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RNA silencing-mediated resistance is related to biotic / abiotic stresses and cellular *RdRp* expression in transgenic tobacco plants

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The discovery of RNA silencing inhibition by virus encoded suppressors or low temperature leads to concerns about the stability of transgenic resistance. RNA-dependent RNA polymerase (RdRp) has been previously characterized to be essential for transgene-mediated RNA silencing. Here we showed that low temperature led to the inhibition of RNA silencing, the loss of viral resistance and the reduced expression of host RdRp homolog (*NtRdRP1*) in transgenic T4 progeny with untranslatable *potato virus Y* coat protein (*PVY-CP*) gene. Moreover, RNA silencing and the associated resistance were differently inhibited by *potato virus X* (PVX) and *tobacco mosaic virus* (TMV) infections. The increased expression of *NtRdRP1* in both PVX and TMV infected plants indicated its general role in response to viral pathogens. Collectively, we propose that biotic and abiotic stress factors affect RNA silencing-mediated resistance in transgenic tobacco plants and that their effects target different steps of RNA silencing. [BMB reports 2008; 41(5): 376-381]

INTRODUCTION

RNA silencing provides plants with a high level of resistance by specific targeting to the cognate viral RNA. However, the discovery of RNA silencing inhibition by virus encoded suppressors or low temperature environment leads to concerns about the stability of transgenic resistance. Silencing may be partially (e.g., CMV), or even totally overcome (e.g., TVCV and TuMV) by viral encoded factors (1). Furthermore, RNA silencing-mediated viral resistance in transgenic plants can also be abrogated by abiotic factor as evidenced by the inhibition of siRNA accumulation upon cold stimulation (2, 3). These observations challenge the stability and durability of the engineered RNA silencing-mediated resistance in transgenic crops which are inevitably subjected to various stress conditions in fields.

Plant RdRps are components of the RNA silencing pathway and are implied in plant antiviral activities (4). Without *NtRdRP1*, plants are more susceptible to TMV infection than their wild-type counterparts (5). In transgenic plants lacking inducible *NtRdRP1*, PVX mutants rescue their ability to spread locally and systemically. Similarly, the RdRp-like SGS2 is required for RNA silencing and mutants of *Arabidopsis* exhibit enhanced susceptibility to CMV but not to TuMV or TVCV infection (1). In addition, SDE1, the putative RdRp in *Arabidopsis*, has been found to be indispensable for maintaining the PTGS of transgenes (6). These results raise the question that whether RdRps have a general role in plant antiviral response.

Previously we have reported the construction of highly resistant transgenic tobacco plants carrying an untranslatable *PVY-CP* gene, and that this resistance is mediated by transgene-induced RNA silencing (7, 8). In this study, we used the T4 transgenic progeny to investigate the role of host RdRp in transgene-mediated RNA silencing, as well as to test the stability and durability of the RNA silencing-mediated resistance in transgenic lines under biotic and abiotic stresses. The results may enhance our understanding of the stability of the engineered resistance in transgenic crops in fields under various biotic and abiotic stress environments.

RESULTS

RNA silencing was related to *NtRdRP1* gene expression in T4 progeny

We have determined that the high resistance to PVY in T4 progeny of M11 was mediated by transgene induced RNA silencing (Supplement Fig. 1). Northern hybridization was performed to assess the relationship between *NtRdRP1* expression and transgene-mediated RNA silencing. As expected, it was evident that much higher level of *NtRdRP1* mRNA was accu-

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mulated in silence-occurring M11 plants, while relatively lower level was associated with silence-lacking M53 plants. There was no obvious difference of *NtRdRP1* gene expression between M53 and WT (Supplement Fig. 1). Since M11 differed from M53 only in the number of transgene copies, we concluded that RNA silencing in M11 was highly related to the *NtRdRP1* gene expression in T4 transgenic plants.

Low temperature inhibited RNA silencing and abrogated resistance in transgenic plants

To test the effect of temperature on transgene silencing-mediated resistance to PVY, transgenic T4 progeny of line M11 were inoculated with PVY and grown at 10, 15, 25, and 30°C respectively. Line M53 and WT plants were used as controls. Mock inoculation was performed with phosphate buffer. As expected, typical PVY symptom was evident as early as 7dpi and was clearly visible at 14dpi for all inoculated plants grown at 15°C, including line M11 plants. However, those grown at 10°C, including M11 plants did not appear any PVY symptom until 12dpi. The delay of the symptom appearance might be attributed to the inhibition of viral replication or / and cellular metabolism due to the low temperature. Severe PVY symptom developed in M11 plants at 10 and 15°C, like M53 and WT (Supplement Fig. 2B). In contrast, M11 plants at higher temperatures (25 and 30°C) exhibited no observable symptom. Interestingly, symptoms in M53 and WT plants were significantly attenuated at 30°C, a phenomenon known as 'heat masking' (3). At 28 dpi, virus infection was assessed by ELISA. As shown in Fig. 1A, symptom severity correlated well with PVY titers in plants (3, 9). Based on these results, we concluded that the gene silencing-mediated resistance to PVY was temperature-dependent, with the antiviral activity being promoted at high temperature and being inhibited at low temperature.

Moreover, we verified that the effect of transgene-mediated RNA silencing was also regulated by temperature. As shown in Fig. 1B, abundant transgenic *PVY-CP* mRNA was accumulated at 10 and 15°C (lane 1 and 2) in M11 plants, while its expression reduced to a non-detectable level at 25 and 30°C (lane 3 and 4). In contrast, although declined slightly at 30°C, relatively higher concentration of *PVY-CP* mRNA was maintained (lane 5 to 8) in the RNA silencing-absent M53 plants. The reduced RNA silencing at low temperature would be responsible for the frequent outbreaks of plant virus diseases in the unusual cold seasons (10).

To test whether *NtRdRP1* was involved in the temperature-dependent transgene silencing, the same RNA samples were subjected to rehybridization with an *NtRdRP1* specific probe. As shown in Fig. 1B, *NtRdRP1* expression correlated well with temperature alterations. Little *NtRdRP1* mRNA was detected at 10 and 15°C, and the expression increased significantly at 25 and 30°C. This revealed that the expression of *NtRdRP1* gene was temperature-dependent. Specifically, its expression was suppressed at low temperature and was facilitated with a rise in the temperature.

RNA silencing was differently suppressed by PVX and TMV infection, both resulting in the inhibition of resistance in transgenic plants

The stability of RNA silencing-based resistance in transgenic plants is frequently challenged by the suppressor factors expressed by plant viruses (11). To investigate the stability of the transgene-mediated silencing and its consequent viral resistance, two unrelated viruses of PVX (potexvirus) and TMV (tobamovirus) were chosen to challenge M11 plants. Both viruses were reported to be capable of suppressing RNA silencing (12). To assess the effect of PVX on silence suppression, M11 plants were inoculated with PVX, PVY and PVX followed by PVY, which were designated as X, Y and X + Y respectively. The same in-

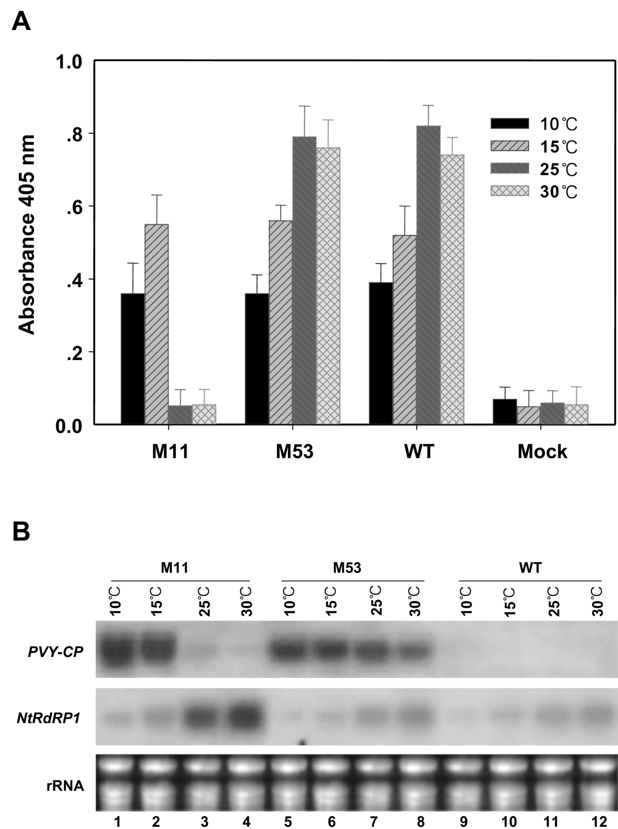

Fig. 1. Effect of temperature on *NtRdRP1* expression, transgene-mediated RNA silencing, and resistance in T4 transgenic plants. (A) ELISA to assess PVY accumulation in leaves of highly resistant line M11, susceptible line M53, wild type (WT) plants, and WT plants inoculated with phosphate buffer (Mock) at 28 dpi. Mean values from 10 plants were used here. (B) Total RNA extracted from the 28-day-old leaves of T4 progeny of M11, M53 or WT plants grown at 10, 15, 25, and 30°C respectively was subjected to Northern blot hybridization. Ethidium bromide-stained rRNA was shown as loading control (bottom). Representative results were presented from three independent experiments.

oculated M53 and WT plants worked as controls. Similar protocol was adopted for TMV, with the corresponding designation as T, Y, and T + Y.

All the X and X + Y plants were infected with PVX as early as 10 dpi with typical PVX symptom (confirmed by ELISA, data not shown). The severity of PVX symptom varied distinctly between different treatments at 28 dpi. The double-inoculated controls, and the X + Y_{10}N inoculated M11 plants displayed severe mosaic, leaf malformation as well as stunt (Supplement Fig. 2C), which was due to the synergism between the two viruses (13). Only in the case of X + Y_{10}N, would PVY establish a stable infection in M11 plants. Here the symptom was milder and was developed much later (delayed 6 to 7 d) compared with M53 and WT plants (Fig. 2B). Other double-inoculated M11 plants only showed PVX symptom. The symptom severity correlated well with the viral titers detected by ELISA (Fig. 2A, B). These results indicated that PVX infection was capable of inhibiting RNA silencing-mediated resistance in nascent leaves, but not in old leaves.

For TMV, dark green mosaic became evident in all T and T + Y plants at 7 dpi, and the infections were also confirmed by ELISA (data not shown). At 28 dpi, all T + Y control plants exhibited both TMV and PVY symptoms, and were further confirmed by ELISA (Fig. 2C). In the double-inoculated M11 plants, only the T + Y_0 showed typical TMV symptom. Although less severe than the controls, the others displayed symptoms of both TMV and PVY (Supplement Fig. 2D). The results of ELISA were consistent with the viral symptoms (Fig. 2C, D). Furthermore, it was noted that, for T + Y_5, T + Y_{10}O and T + Y_{10}N, although all M11 plants were infected with PVY, viral titer in T + Y_5 was the lowest. This was in contrast with M53 and WT plants, where the T + Y_5 resulted in the highest PVY titers (Fig. 2D). Accordingly, TMV was able to inhibit RNA silencing-based resistance in both old and nascent leaves.

We further proved that inhibition of resistance was caused by the suppression of RNA silencing in PVX and TMV infected

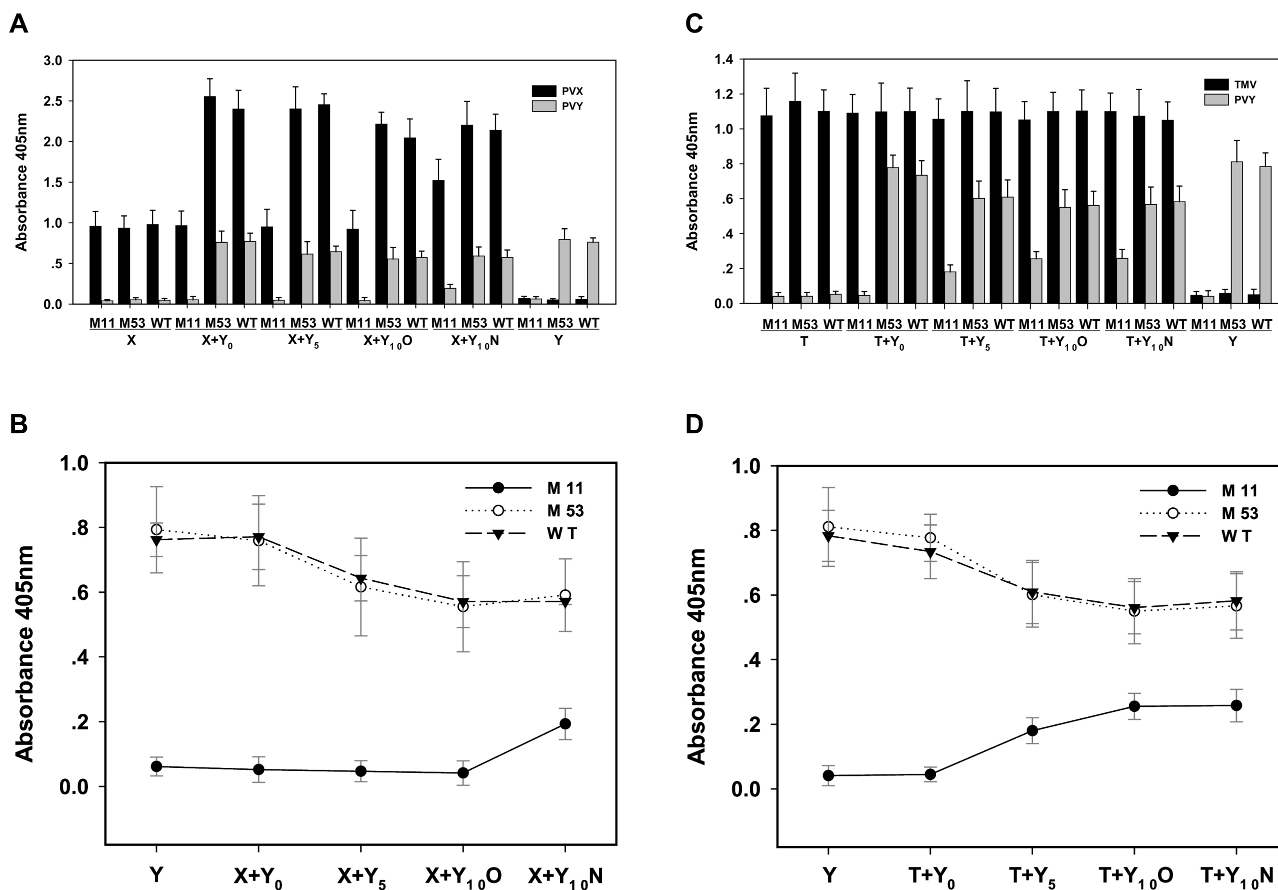

Fig. 2. Suppression effect of PVX and TMV infection on RNA silencing-mediated resistance in T4 progeny. (A) and (B) ELISA of PVX and PVY accumulation in topmost systemic leaves from highly resistant line M11, susceptible line M53 and wild type (WT) plants 28 dpi with PVX, and 28 dpi with PVY. (C) and (D) ELISA of TMV and PVY accumulation in topmost systemic leaves from M11, M53 and WT plants 28 dpi with TMV, and 28 dpi with PVY. The mean values from 10 plants were used.

plants. M11, M53 and WT plants were inoculated with either PVX or TMV, respectively, and were grown at 25°C. Total RNA derived from both the old and nascent leaves at 28 dpi was subjected to Northern analysis with *PVY-CP* probe. As shown in Fig. 3, *PVY-CP* transgene accumulation was differently regulated in old and nascent leaves post PVX infection. In old leaves, PVX affected little on *PVY-CP* mRNA accumulation in M11 plants (Fig. 3A, top, compare lane 4 with lane 1 and 7). Whereas in new leaves, although not strikingly, the *PVY-CP* level increased compared to non- and mock inoculated individuals (Fig. 3B, top, compare lane 4 with lane 1 and 7). There was no significant difference in *PVY-CP* mRNA accumulations in old and new leaves in M53 plants (Fig. 3A, B, compare lane 5 with lane 2 and 8). These results suggested that PVX infection of M11 plants alleviated RNA silencing in nascent leaves, but not in old leaves. This was consistent with the fact that the RNA silencing-mediated resistance was absent in nascent leaves but was maintained in old leaves (Fig. 2A, B).

However, in TMV infected M11 plants, *PVY-CP* mRNA elevated remarkably compared with non- and mock inoculated plants in both old and nascent leaves (Fig. 3C, D, compare lane 4 with lane 1 and 7). There was no difference in old and new leaves in M53 plants for all treatments (Fig. 3C, D, compare lane 5 with lane 2 and 8). These results demonstrated that RNA silencing in both old and new leaves was suppressed by TMV infection. This inactive status of RNA silencing coincided with the resistance attenuation in T + Y_5 and T + Y_{10} M11 plants (Fig. 2C, D). Collectively, RNA silencing was differently suppressed by PVX and TMV infections, although both resulted in the abrogation of resistance in transgenic plants.

To find out whether *NtRdRP1* was suppressed in the plants where the silencing was inhibited by PVX and TMV infections, we conducted Northern blot analysis on the same RNA samples with an *NtRdRP1* probe. Consistent with previous studies (5), our results showed that *NtRdRP1* expression was elevated in both the old and nascent leaves post both infections (Fig. 3A-D, middle, compare lane 4-6 with the rest lanes). Moreover, *NtRdRP1* mRNAs accumulated to a relative higher level in differently treated M11 plants than that in M53 and WT plants correspondingly (Fig. 3A-D, middle, compare lane 1, 4 and 7 with the rest lanes). The similar pattern induced by the two unrelated viruses implied that this response to viruses would be a general feature of *NtRdRP1*.

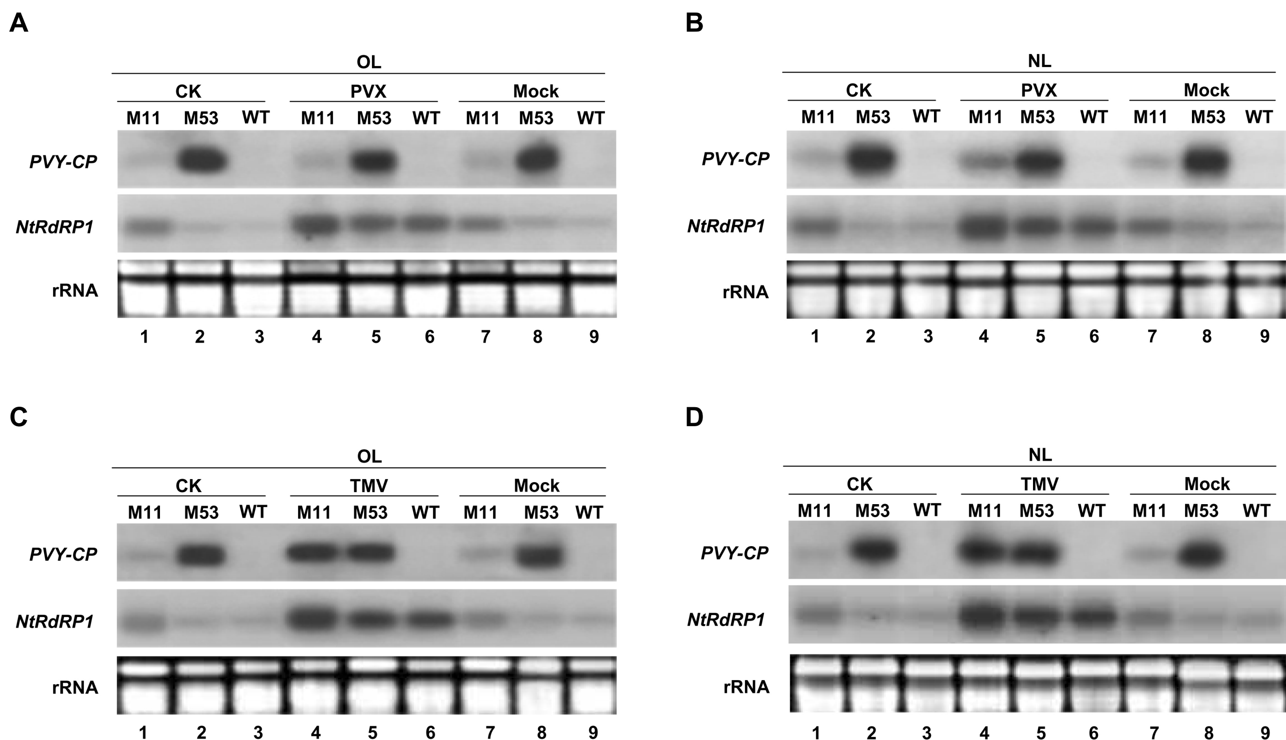

Fig. 3. Effect of PVX and TMV infections on RNA silencing and *NtRdRP1* expression in T4 progeny. (A) and (B) Northern blot analysis of PVX infected highly resistant line M11, susceptible line M53 and wild type (WT) plants in either old leaves (OL) already present before PVX inoculation (A) or nascent leaves (NL) emerged post PVX inoculation (B). (C) and (D) Northern blot analysis of TMV infected M11, M53 and WT plants in either OL (C) or NL (D). Non-inoculated healthy M11, M53 and WT plants were used as control (CK). Ethidium bromide-stained rRNA was shown as loading control (bottom). Representative results were presented from three independent experiments.

DISCUSSION

Our results demonstrate that at low temperature, RNA silencing in T4 progeny is inhibited, the associated resistance is lost, whereas the expression of *NtRdRP1* is reduced. Moreover, RNA silencing and the associated resistance are differently regulated by PVX and TMV infections. PVX only inhibits RNA silencing and viral resistance in nascent leaves in transgenic plants, and does not interfere with that in old leaves; whereas the TMV suppresses the RNA silencing and viral resistance in both the old and the nascent leaves. In addition, the increased expression of *NtRdRP1* in both PVX and TMV infected plants indicates a general role of *NtRdRP1* in response to viral pathogens.

Recent reports have indicated that host RdRps are important in plant antiviral defense (1, 5, 14). In our experiment, we confirm that the increased expression of *NtRdRP1* is related to RNA silencing in transgenic plants. Transgenic plants generally show accumulations of aberrant transgenic RNA, which is recognized by host RdRps as templates to synthesis antisense RNA and dsRNA for sequence-specific RNA degradation (15). The transgene silencing is complicated by many cooperating mechanisms, e.g., DICER activity is compromised at low temperature to inhibit transgene silencing-mediated defense by down-regulation of siRNA (3). Our finding of temperature-dependent expression of *NtRdRP1* provides an alterative explanation for the inhibition of the defense at low temperature. Since host RdRps synthesize dsRNA to generate siRNA (15, 16), RdRp suppression at low temperature will inevitably lead to a decline in dsRNA synthesis, and finally the inhibition of the transgene-mediated defense.

Suppression of RNA silencing is widely used as a counter strategy (17) through the specific suppressor proteins encoded by most plant viruses (18, 19). In this work, we proved that RNA silencing and the associated resistance were suppressed by two distinct viruses. We showed that PVX infection only slightly alleviated the silencing in nascent leaves (Fig. 6B). Comparing with the strong suppressors from PVY, CMV, and many other viruses (17, 20), suppression effect of PVX is negligible (12, 21).

Previous reports have revealed that TMV possesses the ability of suppressing GFP transgene silencing (17, 22). Our results reveal that TMV infection suppresses the RNA silencing and the subsequent resistance in both old and nascent leaves. Overall, the varied effects of PVX and TMV on silence suppression provide further support to the idea that various viral encoded suppressors may target different steps of RNA silencing (17).

It seems conflicting that *NtRdRP1* expression is down-regulated at low temperatures, and is up-regulated upon viral infections, yet both lead to the same outcome of suppression of silencing and resistance. This may reflect that the suppression activity of cold stimulation and viral infections may target different steps of RNA silencing. The former may be upstream of the *NtRdRP1* activity, while the later one downstream.

In conclusion, we propose that biotic (viral infections here) and abiotic (cold stimulation here) stress factors affect RNA silencing-mediated resistance in transgenic tobacco plants and that their effects target different steps of RNA silencing. The results may enhance our understanding of the stability of the engineered resistance in transgenic crops in fields under various biotic and abiotic stress environments.

MATERIALS AND METHODS

Plants and treatments

The T4 progeny of highly resistant M11 and susceptible M53 of *N. tabacum* cv. NC89 plants were germinated and grown in greenhouse under physical containment, and the transgene was confirmed by PCR. The 4-leaf-stage plants mentioned above were treated differently (10, 15, 25 and 30°C) in plant growth chambers with a light-dark cycle of 14 h / 10 h.

Virus inoculation and detection

For mechanical inoculation, 1 g of leaves from PVY, PVX or TMV infected *N. tabacum* plants were ground in 0.1 M phosphate buffer (pH 7.0). The sap was rubbed onto carborundum-dusted systemic leaves of the 4-leaf-stage plants. In the double inoculations, PVY was inoculated at the same day, 5 days, or 10 days post PVX / TMV inoculation (designed as $X + Y_0 / T + Y_0$, $X + Y_5 / T + Y_5$ and $X + Y_{10} / T + Y_{10}$ respectively; for details see supplement Fig. 3). Viral replication post inoculation was confirmed by double antibody sandwich ELISA (23).

Northern blot analysis

Total RNA was extracted from leaves with RNeasy Plant Kit (Qiagen) according to the manufacturer's instructions. For each sample, 20 μg total RNA was separated on 1% formaldehyde agarose gel and then transferred onto Hybond-N^+ nylon membrane for hybridization with the *PVY-CP* (24) or *NtRdRP1* probe. The *NtRdRP1* probe was 995 bp in size and was obtained through RT-PCR based on *NtRdRP1* sequence (accession number AJ011576; Fd : 5′-TTGCACCACATGGTTG ATTG-3′; Rev : 5′-CAGTTGACTCCCGAGTGAAGA-3′). Prehybridization was carried out at 65°C for 24 h with 6 × SSC, 5 × Denhardt's buffer, 0.5% SDS and 0.1mg / ml ssDNA. Hybridization was performed with [α-^{32}P]-labeled probes for 48 h in the same conditions as prehybridization. After washing twice, membranes were exposed to X-ray films for 5 d at -80°C with two intensifying screens. Each analysis was repeated at least three times with independent samples and the representative results were presented below.

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