FIELD ASSESSMENT OF VIRUS RESISTANCE IN TRANSGENIC *NICOTIANA TABACUM* CV. BURLEY 49 PLANTS EXPRESSING TOBACCO ETCH VIRUS SEQUENCES

TOBACCO SCIENCE

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Nicotiana tabacum cv. Burley 49 germplasm, transformed with mutated versions of the tobacco etch virus (TEV) coat protein gene, was tested under field conditions for tolerance and resistance to TEV. Twenty-four independent transgenic lines and eight different mutated versions of the TEV coat protein gene were examined. The test was conducted at the same field location for two successive years. Transgenic plants expressing an mRNA, which was translated into a full length TEV coat protein, became infected when challenged with TEV but then recovered from TEV infection 3 - 4 weeks later. The same response was displayed by plants containing a gene lacking the codons for 29 amino acids at the amino terminus of the TEV coat protein. Plants containing a gene that expressed an antisense RNA version of the TEV coat protein sequence displayed little tolerance to TEV. A similar response to TEV was observed with plants containing only regulatory gene sequences and no TEV-derived sequences, or with plants having no foreign gene sequences at all (Burley 49). Selected entries expressing untranslatable versions of the TEV coat protein mRNA never became infected with TEV, and they displayed a high level of resistance. This study confirms the use of transgenic plants expressing an untranslatable sense RNA as a promising approach in the development of potyvirus-resistant tobacco germplasm.

Additional key words: Potyvirus, transgenic plants, RNA-mediated resistance, Burley tobacco.

INTRODUCTION

The concept of pathogen derived resistance (PDR) suggests that the genome of the pathogen may be a convenient source of resistance genes (30). Pathogen genes could be isolated and altered such that they are expressed in a host organism. Expressing a pathogen's gene at the wrong time, at an elevated level, or in a mutated form could interfere with the highly evolved and coordinated pathogen-host interaction, and this may result in an alteration or abortion of infection.

A number of PDR approaches to control plant viruses and the losses they incur have been tested (1, 3, 11). Many studies demonstrated that the expression of a plant virus coat protein is usually an effective way to generate virus-resistant plants (3). Other approaches have expressed other parts of the viral genome (4, 13, 22) or have expressed non-viral nucleic acids associated with certain virus infections (12, 17). In general, the results of these studies suggest PDR can be implemented in a variety of ways to generate virus-resistant plants.

The resistance generated by these various PDR approaches typically is manifested as a delay in the appearance of symptoms, an attenuation of symptoms, a decrease in virus concentration (24), or in limited cases, a highly resistant state in which no virus can be detected (13, 19, 20). Most of these assessments were made in greenhouse tests under physical confinement. However, over the past five years, a limited number of field studies have been conducted to assess the effectiveness of PDR (18, 29). Many of these studies have confirmed greenhouse results, and they suggest PDR will be an efficient way to generate virus-resistant plants once regulatory concerns are addressed.

Various PDR approaches have been used in attempts to engineer resistance to potyviruses (4, 9, 19, 20, 21, 23, 27, 31). We generated a series of transgenic plants that express various mutated versions of the tobacco etch virus (TEV) coat protein (19, 20). TEV, potato virus Y (PVY), and tobacco vein mottling virus are members of the potyvirus group, and they represent commonly encountered viral pathogens of tobacco (2, 8, 28). Our transgenic plant lines displayed a variety of resistance levels to infection with TEV in greenhouse trials (19). The present study was conducted to answer two questions about the transgenic material: would resistance to TEV be manifested under field conditions and was plant type affected by the transformation, regeneration, and transgene expression? This two-year study suggests excellent virus resistance can be generated, but that plant type can be affected by the expression of selected viral gene products.

MATERIAL AND METHODS

Transgenic Plant Germplasm

Nicotiana tabacum cv. Burley 49 was selected for transformation because of the severe reaction this cultivar displays when inoculated with TEV (16). The construction of the transgenes, transformation procedures and other characteristics of these transgenic plant lines have been described (19). The various transgenes (and their nomenclature) expressed in plants examined in this field study are described in **Figure 1.**

Field Plot Design

In 1992, seeds of 16 different entries were sown on a commercial potting mix. When plants reached a height of 5 - 8 cm, they were transferred to styrofoam trays in a greenhouse float system and grown to transplanting size. Prior to transplanting, each entry was inoculated (as described later) with a local isolate of TEV. Plants were transplanted with a commercial transplanter into a field located on the north side of the University of Florida Agronomy Farm (Green Acres) northwest of Gainesville, FL. Plants were planted 40 cm apart in rows that were 125 cm apart. Each sub-plot consisted of 10 plants.

A split-plot, randomized complete-block design with 4 replicates was used in 1992. Time of inoculation was the main plots and entries were the sub-plots. There were three main plots consisting of inoculation at transplanting, inoculation when plants were 40 cm high, and a control (no inoculation). In 1993, a similar design was followed with two exceptions: 21 entries were examined and the treatment for inoculation when plants were 40 cm high was omitted because TEV spread to uninoculated plants quickly and uniformly. The sixteen entries for 1992 were re-examined along with five additional lines in 1993.

Inoculum Preparation and Procedure

The TEV isolate used was obtained from tobacco grown in a commercial Florida field. Inoculum was prepared using systemically infected leaves from Burley 21 plants. Infected leaves were homogenized in 0.05M Na_2HPO_4 / KH_2PO_4 buffer (pH 7.2) (1 g tissue / 2 mL of buffer) and the homogenate was clarified by expressing through cheesecloth.

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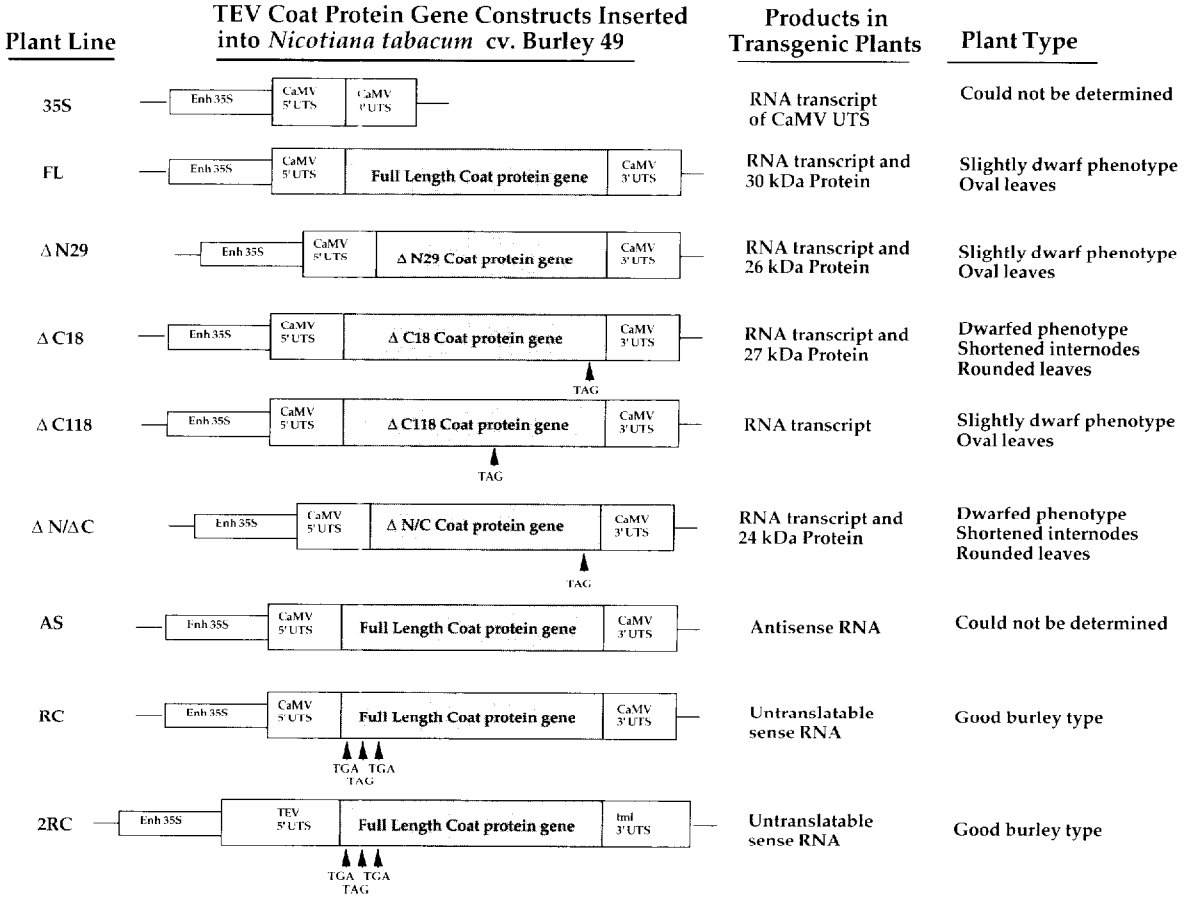
Figure 1. Summary of transgenic plant lines and the transgenes they express. *Nicotiana tabacum* cv. Burley 49 was transformed with 9 different transgenes derived from tobacco etch virus (TEV) sequences. All transgene constructions contain the enhanced cauliflower mosaic virus (CaMV) 35S (Enh 35S) promoter, a 5′ untranslated region (5'UTR) [derived from CaMV or TEV], and a 3′ untranslated region (3'UTR) [derived from CaMV or the *Agrobacterium tumefacians tumor* morphology large gene (tml)]. The nomenclature used to describe the transgenic plant lines is presented along with the gene products detected in those plant lines. In the far right column, burley plant type is given. Abbreviations are as follows: 35S, transgenic plants containing the CaMV 35S promoter and 5′ and 3′ UTS only; FL, ΔN29, ΔC18, ΔC118, ΔN/ΔC, transgenic plants containing the transgene coding for full length TEV CP, TEV CP lacking the amino-terminal 29 amino acids (aa), TEV CP lacking the carboxy-terminal 20 aa, TEV CP lacking the carboxy-terminal 118 aa, or TEV CP lacking both the amino-terminal 29 aa and carboxy-terminal 20 aa, respectively. AS and RC transgenic plants contain the transgene expressed as an antisense form of the TEV CP gene or an untranslatable sense form of the TEV CP gene, respectively. The transgene in the 2RC lines also codes for an untranslatable RNA similar to the RC lines; however, the 5′ and 3′ UTRs are different. Stippled areas represent various forms of the TEV CP nucleotide sequence.



One gram of 600-mesh carborundum was added to each 100 mL of inoculum. The inoculum was stored in a polyethylene bottle in an ice bath, and it was used within 24 h of preparation. Inoculations were made at transplanting by water soaking a small area on two leaves per plant with inoculum. Inoculum soaking was effected with an airbrush (Thayer & Chandler Model E) with a 500 mL reservoir. Inoculum was propelled using carbon dioxide at a pressure of 414 kPa. A young leaf, 8-10 cm in length, near the top of the plant and the next oldest leaf were inoculated on each plant. The inoculated area of each leaf was about 2 cm from the tip of the leaf on the abaxial surface and centered over the mid-vein. The orifice-to-leaf distance of the airbrush was about 1 cm, and this resulted in rapid water soaking of an area of about 1.5 cm in diameter (14).

Plant Evaluation

Plants were visually monitored daily for symptoms associated with TEV infection. Additionally, double diffusion immunoassays (15, 25) and inclusion body analyses (6) were performed on the test entries at selected times. Plant morphology was also noted, and near the end of each growing season, green weights were obtained from three representative plants that were removed from each plot. Each of the three plants was serologically assayed using an enzyme-linked immunosorbant assay (ELISA) for TEV and PVY. Of the seven plants remaining after weighing, four were examined four weeks later for root diseases that may have affected above-ground weight determinations. Although some root galling was observed, it occurred on all entries and it was not believed to be of sufficient severity to affect plant growth before weight determinations. At the end of the test, all transgenic plant material was destroyed.

Cultural Practices

The same experimental site was used for both years. The soil of the experimental area was turned with a bottom plow approximately a month before the tobacco was to be transplanted. In 1992, the herbicides, pendimethalin (1.12 kg [a.i.]/ha) and penulate (4.5 kg [a.i.]/ha); the insecticide, chlorpyrifos (2.2 kg [a.i.]/ha); and the fungicide, metalaxyl (0.56 kg [a.i.]/ha) were applied broadcast and incorporated into the soil with a disk harrow. In 1993, the nematicide, fenamiphos (6.7 kg [a.i.]/ha) was included with the above pesticides. The fumigant nematicide, dichloropropene (56 L/ha) was injected in the row at bedding in 1992, but it was

applied broadcast as the soil was turned in 1993. Thus, the dichloropropene followed the application of other preplant pesticides in 1992 but preceded them in 1993. Two nematicides were used in 1993 because nematodes were expected to be at a high population, and increasing the rate of the fumigant nematicide would increase the risk of phytotoxicity.

Approximately 2,000 L/ha of water was applied during transplanting. Acephate was added to the transplant water to provide 0.8 kg [a.i.]/ha. Also during transplanting, a 6-6-18 fertilizer at the rate of 450 kg/ha was banded to the side of the plants. The experimental area was irrigated by overhead sprinklers after transplanting.

Acephate (0.8 kg [a.i.]/ha) was applied as a foliar spray approximately every two weeks after transplanting. A 6-6-18 fertilizer (860 kg/ha at each of three applications) was side dressed approximately every two weeks after transplanting. When rainfall was insufficient to maintain plant growth, irrigation by overhead sprinklers was used.

RESULTS

Description and plant evaluation of entries

The plant nomenclature, the gene expressed in the transgenic plants, and general plant type are listed in **Figure 1.** Plant growth and morphology were consistent each year and many transgenic lines had a distinct morphology correlated with the transgene expressed. Transgenic plants expressing either a ΔC18 or a ΔN/ΔC version of the TEV coat protein were dwarfed and leaves were rounded in appearance. The internodal distance was shorter than in other entries. Plants expressing the FL, ΔN29, or ΔC118 version of the TEV coat protein were stunted slightly, and the leaves were more typical of Burley tobacco. Transgenic plants expressing the RC or 2RC untranslatable version of the TEV coat protein gene generally possessed a morphology typical of a Burley tobacco plant. Other lines could not be evaluated for plant type because virus infection resulted in severe stunting.

Assessment of Virus Resistance

Different transgenic lines responded to virus infection in an entry-specific manner. The formation of symptoms and the presence of a suspected viral infection was confirmed by cytoplasmic inclusion analysis or by using virus-specific polyclonal antibodies in ELISA. The results are summarized in **Table 1.**

In general, four different plant responses were noted among the different transgenic lines. Some entries became infected with TEV and displayed severe symptoms. These plants were stunted and showed significant etching (necrosis) and extreme chlorosis. All plants of the following entries displayed this response: Burley 49, 35S-4.7, AS-6.1, AS-7.2, and RC-9.1. A second phenotype observed in some lines following TEV infection was characterized by attenuated systemic symptoms, often in the form of localized chlorotic spots on the leaves. Transgenic lines ΔN/ΔC-6.9, ΔN/ΔC-6.14, ΔC18-7.9, and ΔC18-15.7 displayed this attenuated symptom phenotype. The third generalized type of symptomatology was exhibited by all TEV-infected FL- and ΔN29-lines and by some 2RC lines. These transgenic plants initially became infected and displayed typical TEV-induced symptoms, but plants gradually recovered from the infection. As new leaves emerged, symptoms were restricted to interveinal areas. Subsequent leaves possessed less symptomatic tissue until, finally, leaves developed that were totally devoid of symptoms and virus. Once these plants had recovered from TEV infection, the upper leaf tissue was free of virus and symptoms and it never became re-infected with TEV. A fourth response to TEV infection was displayed by selected RC and 2RC lines and one ΔC118 line. Transgenic lines RC-5.02, RC-7.16, 2RC-6.13, 2RC-1.8, and ΔC118-18.1 never displayed any symptoms after infection with TEV via initial mechanical inoculation or subsequent aphid transmission. **Figure 2** shows the dramatic difference in plant growth between infected untransformed Burley 49 plants and highly resistant Burley 49 germplasm expressing an untranslatable RNA molecule (2RC-6.13). Both entries were inoculated with TEV approximately 10 weeks before the photograph was taken.

Table 1. Plant response to infection with tobacco etch virus (TEV).

Entry[a]	Average Symptom Intensity[b]	Average Stunting[c]	Recovered Plant Phenotype[d]
Burley 49	4	4	No
35S-4.7	4	4	No
FL-3.3	3->0	1	Yes
FL-24.3	3->0	1	Yes
ΔN29-2.12	3->0	1	Yes
ΔN29-8.1	3->0	1	Yes
ΔC18-7.9	2	2	No
ΔC18-15.7	2	2	No
ΔN/ΔC-6.9	2	2	No
ΔN/ΔC-6.14	3	2	No
ΔC118-18.1	0	0	N/A
AS-6.1	4	4	No
AS-7.2	4	4	No
RC-5.02	0	0	N/A
RC-7.16	0	0	N/A
RC-9.1	4	4	No
2RC-6.13	0	0	N/A
2RC-1.8	0	0	N/A
2RC-3.3	2->0	0	Yes
2RC-5.2	2->0	0	Yes
2RC-8.13	2->0	0	Yes

[a] Plant entries and their nomenclature are described in Figure 1. Number after line identification (i.e., 4.7 of 35S-4.7) indicates a particular line derived from a single transformed parent plant.

[b] Average symptom intensity was a subjective analysis of TEV-induced symptoms. The rating scale was from 0 to 4, with 0 = no symptoms, 1 = mild chlorosis and mosaic, 2 = moderate mosaic, 3 = severe mosaic and some chlorosis and etching of leaves, 4= severe chlororis and etching of leaves. For plants that displayed the recovered phenotype, ratings are given for initial and final symptoms (i.e., 3->0).

[c] Average stunting was an estimate of how TEV infection impacted plant growth. The rating scale was from 0 to 4 with 0 being no obvious stunting and 4 being severe stunting typical of TEV infection of *N. tabacum* cv. Burley 49.

[d] Some of the transgenic lines displayed a recovery phenotype in which the plant was able to 'outgrow' the infection. Older leaves displayed TEV-induced symptoms while younger leaves possessed no symptoms and virus. N/A, not applicable.

The engineered resistance was TEV specific. None of the lines tested displayed any resistance to PVY or tomato spotted wilt virus, as evidenced by a limited number of plants that were naturally infected with these viruses in the field over the two-year study.

In addition to mechanically transmitted TEV, the test plot was assessed for virus resistance against aphid-vectored TEV (26). No colonizing aphid populations were detected, yet 100% of the uninoculated Burley 49 and susceptible transgenic plants became infected 2-3 weeks after transplanting. Virus movement was likely due to transient, non-colonizing aphid species during this study. Under these conditions, the TEV-resistant RC, 2RC, and ΔC118 lines remained free of TEV.

Green weights of representative plants were also determined as an estimate of virus resistance. In 1992, these measurements were taken when ca. 75% of the plants were flowering. This was two weeks after the highly resistant RC and 2RC lines had flowered. In 1993, green weight measurements were taken when 10% of the plants were flowering. Only plots mechanically inoculated with TEV were weighted. Seven to 10-fold differences in average green

Figure 2. Comparison of tobacco etch virus (TEV) infection of transgenic and untransformed *Nicotiana tabacum* cv. Burley 49 plants. Two entries are shown. Plants of transgenic line 2RC-6.1 are shown in the background. This line expresses an untranslatable version of the TEV coat protein gene. In the foreground are untransformed *N. tabacum* cv. Burley 49 plants. Both lines were mechanically inoculated with a local Florida isolate of TEV concurrent with transplantation into the field. The photograph was taken approximately 10 weeks after transplanting.

weight were readily apparent between protected lines and untransformed Burley 49 tissue. These results are presented in **Table 2**.

DISCUSSION

Results from this field study demonstrate that genetically engineered resistance can be effective in preventing losses incurred by TEV infection.

Two major conclusions were drawn from this study. First, effective virus resistance was displayed in different resistance phenotypes, and resistance could be generated by exploiting different genetic approaches. Second, expression of particular transgenes could alter plant type.

Resistance to TEV was manifested in a variety of phenotypes. Lines producing an untranslatable version of the TEV coat protein gene (RC and 2RC lines) afforded superior protection. Many of these lines never became infected in the two-year field trial. This was in agreement with previous greenhouse studies (19, 20) and was remarkable in view of the inoculum potential provided by endemic TEV and migratory aphid populations. All uninoculated control Burley 49 plants became infected 2 - 3 weeks after transplanting. Therefore, the highly resistant RC and 2RC lines withstood mechanical inoculation and severe aphid-vectored TEV pressure. Transgenic lines expressing these transgenes resulted in green weight difference that were, on average, 10-fold higher than susceptible Burley 49 tissue (**Table 2**).

Another form of resistance manifested itself in a phenotype in which plants recovered. ΔN29, FL, and selected 2RC plant lines initially became infected with TEV but recovered. Newly emerging leaves were free from TEV and TEV-induced symptoms. Of particular interest was the observation that these plants never became re-infected with TEV during the remainder of the field test even under pressure from aphid-transmitted TEV. Laboratory studies suggested that an anti-TEV state is induced in these plants, and therefore, recovered plants are completely resistant to subsequent TEV infection (unpublished observations). These plants also may offer a useful form of resistance. Transgenic plants expressing ΔN/ΔC and ΔC18 forms of the TEV coat protein also displayed resistance to TEV infection, although it was inferior to resistance described above and permitted a mild systemic infection. Our studies also suggested that the 35S and AS transgenic plants did not provide a useful form of resistance to TEV.

The second major conclusion from this study was that the expression of particular transgenes affects plant type. A recent study by Brandle & Miki (5) showed that transformed tobacco plants may have altered agronomic characteristics. In our study, a dwarfed appearance was consistently associated with all plants expressing a ΔC18 or ΔN/ΔC transgene. This phenotype was observed regardless of transgene expression levels, which suggests that the transgene transcript or protein product was responsible for the altered plant morphology. Plants expressing an untranslatable RNA and displaying superior resistance (selected RC and 2RC) had good Burley plant characteristics. These transgenes and their products did not appear to impact Burley growth characteristics.

In summary, extremely effective virus resistance can be generated using a variety of transgenic approaches. Plants expressing an untranslatable sense (or genome sense) RNA will be the transgenic material of choice to deploy. There are several reasons for this conclusion. The virus resistance was superior in these lines. TEV was not detected in these lines over the two- year study. Second, expression of these transgenes did not obviously affect Burley plant type. Third, expression of a gene in the form of an untranslatable RNA molecule may address relevant concerns regarding the

Table 2. Average green weight of plants inoculated with TEV.

Entry[a]	Average green weight (grams/plant)[b] 1992	1993
Burley 49	286	121
35S-4.7	140	91
FL-3.3	1893	-
FL-24.3	1636	-
ΔN29-2.12	1944	-
ΔN29-8.1	1598	-
ΔC18-7.9	2094	-
ΔC18-15.7	1958	-
ΔN/ΔC-6.9	1640	-
ΔN/ΔC-6.14	1682	-
ΔC118-18.1	-	1329
AS-6.1	761	-
AS-7.2	874	-
RC-5.02	1648	1206
RC-7.16	1639	1303
RC-9.1	438	110
2RC-6.13	1487	1123
2RC-1.8	-	1382
2RC-3.3	-	1321
2RC-5.2	-	1121
2RC-8.13	-	848

[a] Plant entry and nomenclature are described in Figure 1.

[b] Three representative plants from each plot were harvested and weighed. The plot was replicated four times and the average green weight for these four replicates is presented. In 1992, green weight was taken after most plants had flowered. The RC and 2RC plants flowered 2 - 3 weeks earlier, presumably because they were free from TEV the entire growing season. Therefore, their green weights are probably lower than the maximum weight achieved. In 1993, green weight measurements were taken as plants began to flower.

potential wide-spread application of PDR practice (7). It has been suggested that transencapsidation of viral RNAs by transgene products may generate virions with expanded host range or new insect vector association. Transencapsidation occurs naturally and recently has been shown to occur in transgenic plants (10). Additionally, RNA recombination between viral genomes and transgene transcripts might increase the possibility of generating new viral entities. Transgenes that express untranslatable RNAs should minimize or essentially negate these concerns. Future questions will focus on the durability of transgenic resistance and the inheritance of this resistance.

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