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Short communication

Silicon delays *Tobacco ringspot virus* systemic symptoms in *Nicotiana tabacum*

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ABSTRACT

Soluble silicon (Si) provides protection to plants against a variety of abiotic and biotic stress. However, the effects of Si on viral infections are largely unknown. To investigate the role of Si in viral infections, hydroponic studies were conducted in *Nicotiana tabacum* with two pathogens: *Tobacco ringspot virus* (TRSV) and *Tobacco mosaic virus* (TMV). Plants grown in elevated Si showed a delay in TRSV systemic symptom formation and a reduction in symptomatic leaf area, compared to the non-supplemented controls. TRSV-infected plants showed significantly higher levels of foliar Si compared to mock-inoculated plants. However, the Si effect appeared to be virus-specific, since the element did not alter TMV symptoms nor did infection by this virus alter foliar Si levels. Hence, increased foliar Si levels appear to correlate with Si-modulated protection against viral infection. This is all the more intriguing since *N. tabacum* is classified as a low Si accumulator.

Introduction

Silicon (Si) is the second most abundant element in the lithosphere and is absorbed by plants to varying degrees (Frantz et al., 2010; Marschner, 2002). The range of Si uptake in dicot plants varies substantially from more than 12,000 mg Si per kg of dry tissue for plants such as *Zinnia elegans* (a high Si accumulator), to <300 mg Si per kg of dry tissue in plants such as *Nicotiana tabacum* (a low Si accumulator). Si provides many benefits to stressed plants including protection against attack from certain fungal and bacterial pathogens. For example, treatment of tomato varieties and *Cucumis sativus* with Si reduced infection by *Ralstonia solanacearum* and *Podosphaera xanthii*, respectively (Dannon and Wydra, 2004; Datnoff et al., 2007; Liang et al., 2005). Interestingly, defense enzyme activity did not change in plants in the absence of the pathogen, whether or not Si was applied. This suggests that the effect of Si on host gene expression only occurs when plants are stressed (Fauteux et al., 2006; Liang et al., 2005).

While Si protects plants against fungal and bacterial pathogens, the effects of this element on viral infections are unclear. Si was shown to *increase* viral incidence in tobacco infected with *Belladonna mottle virus* (BMoV) (Bengsch et al., 1989). However, *N. tabacum* are susceptible to a wide variety of viruses (Brunt et al., 1997) and it is unclear if Si would have the same effect on all of these pathogens. *Tobacco ringspot virus* (TRSV) is a single-stranded, positive-sense, RNA nepovirus that causes systemic chlorotic and necrotic ringspots in *N. tabacum* (Rezaian and Francki, 1973). *Tobacco mosaic virus* (TMV) is another single-stranded, positive-sense RNA virus that infects *N. tabacum* (Brunt et al., 1997; Chapman, 1998). However, TMV belongs to the tobamovirus family and is only distantly related to TRSV. In this study, the ability of Si to influence TRSV and TMV infection of *N. tabacum*, was investigated. Our hypothesis was that like BMoV, Si treatment of *N. tabacum* would render the plants more susceptible to TRSV and TMV infection. In addition, we expected Si concentrations in leaves to remain unchanged in response to either virus since *N. tabacum* is a low Si accumulator (Frantz et al., 2010).

Materials and methods

Propagation of viruses and N. tabacum plants

All experiments were performed using *Nicotiana tabacum* L. cv. Wisconsin 38 as the viral host. TRSV Rubus strain (American Type Culture Collection PV-172) was propagated in *N. tabacum* by serial passage and virus was purified as described by Rezaian and Francki (1973). TRSV particles were mixed with washed celite and used for inoculation. TMV was propagated in that same *N. tabacum* cultivar by serial passage and virus was isolated as described by Chapman (1998). TMV was resuspended in sodium phosphate buffer (pH 7.0), mixed with washed celite and used for inoculum.

Abbreviations: AUDPC, Area under the disease progress curve; C, Control (0.1 mM soluble silicon, K_2SiO_3); DPI, days post-inoculation; ICP-OES, Inductively coupled plasma-optical emission spectroscopy; PCR, polymerase chain reaction; RT, reverse transcriptase; RT-PCR, reverse transcriptase-polymerase chain reaction; SEM, standard error of the mean; Si, silicon; Si+, 1.0 mM soluble silicon (K_2SiO_3); TMV, tobacco mosaic virus; TRSV, tobacco ringspot virus; HSD, honestly significant difference.

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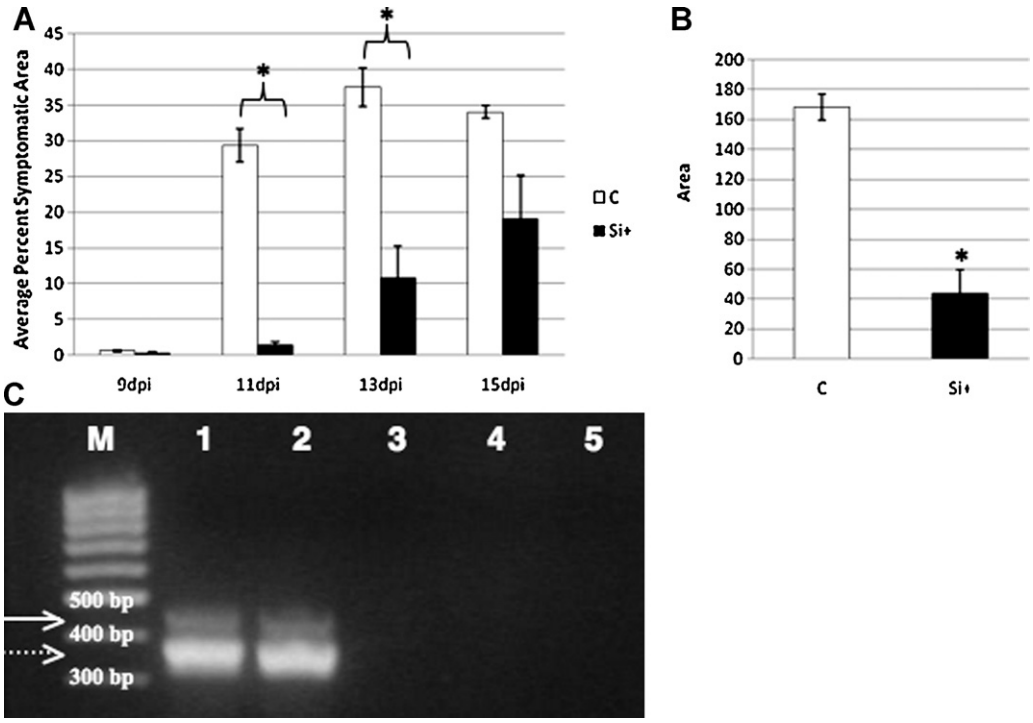

Fig. 1. TRSV systemic symptom spread and detection in *N. tabacum*. (A)Average percent TRSV symptomatic leaf area (Y-axis) on *N. tabacum* treated with 0.1 mM (C, white) or 1.0 mM K_2SiO_3 (Si+, black) at 9, 11, 13, and 15 DPI; the time points are indicated in the figure (*X*-axis). Average values and SEM are given, asterisks indicate significant difference between the C and Si+ plants at a particular time point ($p < 0.05$). Df = 1; *F* values are 0.663 for 9 DPI, 34.85 for 11, 6.555 for 13 and 1.4 for 15. Other statistical parameters are given in Supplementary Table 1A. (B) AUDPC average and SEM is given for C (white) and Si+ plants (black); asterisk indicates a significant difference between the values ($P < 0.05$) with Df = 1 and an *F* value of 12.127, as given in Supplementary Table 1B. (C) Detection of TRSV by RT-PCR in infected *N. tabacum* plants. grown under C (lanes 1 and 3) or Si+ (lanes 2 and 4) conditions. Note, lanes 1 and 2 were from plants infected with TRSV, while 3 and 4 were from mock-inoculated controls. A marker (exACTGene100 bp DNA ladder; Fisher Scientific, Pittsburgh, PA; lane M) and no cDNA PCR control (lane 5). The solid and dashed arrows indicate TRSV RNA 1 and RNA 2 PCR products, respectively.

N. tabacum seeds were germinated hydroponically in nutrient solution containing 0.1 mM soluble K_2SiO_3 as described by Li et al. (2008). Plants were hydroponically propagated in a growth chamber at 20 °C under a 16 h light: 8 h dark photoperiod at a light intensity of 70 μmol m^{-2} s^{-1} of photosynthetically active radiation. After reaching the four-leaf stage, seedlings were transferred to 4 L buckets containing nutrient solution. Immediately prior to virus inoculation, K_2SiO_3concentrations were changed to 1.0 mM (Si+), or maintained at 0.1 mM (C) for elevated Si and control hydroponic conditions, respectively. Three leaves per plant (at the five to six leaf stage) were rub-inoculated with purified TRSV or TMV. Plants were propagated in a growth chamber under the conditions described above. Nutrient solution was changed every 5–7 days. The solution pH was monitored weekly and did not change during the course of the experiments. Plants were examined daily for the formation of symptoms up to 15 days post-inoculation (DPI). Seven to nine plants were examined per treatment.

Analysis of viral infection and Si content

Digital images were taken of the plants (Nikon D40; resolution of 3008 × 2000 pixels), once systemic viral symptoms appeared (about 9 DPI). Percent symptomatic leaf area was calculated using the Assess Program (American Phytopathological Society Press). The pixel value for the sum of local lesions present was divided by the pixel value of the entire leaf area and converted to percentage. Digital image analysis was repeated three times for each leaf image. The total symptom coverage of each plant was determined by dividing the sum of the percent symptom area of each leaf by the total number of leaves present on each plant. Each individual plant was treated as a sample. The average and standard error of the mean (SEM) values for each set of treatments as well as ANOVA were calculated (R program; R Development Core Team, 2005). Tukey's HSD test was then used to indicate significant differences among treatment means. The *F*, Df and *P* values were given in Fig. 1 legend and provided in Supplementary Table 1. Area under the disease progress curve (AUDPC) for the time points indicated in Fig. 1 was calculated by the method described by Sparks et al. (2008).

To detect TRSV, total RNA was isolated from systemic leaves of *N. tabacum* plants at 15 DPI using the RNeasy Kit and reverse transcriptase (RT) reactions were performed with the Moloney murine leukemia virus enzyme according to the manufacturer's specifications (Promega, Madison, WI). Duplex polymerase chain reactions (PCR) were then performed using the GoTaq Master Mix (Promega) according to the manufacturer's specifications. In the same PCR reaction mixtures the TRSV-RNA1-nsp1F (5′-CCGCGAGGAGGGTCTTTCTTTTAG-3′), TRSV-RNA1-nsp1R (5′-CGGGGTGGCAGCGGTCTTC-3′), TRSV-RNA2-nsp1F (5′-AAGGCGCTCCGGGCTGCTCT-3′), and TRSV-RNA2-nsp1R (5′-CATGAAGGCGGGCTGCTGAA-3′) (synthesized by Integrated DNA Technologies Inc., Coralville, IA) were added. The TRSV-RNA1-nsp1F and TRSV-RNA1-nsp1R primers were designed based on the TRSV RNA 1 sequence (GenBank accession number: NC_005097) to amplify a 471 base pair (bp) fragment (from nucleotide positions 166–637) in PCR reactions. The TRSV-RNA2-nsp1F and TRSV-RNA2-nsp1R primers were designed based on the TRSV RNA 2 sequence (GenBank accession number: NC_005096) to amplify a 383 bp fragment (from nucleotide positions 414–797). PCR reactions were performed in a BioRad (BioRad Laboratories Inc, Hercules, CA) iCycler Personal using an initial thermal denaturation step at 92 °C for 1 min, followed by 25 cycles each of: a

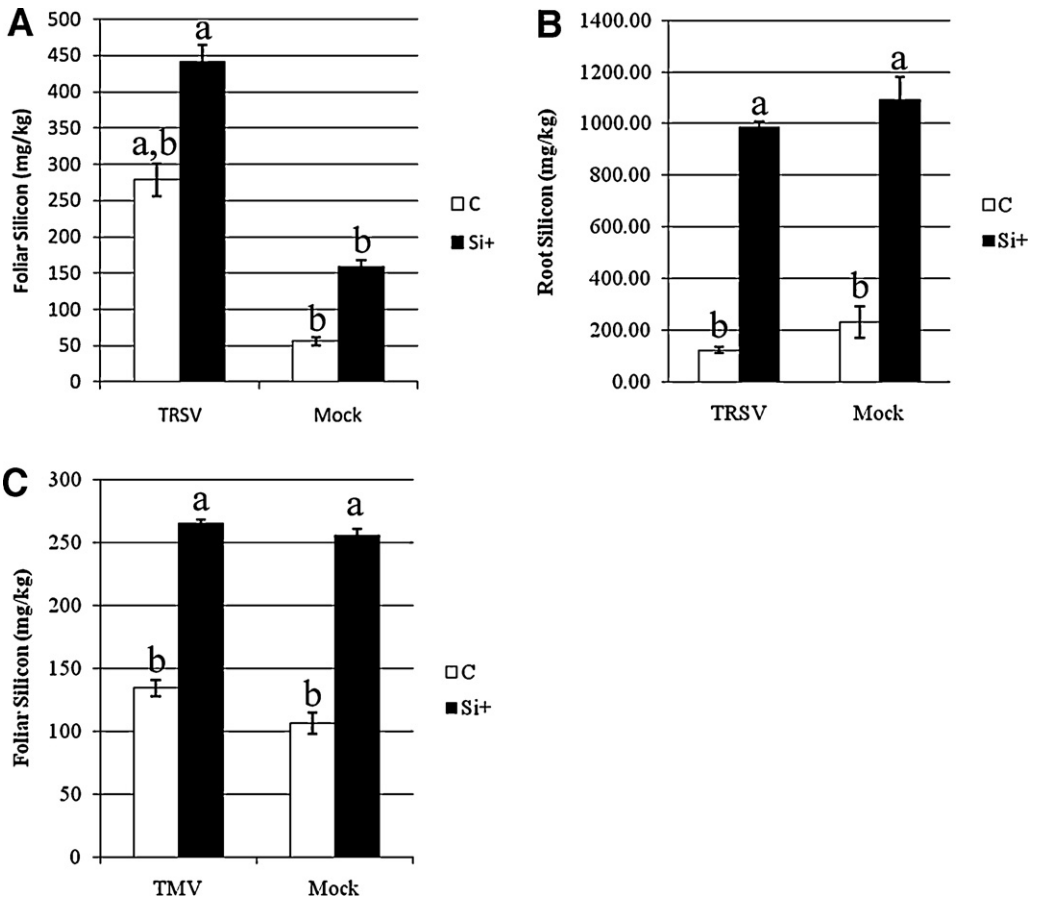

Fig. 2. Si concentration (mg/kg) within virus-infected plants determined by ICP-OES. Plants infected with TRSV (A, B) or TMV (C). Si levels in leaves (A, C) and roots (B) of *N. tabacum*; white bars, control plants (0.1 mM K_2SiO_3); black bars, treated with elevated Si (1.0 mM K_2SiO_3). Bars indicate average, error bars, SEM; letters indicate statistically significant differences as determined by Tukey's HSD test ($p < 0.05$). Other statistical parameters are given in Supplementary Tables 2A–C.

30 s denaturation step at 92 °C, a 30 s annealing step at 50 °C, and an elongation step of 72 °C for 30 s. PCR products were separated by electrophoresis through a two percent agarose gel, stained with ethidium bromide and photographed under ultraviolet light.

The influence of Si on the stability/infectability of purified TRSV preparations was tested by incubating the inoculum overnight at 4 °C with 0.1 mM or 1.0 mM K_2SiO_3. The pH did not change with the addition of the higher level of K_2SiO_3. The next day, washed celite was added to the inoculum and three *N. tabacum* plants (three leaves per plant) were rub-inoculated as before. Plants were then observed for symptoms daily.

For Si measurements, plants were harvested at the end of each experiment (18 DPI) and separated into leaf and root tissue, dried in an oven at 60 °C for 7 days, and ground. Si concentrations in tissue samples were determined as described in Frantz et al. (2008). ANOVA was performed on the averages for each of the treatments, plant organs were analyzed separately and statistical analyses were performed based on Tukey's HSD test as above. Statistical parameters are included in Fig. 2 legend and provided in Supplementary Tables 2A–C.

Results and discussion

The effects of Si on TRSV systemic symptom spread in N. tabacum

At nine DPI, viral systemic symptoms just started to appear on most of the control (C, 0.1 mM K_2SiO_3) plants and some of the tobaccos treated with elevated Si (Si+, 1.0 mM K_2SiO_3) (Fig. 1A). However, by 11 DPI, an obvious difference was observed between the C and Si+ plants. Systemic TRSV symptoms covered about 30% average leaf area on C plants compared to approximately 2% leaf coverage in Si+ plants. By 13 DPI, C plants averaged about 37% systemic leaf coverage, while that of the Si+ tobaccos averaged about 10%. By 15 DPI, C plants showed approximately 34% leaf coverage, while the Si+ plants showed on average approximately 19% leaf symptoms. Overall, the majority of the Si+ plants never exhibited levels of symptomatic leaf coverage to the same extent as the controls. To examine the effectiveness of Si on the control of TRSV systemic infection, the area under the disease progress curve (AUDPC) was calculated for the four time points (Fig. 1B). The AUDPC values show that Si confers a beneficial effect on tobacco by reducing the systemic TRSV symptomatic leaf area. Against our hypothesis, treatment of tobacco with increased Si lead to a reduction in TRSV systemic symptom distribution in infected plants. Systemic viral symptoms eventually appeared on the Si treated plants. Therefore, Si does not eradicate TRSV, but likely helps tobaccos mount a more effective defense delaying both the onset and appearance of systemic symptoms. To be certain that symptomatic plants were infected with TRSV, total RNA was isolated from all of the *N. tabacum* and analyzed by RT-PCR. All samples from infected plants were positive for TRSV (Fig. 1C).

The mechanism(s) by which Si delays viral systemic symptom formation is/are unclear. One possibility was that the element destabilized TRSV particles. To test this, isolated TRSV was co-incubated with 0.1 or 1.0 mM K_2SiO_3 and inoculated to *N. tabacum*. The onset and spread of TRSV symptoms in tobaccos was unchanged by the co-incubation.

Since Si does not directly influence the infectivity of TRSV particles, it seemed more likely that the element modulates host

defenses. *Arabidopsis* plants provided with Si induce a variety of genes in response to mildew that are not up-regulated in the absence of the element (Fauteux et al., 2006). Perhaps Si enhanced tobacco defenses to provide protection against TRSV. Differences in defenses modulated by Si could lead to variations in plant physiological responses. One possibility is that Si may facilitate its own uptake into infected leaves. Therefore, at 18 DPI, leaves and roots of mock-inoculated and TRSV infected plants were harvested, dried, and analyzed for Si content.

Leaf Si levels were influenced by TRSV infection. Plants inoculated with TRSV and supplemented with Si contained four-fold higher Si concentrations (approximately 440 mg Si per kg leaf dry weight) compared to Si-supplemented, mock-inoculated tobaccos (approximately 110 mg per kg) (Fig. 2A). Since leaf Si levels in Si+ TRSV-infected tobaccos were significantly higher than mock-inoculated plants, this suggests that foliar accumulation of Si is regulated in tobacco and may be part of a defense response in tobacco to TRSV. This is intriguing since tobacco is a low Si accumulator (Frantz et al., 2010). Leaves of TRSV-infected plants provided with control levels of Si (0.1 mM) contained an average of approximately 280 mg Si per kg dry weight compared to 80 mg per kg in mock-inoculated controls.

In contrast, *N. tabacum* root Si levels were dependent upon the supply of Si but independent of TRSV-infection (Fig. 2B). TRSV and mock-inoculated plants supplemented with Si contained about 990 and 1100 mg Si per kg root dry weight, respectively, which were not significantly different. However, root Si levels in plants not supplemented with Si were approximately 120 and 230 mg per kg in TRSV and mock-inoculated control plants, respectively. One might speculate that virus-infected leaves produce a signal that is sent to the roots, either releasing Si from internal stores or inducing an increase in root uptake for transport to the leaves.

To determine if the beneficial effects of Si on TRSV were virus-specific, the effects of this element were tested on TMV infection. Interestingly, no obvious differences were observed in TMV systemic spread or symptom distribution between the control plants and those provided with additional Si (data not shown). Si accumulation in TMV-infected leaves at 18 DPI was entirely dependent upon Si application but independent of viral infection (Fig. 2C). The fact that Si did not protect plants against TMV infection suggests that the role of Si in virus protection is specific to TRSV. Such pathogen selectivity has also been shown for certain fungal infections (Rodgers-Gray and Shaw, 2000, 2004). Our data suggest that leaf Si uptake is part of the responses that tobaccos use as a specific defense mechanism against TRSV but these mechanisms are different from those to defend the plant against TMV. In summary, Si specifically delays TRSV systemic symptom formation and this response correlates with higher Si levels in virus-infected leaves.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jplph.2011.04.002.

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