Complementary functions of two recessive R-genes determine resistance durability of tobacco 'Virgin A Mutant' (VAM) to *Potato virus Y*

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ABSTRACT

Tobaccos VAM and NC745 carry the recessive *va* gene that confers resistance to PVY_{NN}. However, they exhibit different levels of resistance durability. Upon virus inoculation, only NC745 developed sporadic systemic symptoms caused by emerging resistance-breaking variants that easily infected both NC745 and VAM genotypes. To identify the differential host conditions associated with this phenomenon, cellular accumulation, cell-to-cell movement, vascular translocation, and foliar content of PVY_{NN} were comparatively evaluated. Virus cell-to-cell movement was restricted and its transit through the vasculature boundaries was completely blocked in both tobacco varieties. However, an additional defense mechanism operating only in tobacco VAM drastically reduced the *in situ* cellular virus accumulation. Genetic analyses of hybrid plant progenies indicate that VAM-type resistance was conditioned by at least two recessive genes: *va* and a newly reported *va2* locus. Moreover, segregant plant progenies that restricted virus movement but permitted normal virus accumulation were prone to develop resistance-breaking infections.

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Introduction

Potyviridae is the largest family of plant viruses with more than 200 species classified into six genera that differ in vector types, modes of transmission, and number of particles encompassing the complete genome (ICTVdB, 2006). *Potato virus Y* (PVY), the type species of the *Potyvirus* genus, is transmitted by many species of aphids in a nonpersistent manner. Its virion is a long flexuous rod (730×11 nm) made up of about 2000 subunits of a single species of capsid protein (CP) arranged helically around a positive sense RNA molecule of 9703–4 nucleotides. This viral RNA has a 5′-genome linked protein (VPg) and a 3′-polyadenylated tail (Robaglia et al., 1989; Shukla et al., 1994; Thole et al., 1993). At the beginning of the infection process, the viral RNA is translated into a large polyprotein precursor of 340-350 kDa, which is processed by three virus-encoded proteases into ten mature multi-functional proteins (Revers et al., 1999).

PVY exhibits an unusually high degree of genetic variability. Numerous strains have been isolated from potato, tobacco, tomato, and pepper plants around the world. Strains from tobacco, collected by Gooding and Tolin (1973) from the Southeastern United States, were designated PVY_{MM}, PVY_{MN}, or PVY_{NN} based on their reactions (Mosaic or Necrosis) in the tobacco varieties McNair 12 and NC 95, respectively. Genomic sequence analyses indicate that PVY_{NN} is most closely related to the potato PVY^N group of strains (Chachulska et al., 1997; Sudarsono et al., 1993; Tordo et al., 1995), which also produce veinal necrosis in tobacco.

The resistance of tobacco Virgin A Mutant (VAM, TI 1406) against several potyviruses was obtained after UV-induced mutagenesis (Koelle, 1961). This character is inherited as a single recessive gene (*va*) located on the chromosome E (Gupton and Burk, 1973), but its complete inheritance is suspected to be more complicated (Stavely, 1979). The *va* allele has been introgressed into several tobacco genotypes by traditional plant breeding, such as in Tennessee 86 (TN86, Athow et al., 1987; Gupton, 1980), or by *in vitro* regeneration of double haploids, such as in NC744 and NC745 (Burk et al., 1979; Chaplin et al., 1980). TN86-resistance to *Tobacco veinal mottling virus* (TVMV) is expressed as a low rate of virus accumulation in the initially infected epidermal cells, reduced virus intercellular movement, and no systemic infection (Gibb et al., 1989). Similarly, the infection of PVY in tobacco VAM is limited to the inoculated leaves where virus cellular accumulation and cell-to-cell movement are restricted (Acosta-Leal, 1999; Masuta et al., 1999).

The TVMV-encoded VPg protein was identified as the determinant for overcoming *va*-mediated resistance (Nicolas et al., 1997). Meanwhile, Wittmann et al. (1997) found that a potyviral VPg interacted *in vivo* with the translational eukaryotic initiation factor eIF(iso)4E.

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Since then, several recessive R-genes have been found to encode isoforms of these host factors. Additional viral strains capable of overcoming these R-genes have since been found to contain mutations either in VPg (review in Kang et al., 2005) or in the untranslated regions of viruses lacking the VPg protein (Nieto et al., 2006; Yoshii et al., 2004). Moreover, disruption of the VPg-eIF4E interaction has been associated with the resistant phenotype (Ruffel et al., 2006; Yeam et al., 2007). Thus, a compatible isoform of eIF4E is presumably required by some plant viruses for their genome translation, replication, stabilization, or intercellular transport (recently reviewed by Robaglia and Caranta, 2006). Although the identity of the *va* gene product has not been uncovered, based on its phenotypic similarity to the cloned recessive R-genes, *va* might also encode a defective eukaryotic translation initiation factor.

Gooding (1985) observed that, while tobacco VAM was completely resistant to PVY$_{NN}$, tobacco NC744 facilitated the emergence of resistance-breaking (RB) variants. Thus, this phenomenon offers an opportunity to explore the basis of resistance durability and the evolution of viruses to overcome resistance. In this work, we tested the hypothesis that an allelic variation, rather than multigenic resistance, is responsible for the aforementioned difference in the resistance durability. Evidence obtained through this study supports the multi-locus resistance hypothesis. We found that each of the functional components of tobacco VAM resistance, cellular accumulation and cell-to-cell movement, were independently controlled by two different, segregating recessive genes. We propose *va* as being responsible for potyvirus cell-to-cell movement and the newly discovered *va2* locus as the determinant for potyviral cellular accumulation. This is the first demonstration that two independent resistant mechanisms complement each other in a plant to increase resistance durability against viruses.

Results

Tobacco NC745 facilitates the emergence of RB variants

A large number of tobacco VAM and NC745 plants were sap-inoculated at the 2–3 leaves stage with the avirulent PVY$_{NN}$ strain and observed for symptoms for up to 70 days. Plants of the susceptible Burley 21 variety were also inoculated simultaneously as a positive control. Burley 21 generally produced 90 to 100% symptomatic plants during the first 7 days post-inoculation (dpi). In contrast, none of the 1132 inoculated VAM plants showed symptoms within 70 dpi (Table 1), and no virus could be detected by Northern or Western dot blotting in approximately 5% of the inoculated plants selected at random. Similarly, no symptomatic infections were observed in 57 VAM plants inoculated with partially purified virions (50 µg/plant), nor in ten plants inoculated with purified viral RNA (4 µg/plant).

While the resistance in tobacco VAM remained highly stable, it broke down in some NC745 plants that eventually developed diverse

viral syndromes (Table 1). The virus recovered from these symptomatic NC745 plants produced symptomatic infections in newly inoculated NC745 and VAM plants, as well as in the asymptomatic plants previously inoculated with PVY$_{NN}$. These isolates were considered RB variants because of their ability to systemically infect NC745 and VAM plants within the first 10 dpi. As we did not find any symptomatic NC745 plants that were not infected by an RB variant, all the symptomatic infections produced in resistant tobaccos after PVY$_{NN}$ inoculation were considered RB-infections.

Two contrasting syndromes produced by RB variants were observed in NC745: a mild vein clearing (MVC) syndrome, characterized by mild vein mosaic followed by a temporary symptom remission, and a severe vein necrosis (SVN) syndrome accompanied by yellow spotting, mosaic, and growth atrophy. These syndromes remained unchanged after several host passages in NC745. The positive detection of PVY by Northern blotting in plants showing these syndromes suggests that variants of PVY, rather than unrelated viruses, were the causal agents.

Tobacco VAM cells suppress virus accumulation

Time-series experiments were conducted with transfected protoplasts to estimate the rate of virus accumulation in individual cells of resistant tobaccos. Protoplasts of VAM, NC745, and susceptible Burley 21 tobaccos were transfected with PVY$_{NN}$ RNA during the same assay. This experiment was performed on three different occasions with comparable results. The protoplast printing technique was used to monitor the progression of virus infection in the transfected cells. The Fig. 1A shows areas of the protoplast printing blots where large amount of positive cells were observed. These micrographs revealed that the intensity of the detection reaction (i.e., darkness of the purple color) was similar in the three cell genotypes or slightly stronger in NC745. The percentage of positive cells was also slightly higher in NC745 (Fig. 1B).

To obtain an obvious qualitative difference in the rate of cellular virus accumulation between tobacco genotypes, soluble proteins were extracted from the transfected protoplasts and analyzed by Western blotting under stringent conditions. The precision of the estimation of viral accumulation per infected cell was increased by normalizing the amount of total proteins loaded into each lane according with the proportion of infected cells per variety and time point. This variable was previously quantified for each sample by protoplast printing. After SDS-PAGE electrophoresis, weak bands of PVY CP were detected in Burley 21 and NC745 protoplasts at 48 h post-transfection (hpt). The bands became stronger in both cell genotypes at 96 hpt. In contrast, the CP was detected in VAM cells only at 96 hpt through a weak signal barely visible in the radiogram (Fig. 1C). Thus, whereas the protoplast printing assays indicated that increasing amounts of individual cells were expressing virus infection in all the tobacco genotypes (Fig. 1B), the Western assays showed that the rate of virus accumulation per cell was restricted only in tobacco VAM cells.

The viral cell-to-cell movement is similarly impaired in both resistant tobaccos

Given the lack of an infectious clone of PVY$_{NN}$ expressing a molecular marker, the spatiotemporal distribution of infected cells was monitored on mechanically inoculated leaves by a highly sensitive, localized protoplast mini-extraction (LPmE) assay. In brief, positive protoplasts isolated from leaf disks spatially referenced on the leaf surface were quantified by protoplast printing and recorded with their X–Y coordinates for graphical representation. After mechanical inoculation of 10 µg of PVY$_{NN}$-virion per half-leaf, foci of infected cells were detected in the inoculated Burley 21 leaves processed as soon as 2–3 h post-inoculation (hpi, launching phase, Fig. 2). Then, the CP became almost undetectable in leaves sampled 6–8 h later (masked

Table 1
Incidence of symptomatic virus infections in resistant VAM and NC745 tobaccos caused by resistance-breaking variants after sap-inoculation with the avirulent PVY$_{NN}$ strain

Observation period (dpi)	Resistant Tobacco[a]	
	VAM	NC745
11–20	0/528	48/731 (6.6)
21–30	0/503	223/998 (22.3)
31–50	0/35	24/80 (30.0)
51–70	0/66	49/170 (28.8)
Total	0/1132	344/1979 (17.4)

[a] Total number of symptomatic/inoculated plants (percentage) compiled from five independent inoculation experiments and recorded at the end of the observation period (days post-inoculation).
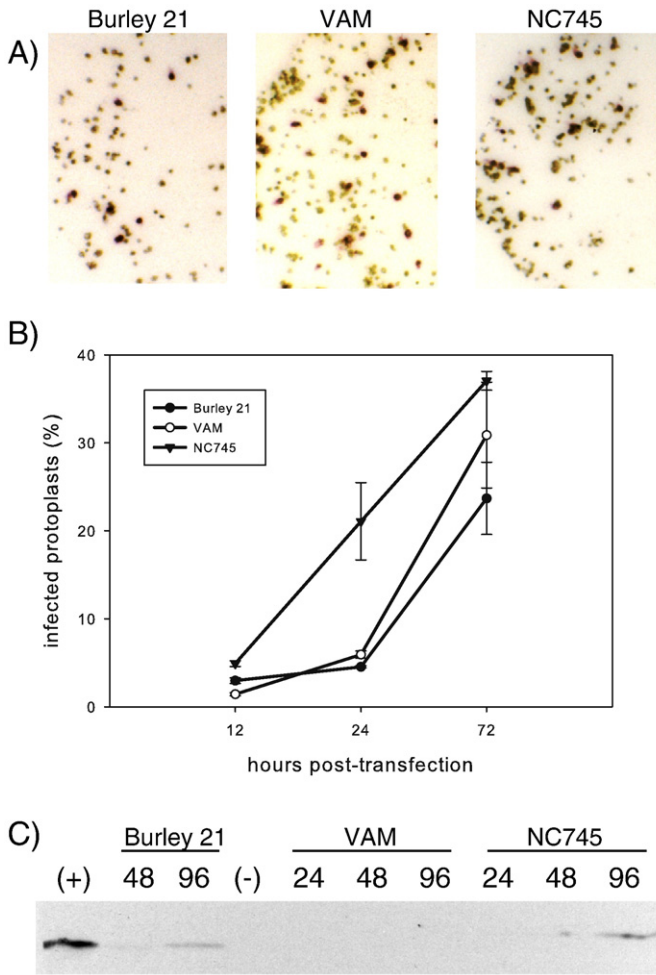
Fig. 1. PVY$_{NN}$-accumulation in protoplasts of resistant VAM and NC745 tobaccos and susceptible control Burley 21 transfected with viral RNA (6×10^4 cells μg^{-1} RNA). (A) Protoplasts expressing the CP (purple cells) at 24 h post-transfection (hpt) detected by protoplast printing. (B) Kinetics of the expression of virus infection in transfected cells (experiment concluded at 72 hpt. Bars = SE), and (C) Detection of viral CP by Western blotting. Soluble proteins were extracted at different hpt from 1.5–2.0×10^5 cells per sample, and the extracts were loaded in a discontinuous SDS-polyacrylamide gel in amounts proportional to the percentage of infected cells.

phase). After that, the number of Burley 21 infected cells increased gradually (spreading phase), with at least one positive cell per leaf-disk at 24 hpi.

In both resistant tobaccos, The LPmE data clearly showed that the overall rate of PVY cell-to-cell movement was significantly slower than in the susceptible Burley tobacco (Fig. 2). In the resistant NC745, the number of initially infected sites, the number of infected cells per infected site, and timing of the launching phase were surprisingly similar to those in Burley 21. In contrast, and consistent with the reduced rate of cellular virus accumulation observed earlier, unambiguously positive VAM cells were evidenced only after a prolonged period of latent virus infection (around 5 dpi). The masked phase occurred later and lasted longer (12–14 h) in NC745 than in Burley 21 and was unclear in VAM cells. During the spreading phase, the proportion of infected leaf disks per leaf-half was around 0.2 for both resistant tobaccos and remained unchanged during the observation period (up to 15 dpi in tobacco VAM). Interestingly, in both resistant tobaccos, the number of infected cells per infected disk steadily increased and eventually reached similar maximum proportions of positive cells, though it took 6 to 9 more days in VAM than in NC745. Taking into consideration the reduced rate of virus accumulation and, consequently, the five or more days of delayed detection of positive

cells in VAM tobacco, these observations suggest that PVY$_{NN}$ moved from cell to cell at a comparable rate in both resistant genotypes.

PVY$_{NN}$ can travel through the vasculature of resistant tobaccos

Although PVY$_{NN}$ exhibited a slow spread in the inoculated leaves of resistant tobaccos, it could eventually reach the vascular system and possibly be transported through the sieve elements. To test this hypothesis, a receptor tobacco VAM shoot was grafted into Burley 21 plants, and then PVY$_{NN}$ was mechanically inoculated in a newly developed VAM leaf. The absence of symptoms in the susceptible Burley 21 grafted plant indicate that the virus infecting VAM leaf tissue was unable to move into the VAM vasculature to reach the susceptible Burley 21 plants (Fig. 3). The graft union did not appreciably affect the systemic PVY$_{NN}$-translocation in Burley 21 plants grafted with Burley 21 scions used as controls. In another treatment the reciprocal scion-grafted plant combination was tested, and only the inoculated Burley 21 scion developed severe symptoms. The VAM rootstocks remained asymptomatic, and no virus could be detected by Northern dot blot or by back-inoculations. A third treatment consisted of the grafting of two Burley 21 scions to the same VAM plant. The scions were separated from each other by at least 10 cm (Fig. 3). PVY$_{NN}$ was inoculated in the lower Burley 21 scion, and the upper Burley 21 scion was used as an indicator for viral transport through the VAM vasculature. Typical PVY symptoms developed in the indicator Burley 21 scion as early as 9 dpi. The pathogenic properties of the recovered virus were the same as those of the parental PVY$_{NN}$ strain. Similar results were obtained with NC745-Burley 21 heterovarietal combinations (data not shown). These data indicated that the PVY$_{NN}$ progeny (the majority presumably generated in the inoculated Burley 21 scion) was able to easily travel through the vascular system of resistant tobaccos without irreversible changes in its genetic composition. However, the virus entry into and exit from the vasculature of resistant plants was blocked somewhere in the vascular boundaries.

Cellular virus accumulation is conditioned by a second recessive gene that segregates independently of va

Viral RNA transfected protoplasts and LPmE assays indicated a significantly higher level of virus accumulation in NC745 than in VAM tobacco. To determine the genetic factor(s) governing this differential rate of virus accumulation, and whether the same recessive locus conditions the resistance to PVY$_{NN}$ in both resistant tobaccos, crosses between tobacco varieties Burley 21, VAM, and NC745 were made.

The F$_1$ progeny of reciprocal crosses between VAM and NC745 was resistant to PVY$_{NN}$ as evidenced by the absence of viral symptoms within the first 13 dpi (data not shown). However, longer subliminal infections resulted in sporadic symptomatic RB-infections. In this regard, the hybrids behaved as the parental NC745 genotype. The occurrence of RB variants was consistently lower when VAM was the ovule parent than in its reciprocal (5% versus 13% in a first assay and 13% versus 43% in a second assay), suggesting the participation of an inherited cytoplasmic factor affecting the frequency of RB-infections.

F$_1$ through F$_3$ lines derived from Burley 21 × VAM reciprocal crosses were evaluated for susceptibility to PVY$_{NN}$, incidence of RB-infections, and virus titer or percentage of locally infected cells detected by LPmE. Thus, all the F$_1$ (Burley 21 × VAM) progenies were susceptible to PVY$_{NN}$ without any significant differences between reciprocal crosses. Interestingly, these hybrids developed a milder mosaic 1–2 days later than Burley 21. This attenuation of disease severity was accompanied by a statistically significant reduction in virus accumulation as measured by the amount of virions purified from systemically infected tissues at 18 and 45 dpi (data not shown). No RB isolates were ever recovered from the passage of PVY$_{NN}$ through any of these F$_1$ genotypes.
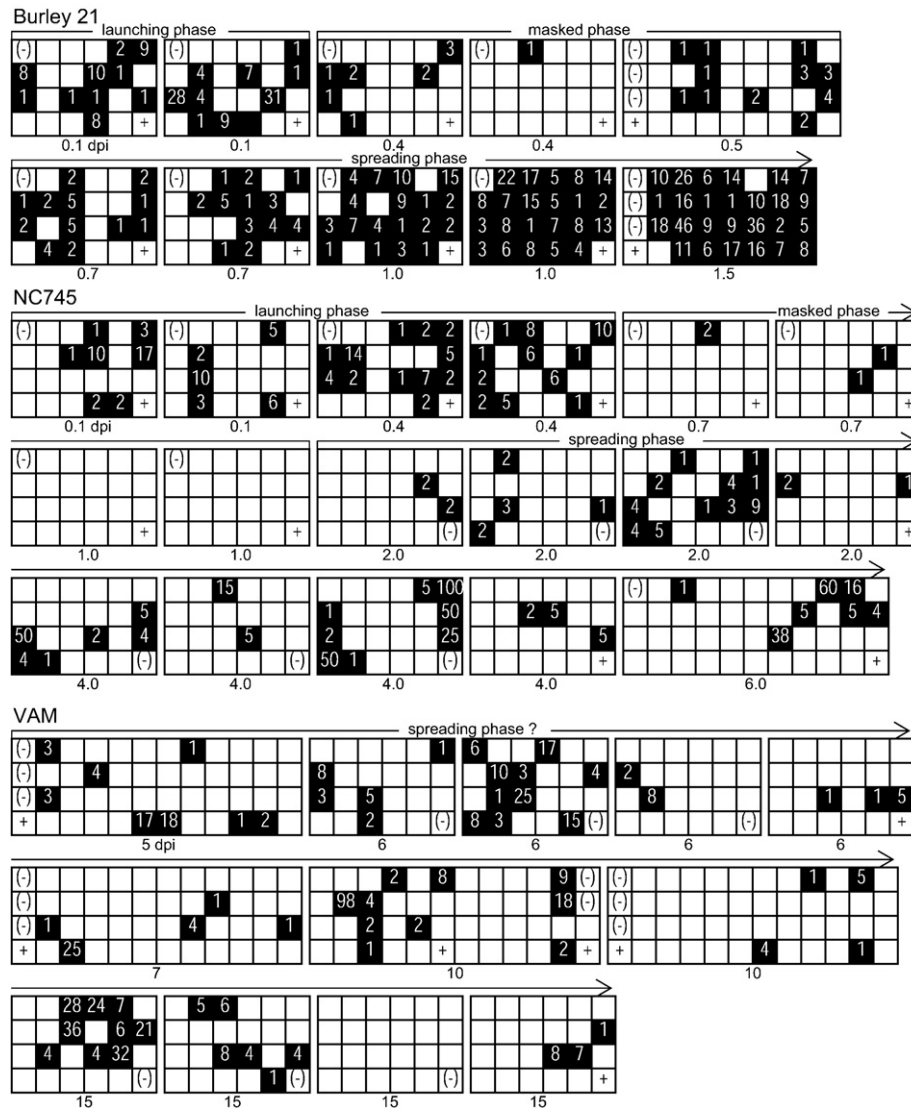
Fig. 2. Localized protoplast mini-extractions at different days post-inoculation (dpi) from leaf halves of tobacco Burley 21, NC745, and VAM mechanically inoculated with 50 µL of 0.2 mg/ml of PVY$_{NN}$ suspensions. Each medium size rectangle represents a leaf-half, while the small squares illustrate the sampled sites, each one consisting of a leaf core of 6 mm diameter. Positive reactions and number of infected cells are indicated in dark squares. Empty white squares represent sites with none positive cells out of around 500 printed cells per leaf-core. Positive (+) and negative (−) controls corresponded to blots of protoplast extracted from severely PVY-infected or mock-inoculated Burley 21 leaves, respectively.

Randomly selected F$_1$ (Burley 21×VAM) plants were backcrossed with the homozygous recessive parent VAM (testcross) or self-pollinated. After PVY$_{NN}$ inoculation, all the F$_2$ segregant populations exhibited susceptible-resistant (S:R) ratios of 1:1 for the test-crossed or 3:1 for the self-pollinated progenies when they were evaluated at 13 dpi. These results are expected when the resistance is controlled by a single recessive gene. Surprisingly, evaluations at later dates revealed that a fraction of the PVY$_{NN}$-resistant plants started to develop systemic symptoms caused by emerging RB variants. This phenomenon was observed in both reciprocal crosses. As a result, the S:R ratio observed under conditions that allowed almost full development of RB-infections (high inoculum concentration and/or long observation periods) was close to 15:1 in the F$_2$ (Burley 21×VAM) progenies (Table 2). This ratio indicated that two unlinked recessive genes were conditioning the stronger VAM-type resistance (i.e., the long resistance durability phenotype rather than just resistance to PVY$_{NN}$). Herein, the second recessive gene is referred to as va2 and the original recessive locus is renamed as va1.

To further understand the genetics of PVY resistance durability, NC745-type or VAM-type resistant F$_2$ (Burley 21×VAM) plants were

self-pollinated to produce F$_3$ families. These F$_3$ plants were mechanically inoculated with PVY$_{NN}$-virion suspensions, and the number of infected cells in the inoculated leaves was determined by LPmE at 5 dpi. Previously, we observed that, in resistant plants, the percentage of locally infected cells was proportional to the rate of cellular virus accumulation (data above). The incidence of RB-infections in these hybrids was also evaluated between 26 and 47 dpi. Thus, the percentage of locally infected cells varied among F$_3$ families ranging from 4 to 97% (Table 3). These values were directly proportional with the incidence of RB-infections observed later on (Pearson coefficient=0.8). The F$_3$-family BA3, derived from an NC745-type parent, displayed the greatest percentage of PVY$_{NN}$-infected cells and the highest incidence of RB-infections. In contrast, the F$_3$-family AB8, derived from a VAM-type parent, showed the fewest locally infected cells (statistically identical to tobacco VAM, $P≤0.05$) and one of the lowest incidence of RB-infections. Consequently, the suspected genotypes at the va2 locus for BA3 and AB8 were dominant-homozygous and recessive-homozygous, respectively (i.e., BA3= va1va1-Va2Va2 and AB8=va1va1-va2va2). The rest of the F$_3$ families most likely were heterozygous for the va2 locus. Hence, by keeping as
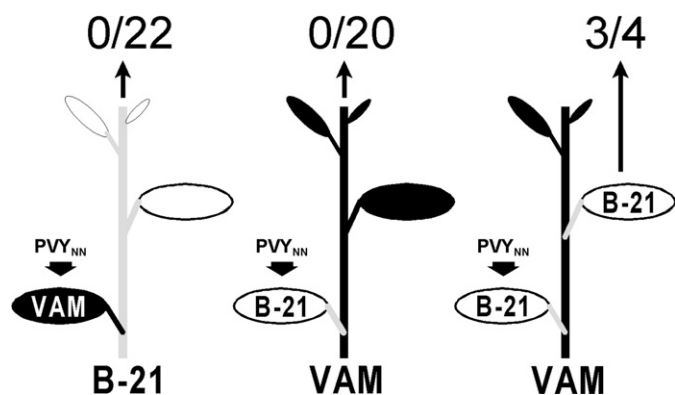
Fig. 3. Vascular translocation of the avirulent PVY$_{NN}$ strain in heterovarietal grafted plants of tobacco. The susceptible control Burley 21 (clear plant) or the resistant VAM (dark plant) were single or double grafted with young shoots of the opposite variety according with the scheme. Around 1 week later, 50 µL of partial purified virus suspension (0.1 mg ml^{-1}) was mechanically inoculated in the distal half of one leaf sprouted from the grafted scion. The results are recorded by the number of positive PVY-infected grafted plants over total plants inoculated on the grafted scion (single-graft treatments). In the double-graft treatment, the numbers indicate the same just that positive infection was detected only in the upper uninoculated scion.

a constant the *va1*-mediated resistance in all the evaluated F$_3$ families, the extreme differences among F$_3$ families in relation with the *va2*-mediated resistance suggest an independent segregation between these two loci.

Discussion

By taking in consideration the evidences presented in this study that support: a) lower resistance durability in NC745 than in VAM, b) restriction of virus accumulation only in VAM cells, c) similar impairment of cell-to-cell and vascular transports in both resistant tobaccos, d) a 15:1 ratio of S:R in F$_2$ (Burley 21×VAM) progenies, and e) genetic segregation of the described plant resistance components, we have proposed a model to explain the differential resistance durability to PVY. In this model, at least two specific resistance mechanisms governed by two independent recessive genes operate in

Table 2
Mendelian inheritance of the susceptible (S) and resistant (R) phenotype in F$_2$ (Burley 21×VAM) segregants under conditions that facilitated the expression of resistance-breaking (RB) variants after PVY$_{NN}$ inoculation

Assay[a]	Conditions given to facilitate RB-infections[b]	S : R[c]		
		Observed	Expected at 3:1	Expected at 15:1
1	Observation for 100 dpi	27:3	23:7	28:2
2	Observation for 83 dpi	38:3	31:10	38:3
3	Observation for 83 dpi	27:1	21:7	26:2
4	High inoculum concentration	33:3	27:9	34:2
	TOTALS	125:10	102:33	126:9
Statistical tests (*P* value):				
Pearson χ^2			0.0001	0.8119[*]
Fisher exact (two-tail)			0.0002	1.0000[*]

[a] Four different F$_2$ lines, each one derived from a different single pollinated plant, were used indistinctly in these assays.

[b] The conditions that facilitated the expression of RB-infections were an extended observation period or inoculation of the plants with a highly concentrated virion suspension (20 µg of virion/plant). No statistical differences were found among assays (*P*=0.82).

[c] The null hypothesis states that there are not significant differences between the observed and expected values. It was rejected only when the probability of making an error by this decision was ≤5% (*P*≤0.05). The tested models were inheritance of the VAM-type resistance by a single recessive gene (expected 3:1 ratio) or by two recessive genes (expected 15:1 ratio) using the total values.

[*] Only under this model, the null hypothesis was not rejected indicating that the observed and expected ratios were statistically identical with a confidence greater than 99%.

Table 3
Relation among the parent phenotype, the number of locally infected cells, and the frequency of resistance-breaking (RB) infections after PVY$_{NN}$-inoculation in resistant F$_3$ families

F$_3$ family[a]	Parent phenotype	Infected cells[d] (%±SE)	RB-infections[d] (%±SE)
BA2	VAM-type	4.4±0.4[b]	32.9±5.2[b]
AB8	VAM-type	1.6±0.2[a]	13.9±2.3[b]
BA3	NC745-type	97.3±0.9[d]	84.3±2.4[d]
AB7	NC745-type	4.1±0.6[b]	24.1±3.9[b]
–	VAM (control)	0.51±0.3[a]	0[a]
–	NC745 (control)	33.9±1.2[c]	39.1±3.2[c]

[a] F$_3$ families were produced by self-pollination of individual plants that did (NC745-type) or did not develop RB-infections (VAM-type).

[b] Number of infected cells was determined in three plants per family by protoplast printing. The protoplasts extracted from each plant were pooled together and four 10-µL blots (200–500 cells per blot) were counted.

[c] In three independent assays, 12 plants per F$_3$ family were mechanically inoculated and the incidence of RB-infections was recorded between 26 and 47 dpi. The identity of RB variants was tested by back-inoculation in resistant tobaccos.

[d] Tobacco genotypes with same letter within the same column were statistically identical (Tukey HSD Test, *P*≤0.05).

tobacco VAM. One of the mechanisms, which we propose being conditioned by *va1*, restricted the intercellular movement of the avirulent PVY$_{NN}$. The other mechanism reduced virus accumulation at the single cell level and was inherited by an unlinked recessive gene designated *va2*. When *va1* was the only operating resistance gene, as in the case of tobacco NC745 and some F$_3$-lines (*va1va1-_Va2* genotype), the plant was resistant to PVY$_{NN}$ but prone to developing spontaneous RB-infections.

Genetic interaction among two or more loci conferring plant resistance has been found in many plant systems (Kang et al., 2005 and references therein). The coordinated actions of multiple loci to enhance resistance are becoming better understood recently. For instance, Ruffel et al. (2006) found that two loci in pepper, *pvr2* and *pvr6*, which confer resistance to *Pepper veinal mottle virus* (PVMV) only when both are in a recessive-homozygous condition, encode two defective isoforms of translational eukaryotic initiation factors (eIF). They suggest that PVMV can interchangeably make use of any the functional molecules encoded by either *Pvr2* or *Pvr6* to overcome resistance. However, another plausible scenario, based on the work we are presenting, is that PVMV populations breaking each resistance gene individually might be in fact distinct RB variants that easily overcame distinct cellular restrictions.

Phenotypic expression of the resistance components

The observation that RB variants isolated from NC745 plants caused diverse syndromes suggests that the subliminally infecting PVY populations underwent rapid and divergent mutations in order to overcome the *va1*-mediated resistance. This is also suggested by a similar situation found in avirulent *Beet necrotic yellow vein virus* (BNYVV) populations subliminally infecting resistant sugarbeet cultivars (Acosta-Leal et al., 2008). In this incompatible interaction, the BNYVV populations were 2–3 times more diverse than resistance-breaking populations isolated from diseased plants. Therefore, cloning and sequencing PVY populations infecting susceptible and resistant tobaccos might reveal its molecular strategies to overcome resistance.

The protoplasts printing technique (Jung et al., 1992) proved to be useful in estimating the proportion of virus infected cells in both transfected protoplasts and mechanically inoculated leaf tissues. However, a normalized Western blot was required to detect the reduction of virus accumulation in viral RNA-transfected VAM cells (Fig. 1). These *in vitro* results were more accentuated in VAM cells infected *in situ* (Fig. 2) where a dramatic 5-day delay of PVY accumulation was observed. Therefore, the *va1*-mediated resistance might not be fully expressed in VAM cells transfected *in vitro*. A similar

behavior was previously reported in resistant cowpea cells transfected with avirulent *Cowpea chlorotic mottle virus* (Wyatt and Wilkinson, 1984). Based on these observations, the LPmE approach might have provided a more realistic picture of the resistance mechanisms operating in both resistant tobaccos.

The six-to nine-day delay in detecting the spread of PVY$_{NN}$ in neighboring VAM cells by LPmE, as compared to those in NC745 (Fig. 2), could be interpreted as a slower intercellular virus movement in VAM. This may have resulted from a stronger restriction of the virus to move from cell to cell or as an indirect consequence of a lower rate of cellular virus accumulation. The second possibility is unlikely based on an observation that an RB variant was able to efficiently move cell-to-cell in tobacco VAM even though its expression in infected cells was barely detectable (Acosta-Leal, 1999). Also, the lack of correlation between virus accumulation and the rate of intercellular virus movement has been documented in other incompatible plant-virus interactions (Schaad et al., 1997; Wyatt and Wilkinson, 1984). A stronger restriction of the virus to move in VAM is plausible but not supported by the following observations. First, PVY$_{NN}$ in VAM cells infected *in situ* required around 5 days to be detectable, and second, both resistant tobaccos reached similar proportions of infected cells per sampled sites at the end of the LPmE assays. Therefore, a more likely explanation is that trace amounts of virus particles below our detection threshold were moving cell-to-cell in VAM at a similar rate as in NC745. However, the virus in the infected VAM cells accumulated at a slower rate due to the effect of *va2* and therefore reached comparable levels 6 to 9 days later than in NC745.

The functional gene product of *Va1*, *Va2*, or both loci, like most of the host factors interacting with VPg, might be a subunit of the eIFs (reviewed by Diaz-Pendon et al., 2004; Robaglia and Caranta, 2006). The VPg-eIF interaction is not highly specific because different potyviruses might recruit the same isoform of an eIF subunit (Nicaise et al., 2007; Sato et al., 2005). These host factors apparently are required by the virus for multiple purposes, but none of them has been clearly demonstrated. Generally, the interference of the VPg-eIF interaction in resistant plants restricts both cellular virus accumulation and intercellular virus movement (Candresse et al., 2002; Gao et al., 2004; Keller et al., 1998; Murphy et al., 1998; Nicaise et al., 2003; Ruffel et al., 2002). Consequently, this pleiotropic effect has complicated the identification of the key step where the virus infection cycle is interrupted. The dissection of these two resistant components in the present work offers a plant-virus system where specific functions could be assigned to specific host factors. However, considering that potyviral VPg has also been found to interact with a cysteine-rich plant protein that potentiates virus cell-to-cell movement (Dunoyer et al., 2004), the search for the *Va* and *Va2* encoded elements should not be limited to genes of the cellular translational machinery.

Inheritance of the tobacco VAM resistance components

The original evidence of a single recessive gene conditioning tobacco VAM resistance was probably based on the ratios of S:R progenies of crosses between VAM and a susceptible parent evaluated during the first couple of weeks after inoculation (Gupton and Burk, 1973; Koelle, 1961). However, when late systemic infections were included in the evaluations of F$_2$ progenies, a 15:1 ratio, typical of characters controlled by two recessive genes, was revealed. The possibility that these late systemic infections were caused by virus contamination is unlikely since mock-inoculated Burley 21 plants never developed virus symptoms during the extended observation periods. Hence, one way to interpret these results is as follows. The tobacco resistance to a specific strain of PVY, in this case PVY$_{NN}$, is conditioned by a single recessive gene; however, the resistance to the parental strain and many other possible variants generated during the course of a virus infection (i.e., resistance durability) is conditioned by two major recessive genes.

The resistant phenotype of F$_1$ (VAM×NC745) reciprocal crosses indicated that *va1* is localized in the same locus in both plant genotypes. However, the occurrence of late RB-infections in the progeny indicated that the NC745-type character was dominant over the VAM-type. Interestingly, the incidence of RB-infections in F$_1$ (VAM×NC745) plants was consistently lower when tobacco VAM was the ovule parent, suggesting the participation of a cytoplasmically inherited host factor. Most potyviruses require an isoform of host eIFs to complete their infection cycle (reviewed by Diaz-Pendon et al., 2004; Robaglia and Caranta, 2006). Recently, Nicolai et al. (2007) demonstrated that nuclear mRNA encoding eIF4E is sequestered by the chloroplast, perhaps as a mechanism to regulate translation. Consequently, a hypothesis that could explain the observed cytoplasmic effect is that VAM chloroplasts might deprive PVY of this host protein more efficiently than NC745 chloroplasts.

The strongest evidence for independent segregation of *va1* and *va2* was obtained from evaluations of F$_3$ lines that were resistant to PVY$_{NN}$ systemic infection (*va1* homozygous) but highly variable in local virus accumulation. The rationale of this test was that, by keeping the *va1*-mediated phenotype (i.e., the absence of systemic infections caused by avirulent PVT$_{NN}$ in all F$_3$ lines) constant, the differential expression of *va2* could reveal loci segregation. Indeed, the expression of *va2*-mediated resistance went from the lowest amount of subliminal local infection in AB8, which was translated into fewer RB-infections, to extremely high amount of locally infected cells in BA3 and, consequently, the highest incidence of RB-infections. Between them, some apparently heterozygous genotypes were expressed. By assuming that the two recessive genes model better explained the observed ratios of S:R plants, the most likely explanation of the contrasting *va2*-mediated phenotypes under invariable *va1*-mediated resistance is that these suspected loci exhibited independent segregation.

Additional support for the two recessive genes model was obtained by studying the behavior of the heterozygous F$_1$ (Burley 21×VAM) plants in a time series experiment. They were susceptible to PVY$_{NN}$ (*va1Va1*) but produced intermediate virus titer (suspected allelic dose effect in *va2Va2*). Also, we found by protoplast printing that an RB variant infecting tobacco VAM was able to overcome *va1* and consequently spread in the plant tissue, but its titer was too low to produce a strong signal in protoplast printing and induce discernable symptoms. Apparently, this RB variant was still sensitive to *Va2*-mediated resistance. The proposed model also predicts that plants with *Va1_-va2va2* genotype should be susceptible to PVY$_{NN}$ systemic infection and resistant to virus accumulation. In fact, some F$_2$ plants with attenuated systemic symptoms that were observed during the evaluations might have had this genotype. Unfortunately, these plants were not recorded separately and further evaluated.

The present study provides a strategy to design multigenic resistance against plant viruses through rational gene pyramiding. Also, this and other reports (Acosta-Leal and Rush, 2007; Andrade et al., 2007; Hebrard et al., 2006; Kuhn et al., 1981) demonstrate that RB variants are easily generated and/or selected in incompatible plant-virus interactions where viruses accumulate to some extent in the initially infected cells, but their intercellular transport is restricted. This may explain why multigenic recessive resistance against potyviruses has been particularly favored in nature.

Materials and methods

Virus and plant materials

A single lesion isolate of PVY$_{NN}$ and tobacco Burley 21, VAM, and NC745 seeds were provided by Dr. G.V. Gooding Jr. (North Carolina State University). Upon initial virus amplification in tobacco Burley 21, stocks of purified virion suspensions, viral RNA, or dry tissue were used as starting inocula for this work. A standard inoculum for

mechanical inoculation was prepared by macerating 1 part of PVY-infected leaf tissue with 9 parts (w/v) of 0.1 M phosphate buffer pH 7.2 containing 0.5% celite. The viral RNA inoculation buffer (pH 9.2) consisted of 50 mM glycine, 30 mM K_2HPO_4, 1% bentonite, and 1% celite (Xiong and Lommel, 1991). All of the plant genotypes generated during this research were derived from single-plant crosses. Cross pollination was performed by supplying emasculated flowers with pollen taken from a selected plant. These emasculated flowers were selected before they were self-pollinated and prepared by removing their immature anthers. For the production of self-pollinated progenies, immature flowers were covered with a paper bag until fruit maturation.

Virion and viral RNA purification

Virions were purified following a modified version of the procedure described by Lommel et al. (1982). In brief, infected leaf tissue, without its central vein, was blended in 0.2 M sodium acetate buffer (pH 5.0, 2.5 ml g^{-1} of tissue) containing 0.25% of 2-mercaptoethanol. The filtered extract was centrifuged at 10,000 g for 15 min. Virus particles were subsequently precipitated from the supernatant with 8% PEG-8000, 0.2 M NaCl and 0.5% Triton X-100 by stirring for 1 h at 4 °C. The pellet, collected after centrifugation at 10,000 g for 15 min, was resuspended in 1/10 (v/v) of resuspension buffer (0.1 M Tris pH 6.5, 32 mM sodium citrate, and 0.5% Triton X-100), and centrifuged through a 30% sucrose cushion at 100,000 g for 2 h. The final virus pellet was gently resuspended in 0.5 ml of the resuspension buffer per 100 g of initial leaf tissue. Virions were destabilized by proteinase K-SDS treatment at 60 °C for 2 min, and viral RNA was recovered by phenol-chloroform extractions as previously described (Carrington and Morris, 1984).

PEG-mediated protoplast transfection

Protoplasts were extracted from leaves of *in vitro*-cultured tobacco plantlets. The extraction procedure was a combination of the methods described by Galbraith (1990), and Shillito and Saul (1988). Viral RNA transfection proceeded only when the concentration of protoplasts was at least 2×10^5 rounded cells/ml and when at least 80% of them were viable after the enzymatic digestion. The protoplasts were collected by centrifugation at 60 g for 5 min and resuspended in the appropriate volume of transfection buffer (0.5 M mannitol, 15 mM $MgCl_2$ $6H_2O$, and 10% MES, pH 5.7) to obtain a concentration of 1.5–2.0×10^6 cells/ml. Protoplast aliquots (300 µL) were mixed with 10 µL of viral RNA (1–1.5 µg $µL^{-1}$), and immediately treated with an equal volume of 40% PEG-4000 (pH 8.0) as described by Negrutiu et al. (1987). Osmotically stressed cells were gradually diluted with 10 ml of W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, and 5 mM D-glucose, pH 5.8) to complete the transfection process. After sedimentation at room temperature for 10 min, the protoplasts were collected by centrifugation at 60 g for 5 min at 15 °C, and the pellet was resuspended in 3 ml of Gamborg's culture media (Gamborg et al., 1968). These cultures were maintained in the dark at 26 °C until harvested.

Localized protoplast mini-extraction and protoplast printing

Virus- and mock-inoculated tobacco leaves were rinsed with tap water, immersed in 70% ethanol for 30 s, and rinsed again with water. Then, the lower leaf surface was gently abraded with celite to remove most of the cuticle and epidermis. The sampled sites were leaf disks of 6 mm diameter punched out with a cork borer. They were evenly distributed on the surface of the treated leaf (spaced 1–1.5 cm) following a grid pattern. Each spatially referenced disk was floated on 200 µL of simplified enzymatic media (0.6% Cellulase R10, 0.2% Macerozyme R10, 0.5 M mannitol, 1 mM KCl, 1 mM $CaCl_2$ $2H_2O$, and

5 mM MES pH 5.8) contained in each well of a microtitre plate and incubated in a dark humid chamber at 26°C for 16–18 h. An aliquot of 100 µL of W5 solution per well was added. A 5 µL sample of the gently resuspended protoplasts in each well was blotted onto a piece of nitrocellulose membrane. This membrane was further processed for capsid protein immunodetection following the protoplast printing technique (Jung et al., 1992).

Virus detection by Northern and Western blotting

A single stranded cDNA probe, labeled with 10% Dig-11-dUTP, was synthesized by reverse transcription of PVY RNA (10 µg) primed with 50 ng/µL of oligo $(dT)_{12-18}$. Total nucleic acids were extracted from 0.1 g of leaf tissue samples according to a procedure described by Crespi et al. (1991), but including a phenol-chloroform (1:1) extraction step after partial protein precipitation with 5 M potassium acetate. Total nucleic acid extractions were diluted 1:10 and denatured before being blotted by vacuum infiltration to a positively charged nylon membrane. Chemiluminescent detection of UV-crosslinked viral RNA was carried out basically as described in the Genius System User's Guide (Boehringer Mannheim). Prehybridization and hybridization with denatured Dig-cDNA probe (1 µL ml^{-1}) were performed at 65 °C for 2 h and 42 °C for 12–16 h, respectively.

Tobacco cells, collected at different times after transfection (3 ml cell culture), were washed with W5 solution, and the integral cells were collected from the interface after centrifugation in a 25% sucrose cushion. These cells were resuspended in 2 ml of W5 to estimate their concentration. Then, the cells were precipitated by centrifugation and the pellets were stored at −80 °C. For capsid protein detections, the pellets were thawed and resuspended in 100 µL of protein extraction buffer (25 mM Tris, pH 7.4, 137 mM NaCl, 3 mM KCl, 0.1% SDS, and 0.1% 2-mercaptoethanol). The cells were disrupted by 2 cycles of freezing, thawing, and shaking before the separation of the soluble proteins from the cellular debris by centrifugation. Ten microliters of the supernatant were mixed with an equal volume of 2×Laemmli loading buffer and boiled before fractionation by denaturing SDS-PAGE as described by Laemmli (1970).

After electrophoresis, the fractionated PVY capsid protein was electroblotted onto a nitrocellulose membrane following the method of Salinovich and Montelaro (1986) and subjected to immuno-chemiluminescent detection (Amersham, ECL System) as follows: the membrane was incubated in the blocking solution (25 mM Tris Cl, pH 7.4, 137 mM NaCl, 3 mM KCl, 0.5 Tween-20, and 5% skim milk) for 1 h, and then one more hour in the same solution supplemented with anti-PVY antibody (2 mg/ml of γIgG, Agdia) at a concentration of 0.4 µg/ml. The chemiluminescent reaction was catalyzed by an anti-rabbit IgG peroxidase conjugate diluted with the blocking solution at 1/40,000.

Heterovarietal plant grafting

Immature tobacco plants of at least 10 in. tall were grafted on their stem with a leaf shoot taken from a different plant by the following procedure. A downward cut was done in the stem cortex of the stock plant starting from a leaf node. Then, a wedged shoot was inserted into the wound. Dehydration of the grafted scions was prevented by sealing the cuts with parafilm and covering the entire plant with a polypropylene bag that was gradually removed a few days later.

The grafted scions were apical or lateral leaf shoots induced in plants where the apical dominance was mechanically removed. These shoots consisted of the stem, its apical meristems, and few undeveloped leaves. Later, a partially expanded distal leaf of the grafted scion was used for virus inoculation. This scion was defined as the virus receptor. A partial purified virus suspension (0.1 mg ml^{-1}) was used to inoculate the scion approximately 1 week later. By the time of inoculation, the reestablishment of vascular connections was

evidenced by the turgidity and growth of the grafted tissue. In some treatments, an upper non-inoculated grafted scion was used as indicator of systemic virus translocation. Grafted plants and scions were allowed to develop new tissue. In this way, different rootstock-scion combinations of susceptible Burley 21 and resistant tobaccos were created, including controls grafted with the same variety.

Evaluation of F3 families

To determine the number of cells infected in the inoculated leaves, three plants from each F_3 line derived from Burley 21 × VAM and PVY_{NN}-resistant controls, NC745 and VAM, were mechanically inoculated with 2 mg ml^{-1} of partial purified virus suspension on the most basal two true leaves (100 μL/leaf-half). The inoculated leaves were then processed together for protoplast printing at 5 days post-inoculation. Protoplast suspensions were diluted to $1.5–2.0 × 10^6$ cells/ml, and four 10 μL dots were blotted onto a nitrocellulose membrane. To determine the percentage of RB-infected plants, each genotype was PVY_{NN}-inoculated, and the incidence of systemic infections was recorded between 26 and 47 dpi in three independent assays. The recovered virus was evaluated for its ability to overcome resistance by back-inoculation to tobacco VAM. Tukey HSD test from the SYSTAT software (Berk, 1994) was used to evaluate the significance in the percentage of infected cells and the frequency of emerging RB variants among tobacco genotypes. Pearson correlation analysis was used to evaluate correlation between the percentage of infected cells and the frequency of RB-infections.

Acknowledgments

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