut lines (R_2 generation). Expected PCR profiles for each set of primers are shown in the lane labeled

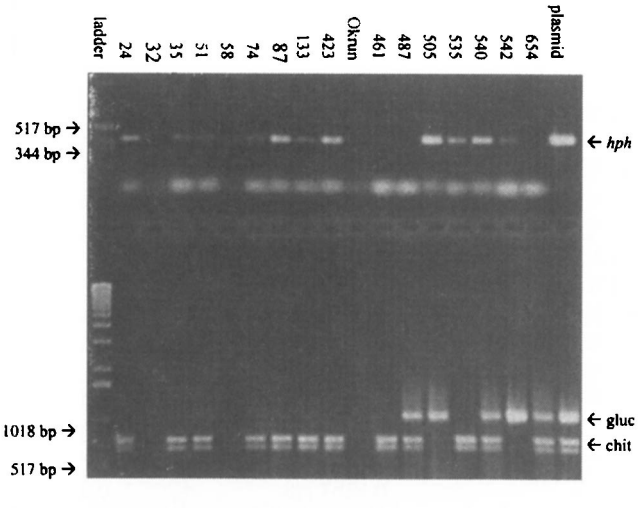

Fig. 1. PCR analysis of DNA from 16 representative transgenic peanut lines (R_2 generation), identified by the numbers above individual lanes. The upper set of lanes contain the 400-bp fragment generated from primers specific to the hygromycin resistance (*hph*) marker gene, while the lower set of lanes (same plant line order as upper lanes) contain fragments generated from the same DNA by primers specific for either the chitinase gene (750-bp fragment), the gluncanase gene (1100-bp fragment), or the multiplex pattern generated from primers for both genes.

'plasmid'. The upper plasmid lane contains the PCR product from the amplification of the *hph* gene, while the lower plasmid lane illustrates the multiplex pattern generated by amplification of both the chitinase and glucanase genes. All of these transgenic lines tested positive for the rice chitinase, the alfalfa glucanase, or both transgenes when subjected to PCR analysis (representative lines shown in Fig. 1, lower lanes), giving a 100% cotransformation frequency of the *hph* and hydrolase transgenes. Parent R_0 transgenic plants were originally screened and subsequent progeny continued to test positive for the presence of the transgene(s) through the R_2 generation (Fig. 1), suggesting stable integration of the transgene(s) into the peanut genome through two generations had been achieved.

Transgenic lines testing positive for a hydrolase transgene(s) were tested for increased enzymatic activity due to transgene expression. The assay background level is indicated by the activity observed in the nontransformed Okrun control. Using the conservative Dunnett's test (22), least squared mean (LSM) values along with probability values were calculated for each transgenic plant line as compared to the nontransgenic Okrun control (Tables 2 and 3). Twenty-nine lines were assayed for increased chitinase activity (Table 2). Increased enzyme activity levels in transgenic lines containing the rice chitinase gene ranged from 0-37% above background at the 120-min time point. Statistical analysis showed that 4/29 (14%) of transgenic lines had levels of chitinase activity significantly higher than the nontransgenic control at $P \leq 0.001$, 1/29 (3%) had significantly increased activity at $P \leq 0.01$, and 7/29 (24%) also had significant activity levels ($P \leq 0.05$).

Similar assays were performed on the six transgenic lines containing the alfalfa glucanase gene. Estimated LSM values for glucanase assays are shown in Table 3. Glucanase activity levels in transgenic plant lines ranged from 0-35% above background at the 120-min assay point. One transgenic line (542) demonstrated glucanase activity significantly higher than the Okrun control at $P \leq 0.001$, while three other lines had increased activity levels significant at $P \leq 0.01$.

Average slope and curve area estimates also were calculated for each transgenic plant line compared to the nontransgenic Okrun control (Tables 4-6). Slope estimates for chitinase activity are shown in Table 4 with 10/29 (34%) of transgenic plant lines having significantly increased slope values ($P \leq 0.001$), 7/29 (24%) were significantly higher at $P \leq 0.01$, and 5/29 (17%) were higher at $P \leq 0.05$. Area under the curve estimates for chitinase activity in transgenic lines (Table 5) show that 4/29 (14%), 1/29 (3%), and 2/29 (7%) are significantly higher than the nontransgenic control at the 0.001, 0.01, and 0.05 probability levels, respectively. Similar analysis for transgenic plants assayed for increased glucanase activity are shown in Table 6, with lines 487 and 505 having an increased slope and curve area significant at the 0.001 and 0.01 probability levels, respectively.

Discussion

Transformation of many plant species has been achieved easily by *Agrobacterium tumefaciens*-mediated transfer of desired genes (12). However, susceptibility of peanut to infection by *A. tumefaciens* and subsequent regeneration of transgenic plants are highly genotype dependent and are currently limited to the cultivar New Mexico Valencia A (7, 8, 9, 27). Thus, microprojectile bombardment of embryonic tissue is currently the only means by which to transform the runner-type peanut cultivars commonly grown in the Southwest U.S. Yang *et al.* (29) recently reported the successful transformation of three runner-type cultivars—Florunner, Georgia Runner, and MARC-1—with the nucleocapsid protein of tomato spotted wilt virus (TSWV) via microprojectile bombardment. The results presented here demonstrate the successful incorporation of two antifungal hydrolase genes into the runner peanut cultivar Okrun. Hygromycin resistance proved to be an efficient means by which to select for transformants. Of approximately 300 embryonic clusters bombarded, 37 hygromycin-resistant, putative transformants were regenerated. Eighty-six percent (32/37) of those plants tested positive for the *hph* gene via PCR analysis. Although embryo culture conditions were the same for each bombardment experiment, embryo age variation may account for the fact that 81% (26/32) of the plant lines regenerated were produced from one experiment (E2). The cotransformation rate of 100% for the *hph* and hydrolase gene(s) is comparable to that found by others for covalently linked genes (29).

Table 2. Estimated least squared mean (LSM) values and probability level determined for chitinase activities over time among transgenic plant lines compared to the nontransgenic Okrun control.

	Time (min)			
	30	60	90	120
Plant line	LSM est.	LSM est.	LSM est.	LSM est.
Okrun	0.1501	0.2770	0.4596	0.5263
24[a]	0.1911 NS	0.3523 NS	0.5904 NS	0.6694 *
33[a]	0.1803 NS	0.3214 NS	0.5796 NS	0.6237 NS
34[a]	0.1960 NS	0.3685 NS	0.6052 NS	0.6636 *
35[a]	0.1882 NS	0.3387 NS	0.5814 NS	0.6438 NS
51[a]	0.1963 NS	0.3551 NS	0.6123 *	0.6584 NS
57[a]	0.2128 NS	0.3350 NS	0.5912 NS	0.6392 NS
74[a]	0.2182 NS	0.3567 NS	0.6144 *	0.6431 NS
81[a]	0.2326 NS	0.3531 NS	0.5993 NS	0.6532 NS
87[a]	0.1920 NS	0.3465 NS	0.5950 NS	0.6484 NS
90[a]	0.2649 NS	0.3980 NS	0.7780 ***	0.7296 ***
133[a]	0.2307 NS	0.3605 NS	0.6890 ***	0.6711 *
135[a]	0.2258 NS	0.3426 NS	0.6006 NS	0.6447 NS
139[a]	0.2205 NS	0.3586 NS	0.6294 **	0.6524 NS
145[a]	0.3186 **	0.4705 ***	0.7268 ***	0.7826 ***
146[a]	0.1535 NS	0.2854 NS	0.5181 NS	0.5718 NS
157[a]	0.2430 NS	0.3963 NS	0.6716 ***	0.6696 *
188[a]	0.1857 NS	0.3263 NS	0.5847 NS	0.6370 NS
412[a]	0.1794 NS	0.3216 NS	0.5471 NS	0.5985 NS
416[a]	0.1632 NS	0.2969 NS	0.5108 NS	0.5745 NS
423[a]	0.2179 NS	0.3495 NS	0.5964 NS	0.6407 NS
461[a]	0.2042 NS	0.3750 NS	0.6315 **	0.6639 *
487[b]	0.2920 *	0.4484 **	0.7617 ***	0.7384 ***
511[a]	0.2342 NS	0.3915 NS	0.6229 **	0.6613 *
514[a]	0.3321 ***	0.5395 ***	0.8065 ***	0.8282 ***
517[a]	0.2205 NS	0.3388 NS	0.6064 *	0.6219 NS
531[a]	0.1761 NS	0.3858 NS	0.5451 NS	0.6072 NS
535[c]	0.2216 NS	0.3716 NS	0.5821 NS	0.6310 NS
540[b]	0.2508 NS	0.4267 *	0.6320 **	0.6669 *
654[b]	0.2199 NS	0.3880 NS	0.6503 ***	0.6669 *

[a]Derived from experiment E2.

[b]Derived from experiment E12.

[c]Derived from experiment E13.

*,**,***Significant at the 0.05, 0.01, and 0.001 probability levels, respectively. NS = not significant at $P \leq 0.05$.

Although the majority of regenerated plant lines resulted from a single experiment, all were considered to have arisen as independent transformation events given the fact that each line was regenerated from a separate embryonic cluster.

During pathogen attack, most plants respond by synthesizing an assortment of defense proteins as a means of defense. Both chitinases and endo-β-1,3-glucanases belong to one group of these proteins known as pathogen-related (PR) proteins. Chitinases hydrolyze chitin, a polysaccharide foreign to the plant but comprising a significant portion of the cell wall of most filamentous fungi except the Oomycetes (25). Purified chitinases have been shown to degrade the cell walls of many types of phytopathogenic fungi (11, 14). Endo-β-1,3-glucanases have been shown to possess antifungal properties similar to those of chitinases (1, 18, 19, 20). The activity of these hydrolases makes them attractive candidates for overexpression in transgenic plants, possibly producing fungal-resistant crop varieties of agronomical importance.

Chitinases are found in a wide variety of plant species, including peanut. Peanut has been shown to contain an endogenous family of chitinase genes, of which two class II chitinases have been well characterized (13). Endogenous levels of chitinase activity were observed in the nontransformed Okrun plants included in this study.

By introducing a class II chitinase from rice into the peanut genome under the constitutive 35S promoter, we were able to elevate total chitinase activity in most transgenic lines produced. Chitinase activity levels among independent transformants significantly different than nontransformed controls averaged an increase of 25%. Increased chitinase activity was consistently significant for three plant lines (90, 145 and 487) flagging them for

Table 3. Estimated least squared mean (LSM) values and probability levels determined for glucanase activity levels of transgenic plant lines over time compared to the nontransgenic Okrun control.

	Time (min)			
	30	60	90	120
Plant line	LSM est.	LSM est.	LSM est.	LSM est.
Okrun	0.1234	0.2645	0.3877	0.6195
487[a]	0.2595 *	0.4467 ***	0.6164 ***	0.8484 **
503[b]	0.1740 NS	0.3796 *	0.5560 **	0.8208 **
505[b]	0.2070 NS	0.3997 **	0.5504 **	0.8047 **
540[a]	0.1534 NS	0.3311 NS	0.4540 NS	0.7379 NS
542[c]	0.1849 NS	0.4009 **	0.5883 ***	0.9558 ***
654[c]	0.0997 NS	0.2363 NS	0.3399 NS	0.5585 NS

[a]Derived from experiment E13.

[b]Derived from experiment E14.

[c]Derived from experiment E15 .

*,**,***Significant at the 0.05, 0.01, and 0.001 probability levels, respectively. NS = not significant at $P \leq 0.05$.

Table 4. Average slope estimates and probability levels determined for chitinase activity in transgenic plant lines compared to the nontransgenic Okrun control.

Plant line	Avg slope est.[a]	Plant line	Avg. slope est.[a]
Okrun	4.500	188[b]	5.550 *
24[b]	5.760 ***	412[b]	5.176 NS
33[b]	5.451 NS	416[b]	4.971 NS
34[b]	5.750 ***	423[b]	5.513 *
35[b]	5.567 **	461[b]	5.819 ***
51[b]	5.740 **	487[d]	6.446 ***
57[b]	5.489 *	511[b]	5.740 **
74[b]	5.579 **	514[b]	5.678 ***
81[b]	5.552 *	517[b]	7.078 NS
87[b]	5.637 **	531[b]	5.393 NS
90[b]	6.534 ***	535[c]	5.269 NS
133[b]	5.951 ***	540[d]	5.382 **
135[b]	5.516 *	654[d]	5.699 ***
139[b]	5.679 **		
145[b]	6.546 ***		
146[b]	4.985 NS		
157[b]	5.861 ***		

[a]Average slope estimates reported $\times 10^{-3}$.

[b]Derived from experiment E2.

[c]Derived from experiment E12.

[d]Derived from experiment E13.

*,**,***Significant at the 0.05, 0.01, and 0.001 probability levels, respectively. NS = not significant at $P \leq 0.05$.

Table 5. Average area under the curve estimates and probability levels determined for chitinase activity in transgenic plant lines compared to the nontransgenic Okrun control.

Plant line	Avg slope est.	Plant line	Avg. slope est.
Okrun	34.58	188[a]	42.51 NS
24[a]	44.13 NS	412[a]	40.51 NS
33[a]	41.88 NS	416[a]	37.78 NS
34[a]	45.13 NS	423[a]	44.57 NS
35[a]	42.99 NS	461[a]	46.35 NS
51[a]	44.87 NS	487[c]	56.14 ***
57[a]	43.83 NS	511[a]	47.43 NS
74[a]	45.39 NS	514[a]	62.82 ***
81[a]	45.40 NS	517[a]	44.39 NS
87[a]	43.80 NS	531[a]	42.34 NS
90[a]	54.26 ***	535[b]	44.79 NS
133[a]	48.00 *	540[c]	49.33 *
135[a]	59.00 NS	654[c]	47.86 NS
139[a]	44.81 NS		
145[a]	46.11 ***		
146[a]	57.31 NS		
157[a]	49.44 **		

[a]Derived from experiment E2.

[b]Derived from experiment E12.

[c]Derived from experiment E13.

*,**,***Significant at the 0.05, 0.01, and 0.001 probability levels, respectively. NS = not significant at $P \leq 0.05$.

further analysis. Differences among chitinase activity levels in other plant lines may be attributed to variations in site of transgene insertion. It was not determined whether gene silencing was responsible for lines lacking heightened chitinase activity. We recognize that our experiments were not conducted under conditions of disease pressure which, in most cases, induces the expression of endogenous PR genes. Therefore, it is reasonable to conclude that, when under fungal attack, total chitinase activity in control and transgenic lines would be higher than the levels recorded in these studies.

Although there have been no reports of an endogenous endo-β-1,3-glucanase gene in peanut, some degradation of substrate was observed during glucanase

Table 6. Average slope and area under the curve. Estimates with probability levels calculated for glucanase activity in transgenic plants compared to the nontransgenic Okrun control.

Plant line	Avg slope est.[b]	Avg area est.
Okrun	49.99	32.58
487[b]	68.63 **	52.42 ***
503[c]	67.34 ***	45.62 *
505[c]	64.85 **	46.84 **
540[b]	59.02 NS	39.27 NS
542[d]	77.03 ***	49.59 **
654[b]	45.15 NS	28.67 NS

[a]Average slope estimates reported × 10^{-3}.
[b]Derived from experiment E2.
[c]Derived from experiment E12.
[d]Derived from experiment E13.
*,**,***Significant at the 0.05, 0.01, and 0.001 probability levels, respectively. NS = not significant at $P \leq 0.05$.

assays on nontransformed controls. Further investigations are required to determine whether this is actual glucanase activity or a property of the cell-free extract used in the assays. Of the transgenic lines possessing the alfalfa glucanase transgene, activity levels averaged 25% higher in those lines significantly different than background levels. Increased glucanase activity in plant line 487 was consistently significant in all statistical analyses, again flagging this line for further testing.

The levels of hydrolase activity observed in these transgenic peanut lines (0-37% above background) is comparable to that recorded for plant varieties with elevated fungal resistance. Lozovaya *et al.* (18) reported that β-1,3-glucanase activity levels were 33% higher in maize genotypes resistant to *A. flavus* infection than in those considered susceptible. Similar observations were reported by Neucere *et al.* (23) when examining glucanase levels in mature kernels of *A. flavus* susceptible and resistant genotypes. Others have been successful in generating transgenic plants with resistance to fungal infection through the introduction of a foreign chitinase gene (5, 16, 17). Broglie *et al.* (5) reported an increased resistance to *Rhizoctonia solani* infection in the roots of tobacco containing a chitinase transgene.

Many host plant resistance strategies are based on multiple genes encoding a diverse set of resistance factors, a system that may help prevent pests from overcoming such resistance. Chitinases and glucanases have been shown to act synergistically to inhibit the growth of several genera of fungi *in vitro* (19, 24, 31). Graham and Sticklen (11) cited reports of various fungal species that are sensitive to chitinase or glucanase degradation. Of the transgenic lines produced in this study containing both a chitinase and a glucanase transgene, one (487) shows elevated enzymatic activities for both hydrolases that were similar to those seen in fungal-resistant peanut cultivars (24).

If transgenic peanut containing foreign hydrolase genes reacts to fungal infection as other plant species transformed with antifungal genes, it is possible that the transgenic lines with high transgene expression would exhibit some level of resistance to a broad range of fungal pathogens. The results from this study have advanced the identification of transgenic plant lines warranting disease resistance analysis. The introduction of genes responsible for disease control directly into the plant genome provides a stable, alternative management strategy to that of using pesticides or biocontrol agents.

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