Genetic Transformation of a Runner-Type Peanut with the Nucleocapsid Gene of Tomato Spotted Wilt Virus

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ABSTRACT

Peanut (*Arachis hypogaea* L.) lines containing the nucleocapsid protein (N) gene of tomato spotted wilt virus (TSWV) were generated via microprojectile bombardment of somatic embryos from the cultivar Okrun, a runner-type peanut primarily grown in the southwestern U.S. The gene for hygromycin resistance, *hph*, was included on the plasmid containing the N gene as a selectable marker. PCR and ELISA results indicated that eight of 10 primary transformant lines were positive for incorporation and expression of the TSWV N transgene. PCR and ELISA analyses of T_1 progeny indicated that two of the eight transgenic lines demonstrated a 3:1 segregation ratio, indicative of incorporation of a single copy of the TSWV N transgene. Transgene expression levels remained constant through the T_1 generation.

Key Words: Transformation, virus, peanut, resistance.

Cultivated peanut (*Arachis hypogaea* L.) is an economically important crop throughout the tropics and semi-tropics. First observed in Brazil in 1941 (27), tomato spotted wilt virus (TSWV), which has a broad host range including over 500 species of plants (17, 21), is a significant peanut pathogen in the southeastern U.S. (7). The virus is exclusively vectored by thrips (Thysanoptera: Thrypidae) (17) and is member of the *Tospovirus* genus which comprises the plant members of the Bunyaviridae family (25). Although not yet a significant problem to all peanut production areas in the southwestern U.S., TSWV has been responsible for extreme losses in areas of south Texas (14) and has recently been noted in areas of northern Texas and southern Oklahoma (H.A. Melouk, pers. commun.).

Control of the virus through pesticide application is not possible due to the sporadic dispersion of the thrips vector. Thus, host plant resistance offers the most effective solution to TSWV control. Traditional breeding practices and germplasm screening have primarily been focused on developing TSWV-resistant cultivars for production in the southeastern U.S. where the disease is prominent (27). As a result, such practices have produced only a few cultivars with partial TSWV resistance suitable for production in the southwest U.S.

Genetic transformation of peanut with a TSWV resistance gene offers an alternative to time-consuming traditional breeding techniques. Most efforts to achieve genetically engineered virus resistance have involved the introduction of a virus coat protein into the plant host genome so as to simulate cross protection. Coat protein-mediated resistance or protection against virus infection in plants has been well documented (3, 4, 25, 30, 31, 35). Plant hosts expressing viral coat proteins as transgenes have resulted in various degrees of protection ranging from delay in viral symptoms to restriction of virus movement outside a local lesion (22, 31, 35). Many studies reporting on coat protein-mediated resistance have involved RNA viruses (39). TSWV is an enveloped RNA virus with a tripartite genome. The nucleocapsid protein, a small protein of 29 kd, encapsidates all three genomic RNAs (10, 12, 13, 15, 17). The nucleocapsid protein has been shown to act as a controlling component of virus replication as well as take part in virion assembly (5, 6). The expression of the TSWV N protein has been used as a strategy for producing transgenic plants with virus resistance by other researchers (11, 19, 22, 24, 33, 36, 37, 42, 43). Peanut lines have been generated from cultivars grown in the southeastern U.S. which contain a TSWV N transgene (22, 24, 43). The objective of this study was to introduce the TSWV N gene into the genome of the peanut cultivar Okrun, a runner-type peanut commonly grown in the southwestern U.S.

Materials and Methods

Plant Tissue Culture. Somatic embryos were initiated using seed from the cultivar Okrun following procedures similar to those previously reported (9, 32). Individual embryos were excised from Okrun seed, surface sterilized, and placed on petri plates (100 x 15 mm) of PIC medium (MS salts (29), 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 pBIN (obtained from Dr. Tom German, Univ. Wisconsin) was originally constructed by replacing the β-glucuronidase gene (*Bam*HI/*Sst*I digestion) in plant transformation vector pBI121 (Clonetech, Palo Alto, CA) with the N gene from a Hawaiian TSWV isolate collected on the island of Maui (TSWV-MT2; 41). The expression cassette containing the CaMV 35S promoter, the N gene, and a NOS terminator was excised via *Pst*I/*Eco*RI digestion and subcloned into the plasmid pAB2.5 (9) containing the selectable marker gene hygromycin phosphotransferase (*hph*) which confers hygromycin resistance. The resulting plasmid is referred to as pAB-N.

Microprojectile Bombardment and Regeneration of Transgenic Plants. Four bombardment experiments

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were carried out using DNA from pAB-N. Each experiment consisted of three to seven plates, each containing eight to 10 embryonic clusters 2-3 cm in diameter. 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. Viable embryonic tissue was then transferred to shoot regeneration (SR) plates [MS basal medium (29), 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 in the designated plant line number.

PCR Analysis of Putative Transformants. DNA was isolated from leaves of putative transformants by harvesting 2 g of fresh leaf tissue from 10 wk old plants 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 12,000 Xg 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 re-suspended in 2 mL sterile TE buffer. Preliminary screening of primary transformants was done by PCR for the presence of the *hph* gene. A 480 bp region of the *hph* gene was amplified using primers F6 5' CGCAAGAATCGGTCAATACACTAC 3' and B11 5' TCCATACAAGAAAACCACGG 3'. A 800 bp region of the TSWV N gene was amplified using primers N1 5' TAGTCACGAGTCATATGTCTAAGGTTAAGCTC 3' and N2 5' TAATATAGGATCCTCAAGCAAGTTCTGCGAG 3'. 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, 1 min at 72 C, for 40 cycles. PCR products were visualized via electrophoresis on a 1% agarose gel in 1X Tris-acetate-EDTA (TAE) buffer and subsequent staining with ethidium bromide (1). The marker used for PCR analysis was a 1 kb ladder (Promega, Madison, WI). PCR was performed on DNA from both the T_0 and T_1 generations.

ELISA Analysis. Detection of TSWV N protein was performed per the manufacturer's protocol using the TSWV Pathoscreen Kit (Agdia, Inc., Elkhart, IN). Plant extracts for ELISA analysis were prepared by grinding 0.5 g young leaf material from 10 wk old plants in 1.0 mL phosphate buffered saline (PBS). ELISAs were performed on both the T_0 and T_1 generations in triplicate to ensure accuracy. LSD analysis was performed on ELISA data using SAS version 8.2 (40).

Results and Discussion

All transgenic plant lines evaluated in this study were considered to have arisen as independent transformation events since each line was taken from a separate embryonic cluster. Eight of 10 putative transformants were positive for incorporation of both the selectable marker gene *hph* and the N gene from the Hawaiian isolate of TSWV when tested by PCR (Table 1; Fig. 1) with primers specific for those genes. All plant lines positive for the *hph* gene were also positive for the N gene, indicating an expected 100%

Table 1. Bombardment experiments from which hygromycin-resistant peanut lines were regenerated and number of lines positive for transgenes by PCR (*hph* or N) and ELISA (N) analyses.

Experiment	Hyg^R	PCR^{hph}	PCR^N	ELISA
E-N5	1	1	1	1
E-N8	2	2	2	2
E-N10	2	2	2	2
E-N11	5	3	3	3
Total	10	8	8	8

co-transformation frequency for the covalently linked genes. The use of hygromycin resistance as a selectable marker in peanut transformation studies has been well established (9, 22, 24, 43). Plant lines E-N11-3 and E-N11-7 which were hygromycin-resistant but PCR-negative were considered putative escapes. The two lines were not analyzed via a second DNA isolation from T_0 plants because ELISA results were also negative (Table 2). However, T_1 generation plants from these lines were analyzed and considered negative controls along with non-transformed Okrun plants. Ten progeny from each of the primary transformant plant lines were tested via PCR and ELISA to determine segregation patterns of the transgene(s) and expression levels. Of the eight plant lines testing positive for the transgene(s) in the T_0 generation, two lines (E-N10-7 and E-N11-4) demonstrated a segregation pattern of 3:1, suggesting that a single copy of the transgene was inserted into the peanut genome. Future Southern blot analysis will determine the exact copy number of the transgene present for individual plant lines and whether or not the copy number can be correlated with transgene expression level.

ELISA analysis of transgenic plant lines revealed various levels of transgene expression. Plants were considered negative for transgene expression if their A_{405} value was less than 0.10. Levels of expression among T_0 plants ranged from 1.7-28X background levels present in non-transgenic Okrun. Statistical analysis of ELISA analysis indicated five of 10 transgenic plant lines had levels of transgene expression that were significant at $P \leq 0.05$, ranging from 20-28X background levels. ELISA absorbance values of T_1 plants ranged from 0-15X background levels. Eight of the 10 transgenic plant lines were statistically different from non-transgenic controls at the 0.05 probability level, ranging from 4.5-15X background levels. For most transgenic plant lines, transgene expres-

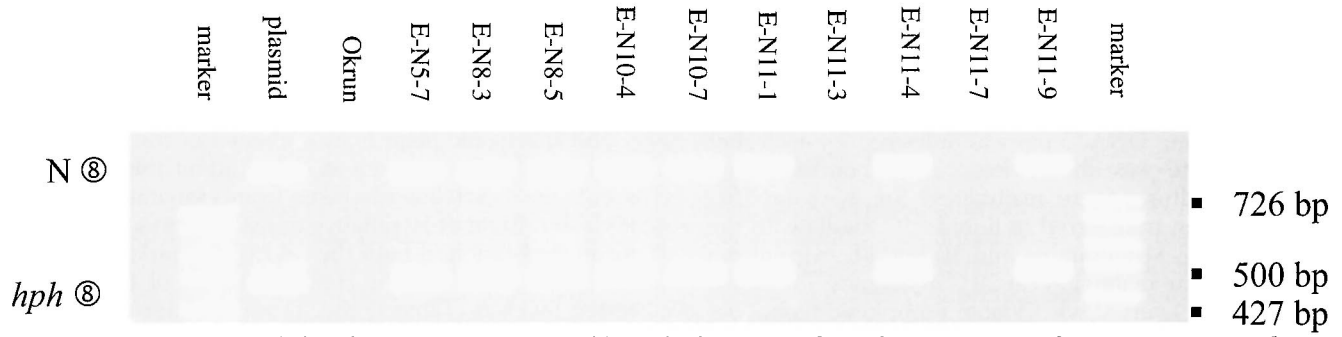

Fig. 1. Amplification of the *hph* and N transgenes generated by multiplex PCR analysis of T_0 putative transformants. Positive and negative control lanes are labeled *plasmid* and *Okrun*, respectively, while numbered lanes represent individual transgenic plant lines.

sion decreased from generation T_0 to generation T_1, with line E-N11-9 having the largest reduction of 84%. Transgene expression in plant lines E-N10-7 and E-N11-4 remained constant for both generations analyzed. These lines also demonstrated a 3:1 transgene segregation ratio, a characteristic more commonly reported for plant lines containing a single transgene copy (23, 43). The decrease in transgene expression seen in all other plant lines may be a result of transgene silencing which has been commonly observed in transgenic plants containing multiple transgene insertions (20, 43). Host genome defense mechanisms have been shown to silence genes through methylation of promoter regions of multiple copy transgenes present at different insertion sites (2, 16, 26, 28) while multiple transgenes inserted at a single locus tend to remain active (8). Northern blot analysis of these transgenic plant lines may determine whether a gene silencing mechanism is acting to decrease transgene expression.

TSWV N protein expression levels in the transgenic peanut lines included in this study are lower than those generally found in infected peanut plants (24) and transgenic peanut plants produced by other researchers (24, 43). However, it has been reported that protection of transgenic plants expressing viral coat proteins may be RNA and/or protein mediated (11, 33, 34, 39, 42). Prins and co-workers reported that TSWV resistance in transgenic plants is sequence specific and requires expression of the N or NsM gene sequences (37, 38). Resistance to TSWV in transgenic tobacco has been shown to be largely post-transcriptional (11, 33, 42) where non-functional or anti-sense N genes provided resistance to TSWV infection. Recently, field resistance to TSWV infection was reported for transgenic tobacco expressing the TSWV N gene (19) and for transgenic peanut plants expressing an antisense N gene sequence (24). Thus, it is possible to achieve protection from TSWV infection in transgenic plants without high levels of N protein expression.

Genetic engineering of host plants for TSWV has proven to be a potential solution for controlling virus infection and spread. Efforts to combine transgenic and natural resistance could offer an even more stable form of TSWV control and possibly a broad resistance to infection by other tospoviruses (18, 36). The results reported here demonstrate the successful incorporation of the TSWV N gene into the runner peanut cultivar Okrun. Future analysis of the plant lines generated in this study will determine their utility for controlling TSWV infection of peanut in the southwestern U.S.

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Table 2. ELISA A_{405} readings averaged from three independent tests for Okrun, T_0 and T_1 hygromycin-resistant plant lines. LSD estimates and P values for plant lines as compared to the non-transgenic Okrun control are also listed.

	Generation					
	T_0			T_1		
Plant line	ELISA	LSD	P value	ELISA	LSD	P value
Okrun	0.0147	–	–	0.0220	–	–
E-N5-7	0.0530	0.0383	0.7723	0.2293	0.2073	0.0001
E-N8-3	0.2923	0.2777	0.0453	0.3127	0.2907	0.0001
E-N8-5	0.3120	0.2973	0.0332	0.2483	0.2263	0.0001
E-N10-4	0.3287	0.3140	0.0253	0.2780	0.2560	0.0001
E-N10-7	0.2650	0.2503	0.0688	0.3303	0.3083	0.0001
E-N11-1	0.3520	0.3373	0.0171	0.2747	0.2527	0.0001
E-N11-3[a]	0.0250	0.0103	0.9378	0.0387	0.0167	0.5230
E-N11-4	0.1193	0.1047	0.4322	0.1393	0.1173	0.0002
E-N11-7[a]	0.0723	0.0577	0.6637	0.0147	-0.0073	0.7779
E-N11-9	0.4073	0.3927	0.0066	0.0987	0.0767	0.0068

[a]Plant lines not positive for *hph* or N genes via PCR analyses.

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