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Characterization of a gametoclonal variant controlling virus resistance in tobacco *

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Summary. Potato virus Y (PVY), susceptible tobacco (*Nicotiana tabacum* L.) cultivar, McNair 944, was subjected to in vitro anther culture to determine if genetic variability for virus resistance could be induced among resulting haploids. Five hundred and forty-five haploids were produced and inoculated with a highly necrotic strain (NN) of PVY. One haploid plant survived, even though it was infected with the virus. Selfed progenies of a chromosome-doubled plant of this variant, designated NC 602, proved to be highly resistant to the necrotic effects of the virus. An investigation into the genetic nature of this variant showed the resistance mechanism to be controlled by a single gene exhibiting incomplete dominance. Cytoplasmic and maternal effects were not involved in the disease resistance reaction. The variant was challenged with ten additional strains of PVY from an international collection, and it proved to be resistant to three (VAM-B, MM, and Spanish) strains. NC 602 was evaluated for five agronomic traits and concentrations of total alkaloids as nicotine and reducing sugars in cured leaf. The gametoclonal variant differed from McNair 944 only for cured leaf yield, where an 18.4% reduction was measured.

Key words: Gametoclonal variation – Anther culture – Potato virus Y – Tissue culture – Haploidy

Introduction

Potato virus Y (PVY) is distributed worldwide, and it causes economic losses to numerous solanaceous crop species including tobacco, *Nicotiana tabacum* L. The virus is highly variable and numerous strains, causing differential disease reactions on selected host genotypes, have been described (Gooding 1985). Gooding and Tolin (1973) described three strains of PVY isolated from flue-cured tobacco in the United States and characterized disease development on an array of tobacco genotypes. Isolate NC 78 (strain NN) produced severe necrosis and/or death of young tobacco seedlings approximately 3 weeks following inoculation. All American flue-cured tobacco germ plasm has proven to be susceptible to this PVY strain.

Two sources of host resistance to PVY strain NN have been described in tobacco. Virgin A Mutante, an irradiation-induced resistance source produced by Koelle (1961), is highly resistant to strain NN, and resistance is conditioned by a single recessive gene. Cigar cultivar Havana 307 is also resistant to strain NN (Gooding et al. 1985). Although the mode of inheritance of this resistance mechanism is not complex, it may not be controlled by a single gene (Rufty et al. 1988).

Anther culture, like other tissue culture systems, can generate genetic variability in *Nicotiana* (Burk and Matzinger 1976; De Paepe et al. 1981; Brown and Wernsman 1982). Evans et al. (1984) designated gametic-derived variation via anther culture as gametoclonal variation. This source of variability could be of potential value in crop improvement (Schnell and Wernsman 1986).

Anther culture has been used in tobacco for haploid production and, upon doubling the chromosome complement, homozygous genotypes can be obtained in a single generation. Unexpected and unusually large amounts of genetic variation have been observed among anther-derived doubled haploids (ADHs). Burk and Matzinger (1976) reported large differences among ADH

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families derived from a highly inbred cultivar, Coker 139, for nine agronomic traits. Collins et al. (1972) reported significant differences between conventionally developed and ADH lines from a similar parental source.

ADH tobacco variants generally result from nuclear rather than cytoplasmic changes (Brown and Wernsman 1982), although cytoplasmic variants have been observed (Matzinger and Burk 1984). Tobacco anther culture haploids develop from the vegetative nucleus rather than from the generative nucleus of the microspore or pollen (De Paepe et al. 1977). Dhillon et al. (1983) reported DNA amplification without chromosome number changes in ADH lines, and an average increase of 12% heterochromatin. In *Drosophila melanogaster*, phenotypic expression of a gene can be affected by the proximity of heterochromatic regions to the gene (Baker 1968). Therefore, the increase in heterochromatin may contribute to the observed variants derived from ADHs of tobacco.

Objectives of the current study were: to genetically characterize a gametoclonal tobacco variant providing resistance to potato virus Y strain NN (PVY-NN), to test this variant for resistance to other strains of PVY, and to determine the agronomic utility of this germ plasm.

Materials and methods

Anthers from a single plant of PVY-susceptible cultivar McNair 944 (McN 944) in the S_{17} generation of inbreeding were cultured on Nakata and Tanaka (1968) medium and 545 haploids were recovered. The parental plant was also self-pollinated, and resulting progeny are referred to hereafter as McN 944. Haploid plants with leaves 50 mm in diameter, along with diploid McN 944 as a control, were inoculated with PVY-NN. Inoculum was prepared by grinding 1 g tissue from a systemically infected leaf of Burley 21 in 2-ml of buffer (0.05 *M* Na_2HP_4-KH_2PO_4, pH 7.2) with mortar and pestle. Inoculum was applied to leaves using a cotton swab previously dipped into 22×10^{-6} m (600 mesh) carborundum and then into the inoculum. Approximately 18 days after inoculation, all except one of the haploids were dead. This plant contained the virus, but exhibited mild mosaic symptoms and no necrosis. The chromosome complement from this variant was subsequently doubled using the method of Kasperbauer and Collins (1972), cytologically confirmed to possess the $2n = 48$ chromosome number, self-pollinated, and designated NC 602.

Genetic characterization

NC 602 was crossed reciprocally to McN 944 (F_1s) and the F_1 was selfed to obtain an F_2 population. Both F_1s were also backcrossed to NC 602 (BC_1A) and McN 944 (BC_1B), the resistant and susceptible parents, respectively. Progeny of these crosses were inoculated with PVY-NN and evaluated for percent leaf area exhibiting veinal and interveinal necrosis, and for plant stunting. Percent stunting was estimated from comparisons of inoculated plants with uninoculated checks of the same genotype. NC 602 and McN 944 were utilized as resistant and susceptible controls, respectively, in each experiment. Tests were conducted in a greenhouse cool chamber with a mean temperature range of 26 ± 6 °C. A randomized complete block design was employed for the virus evaluations of NC 602, both F_1s, and the F_2 population. Blocking was performed over time due to restrictions on greenhouse space. Five replications (blocks) were used with 14 plants per replication for the evaluation of NC 602 and the F_1s, and 48 plants per replication for the F_2 population. A completely random design was employed for the BC_1A and BC_1B progeny evaluation, and 167 plants of each backcross were inoculated.

Rufty et al. (1983) showed that a recessive gene located on chromosome G providing resistance to a strain of PVY is temperature sensitive such that virus-induced necrosis is severe at 28 °C and absent at 35–40 °C. Therefore, NC 602, McN 944, and the F_1 hybrid were also grown in two temperature regimes of 27.4 to 17.6 °C and 36.6 to 26.4 °C, to determine if the higher temperature might reverse the disease reaction. The genotypes were allowed 4 days to adjust to the temperature before inoculation with PVY-NN. Plants were rated for veinal and interveinal necrosis and stunting 21 days after inoculation.

PVY strain test

Gametoclonal variant NC 602 and McN 944 were evaluated for their reaction to the following strains of PVY: P-US, MM, MN, NN, VAM-B, Spanish, South Korean, South African, European-WG, European-H, and Chilean (Gooding 1985). A completely random design replicated over time was utilized. Each replication consisted of four plants, of which three were inoculated. Uninoculated plants were used as controls to determine the amount of stunting (reduced plant growth). Data were also obtained for percent veinal necrosis, interveinal necrosis, and stunting.

Agronomic evaluation

NC 602 was crossed reciprocally with McN 944 and the two parents, and the F_1 and reciprocal F_1 hybrids were grown in three replications of a randomized complete block design at Whiteville and Rocky Mount/NC in 1986. Genetic entries were planted in single-row, 20-competitive-plant plots, with 0.56 m between plants and 1.2 m between rows. Standard fertilization, management, harvesting, and curing procedures for flue-cured tobacco production in North Carolina were followed.

Data were collected for cured leaf yields, plant height, number of leaves per plants, days from transplanting to flowering, cured leaf quality as measured by grade index (Wernsman and Price 1975), and concentrations of total alkaloids and reducing sugars in the cured leaf lamina (Harvey et al. 1969). Combined location analyses of variance were conducted for all measured characters, and entry sums of squares were partitioned into single-degree-of-freedom comparisons of NC 602 versus McN 944, parents versus F_1 and reciprocal F_1 hybrids, and the F_1 versus reciprocal F_1 hybrid.

Results and discussion

Genetic characterization

Gametoclonal variant NC 602 possessed resistance to the necrotic effects of PVY-NN (Fig. 1). However, NC 602 was stunted by 26% when compared to an uninoculated control (NC 602). Virus was present in inoculated NC 602 plants, but disease was restricted to mosaic leaf symptoms. Hence, the genotype is resistant to the necrotic effects caused by this strain, but only tolerant to mosaic and stunting reactions. McN 944 was highly suscepti-

Fig. 1. Reaction of McN 944, the F_1 hybrid McN 944 × NC 602, and gametoclonal variant NC 602 to potato virus Y strain NN 21 days after inoculation

Table 1. Disease rating for a potato virus Y resistant tobacco variant NC 602, McN 944, the susceptible parent, and reciprocal F_1 hybrids, when inoculated with strain NN

Genotype	Number of plants	Disease rating[a]		
		Veinal necrosis	Interveinal necrosis	Stunting
		% Leaf area		
NC 602	140	0	0	26
McN 944	140	Dead	Dead	Dead
NC 602 × McN 944	140	75	50	88
McN 944 × NC 602	140	76	51	81

[a] Stunting: a visual estimate of the percent reduction in growth compared with uninoculated plants of corresponding genotype. There was essentially no variation among individual plants Necrosis: a visual estimate of total necrotic tissue on a plant Death: 100% necrotic tissue

ble to the virus, necrosis was evident 8 days after inoculation, and the plants eventually died. F_1 and reciprocal F_1 hybrids responded to the virus in an identical manner for veinal and interveinal necrosis and stunting (Table 1). Therefore, cytoplasmic and maternal effects were not involved in resistance expression. The hybrids were intermediate to the parents in their response to the virus, and an indication of additivity for the resistance factor was evident.

Segregating F_2 and backcross progenies could easily be grouped into three distinct classes for their reaction to PVY-NN (Table 2). The classes were a mild mosaic (no necrosis), intermediate necrosis but no plant death (40% total tissue necrosis), and severe necrosis and eventual death (80–100% tissue necrosis). Segregation in the F_2 population fitted the Mendelian genetic ratio 1:2:1 as indicated by a nonsignificant Chi-square value for goodness of fit. The two backcross populations provided a nonsignificant goodness-of-fit test for a 1:1 genetic segregation ratio. It was concluded that the resistance mechanism of NC 602 is controlled by a single gene exhibiting incomplete dominance.

When NC 602 plants were inoculated under mean temperature regimes of 27.4 to 17.6 °C and 32.6 to 26.4 °C, NC 602 gave the same disease response under both temperature ranges (Table 3), while susceptible control McN 944 died 18 days after inoculation in each regime. At the warmer temperature, the F_1 hybrid exhibited 3% veinal and 17% interveinal necrosis; however, the hybrid had 22% veinal and 29% interveinal necrosis at the cooler temperature. Uninoculated control plants grew much more vigorously at the higher temperature range, and the difference in hybrid response under the different temperatures may be a function of growth rate and not the resistance mechanism. The F_1 hybrid exhibited similar degrees of stunting in both temperature regimes.

Table 2. F_2 and backcross progeny segregation ratios for plants inoculated with potato virus Y strain NN

Genotype		Disease symptoms[a]				
		Mild mosaic	Intermediate necrosis	Severe necrosis	Total	Chi-square
		Number of plants				
(NC 602 × McN 944) F_2	OBS	124	235	121	480	
	EXP	120	240	120	480	0.25
(NC 602 × McN 944)	OBS	93	74	–	167	
× NC 602	EXP	83.5	83.5	–	167	2.16
(NC 602 × McN 944)	OBS	–	78	89	167	
× McN 944	EXP	–	83.5	83.5	167	0.73

[a] Mild mosaic: no necrosis, mild mosaic symptoms Intermediate necrosis: mild mosaic plus 40% of total plant tissues necrotic but no plant death Severe necrosis: 80–100% of total plant tissues, necrotic with eventual plant death

Table 3. Temperature sensitivity test for the genetic mechanism providing resistance to potato virus Y strain NN

Temp. range	Genotype	Disease rating[a]		
		Veinal necrosis	Interveinal necrosis	Stunting
		% Leaf area		
32.6–26.4 °C	NC 602	0	2	25
	McN 944	Dead	Dead	Dead
	NC 602 × McN 944	3	17	43
27.4–17.6 °C	NC 602	0	8	22
	McN 944	Dead	Dead	Dead
	NC 602 × McN 944	22	29	48

[a] Stunting: a visual estimate of the percent reduction in growth compared with uninoculated plants. There was essentially no variation among individual plants
Necrosis: a visual estimate of total necrotic tissue on a plant
Death: 100% necrotic tissue

PVY strain test

As seen in the previous study, McN 944 was killed by PVY-NN and NC 602 was only slightly stunted in the PVY strain test (Tables 1 and 4). Significant differences in disease reactions between McN 944 and NC 602 were not observed when inoculated with strains MN, P-US, Chile, South Africa, South Korea, Europe-H, or Europe-WG.

However, the VAM-B strain caused severe necrosis on McN 944 followed by eventual death. VAM-B did not produce necrotic lesions on NC 602, but caused an estimated 15% stunting of the plants. The virus was present in the plant, as mosaic symptoms were exhibited in leaves. The non-necrotic MM strain caused 10% more stunting on McN 944 than NC 602, while the Spanish strain caused 60 and 33% stunting of McN 944 and NC 602, respectively. NC 602 provided resistance to the necrotic effects of strains NN and VAM-B and the reduced stunting caused by MM and Spanish strains.

Table 4. Disease reaction of tobacco genotypes NC 602 and McN 944 inoculated with ten potato virus Y strains of international origin

PVY strain	Disease rating[a]					
	Veinal necrosis		Interveinal necrosis		Stunting	
	McN 944	NC 602	McN 944	NC 602	McN 944	NC 602
			% Leaf area			
MM	0	0	0	0	32	22
MN	0	0	0	0	15	18
NN	Dead	0	Dead	0	Dead	5
P-US	0	0	0	0	7	18
VAM-B	Dead	0	Dead	0	Dead	15
Chile	58	62	7	7	28	20
Spain	38	42	7	5	60	33
South Africa	25	25	13	13	50	45
South Korea	30	26	23	18	33	45
Europe-H	8	10	10	7	7	22
Europe-WG	20	20	0	0	13	15

[a] Stunting: a visual estimate of the percent reduction in growth compared with uninoculated plants. There was essentially no variation among individual plants
Necrosis: a visual estimate of total necrotic tissue on a plant
Death: 100% necrotic tissue

Table 5. Mean agronomic performance and chemical composition of cured leaf of a PVY-resistant gametoclonal tobacco variant, NC 602 hybrids with the parental cultivars, and McN 944

Genotype	Yield	Days to flower	Leaves	Plant height	Grade index	Total alkaloids	Reducing sugars
	kg/ha		no.	cm		mg/g	
NC 602	2954	64	21	111	53	25.9	184
NC 602 × McN 944	3362	63	21	113	51	27.8	198
McN 944 × NC 602	3299	64	21	114	50	26.3	195
McN 944	3620	63	24	115	47	25.1	197
LSD 0.05	511	NS	NS	NS	NS	NS	NS

Agronomic evaluation

NC 602 differed significantly from McN 944 only for cured leaf yield; the gametoclonal variant line produced 18.4% less cured leaf than McN 944 (Table 5). This magnitude of yield reduction was similar to those observed previously for ADH lines of flue-cured tobacco (Burk and Matzinger 1976; Brown and Wernsman 1982). F_1 and reciprocal F_1 hybrids did not differ for any character measured, and genetic changes in NC 602 affecting agronomic performance did not exhibited maternal or cytoplasmic effects. The F_1 and reciprocal F_1 hybrids did not differ from the mean of the parents for any character, and additive effects for differences between NC 602 and McN 944 are implied (Table 5).

Possible allelism of the gene for virus resistance in NC 602 with genes in cultivars Virgin A Mutante (VAM) or Havana 307 (HA 307) is unknown, but seems unlikely. The gene for resistance in NC 602 is expressed additively, while that in VAM is recessive. Furthermore, VAM and HA 307 genes provide resistance to tobacco etch virus and tobacco vein mottling virus as well as PVY. NC 602 is susceptible to these potyviruses (C.H. Yung, personal communication).

Evidence is firmly established for a genetic change in NC 602 that behaves as a single gene providing resistance to the necrotic effects of two virus strains and reduced stunting by two other strains. This gene was not present in the parental McN 944 plant subjected to anther culture. Nevertheless, we do not have definitive data that this genetic change resulted from anther-culture-induced effects per se. The additivity of its effects is similar to most anther-culture-induced changes (Brown and Wernsman 1982; Schnell and Wernsman 1986), yet the lack of temperature sensitivity on expression of this effect is different (unpublished results).

NC 602 represents the first gametoclonal variant derived from in vitro anther culture of tobacco in this laboratory with possible commercial value. The line possesses good resistance to the major necrotic strains of PVY on United States flue-cured tobacco, and leaf quality and chemical composition appear to be acceptable for commercial deployment. Efforts to incorporate the gene conditioning resistance into acceptable yielding genotypes are in progress.

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