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Tobacco ringspot virus persists in the shoot apical meristem but not in the root apical meristem of infected tobacco

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Abstract We analysed the kinetics of virus distribution in *Tobacco ringspot virus* (TRSV)-infected tobacco. Infected plants developed asymptomatic leaves above the symptomatic leaves that exhibited ringspots. We detected virus in the shoot apical meristem (SAM). Time-course immunohistochemical microscopy showed that TRSV continuously distributed in the centres of the SAM and in leaf primordia. In contrast, TRSV infection was transient in the root meristem. In western blot and RT-PCR analyses, TRSV infection occurred only within and around the ringspot tissues of the symptomatic leaf; however, TRSV was not or hardly detected in the asymptomatic leaves of inoculated tobacco plants. These results indicate that TRSV persists in the SAM, but that TRSV infection is transient in the root meristem and asymptomatic leaves.

Keywords Immunohistochemical microscopy · Recovery · Time-course analysis · Viral titre

Abbreviations

CP	coat protein
dpi	days post-inoculation
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
RAM	root apical meristem
RT-PCR	reverse transcriptase-polymerase chain reaction
SAM	shoot apical meristem
TRSV	*Tobacco ringspot virus*

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Plant shoot meristem tissue, including the shoot apical meristem (SAM), escapes infection by many plant viruses (Hull 2002). This absence of viruses in the shoot tissues is of practical importance because virus-free plant clones can be generated by culturing meristem tips. In contrast, some viruses have been observed in the SAM of infected plants, *e.g.*, *Pepper ringspot virus* (Kitajima and Costa 1969), *Potato virus X* (Appiano and Pennazio 1972), *Odontoglossum ringspot virus* (Toussaint et al. 1984), *Barley stripe mosaic virus* (Lin and Langenberg 1984), *Cherry leaf roll virus*, *Strawberry latent ringspot virus,* and *Cucumber mosaic virus* (CMV) (Walkey and Webb 1968). Recently, recovery of SAM from plants infected with CMV (Mochizuki and Ohki 2004) and *Tobacco rattle virus* (TRV) (Martín-Hernández and Baulcombe 2008), which were able to invade the SAM, was reported. These shoot meristem tissues later became virus-free. Thus, the distribution of some viruses in shoot meristem changes with the passage of time. Therefore, elucidating the kinetics of viral infection in meristematic tissues, including the SAM, is of great value.

Tobacco ringspot virus (TRSV) is a member of subgroup A of the genus *Nepovirus* in the family *Comoviridae* (Le Gall et al. 2005). The virus particles are icosahedral and 28 nm in diameter (Rezaian and Francki 1973; Murant et al. 1996). Unlike many plant viruses, TRSV has been detected in the SAM of tobacco plants (Roberts et al. 1970). Tobacco plants infected with TRSV exhibit distinct ringspot symptoms, but ringspot symptoms are absent in the newly developing leaves during the late period of infection; this is termed a recovery phenomenon. The asymptomatic leaves are protected against secondary infection by the same virus, although the leaves contain infectious TRSV (Wingard 1928). The development of asymptomatic leaves has been implicated in the kinetics of viral distribution in the shoot meristem of infected plants. Roberts et al. (1970) suggested that recovery from ringspot symptoms induced by TRSV occurred after viral invasion of the SAM. Ratcliff et al. (1997) also hypothesised that the recovery phenotype of *Nepovirus*, attributable to homology-dependent degradation of viral RNA (*i.e.*, RNA silencing), was correlated with *Nepovirus* invasion of the SAM (*i.e.*, virus invasion of the SAM might activate RNA silencing in the meristem). However, the kinetics of TRSV infection in tobacco plants is not yet fully understood. Therefore, TRSV distribution in the shoot and root meristems as well as in recovered tobacco leaves was analysed over time by immunohistochemical and western blot analyses.

TRSV Ibaraki 1 isolate (named as TRSV-Ib1) (Fukumoto et al. 1982), obtained from the National Institute of Agrobiological Sciences Genebank of Japan (MAFF No. 104031), was purified according to the methods described by Rezaian and Francki (1973). The largest leaf of each five- to six-leaf stage tobacco plants (*Nicotiana tabacum* cv. Xanthi) was mechanically inoculated with purified TRSV-Ib1 (50 μg ml^{-1}). The inoculated plants were then grown under greenhouse conditions at 24–30°C.

The distribution of TRSV-Ib1 in the tobacco shoot meristem was sequentially studied at 4, 7, 10, 14, 18, 21, 24, and 38 days post-inoculation (dpi) by immunohistochemical microscopy (Fig. 1a). Preparation of paraffin wax-embedded sections and immunohistochemical detection using anti-TRSV antiserum were conducted using previously described methods (Mochizuki and Ohki 2004). Five independent experiments were conducted, and five to ten meristem tissue samples were examined in each experiment. TRSV signals were first detected at 14 dpi in the meristem tissues in the TRSV-inoculated tobacco shoot meristem. Interestingly, intense TRSV signals were detected only in the centres of the SAM and leaf primordia; the signals in these locations were consistently detected up to 38 dpi. Limited distribution of TRSV in the centre of the SAM was confirmed in transverse serial sections of the SAM at 18 dpi; TRSV signals were detected in mid-sections, but not in surface sections of the SAM (Fig. 1b). To confirm the timing of the TRSV invasion of SAM, we further observed the distribution of TRSV in sections at 12, 13, 14, and 15 dpi. TRSV signals were not observed at 12 dpi but were detectable in all examined SAM at 13, 14 or 15 dpi (data not shown).

Previous studies have reported that viruses such as TRV are able to infect root apical meristems (RAM) (Valentine et al. 2002, 2004). To elucidate TRSV-Ib1 distribution in the RAM of tobacco, we examined RAM tissues at 10, 18, and 25 dpi by immunohistochemical microscopy using paraffin wax-embedded sections from tobacco root meristem tissue (Fig. 2). TRSV signals were not detected in the root meristem tissues at 10 dpi. TRSV signals were detected at 18 dpi in the centre of the RAM. However, TRSV signals were again undetectable in the root meristem at 25 dpi. To confirm the recovery of root meristem from TRSV infection, we carried out tissue-printing analysis. About 50 root tips were collected from TRSV-inoculated tobacco at 10, 18, or 25 dpi and pressed onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA) that had been treated with 0.2 M $CaCl_2$ prior to blotting. TRSV antigen present on the membrane was detected using anti-TRSV antiserum, as previously described (Ryang et al. 2004). TRSV signals were detected from the tissues representing the upper 60% of root tips at 10 and 18 dpi. However, TRSV signals were only detected in a minority of the root tips (<10%) at 25 dpi. These results clearly indicate that the root meristem of tobacco was initially infected with TRSV, but that it later recovered from TRSV infection. Thus, detailed time-course analysis using immunohistochemical microscopy revealed that the infection kinetics of TRSV-Ib1 was different between the SAM and RAM.

The kinetics of TRSV-Ib1 infection in the inoculated leaf, symptomatic leaves, and asymptomatic

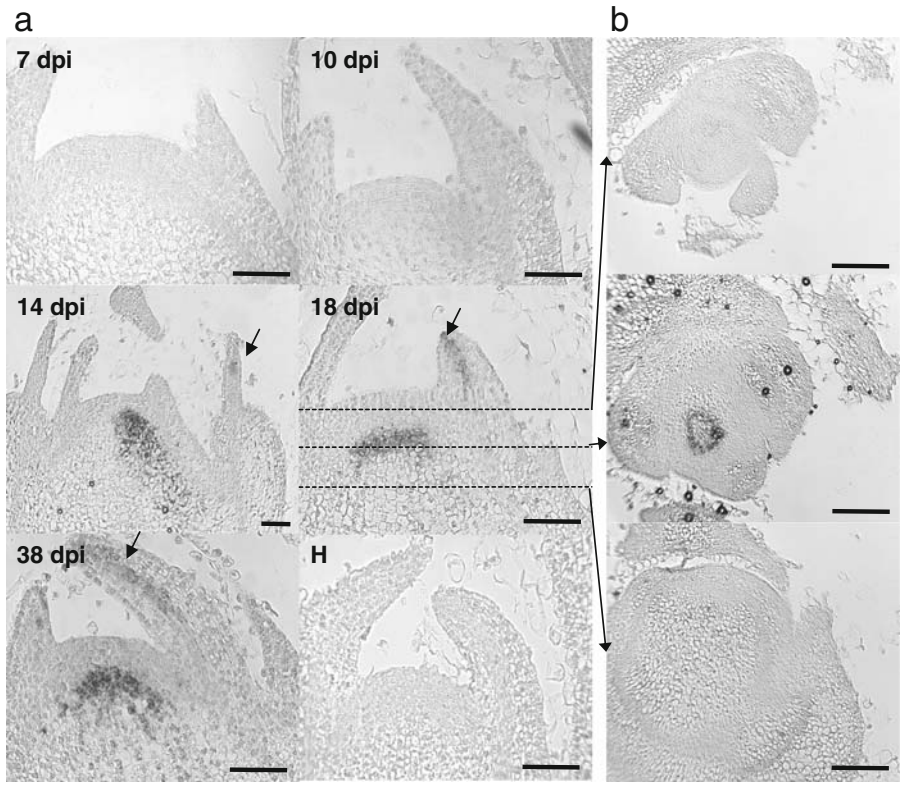

Fig. 1 Time-course analysis of TRSV distribution in the shoot meristem of tobacco plants infected with TRSV. **a** Longitudinal sections of tobacco plant meristem tissues at 7, 10, 14, 18, and 38 days post-inoculation (dpi), treated with an anti-TRSV antiserum. *Dotted lines* in the 18-dpi image indicate the location of the three transverse sections in **b** The cells infected with TRSV are darkly stained. Note that no TRSV signal is detected in the shoot meristem tissue of healthy tobacco. *H*, healthy shoot meristem tissue as a negative control. **b** Serial transverse sections of tobacco plant meristem tissues at 18 dpi. Bars=100 μm

leaves of inoculated tobacco were also investigated. To determine the viral content in these leaves, we performed western blot analysis of leaves at 12 and 18 dpi. At 12 dpi, the inoculated leaf and the 2nd, 3rd, and 4th leaves above the inoculated leaf expressed the ringspot symptoms, but leaves above the 5th leaf did not exhibit symptoms. The ringspot symptoms often appeared on the 5th to 7th leaves, but leaves above the 8th leaf remained symptom-free at 18–20 dpi. In the leaves expressing the ringspots, samples were collected from three parts: ringspot tissues (RS), tissues adjacent to ringspots (aR), and tissues far removed from the ringspot (n). Leaf samples of asymptomatic leaves (as) were also carefully collected, in which the samples should not contain major veins. The levels of TRSV coat protein (CP) in these tissues were then compared by western blot analysis. Western blot analysis with anti-TRSV antiserum was performed according to the procedures previously described by Kubota et al. (2003). TRSV CP was detected in three parts (RS, aR, and n) of the inoculated leaf at 12 and 18 dpi, indicating that TRSV invaded the entire leaf, including the asymptomatic regions. In the symptomatic leaves, TRSV CP

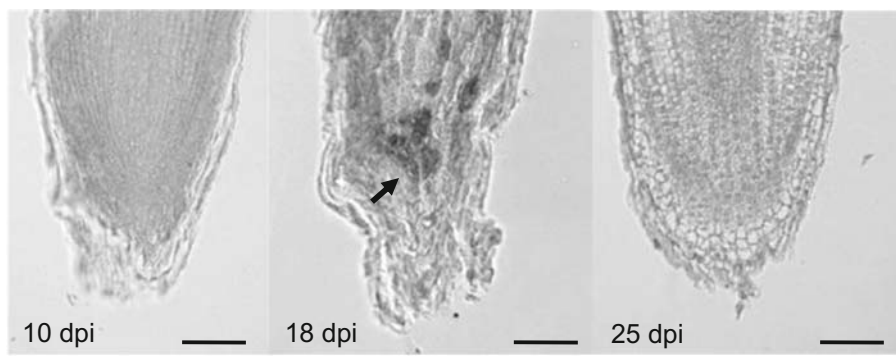

Fig. 2 Time-course analysis of TRSV distribution in the root meristem of tobacco plants infected with TRSV. Longitudinal sections of root meristem tissues at 10, 18, and 25 days post-inoculation (dpi), treated with an anti-TRSV antiserum. The *arrow* in the 18-dpi image indicates the darkly stained infected cells. Bars=100 μm

was detected in RS and aR tissues, but not the n tissues. Accumulation of TRSV CP in the tissues from asymptomatic leaves (as) was not detected by the western blot analysis at 12 and 18 dpi (Fig. 3a). The experiments were repeated twice, and identical results were obtained for each.

We further analysed TRSV-Ib1 infection in the inoculated leaf, symptomatic leaves, and asymptomatic leaves of inoculated tobacco at 20 dpi by reverse transcriptase-polymerase chain reaction (RT-PCR) assay, which is a more sensitive method. Primer TRSV2 (5′-GGACAAACACGACAC-TAGG-3′), which binds to the conserved 3′-terminal region of RNA 1 and RNA 2 (GenBank/EMBL/DDBJ accession nos. RNA 1: NC_005097 and RNA 2: NC_005096), was used in the reverse transcription reaction. Primer pair TRSVf1 and TRSVr1 (Martin et al. 2009), which amplifies the CP region in RNA 2, was used in the PCR reaction. Total RNA was isolated from the samples with TRI

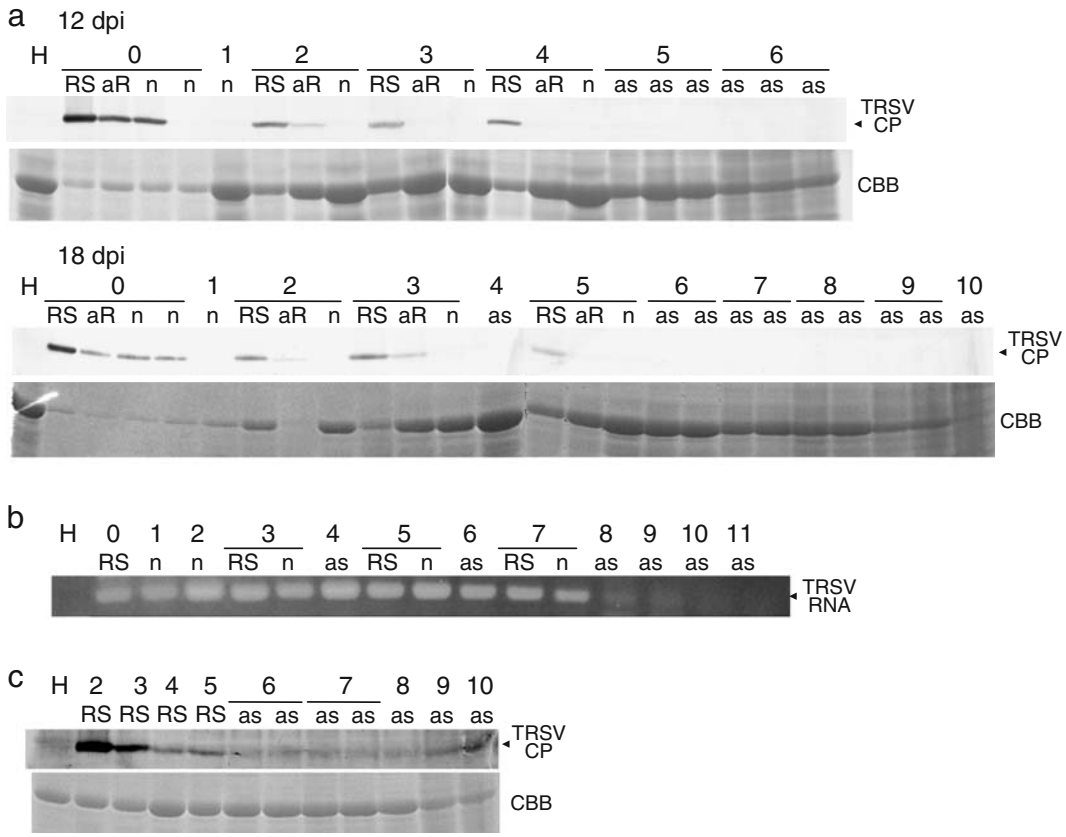

Fig. 3 Accumulation of *TRSV* in an inoculated leaf and un-inoculated upper leaves of tobacco plants infected with *TRSV*. **a** Accumulation of *TRSV* coat protein (*CP*) in each leaf of a tobacco plant at 12 and 18 dpi. *CP* content was analysed by western blot analysis with an anti-TRSV antiserum. Total protein was stained with Coomassie brilliant blue G-250. 1–10; the leaf position of upper leaves above the inoculated leaf (0), *RS*; ringspot tissue, *aR*; tissue adjacent to ringspot *n*; non-ringspot tissue in the symptomatic leaf, as; tissue in the asymptomatic leaf, *H*; healthy tobacco leaf as negative control. Note that the *TRSV CP* is detectable in the samples from inoculated leaf (0) and *aR* tissue of 2nd leaf at 18 dpi, although the total protein loaded was lower than in other samples. **b** Detection of *TRSV RNA* in each leaf of a tobacco plant at 20 dpi by RT-PCR. 1–11; the position of upper leaves above the inoculated leaf (0), *RS*; ringspot tissue, *aR*; tissue adjacent to ringspot *n*; non-ringspot tissue in the symptomatic leaf, *as*; tissue in the asymptomatic leaf, *H*; healthy tobacco leaf as negative control. **c** Accumulation of *TRSV CP* in un-inoculated upper leaves of *Nicotiana benthamiana* at 18 dpi. *CP* content was analysed by western blot analysis. Total protein was detected with Coomassie brilliant blue G-250. 2–10; the position of upper leaves above the inoculated leaf, *RS*; ringspot tissue, *as*; asymptomatic leaf, *H*; healthy leaf as negative control

reagent (Molecular Research Centre Inc., Cincinnati, OH). cDNAs were prepared by reverse transcription with ReverTra Ace (Toyobo, Tokyo, Japan), and the PCR was performed using Phusion DNA polymerase (Finnzymes, Epsoo, Finland). The TRSV RNA-specific bands were detected in both ringspot tissues (RS) and non-ringspot tissues (n) of the 1st to 7th upper leaves, as well as in the inoculated leaf (0). However, a very faint TRSV RNA-specific band was detected in tissues of the 8th and 9th asymptomatic leaves (as) and was not detectable in tissues from the 10th to 11th asymptomatic leaves (Fig. 3b). These results demonstrate that the large amount of TRSV-Ib1 accumulated only in and around the ringspot tissues of the symptomatic leaf; TRSV-Ib1 infection was not or hardly detected in the asymptomatic leaves of inoculated tobacco plants. Although TRSV CP was consistently detected in leaf primordia at 14, 18, and 38 dpi (Fig. 1a), these tissues might recover from TRSV infection during their development.

In contrast to our results in tobacco plants (Fig. 3a and b), Jovel et al. (2007) reported *Tomato ringspot virus* (ToRSV, a subgroup C *Nepovirus*) to accumulate at high concentrations in the youngest asymptomatic leaves at the shoot apex of *Nicotiana benthamiana*, as well as in symptomatic leaves. Therefore, we examined the accumulation of TRSV-Ib1 in the asymptomatic leaves of *N. benthamiana*. When fully expanded leaves of the six- to seven-leaf stage of *N. benthamiana* were inoculated with purified TRSV-Ib1 (100 μg ml^{-1}), necrotic symptoms appeared in the 2nd to 5th upper leaves, whereas leaves above the 5th leaf recovered from the ringspot symptoms. The level of TRSV in each leaf was examined at 18 dpi by western blot analysis. In contrast to the result of the experiment using tobacco plants, TRSV CP was detected in all of the upper leaves, regardless of the presence of symptoms in *N. benthamiana* (Fig. 3c); this result is identical to those of Jovel et al. (2007). Thus, infection of TRSV-Ib1 in asymptomatic leaves around 20 dpi was different in tobacco and in *N. benthamiana*.

Wingard (1928) reported that asymptomatic leaves of TRSV-infected tobacco contained a large amount of virus. ToRSV also accumulated at high concentrations in asymptomatic leaves of *N. benthamiana* (Jovel et al. 2007). In contrast, *Tomato black ring virus* (TBRV, a subgroup B *Nepovirus*) was not detectable in asymptomatic leaves of *Nicotiana clevelandii* (Ratcliff et al. 1997). Similarly, TRSV potato calico strain did not accumulate in the uppermost asymptomatic leaf of *N. benthamiana* at 40 dpi (Siddiqui et al. 2008). In our study, TRSV-Ib1 continuously infected the asymptomatic leaves of *N. benthamiana* at 20 dpi, whereas TRSV-Ib1 was not detected in the asymptomatic leaves of tobacco plants. These findings suggest that the infection kinetics of *Nepoviruses* in asymptomatic leaves vary among viral strains, viral species, and host plant species.

In the present study, we did not resolve why the kinetics of viral infection was different among shoot meristem, root meristem, and un-inoculated upper leaves of infected tobacco plants. However, RNA silencing is now implicated in the recovery of SAM, RAM, and leaves from viral infection. Decreased viral distribution in the lateral root meristem, a phenomenon attributable to an RNA silencing-like mechanism, has been previously observed in *Tobacco mosaic virus*-infected *N. benthamiana* (Valentine et al. 2002). Reduction of viral infection in the shoot meristem has also been attributed to antiviral RNA silencing in CMV and TRV (Mochizuki and Ohki 2004; Martín-Hernández and Baulcombe 2008). Ratcliff et al. (1997, 1999) demonstrated that a drastic reduction of viral infection in asymptomatic leaves resulted from RNA silencing in both *N. clevelandii* infected with TBRV and *N. benthamiana* infected with TRV. In addition, differences were reported between leaves and roots in RNA silencing activity against the virus (Andika et al. 2005; Rahim et al. 2007). Recently, Siddiqui et al. (2008) reported that some silencing suppressors affected the temperature-dependent infection pattern of the TRSV potato calico strain in transgenic tobacco plants, although the authors did not describe the effect of silencing suppressors on the recovery phenotype of TRSV-infected tobacco plants. To elucidate the mechanism of the differences in TRSV infection kinetics among shoot meristem, root meristem and un-inoculated upper leaves of the tobacco plant, involvement of RNA silencing in the recovery of RAM and un-inoculated upper leaves from TRSV infection will first need to be verified.

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