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Field resistance to Tomato spotted wilt virus in transgenic peanut (*Arachis hypogaea* L.) expressing an antisense nucleocapsid gene sequence

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Abstract

Peanut (*Arachis hypogaea* L.) lines transgenic for the antisense nucleocapsid (N) gene of a Tomato spotted wilt virus (TSWV) strain isolated from peanut were generated by microprojectile-mediated transformation of repetitive somatic embryos of cultivars VC1 and AT120. The selectable marker (hygromycin resistance) and the N gene were on separate plasmids. A total of 207 VC1 and 120 AT120 hygromycin-resistant lines were produced. Of all the VC1 plants recovered 71% were cotransformed with the N gene (N+), but all plants were sterile. For AT120, 48 of the transgenic cell lines converted into plants. Polymerase chain reaction (PCR) screening showed 15 of the lines were transgenic for the N gene (N+), and two of these lines were fertile. A field test was conducted in 1998 at Ashburn, GA, using seeds from each fertile line, along with segregated and non-transgenic controls. Plants from four randomly selected field plots were examined for symptoms and analyzed by double-antibody sandwich enzyme-linked immunoabsorbent assay and PCR at 10 and 14 weeks after planting. At 14 weeks, 76% of the N+ plants were symptomless, while 2% were severely symptomatic or dead. In contrast, only 42% of the plants lacking the N gene were symptomless and 50% were severely symptomatic or dead. Northern blot analysis of selected field-resistant plants detected transgene RNA, and the transcript level appeared undiminished after viral exposure.

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; DAS-ELISA, double-antibody sandwich enzyme-linked immunoabsorbent assay; MCS, multiple cloning site; N, nucleocapsid; PCR, polymerase chain reaction; TSWV, tomato spotted wilt virus

Introduction

Peanut is an important food, fodder, and oilseed crop in many tropical and subtropical countries. The tomato spotted wilt virus (TSWV) is principal pathogen of peanut in the southeastern United States. TSWV has a broad host range that includes crops such as tomato and tobacco, as well as numerous fruit, vegetable, weed and ornamental species [15]. Infection at early growth stages can lead to total yield loss or even death of the host plant [27]. Vectored exclusively by thrips (Thysanoptera: Thrypidae) [27], TSWV is the type member of the *Tospovirus* genus, comprising plant-infecting members of the arthropod-borne Bunyaviridae family [10]. Control of the virus through

cultural practices such as crop rotation, control of thrips vectors, and the removal of alternate weed hosts has met with only limited success [3,4]. Hence, the development of TSWV-resistant host plant varieties is the most promising means of controlling the disease in the long term. While cultivars with limited resistance to TSWV have been developed via traditional breeding methods, pathogen-derived resistance may be used to introduce resistance in susceptible cultivars, or a complementary, alternate resistance mechanism in resistant cultivars [5].

The nucleocapsid (N) gene of TSWV has been used extensively to impart TSWV resistance to plants. Gielen *et al.* [12] first reported engineered resistance to TSWV in tobacco, although no correlation was observed between resistance and N protein levels. Pang *et al.* [23, 24] showed that the N gene imparted resistance to tobacco and lettuce either through high N protein accumulation or via post-transcriptional silencing activated by N transgene overexpression. Pang *et al.* [25] further showed that TSWV resistance was induced in tobacco plants transformed with a half-length N gene, or one quarter of the gene fused to green fluorescent protein. Vaira *et al.* [25] likewise used the TSWV N gene to transform tobacco and showed that resistance in the resulting transformants was RNA-mediated and correlated to low transgene expression. Kim *et al.* [17] and Ultzen *et al.* [34] observed a similar mechanism in studies on TSWV N gene transformation of tomato. Subsequently, Prins *et al.* [28] used the genes for each protein component of TSWV individually, in whole or in part, to transform tobacco. Only plants expressing the N or NS_M (the putative non-structural movement protein) genes exhibited resistance to TSWV. Resistance was correlated with high nuclear transcription levels, but not to steady-state mRNA concentrations.

There have been attempts to transform peanut for resistance to TSWV, using an N gene obtained from a strain isolated from lettuce cv. Batavia in Hawaii, and known as TSWV-BL or TSWV -L [36]. Brar *et al.* [2] first reported peanut transgenic for this gene construct, but no information was provided regarding resistance of the plants to the virus. Yang *et al.* [37] transformed embryonic cultures of three commercial peanut cultivars using the same N gene, and found divergent levels of transgene expression in the transgenic plants and their progeny. Many of the primary transgenic plants became infected with TSWV and were not able to set seed. No resistance data on the remaining plants were presented. Li *et al.* [19] mechanically inoculated T_1 plants of cv. New Mexico Valencia A, which developed symptoms 10 to 15 days later than non-transgenic controls, and were protected from systemic infection. No field data were recorded.

Sense and antisense orientations of the TSWV N gene were used by Pang *et al.* [23 to transform tobacco, and by Kim *et al.* [17] to produce transgenic tobacco and tomato. In both cases, the orientation of the gene did not affect the level of protection. Likewise, Sherman *et al.* [31] transformed chrysanthemum leaf explants via *Agrobacterium*-mediated transformation with sense and antisense orientations of the N gene and with a construct that would produce a truncated N protein. Resistant plants were associated with all versions of the transgene. The resistant lines expressed low levels of the N transcript, although the same was true for two susceptible lines. Further tests with western analysis and ELISA indicated that if the transformed lines were translating the N protein, the levels were not detectable.

The goal of this research was to produce TSWV-resistant, transgenic peanuts using an N-gene antisense construct, and to test transgenic progeny under field conditions to evaluate the level of resistance to the virus.

Materials and methods

Generation of transformed plant lines

Initial somatic embryogenesis and co-transformation studies were performed with *A. hypogaea* cv. VC1 (AgraTech, Ashburn, GA). All subsequent embryogenic lines were obtained from cv. AT120 (AgraTech), which exhibits no resistance against TSWV.

Establishment of embryonic cell lines

Mature embryo axes were used as starting materials to induce primary embryos [1]. The axes were isolated from seeds and surface-sterilized with 10% commercial bleach (5.25% NaOCl) for 10 min and rinsed three times with autoclaved deionized water. The epicotyls were excised and placed onto medium consisting of MS [22] salts supplemented with B5 vitamins [11], 3% sucrose and 30 mg/l 2,4-D. The pH was adjusted to 5.8, and 0.8% Sigma Agar (Sigma, St. Louis, MO) was added prior to autoclaving. Each 100 mm $\times$ 20 mm plate contained ten epicotyls and was sealed with Nescofilm (Karlan Research Products, Santa Rosa, CA). Plates were cultured in the

dark at ca. 22 °C for six weeks. Small, globular-stage, translucent primary embryos were then selected for establishment in liquid FN medium [9], using a procedure similar to that of Durham and Parrott [7] as follows. Primary embryos were seeded individually into each of 20 125 ml Erlenmeyer flasks containing 40 ml of FN and capped with silicone closures (Bellco Glass, Vineland, NJ) and shaken at 125 rpm. The liquid cultures were maintained at 27–29 °C, a 24 h photoperiod, and a light intensity of ca. 20 μE m^{-2} s^{-1} provided by cool white fluorescent tubes. These cultures were transferred to fresh medium every 2 weeks. Clusters of repetitive globular-stage embryos appeared in one flask after 4 months of culture. These were separated using a scalpel, thus establishing a repetitively embryogenic cell line. This cell line was designated AT120C and used for all the transformation attempts in this study.

Transformation system

N gene

Peanut plants exhibiting symptoms of TSWV were harvested in Georgia. Virus was isolated from infected plants as described by Sreenivasulu *et al.* [32]. A particularly virulent isolate, designated TSWV-11, was selected based on the severity of symptoms in *Nicotiana benthamiana*, when these were inoculated as described by Vaira *et al.* [35]. Viral RNA and the N gene were isolated as described by Sherman *et al.* [31], modified to the extent that a *Xho*I site was attached to the 3′ primer instead of a *Bam*HI site. The resulting 798 bp fragment was cloned into the plasmid using non-unique *Xba*I and *Xho*I sites. The new *Xba*I-*Xho*I fragment was ligated into the corresponding sites of pKYLX80 [30], placing the gene behind a double CAMV 35S promoter. Two *Hin*dIII sites are also present in the construct, one at the MCS immediately downstream of the promoter, and the second in the N gene coding sequence itself. The size of the fragment resulting from *Hin*dIII digestion was then used to determine orientation of the fragment within pKYLX80. A plasmid with the insert in the antisense orientation (Figure 1) was termed pKYLX80-N11, and used for the work in this study.

Plasmid preparation

Plasmid KYLX80-N11 was used for transformation in a 1:1 molar ratio with pTRA140 [38], which contains the *gus*A gene [14] and the *hph* gene in opposite orientations, both driven by 35S promoters. Plasmids were isolated using a Qiagen (Chatsworth, CA) Maxi-Prep according to the manufacturer's instructions.

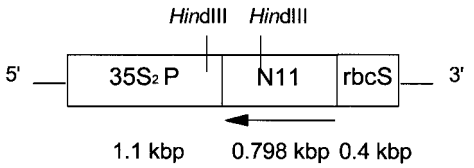

Figure 1. Map of the N11 gene in pKYLX80-N11. The two *Hin*dIII sites which were used to cut the genomic DNA for Southern analysis are indicated by vertical lines.

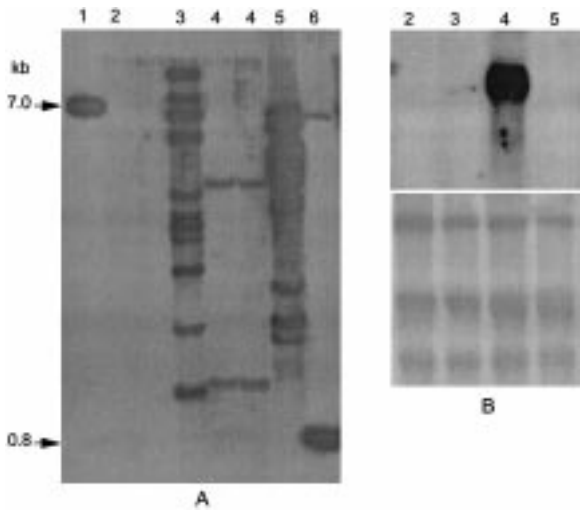

Figure 2. A. Southern analysis on primary transformants. Band sizes above 0.8 kb show N gene integration in the genome. The 0.3 kb band generated by the *Hin*dIII digestion ran off the gel due to its small size. B. Northern analyses of primary transformants. Bottom panel was the same membrane, stripped, washed and probed with a chitinase gene. 1, linearized pKYLX80-N11, using two-genome equivalents; 2, hygromycin-only transgenic; 3, transgenic line which did not produce seeds; 4, line 3uu; 5, line 5ss; 6, N-gene-coding sequence, using two-genome equivalents.

Bombardment

Gold particles 1 μm in diameter (BioRad, Hercules, CA) were used as microcarriers. The particles were ultra-sonicated in absolute ethanol using a Fisher Scientific (Madison, WI) sonic dismembrator model 300 set at 35%. A total of 30 μg of plasmids was added to 2.1 mg of sonicated gold suspended in 35 μl of absolute ethanol contained in a 1.5 ml siliconized Eppendorf centrifuge tube, and co-precipitated by successively adding 250 μl of 2.5 M $CaCl_2$, 50 μl of 0.1 M spermidine, and 220 μl H_2O. The suspension was mixed by pipetting after the addition of each component, and vortexed and sonicated using a jewelry sonicator (Fisher Scientific) between additions.

The resulting suspension was vortexed for 10 min, centrifuged at 500 rpm for 5 min, and the supernatant removed. Final sterilization was performed by resuspension of the gold in 600 μl absolute ethanol, vortexing for 10 s centrifugation at 500 rpm for 5 min, and resuspension in 36 μl of absolute ethanol. The prepared microcarriers were kept on ice until use.

Bombardment was performed two days after subculture of the somatic embryos. Embryos were harvested from liquid suspension culture, and ca. 1.0 g of embryos was arranged in a monolayer about 2.5 cm in diameter on sterile 100 min $\times$ 20 mm polystyrene plates and allowed to desiccate for about 15 min. Ten such plates were bombarded. Each plate was positioned in the third groove from the bottom of the PDS 1000/HE support bracket, while the launcher assembly, with a gap distance of 1.7 cm, was in the second groove from the top. Microcarriers were pressurized to 7585 kN/m^2 at 3.6 kN/m^2 vacuum to carry out the bombardment. Each plate was bombarded once.

The tissues were allowed to recover for 15–30 min subsequent to bombardment, and returned to FN for seven days under conditions as described before. Transient GUS expression in a subsample of culture was visualized after two days as described by Jefferson *et al.* [4]. Selection began on the eighth day after bombardment, at which time the tissues were exposed to 10 mg/l hygromycin, and continued through the next seven days. Beginning on the fourteenth day after bombardment, the level of hygromycin was increased to 20 mg/l. Bombarded tissues were transferred to fresh selection medium on a weekly basis. Resistant tissues were apparent after five weeks of selection on hygromycin. These were isolated into individual flasks and proliferated until ca. 15 mg were available from each cell line.

Conversion of somatic embryos

For plant recovery, the embryonic clusters from each cell line were teased apart into clusters about 2 mm in diameter. These were transferred individually into 125 ml Erlenmeyer flasks containing MSM3liq medium (MS salts, 3% maltose, B5 vitamins, pH 5.8) as the embryo histodifferentiation medium, and shaken for three weeks under the same conditions as described previously. The embryos from each flask were desiccated for one week inside a 100 mm $\times$ 20 mm sterile polystyrene Petri dish sealed with microporous tape (Johnson and Johnson, Skillman, NJ). The desiccated tissues were transferred to 100 mm $\times$ 20 mm Petri dishes containing FNLOS3 medium [29] supplemented with 0.1 mg/l BA and solidified as described before. Shoots started to appear after four weeks, then these were excised and transferred to 60 mm $\times$ 15 mm glass test tubes half-filled with FNLOS3 solidified with agar as described before, and capped using No. 4 polypropylene caps (Magenta, Chicago, IL). Roots started to appear in another two weeks. Plantlets with well-developed root systems were transferred individually to autoclaved 5 cm $\times$ 5 cm plastic pots filled with a 1:1 mixture of sand and Hyponex (Hyponex, Marysville, OH) potting mix contained inside GA7 (Magenta) coupled boxes. Plants were hardened after one week of acclimation in soil by gradual aeration of the assembly. Meanwhile, tissues remaining on plates were continuously transferred to fresh plates every four weeks for three months, and taken through the plant conversion process as described.

The acclimated plants were taken out of the GA7 box assemblies after two weeks. Normal growth rates were apparent after 2 to 4 more weeks, at which point they were planted in the greenhouse on beds consisting of a 2:1 mixture of sand and Promix and fertilized once a week with Peter's 20:20:20. Flowering took place after 2–4 months of greenhouse establishment and seed set started two months thereafter. At maturity, seeds were collected from fertile lines, heat-treated at 45 °C for three weeks, and stored with a dessicant until planting.

Field evaluation

A field test was conducted in 1998 to evaluate the resistance of the transgenic plants to TSWV. Seeds (T_1 generation) collected from the primary AT120 transformants (T_0) were planted in Ashburn, GA, following USDA-APHIS notification. A randomized complete block design was utilized with 5 replications. Non-transgenic AT120 plants were used as borders. Each block consisted of two-row ranges, containing non-transgenic AT120 and GK7 (which also exhibits no resistance to TSWV), hygromycin-resistant transgenics, T_1 plants which had lost the N gene through Mendelian segregation, and T_1 plants transgenic for the N gene. Each row contained 14 to 20 plants. Ten and 14 weeks after planting, leaf samples were collected from four, randomly selected rows within plots, and used for Southern blot, northern blot, and DAS-ELISA analysis. The data obtained were analyzed

using the SAS (SAS Institute, Cary, NC) general linear models and means procedures.

Analysis of transgenic plant lines

PCR analysis

Plants were initially analyzed by polymerase chain reaction (PCR) to confirm the presence of the TSWV N gene before further analyses were performed. One newly expanding tetrafoliolate leaf from each plant line was collected and used as the genomic DNA source. DNA extraction for the transgenic parents was performed using the procedure described by Stewart and Via [33] or a modification thereof for the progeny. The modified procedure consisted of grinding the leaf samples in the presence of liquid nitrogen using plastic pestles fitted for 1.5 mL Eppendorf tubes. CTAB/bME buffer (603 μl) was then added, and the slurry vortexed. The ground samples were kept at 65 °C for 15–60 min and subsequently extracted with 500 μl of a 24:1 mixture of chloroform and isoamyl alcohol. The aqueous phase was transferred into a new tube and precipitated with 0.7 volume of 2-propanol. The tube was inverted several times and the DNA hooked with a bent Pasteur pipet and transferred into a tube containing 1 ml of 70% ethanol. The tube was again inverted several times and the DNA hooked with the same pipet, blotted gently, and allowed to slowly dissolve in 200 μl of TE (10 mM Tris-HCl, 1.0 mM EDTA, pH 8.0). The amount of DNA was quantified using a minifluorometer (Hoefer Scientific, San Francisco, CA).

The PCR reaction cocktail contained 0.2 μM of each primer, 500 μM nucleotides, 2.5 mM $MgCl_2$, 1× reaction buffer, and 0.5 μl *Taq* polymerase in a total volume of 50 μl. A 500 ng portion of sample genomic DNA were then added to each tube. The primer sequences used were 5′-GCTCTAGAGCTTTCAAGCAAGTTCTGCG-3′ for the forward primer and 5′-CCAAGCTTCCCATCATGTCTAAGGTTAAGCTCAC-3′ for the reverse primer. These primers amplified the whole gene and resulted in a 798 bp product. The cycling conditions used were: initial denaturation at 94 °C for 5 min; 35 amplification cycles, each cycle consisting of denaturation at 94 °C for 1 min, annealing at 55 ° for 1 min, and extension at 71 °C for 1 min; and post-extension at 71 °C for 7 min.

Southern blot analysis

For the transgenic parents, DNA was extracted according to the procedure of Kochert *et al.* [18]. For the progeny, DNA extracted for PCR analysis was of sufficient quantity for Southern blot analysis. The genomic DNA was digested with *Hin*dIII to verify transgene integration and copy number. *Hin*dIII liberates the first 350 bp of the 5′ end of the N gene coding sequence, and the 3′ end of the 35S promoter (Figure 1). Ten μg of genomic DNA and either one or two genome equivalents of linearized pKYLX80-N11 plasmid and N gene coding sequence were loaded on each lane and fractionated in an 0.8% agarose gel. The gel was transferred to a Genescreen (DuPont, Boston, MA) membrane by capillary action and fixed by baking under vacuum at 80 °C for 2 h.

Hybridization probes were prepared by labeling 1 ng of the entire 798 bp N gene with [^{32}-P]-dCTP using random priming (Gibco-BRL, Gaithersburg, MD). Prehybridization was performed for at least 2 h, followed by hybridization overnight, both at 65 °C, using the buffers and the washing procedure provided in the Genescreen manual. The washed membrane was exposed to an autoradiography film which was developed after 3 to 5 days.

Northern blot analysis

The level of steady-state mRNA in the transgenic AT120 plants was analyzed. Total RNA was isolated from leaves using the RNeasy kit (Qiagen, Chatsworth, CA). Fresh leaf materials (100 mg) from the transgenic parents and lyophilized materials (20 mg) from representative progeny exhibiting different viral responses were used. Resistant plants that were situated near an infected plant, if available, were selected. Controls included a transgenic progeny grown in a greenhouse free of TSWV, and infected and uninfected non-transgenic progeny. The RNA collected was quantified with a spectrophotometer, and 10 μg from each sample were electrophoresed in 1.2% agarose-formaldehyde denaturing or 1.5% native agarose gel [26]. The gel was transferred to a Genescreen membrane and fixed by baking at 80 °C under vacuum for 2 h. Equal loading of the wells was verified by probing the same membrane with a PCR-amplified 400 bp segment of a peanut chitinase [16] after the N-gene hybridization.

The same procedure used in Southern blot analysis was utilized to label the N and peanut chitinase genes for use as RNA hybridization probes. The membrane was prehybridized for at least 2 h and hybridized

overnight at 42 °C with the buffer specified in the Genescreen manual. The washing steps enumerated in the manual were also followed. The hybridized membrane was exposed to an autoradiography film and developed after 1 to 5 days.

DAS-ELISA

Double-antibody sandwich enzyme-linked immunosorbent assay was used to detect the level of virus infection occurring in field-harvested plants of AT120. The Agdia TSWV alkaline phosphatase kit (Agdia, Elkhart, IN) was utilized according to the manufacturer's instructions. Leaves collected from both sampling times were lyophilized for 24 h and stored in a sealed container at 4 °C until analyzed. For each sample, 50 mg of dried materials were used with 1 mL of extraction buffer, obtaining a 1:20 dilution. A TSWV-infected peanut plant was used as positive control and diluted 1:20, 1:200, 1:2000, 1:6000, 1:60 000 and 1:180 000 times. Wells were also allotted for a peanut negative control diluted 1:20 and the extraction buffer. The difference in the absorbances at 405 and 492 nm was read using an ELISA plate reader.

Results and discussion

Transgenic peanuts generated

Small, compact primary embryos were inoculated in liquid medium, with 5% yielding proliferative embryo cultures. These repetitive cultures have the advantage of being efficient targets for transformation due to their rapid proliferation. Within 2 weeks, 100 mg of starting material that had been seeded in a flask generated ca. 1 g of embryogenic tissues, sufficient for bombardment of 1 plate.

A total of 20 plates containing ca. 1 g each of embryogenic tissues of VC1 and 10 plates of AT120 was bombarded. A total of 207 putative transgenic lines of VC1 was recovered. Of these, 77% were transgenic for the *hph* and *N* genes. However, all plants were sterile. Consequently, further work was focused on a younger line.

From the AT120 line, the first 120 hygromycin-resistant cell lines to appear were isolated, although many more were generated. However, only 48 of the 120 lines were successfully converted into plants. PCR analysis of the recovered plants showed that 15 lines contained the N gene, with the remaining only having the *hph* gene from pTRA140. Thus, the frequency of co-transformation was 31%. This frequency was lower than the 77% frequency obtained with the older VC1 line, where lines were bombarded >24 months after induction compared to 7 weeks in the current study. Hazel *et al.* [13] noted that older lines of soybean are more transformable than younger lines. It may be that older, more transformable tissue is also more amenable to co-transformation.

Only 2 of the 15 lines recovered and found to contain the N gene set seeds. Southern and northern blot analyses were performed on those 2 lines, designated 3uu and 5ss (Figure 2). The Southern blot results showed that in 3uu, the transgene was integrated at two places in the genome, while in 5ss it was incorporated at ten sites. Northern blot analysis indicated detectable levels of the N gene transcript only in line 3uu. No transcript was detected in line 5ss. For line 3uu, the N transcript migrated closely with the 18S rRNA, which is ca. 1.9 kb. This suggests that both sites of integration in the genome may consist of two copies in tandem. No band was found at the expected position of the 800 bp fragment, based on the migration distance estimated using its molecular weight [26].

Between the two lines, 3uu produced approximately four times more seeds, which was comparable to the yield obtained from non-transgenic controls. PCR analysis on the progeny showed that 45 out of 52 plants inherited the N gene. Southern blot analysis indicated that all 45 contained the transgene at both insertion sites characteristic of the parent (Figure 3). The segregation pattern was consistent with the Mendelian ratio for the inheritance of a single dominant gene ($\chi^2 = 1.8$, $P = 0.24$). In line 5ss, none of 20 progeny had the N gene (data not shown). We do not know if the transgene was not passed on to the progeny, if the transgenic event was lethal or produced non-viable pollen, or if the seeds were mislabeled.

Field results

The progeny of lines 3uu and 5ss were planted in the field near Ashburn, GA, known to be heavily infested by thrips, the vector for TSWV. Leaf samples were collected at 10 weeks and 14 weeks after planting to verify any resistance associated with the presence of the gene, and to determine if there was a delay in infection and/or symptom development that could be attributed to it. Absorbance values from DAS-ELISA ranged from slightly less than zero to almost 1.7. The positive control wells showed that, at dilutions less than 1:6000, the virus titer exceeded the capacity of the antibodies loaded into the wells. This

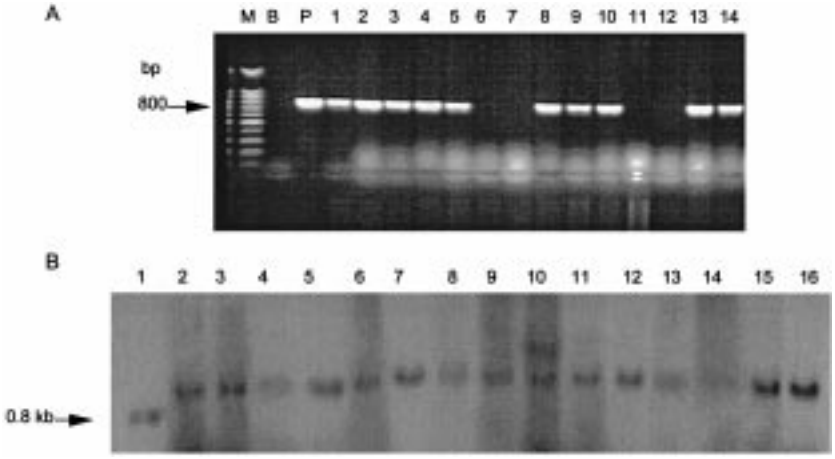

Figure 3. Segregation analysis of T_1 peanut plants. A. PCR amplification of the N (800 bp) to screen for the inheritance of the gene and chitinase (400 bp) as control. Samples which did not amplify the chitinase gene were screened using Southern analysis. M, 100 bp markers; B, blank; P, plasmid control; 1–14, samples. B. Southern analysis. Ten μg of genomic DNA was digested with *Hin*dIII prior to electrophoresis. Lane 1, N11gene, using one-genome equivalent; 2–16, samples.

means that the calibration curve is only linear for the 1:6000, 1:60 000 and 1:180 000 dilutions, and reflects the extreme sensitivity of this assay.

ELISA results for many of the infected plants provided absorbance readings beyond the linear range at the 1:20 dilution rate used; therefore a rating system based on the absorbance reading was devised. A cumulative frequency distribution curve for all the absorbance values acquired from ELISA of the samples collected at both time points was constructed. Break points in the distribution were obtained at absorbance values of 0.03, 0.75 and 1.5 (Figure 4). On this basis, a rating system reflecting the level of infection was formulated, whereby a rating of 0 was used for absorbance readings below 0.03; 1 for readings between 0.03 and 0.75; 2 for 0.76 to 1.5; and 3 for readings above 1.5. The rating 3 reflected an infection level which normally led to the death of the plant, and hence is the most economically significant.

For TSWV, ELISA is a measure of actively replicating virus, so negative results were expected from plants that had died of TSWV infection. Dead plants were common at the second sampling time point. In cases like this, visual observations and the ELISA result for the 10-week time point were used as indications of whether the plant died of TSWV infection. If, based on these criteria, the death of the plant could be attributed to TSWV infection, it was assumed to have a high virus titer, or an infection level rating of 3. Otherwise, that particular plant was excluded from the statistical data pool. Other than Impatiens necrotic spot virus, which is present under greenhouse conditions, it should be noted that other tospoviruses have not been found in Georgia; hence symptomology can be used to identify TSWV infection.

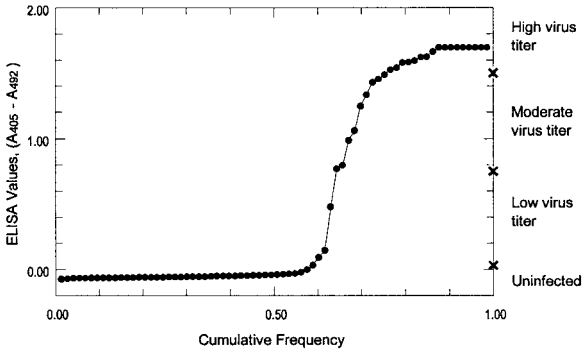

Figure 4. Cumulative frequency distribution of ELISA results and corresponding numerical infection level ratings.

Table 1. Mean infection ratings obtained during field testing of transgenic progeny.

N gene	*n*	Sampling time point, weeks	Infection rating mean ± SE
Positive	45	10	0.04 ± 0.03
	45	14	0.47 ± 0.13
Negative	24	10	0.83 ± 0.23
	24	14	1.63 ± 0.30

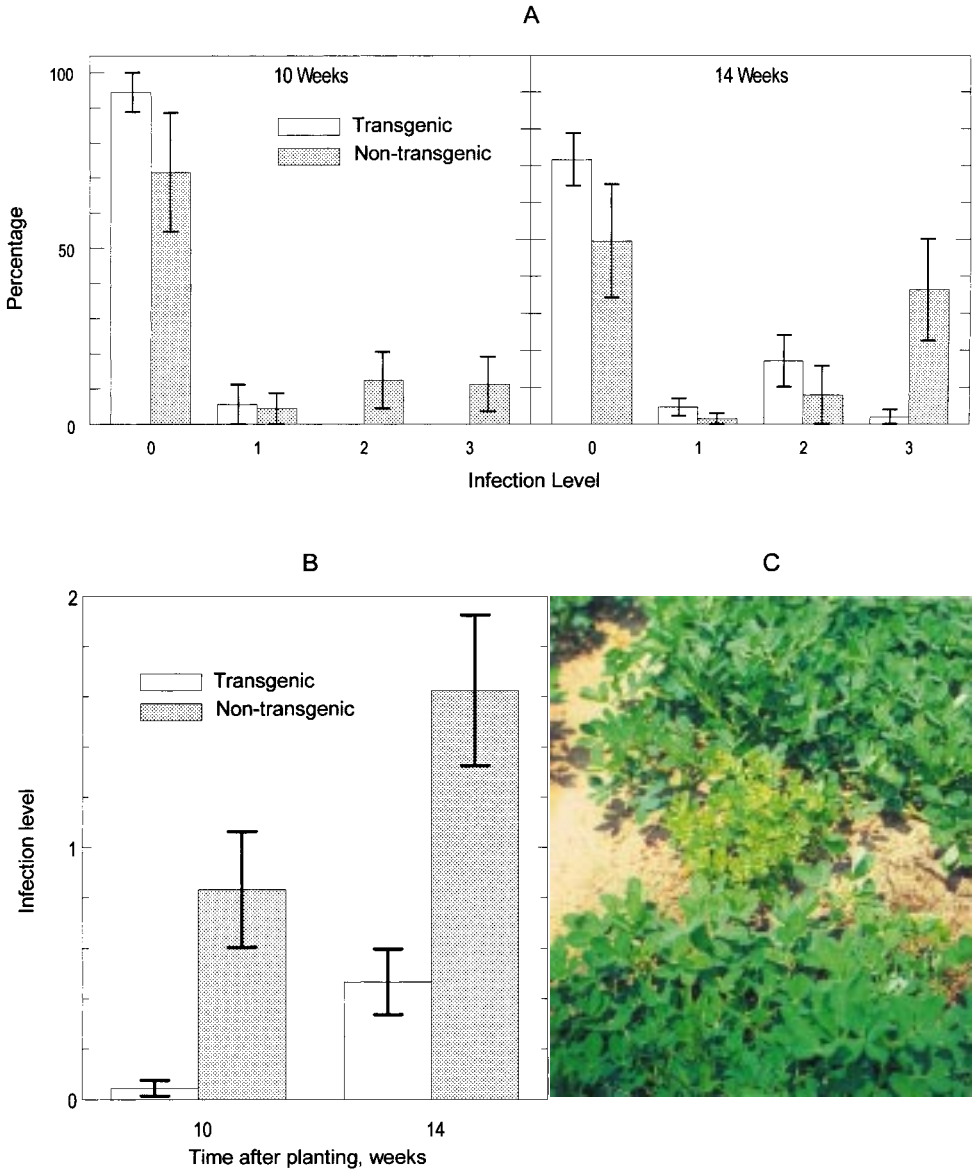

Figure 5. Infection level distribution (percentage and standard error) (A) and infection levels (mean and standard error (B) in test plants, 10 and 14 weeks after planting. C. A TSWV-infected, non-transgenic plant between two uninfected transgenic plants in the field test at 14 weeks.

The mean ratings (Table 1) for the two sampling times were calculated using the SAS (SAS Institute, Cary, NC) procedure means. The mean rating values suggest that the rate of infection of plants that contained the N gene (N+) was lower than that of plants where the N gene was absent (N−). The N− plants included both hygromycin-resistant transgenics and T_1 plants which had lost the N-gene through segregation. At the 10-week sampling point, 94 ± 6% of the N+ progeny displayed a rating of 0, or were uninfected, while only 72 ± 17% of N− plants obtained the same rating. There were no N+ plants with a rating of 2 or 3. At the 14-week sampling point, 75 ± 7% of the N+ plants were uninfected, while 2 ± 2% had a high virus titer or were dead. In contrast, 52 ± 16% of the N− plants were not infected, while 38 ± 14% had a high virus titer or were dead (Figure 5). Based on an analysis of variance, the presence of the N gene had a significant effect on the resistance of the plants to

TSWV infection at the 10-week ($P < 0.0001$) and 14-week ($P < 0.0001$) sampling points.

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