Disruption of Vector Transmission by a Plant-Expressed Viral Glycoprotein

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Vector-borne viruses are a threat to human, animal, and plant health worldwide, requiring the development of novel strategies for their control. *Tomato spotted wilt virus* (TSWV) is one of the 10 most economically significant plant viruses and, together with other tospoviruses, is a threat to global food security. TSWV is transmitted by thrips, including the western flower thrips, *Frankliniella occidentalis*. Previously, we demonstrated that the TSWV glycoprotein G_N binds to thrips vector midguts. We report here the development of transgenic plants that interfere with TSWV acquisition and transmission by the insect vector. Tomato plants expressing G_N-S protein supported virus accumulation and symptom expression comparable with nontransgenic plants. However, virus titers in larval insects exposed to the infected transgenic plants were three-log lower than insects exposed to infected nontransgenic control plants. The negative effect of the G_N-S transgenics on insect virus titers persisted to adulthood, as shown by four-log lower virus titers in adults and an average reduction of 87% in transmission efficiencies. These results demonstrate that an initial reduction in virus infection of the insect can result in a significant decrease in virus titer and transmission over the lifespan of the vector, supportive of a dose-dependent relationship in the virus-vector interaction. These findings demonstrate that plant expression of a viral protein can be an effective way to block virus transmission by insect vectors.

The *Bunyaviridae* is a family of animal and plant viruses that threaten human health, animal husbandry and plant production worldwide, and both new and established species are currently emerging as important pathogens (de Oliveira et al. 2012; Guler et al. 2012; Herder et al. 2012; McMullan et al. 2012; Walter and Barr 2011). The epidemiology of these viruses is dependent on arthropod vectors (the genera *Orthobunyavirus*, *Nairovirus*, *Phlebovirus*, and *Tospovirus*) or rodent vectors (genus *Hantavirus*). The effect of climate change on vector-borne virus distribution and population dynamics suggest that bunyavirus spread and occurrence will continue to increase

(Jones 2009; Kilpatrick and Randolph 2012; Pappu et al. 2009; Walter and Barr 2011). Likewise, increasing human population and the consequent urbanization of wild areas contribute to this problem (Jones 2009; Kilpatrick and Randolph 2012; Pappu et al. 2009; Walter and Barr 2011). Therefore, effective approaches to control these viruses are needed. Here, we present a novel control strategy to interfere with the transmission cycle of *Tomato spotted wilt virus* (TSWV), a plant-infecting virus in the family *Bunyaviridae*, genus *Tospovirus*, that is transmitted by several species of thrips (order Thysanoptera) (Whitfield et al. 2005).

Tospoviruses and their thrips vectors are increasing in importance worldwide. Thrips are economically important as direct plant pests that limit crop production in fields and greenhouses, and some species are expanding their geographical range, helped by, among various factors, increasing international trade and climate change (Morse and Hoddle 2006; Reitz 2009). Tospoviruses are considered emerging diseases because, in recent years, new species have been described and established species are found infecting new hosts or in geographical areas where they were not previously reported (Mandal et al. 2012; Pappu et al. 2009). TSWV is one of the top 10 plant-pathogenic viruses due to the combined effect of the damage it causes to crops and the cost of disease management (Scholthof et al. 2011). The virus has a global distribution and has a wide host range, infecting hundreds of plant species, including important commodities such as tomato, pepper, lettuce, peanut, potato, and many ornamental plants. The losses associated with TSWV and other members of this genus exceed tens of millions of dollars (Culbreath et al. 2003; Mandal et al. 2012; Pappu et al. 2009; Riley et al. 2011).

The control of TSWV and other tospoviruses relies heavily on control of vector thrips. The management of thrips pests has proved very difficult and, even when insect levels are below an economic threshold for plant damage, a few thrips are enough to transmit viruses to plants (Amin et al. 1981; Evans and Gunderson-Rindal 2003; Falk and Tsai 1985; Sutherst 2004). Resistance to certain tospoviruses mediated by *Tsw* and *Sw-5* genes is used in commercial pepper and tomato varieties, respectively. However, virus strains overcoming the resistance have been documented (Lopez et al. 2011; Margaria et al. 2004). Likewise, chemical control of thrips may not reduce tospovirus transmission (Culbreath et al. 2003; Mandal et al. 2012; Morse and Hoddle 2006; Reitz 2009). Among thrips, *Frankliniella occidentalis* is the most efficient TSWV vector and is an economically important pest in greenhouses and field crops (Reitz 2009). Populations of *F. occidentalis* have developed high levels of resistance to many insecticides, leaving few options to control thrips or the tospoviruses they can trans-

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mit (Gao et al. 2012). New control strategies against thrips and tospoviruses are required, and transgenic plants are recognized as promising options for tospovirus management (Mandal et al. 2012; Pappu et al. 2009).

The understanding of the molecular determinants of plant virus-vector interactions allows the development of novel ways to control viruses by interfering with the initial steps in the interaction. Disruption of the acquisition process renders insects incapable of transmitting the virus or greatly reduces transmission efficiency and, thus, stops the transmission cycle (Liu et al. 2010; Shao et al. 2003; Sparks et al. 2011). In the case of TSWV, it was shown that one of the virus glycoproteins, G_N , serves as the attachment protein of the virus (Whitfield et al. 2004). A soluble form of G_N produced in vitro (G_N -S) was able to interfere with the acquisition and transmission of TSWV by *F. occidentalis* when fed exogenously to thrips prior to or simultaneously with TSWV (Whitfield et al. 2004, 2008). These results suggest that G_N -S is a good candidate to deploy against TSWV. Moreover, this approach may be used against other tospoviruses by using the corresponding G_N glycoproteins.

The objective of this work was to generate and characterize transgenic tomato plants expressing a soluble form of TSWV glycoprotein G_N fused to green fluorescent protein (GFP): G_N -S::GFP. We generated the fusion to GFP in order to facilitate characterization of the plants and tracking the recombinant protein. We demonstrated that the recombinant protein (G_N -S::GFP) in the transgenic plants interfered with TSWV acquisition by thrips and, as a consequence, the transgenic plants decreased virus transmission by the corresponding adult thrips. Transgenic plants carrying G_N -S constitute a novel tool to control TSWV spread by means of a transmission inhibition strategy.

RESULTS

Generation of G_N -S transgenic tomato plants.

Putative transgenic tomato plantlets were regenerated in vitro under kanamycin selection after *Agrobacterium*-mediated transformation of tomato calli. In total, 10 independent G_N -S::GFP T0 transgenic plants (events) were obtained after transformation and in vitro culture. Six G_N -S::GFP plants (60%) were confirmed as transgenic plants by enzyme-linked immunosorbent assay (ELISA) with specific antibodies to detect the recombinant protein. Moreover, fluorescent confocal microscopy observations showed the presence of a GFP signal in those plants (Fig. 1). These G_N -S::GFP-expressing transgenic plants did not display deleterious phenotypes (e.g., reduced vegetative growth, flower set, or fruit yield) during growth and development (data not shown).

Seed were harvested from five T0 G_N -S::GFP plants out of the six events which were confirmed to express the recombinant protein and the seed were planted to obtain T1 plants. The segregation of the transgenic phenotype (presence of recombinant protein) was evaluated by ELISA with antibodies against G_N or GFP proteins. For all five T0 G_N -S::GFP events for which seed were planted, some proportion of the T1 seedlings tested positive for the recombinant protein (12 to 85%). Additionally, a commercial double-antibody sandwich (DAS)-ELISA that detects the protein product of the *np1II* gene (kanamycin resistance marker) was used to evaluate T1 plants and confirmed the transgenic nature of the plant lines (71 to 94% of positive seedlings for neomycin phosphotransferase II [NPTII] enzyme). Fluorescent microscopy observations of leaf tissue from these T1 plants confirmed the presence of a GFP signal (Fig. 1).

The amount of recombinant protein accumulating in the transgenic tissue (T1) varied among the different tomato lines. The relative amount of recombinant protein accumulating in different transgenic lines was indirectly determined by quantifying the

amount of GFP protein on those plants. There is a 1:1 molar ratio between G_N -S and GFP molecules due to the fusion nature of the recombinant protein. Therefore, the amount of GFP protein detected in each plant (500 mg of tissue) by a quantitative DAS-ELISA with a GFP standard curve was used to calculate the amount of G_N -S::GFP. The amount of recombinant protein was 22.6 to 2400.8 ng of G_N -S::GFP per milligram of total soluble protein extracted from 17 transgenic T1 plants (Supplementary Fig. S1). The fusion of G_N -S to GFP enabled tracking, visualization, and quantification of G_N -S in planta.

Detection of the recombinant protein by Western blot.

The presence of recombinant protein and its identity was confirmed by Western blot analysis using specific antibodies against G_N and GFP proteins. Bands corresponding to G_N -S::GFP that migrated similarly to the 75-kDa molecular weight marker were detected in several transgenic lines by Western blot using antibodies against G_N (Supplementary Fig. S2A). The predicted molecular weight for G_N -S::GFP based on the nucleotide sequence of the fusion cassette was 63.4 kDa (using the tools available at ExPASy Bioinformatics Resource Portal); however, the calculated size does not account for the glycosylation (Naidu et al. 2004) events that occur during the posttranscriptional processing of the protein. Moreover, the observed size for a previously in-vitro-produced G_N -S was approximately 50 kDa (Whitfield et al. 2004) and the size for GFP is 27 kDa; thus, the 77-kDa band is consistent with mobility of glycosylated G_N -S::GFP. Bands corresponding to G_N -S::GFP were also detected with antibodies against GFP. The commercial GFP antibody had a stronger reaction to the G_N -S::GFP fusion protein than the anti- G_N peptide antibody, and the recombinant protein was detected with anti-GFP antibody in all tested plants. The relative intensity of the bands varied among samples, despite adding equal quantity of protein, suggesting different levels of recombinant protein content among different transgenic lines and generations, and was in agreement with the quantification data. In the TSWV-positive control sample and in several of the transgenic samples, a double band was observed for G_N or G_N -S::GFP, suggesting that the wild-type G_N and also the recombinant G_N -S::GFP protein occurred as two different forms in planta. These two different forms may represent different levels of glycosylation, as was suggested by Naidu and associates (2004), who reported two molecular sizes for G_N , a 58- and a 53-kDa protein mass.

Reaction of G_N -S::GFP transgenic plants to TSWV inoculation and detection of recombinant protein in TSWV-infected plants.

As a requisite to use G_N -S::GFP transgenic plants as a transmission blocking strategy, the recombinant protein must be present during viral infection of the plant. To evaluate the reaction of G_N -S::GFP transgenic plants to virus infection and the co-occurrence of virus and recombinant protein in these plants, 18 transgenic T1 plants were inoculated with TSWV. Overall, ELISA of young leaf tissue from these transgenics revealed that 100% of the T1 plants were systemically infected 15 days after inoculation. There was no apparent association (Fisher exact test of independence, $P = 1.0$) between the presence or absence of TSWV and the presence or absence of G_N -S::GFP protein, regardless of leaf age tested (Supplementary Table S1). In other words, the presence or absence of one had no apparent influence on the presence or absence of the other in the T1 transgenics. Moreover, the proportion of T1 plants with detectable expression of G_N -S::GFP in both old and young leaves for noninoculated (43%) and TSWV-inoculated (33%) transgenics was comparable (data not shown). If the mechanism of inhibition by G_N -S::GFP is competition for binding to

the thrips midgut, these findings indicate that virus and $G_N\text{-}S\text{:GFP}$ co-occur in the tissues that the insect would be feeding on to acquire virus.

The presence of $G_N\text{-}S\text{:GFP}$ was verified using confocal microscopy for a number of plants selected from the plants that tested positive for both TSWV and $G_N\text{-}S\text{:GFP}$ by ELISA. $G_N\text{-}S\text{:GFP}$ fluorescent signal was observed in T1 transgenic plants that were TSWV positive (Supplementary Fig. S3). The fluorescence signal in the infected, transgenic plant tissues was not different from the one observed for noninfected transgenic plants, indicating that TSWV infection and the presence of the native viral proteins did not alter $G_N\text{-}S\text{:GFP}$ expression.

Transgenic $G_N\text{-}S\text{:GFP}$ tomato plants interfere with TSWV acquisition by *F. occidentalis*.

TSWV acquisition assays on TSWV-infected transgenic plants were conducted with the objective of testing the hypoth-

esis that $G_N\text{-}S\text{:GFP}$ expression in planta will interfere with virus acquisition by thrips. The results obtained supported this hypothesis because thrips that fed on TSWV-infected transgenic $G_N\text{-}S\text{:GFP}$ tomato plants had a lower virus titer than thrips fed on TSWV-infected nontransgenic plants ($P < 0.0001$) (Fig. 2). TSWV-infected tissue was confirmed to express the recombinant protein 15 days after virus infection. Transgenic and nontransgenic TSWV-infected plants were used as virus sources to feed 0- to 18-h-old first-instar larval (L1) *F. occidentalis*. The L1 thrips were exposed to virus during an acquisition access period (AAP) of 24 h on tomato leaflets in water agar plates (three replicates for each treatment: transgenic and nontransgenic) and then moved to green beans for another 24 h to allow for virus replication in the insect body. A pooled sample of 15 thrips was picked from each replicate thrips cohort and tested by DAS-ELISA for the presence of TSWV N protein. The ELISA absorbance values for thrips fed on transgenic tissue were consistently lower than those values for thrips that fed on the corresponding nontransgenic tissue. Comparisons of the TSWV titer in the leaf tissue that was used for the thrips AAP in these experiments revealed that the transgenic and nontransgenic leaflets had similar relative virus titers. This finding indicates that the differences in virus titer in the thrips are not due to differences in the amount of virus in the tissue (Fig. 3).

Lower TSWV titers detected in thrips after feeding on transgenic TSWV plants suggested that virus infection also occurred in these groups of thrips but the degree of infection was lower in comparison with thrips fed on nontransgenic, TSWV-infected plants. The lower titer observed for a sample consisting of 15 pooled thrips may indicate i) a lower percentage of thrips infected with the virus in the cohort, ii) a lower level of infection in the individual infected thrips, or iii) a combination of these two variables. Independently of the factors contributing to the lower overall virus titer observed, the results indicate that the transgenic plants interfered with the acquisition of TSWV by *F. occidentalis*.

To confirm the results obtained by ELISA for groups of thrips, normalized real-time quantitative reverse-transcriptase

Fig. 1. A, $G_N\text{-}S\text{:GFP}$ fluorescence on tomato transgenic plant T1 number 9.201. **B,** Control tomato plant. Green channel (488 nm) images. Scale bars represent 50 μm .

Fig. 2. Detection of TSWV nucleocapsid protein (N) in thrips cohorts given a 24-h acquisition access period on TSWV-infected, $G_N\text{-}S\text{:GFP}$ transgenic or nontransgenic tomato leaf tissue. TSWV was detected by double-antibody sandwich enzyme-linked immunosorbent assay in samples of pooled thrips ($n = 15$). The graph depicts 12 data points (replicates) per treatment out of four independent experiments. The horizontal line indicates the median of the data points. Medians were determined as statistically different ($P < 0.0001$) between the two treatments (transgenic or nontransgenic) by the Mann-Whitney test.

polymerase chain reaction (qRT-PCR) of TSWV nucleocapsid (N) RNA to actin RNA was performed on individuals to estimate virus titer. TSWV titer in L1 thrips exposed to TSWV-infected transgenic plants for 24 h was significantly lower ($P \leq 0.0002$) than L1 thrips exposed to nontransgenic plants in all biological replications of the experiment (Fig. 4), indicating a robust capacity of the transgenic plants to inhibit acquisition and infection of the vector by TSWV. We found that the number of L1 thrips infected with TSWV was reduced when fed on transgenic tissue for all three replications of the experiment (6/12, 0/12, and 2/12 versus 12/12, 11/12, and 11/12, respectively). In accordance, TSWV-N cycle threshold values were statistically different between treatments ($P < 0.0001$, Mann-Whitney tests). Therefore, transgenic plants have an effect on the number of L1 thrips infected with TSWV and on the level of infection within individual thrips.

Fig. 3. Detection of TSWV nucleocapsid protein (N) by double-antibody sandwich enzyme-linked immunosorbent assay in tissue used for thrips acquisition access period. The graph depicts 12 data points (replicates) per treatment out of four independent experiments. The horizontal line indicates the median of the data points. Medians between the two treatments (transgenic or nontransgenic) were compared by the Mann-Whitney test.

Fig. 4. Normalized abundance ($\log_{10}$ transformed) of TSWV nucleocapsid protein (N) RNAs in larval thrips fed in transgenic and nontransgenic TSWV-infected plants. Larval thrips (0 to 18 h old) were exposed to a $G_N-S::GFP$ transgenic plant or to a nontransgenic plant, both TSWV-infected, and allowed to feed for a 24-h acquisition access period. A second 24-h feeding period was done on green bean and sampled for quantitative reverse-transcriptase polymerase chain reaction. Twelve thrips per treatment and three independent experiments are shown. Virus abundance in thrips fed on the transgenic plant was significantly different ($P \leq 0.0002$) from thrips fed on nontransgenic plant as determined by the Mann-Whitney test.

F. occidentalis cohorts show reduced transmission of TSWV when acquired from transgenic $G_N-S::GFP$ tomato plants.

Transmission assays were performed to determine whether reduced titers in larval thrips fed on transgenic plants translated into reduced transmission efficiency by adults. Over three independent replications of the experiment, the proportions of transmitting adult thrips (i.e., efficiency of transmission) fed on G_N-S transgenics as larvae were significantly lower ($P = 0.0003$, median = 0%) than those fed on nontransgenic tissues (median > 60%), given a 48-h inoculation access period (Fig. 5; Supplementary Table S2). These results show that the lower virus titers detected 24 h after larval acquisition of the virus from plants resulted in fewer individuals capable of transmitting the virus as adults.

The negative effect of G_N-S transgenics on virus accumulation in larvae persisted to adulthood. Provided only a 24-h access on TSWV-infected transgenics, larvae developed to adults that harbored four-log lower normalized levels of TSWV N RNA (median = 0.0004) compared with those result-

Fig. 5. Efficiency of transmission of TSWV by adult *Frankliniella occidentalis* individuals fed as larvae on transgenic and nontransgenic TSWV-infected plant tissue. Each data point represents the efficiency of transmission for 10 thrips tested individually for TSWV transmission by a 48-h inoculation access period on leaf discs of *Datura stramonium*. Treatments are significantly different at $P = 0.0003$ as determined by the Mann-Whitney test.

ing from larvae fed on nontransgenics (median = 6.81), with an overall significance between treatment groups ($n = 12$, $P < 0.0001$), as well as significant treatment differences within each biological replicate (Fig. 6) ($P < 0.0073$). Significant associations between larval and adult titers (Fig. 7A) ($r_s = 0.98$, $P < 0.0001$) were determined, and larval titer was significantly associated with transmission efficiency as adults (Fig. 7B, $r_s = 0.92$, $P < 0.0001$). Together, these findings support the hypothesis of a dose-dependent relationship between viral load and transmission efficiency in the virus–vector interaction, and underscore the significance of G_N -S transgenics as a possible strategy to control disease spread.

DISCUSSION

Interference with the initial steps of a pathogen–vector interaction provides an opportunity to inhibit transmission of the pathogen. We demonstrated the utility of a plant transgenics-based approach to disrupt the transmission cycle of a plant virus by effectively reducing virus acquisition by the insect vector and subsequent viral loads. The results herein demonstrate that plant-expressed G_N glycoprotein of TSWV interfered with the capacity of the thrips vector, *F. occidentalis*, to harbor and transmit the virus. Additionally, these results provide further support for G_N as an important determinant of the virus–vector interaction (Sin et al. 2005; Whitfield et al. 2004). G_N -S transgenic plants enable a new and promising option to include in integrated management schemes for sustainable and long-term control of TSWV epidemics. Future research resulting from our findings includes the need to study the effect of the transgenic plants in the transmission of other tospoviruses in tomato or transmission of TSWV by other thrips species. Moreover, other susceptible crop species or ornamental plants may also be transformed following this approach.

The cytological and quantitative evaluation of the G_N -S expression in transgenic tissue furthers the understanding of the TSWV–plant interaction and has implications for use of these transgenics for virus control. One possible outcome of expressing recombinant viral RNA in plants and challenging these plants with wild-type virus is resistance triggered by RNA interference and subsequent degradation of wild-type viral RNA (Lindbo and Dougherty 2005). Such a phenotype was not observed in this study, 15 days after TSWV inoculation and, subsequently, when tissue was sampled for acquisition experiments. Transgenic expression of G_N -S had no apparent effect on the stability or pattern of TSWV infection of the trans-

Fig. 7. Relationship between TSWV titer in larval *Frankliniella occidentalis* cohorts and the corresponding TSWV titer or transmission in adult thrips. **A**, TSWV titer was measured as normalized abundance of TSWV nucleocapsid protein (N) transcripts by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) for individual larval and adult thrips, and four individuals (adults and larvae) were analyzed per cohort. Each data point represents the median value (out of four individuals tested) for larvae or adults per cohort; Spearman's rank correlation test, $r_s = 0.9794$ ($P < 0.0001$). **B**, TSWV titer was determined for each cohort by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) from a pooled sample of 15 larvae and efficiency of transmission was determined for each cohort from TSWV transmission by 10 individual adults in a 48-h inoculation access period; Spearman's rank correlation test, $r_s = 0.9152$ ($P < 0.0001$).

Fig. 6. Normalized abundance ($\log_{10}$ transformed) of TSWV nucleocapsid protein (N) RNAs in 24-h post-adult eclosion thrips fed as larvae in transgenic and nontransgenic TSWV-infected plants. Larval thrips (0 to 18 h old) were exposed to a G_N -S::GFP transgenic plant or to a nontransgenic plant, both TSWV-infected, and allowed to feed for a 24-h acquisition access period. Adult thrips (24 h old) reared from these larvae were given a 48-h inoculation access period on *Datura stramonium* leaf discs and then sampled for quantitative reverse-transcriptase polymerase chain reaction. Twelve thrips per treatment and three independent experiments are shown. Virus abundance in thrips fed on the transgenic plant was significantly different ($P \leq 0.0073$) from thrips fed on nontransgenic plant as determined by the Mann-Whitney test.

genics used in acquisition and transmission assays. TSWV N protein was detected 15 days after inoculation in both transgenic and nontransgenic plants and systemic movement and infection occurred in both cases. Virus symptomatology and distribution was similar in the TSWV-infected transgenic and nontransgenic plants. The TSWV glycoproteins and the movement protein, NSm, are encoded on the M RNA segment. The widespread distribution of virus in the transgenic plants suggests that the M genome segment was not functionally silenced. In addition, infection had no apparent effect on accumulation of the recombinant protein in the tissue. Finally, given the plant-to-plant variation in the amount of recombinant protein produced in the transgenic lines tested in this study, it is likely that a higher amount of G_N-S may be achieved by selecting through several generations of the higher-expressing plants to produce more efficacious control.

The results on TSWV acquisition and subsequent transmission by *F. occidentalis* indicate a dose-dependent relationship in the virus–vector interaction, as was previously proposed by Rotenberg and associates (2009). Although few reports study the quantitative aspects of plant virus acquisition and transmission, the data support a quantitative parameter involved in the phenomenon for plant viruses (Ng et al. 2004). In our system, it appears that the number of virus particles that start the infection makes a difference in terms of later virus titer in adults (Fig. 7A) and transmission by the individual thrips (Fig. 7B). We hypothesize that the initial infection of the midgut requires reaching certain titer thresholds to spread and potentially reach the salivary glands during a specific time or development window. The virus–vector interaction at the dose level and the host cellular response to virus replication in this system have just begun to be characterized (Badillo-Vargas et al. 2012; Rotenberg and Whitfield 2010; Rotenberg et al. 2009). Further research is required to understand the interplay between TSWV and thrips host cell responses to it, and how these interactions will define vector competence of thrips, as has been suggested for mosquito vectors of animal-infecting viruses (Keene et al. 2004; Khoo et al. 2010; Sanchez-Vargas et al. 2009). A balance may be reached between the virus replication cycle and thrips defense pathways which may vary depending on the level of initial infection.

We showed that transgenic tomato plants expressing a recombinant form of the ectodomain of TSWV G_N glycoprotein, G_N-S, interfered with TSWV acquisition and transmission by western flower thrips. The results contribute to the hypothesis that G_N is the attachment protein of the virus (Whitfield et al. 2004). Additionally, these transgenic plants are tools for further studying the virus–vector interaction and transmission specificity between virus and vector species. Overall, the results herein exemplify how the understanding of the molecular aspects of plant virus–vector interactions enable exploitation of the elements of the system to develop novel and ecologically sound approaches to control the spread of plant viruses. Moreover, this understanding and application can be broadened and adjusted for the control of other plant viruses and contribute to understanding and control of animal- and human-infecting counterparts: other arthropod-vectored bunyaviruses.

MATERIALS AND METHODS

Construction of G_N-S::GFP expression clone and plant transformation.

The sequence corresponding to the soluble form of TSWV glycoprotein G_N was amplified from plasmid pGF7 (Adkins et al. 1996) with primers *General WtS nt1-31 F* (5'-CACCATGA GAATTCTAAACTACTAGAACTAGTCG-3') and reverse primer *G_N-S fusion nt895-930 R* (5'-AATGCTTTTGAATATT TGATTATGCAATCTCTAAC-3'). G_N soluble (G_N-S) included

only the predicted ectodomain of the glycoprotein, excluding the transmembrane and cytoplasmic domains. A C-terminal fusion to enhanced GFP was done by cloning the amplification product into pENTR/D-TOPO vector and then into the expression vector pSITE-2NB (Chakrabarty et al. 2007) by means of Gateway cloning technology (Invitrogen, Carlsbad, CA, U.S.A.).

Tomato (*Solanum lycopersicum* L.) 'Money-maker' cotyledon and hypocotyls explants were transformed via *Agrobacterium tumefaciens* (strain LBA 4404) carrying the G_N-S::GFP expression clone (Park et al. 2003). Transgenic tomato plants were kept in a greenhouse within a temperature range of 25°C (day) to 23°C (night) at a light cycle of 16 h of light and 8 h of darkness. Tomato fruit were periodically harvested from the plants and stored at 4°C until processing for seed extraction.

Generation of peptide antibodies that recognize G_N.

Three polyclonal peptide antibodies against TSWV G_N were custom made during this work to enable detection of G_N-S by Western blot and triple-antibody sandwich (TAS)-ELISA (GenScript USA Inc., Piscataway, NY, U.S.A.). A polyclonal peptide rabbit anti-G_N was custom made against a 14-amino-acid peptide (SQTPGTRQIREEEES) and two independent goat anti-G_N antibodies against another 14-amino-acid peptide (DKSAKIPKTNKRVR). The goat antibody was designed to recognize a conserved domain in tospovirus G_N proteins and the rabbit antibody to target a peptide unique to TSWV. Both 14-amino-acid peptides localize to the N-terminus region of G_N, starting at residue 78 and 251, respectively, from the start methionine of the polyprotein.

ELISA screening of transgenic plants.

ELISA was used to test transgenic plants for the presence of G_N-S::GFP and NPTII enzyme. Presence of NPTII was assayed by a DAS-ELISA, using a commercial detection kit (Agdia Inc., Elkhart, IN, U.S.A.), following the recommendations of the manufacturer. The presence of G_N-S protein fusion to GFP was tested by TAS-ELISAs using either i) goat and rabbit peptide polyclonal antibodies generated against the ectodomain of G_N protein (GeneScript USA Inc.) or ii) goat peptide polyclonal antibodies against G_N in combination with a rabbit polyclonal antibody against GFP (Invitrogen). In addition, the presence of transgenes and expression of full-length genes was confirmed by PCR and RT-PCR, respectively (data not shown).

Plant material for ELISA confirmation of protein expression consisted of a leaf disc (diameter approximately 13 mm) that was cut from the apical leaflet of the youngest completely developed leaf (leaf node number three) of each tomato plant (3 weeks old). The leaf disc was ground in 1 ml of general extraction buffer (GEB; Agdia Inc.). A working volume of 100 µl per well was used for the different steps of the ELISA. i) For the anti-G_N TAS-ELISA, the polyclonal rabbit anti-G_N antibody was the coating antibody used at a concentration of 0.4 µg/ml. The polyclonal goat anti-G_N antibodies number 163 and 164, at 0.2 µg/ml, were used as secondary antibodies. ii) For the anti-G_N/GFP TAS-ELISA, the polyclonal goat anti-G_N antibodies number 163 and 164, diluted 0.4 µg/ml each, were used as coating antibodies. A commercial polyclonal rabbit anti-GFP (Invitrogen) diluted to 0.8 µg/ml was used as the secondary antibody. In the NPTII ELISA, the cut-off value to declare a sample positive corresponded to two times the mean of negative controls and, for the G_N-S::GFP ELISAs, the cut-off value corresponded to the mean of negative controls plus three times the standard deviation of the negative samples.

Quantification of recombinant protein in transgenic plants.

The amount of recombinant protein (G_N-S::GFP) in 17 T1 transgenic plants was estimated using a quantitative GFP

ELISA (MaxDiscovery green fluorescent protein ELISA kit; Bioo Scientific, Austin, TX, U.S.A.). The T1 plants analyzed were 1 plant from T0 G_N-S::GFP number 3, 2 from G_N-S::GFP number 6, 1 from G_N-S::GFP number 7, 12 from G_N-S::GFP number 9, and 1 from G_N-S::GFP number 11. Per every molecule of recombinant fusion protein there was one molecule of GFP; therefore, the amount of GFP in the plant tissue was an indirect measurement of the amount of the recombinant protein. Transgenic leaf tissue was sampled at 500 mg/plant and ground in 0.5 ml of protein extraction buffer included in the kit. The samples were treated and the ELISA conducted as indicated in the manufacturer's protocol. Total soluble protein per sample was quantified using Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, U.S.A.) and standardized to total soluble protein at 3 mg/ml.

One mole of G_N-S::GFP contains one mole of GFP; therefore, to estimate the amount of G_N-S::GFP protein in each sample, the following formula was used: grams of G_N-S::GFP = grams GFP × 63,416/23,283, where 63,416/23,283 corresponds to the ratio between the formula weight of G_N-S::GFP and GFP, respectively. To compare the relative amount of recombinant protein among plants, the amount (in nanograms) of G_N-S::GFP was expressed per milligram of total soluble protein in the sample.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blots.

Protein expression was confirmed by separation of total protein extracts by sodium dodecyl sulfate polyacrylamide gel electrophoresis and detection by Western blot, as previously described (Whitfield et al. 2004). Primary antibodies were polyclonal peptide rabbit anti-G_N and polyclonal rabbit anti-GFP (Invitrogen), diluted in phosphate-buffered saline (PBS) supplemented with 5% nonfat dry milk (NFD) at 1:2000 and 1:2500, respectively, and incubated for 2 h. Secondary horseradish peroxidase-conjugated antibodies (Bio-Rad, Hercules, CA, U.S.A.) were diluted 1:5000 and incubated for 1 h. Three rinses, each 100 ml of PBS Tween and 5% NFD for 5 min, were done between incubations with antibodies. Western blots were visualized with ECL Plus Western blotting detection system (GE Healthcare, Little Chalfont, BKM, U.K.).

Fluorescence microscopy of transgenic tissue.

The presence of the GFP-fused recombinant protein in the transgenic leaf tissue (abaxial surface) was evaluated by fluorescence microscopy of leaf sections (0.5 by 0.5 cm) mounted in distilled water. The plant leaf tissue was observed by confocal microscopy (Zeiss Axiovert 200M microscope equipped with a Zeiss LSM 510 META system or a LSM 700 Confocal Laser Scanning microscope with Zen Efficient Navigation software).

Challenge of G_N-S::GFP transgenic plants with TSWV.

Three-week-old transgenic G_N-S::GFP tomato plants that tested positive for the presence of the recombinant protein were inoculated with TSWV in order to evaluate the response of the plants to TSWV infection and to evaluate whether G_N-S::GFP is detectable in plant tissue after virus infection. In all, 18 T1 plants and 20 nontransgenic tomato plants were mechanically inoculated. For inoculation, leaf tissue from symptomatic, ELISA-positive tomato plants 3 weeks after inoculation was used as the virus source. Tomato plants were evaluated for virus presence 15 days after inoculation; apparently negative plants were reinoculated immediately and retested 15 days after the second inoculation.

Leaf tissue samples (1.3-cm-diameter disc) were collected from inoculated leaf tissue (old leaf) and from new systemic

tissue (newly emerged leaf) for each plant. The terminal leaflet of the second true leaf (from bottom to top) was used to collect the mechanical inoculated leaf sample or local infection sample, and the terminal leaflet of the second younger leaf (from top to bottom) was collected as the systemic infection sample. Each leaf disc was homogenized in GEB and aliquots were tested separately for G_N-S::GFP and TSWV N presence by TAS-ELISA (described previously) and DAS-ELISA (anti-TSWV reagent set; Agdia Inc.) protocols, respectively. In the case of anti-TSWV DAS-ELISA, samples were considered positive when the absorbance value was higher than three times the mean absorbance value of the negative controls (noninoculated, healthy tomato plants of the same age as the inoculated ones).

TSWV acquisition test on transgenic tissue.

To determine the effect of transgenic tomato plants on TSWV acquisition by thrips, four independent experiments were conducted. For each experiment, leaflets (leaf node six from the top of the plant) from TSWV-infected transgenic and nontransgenic plants were placed in 15% agar plates. For each experiment, each treatment (transgenic or nontransgenic) was replicated three times (plates). Transgenic plants used for acquisition and transmission assays were confirmed to be TSWV positive and to harbor recombinant protein 15 days after inoculation. Moreover, fluorescence observations of the TSWV-infected plants confirmed the presence of G_N-S::GFP in these plants. Plants G_N-S::GFP number 9.302 (a T1 plant derived from T0 plant number 9) and nontransgenic number 113 were used in experiment one, and plants G_N-S::GFP number 9.517 (another T1 plant derived from T0 plant number 9) and nontransgenic-C were used for the other three experiments. Plants G_N-S::GFP number 9.302 and nontransgenic number 113 were mechanically inoculated and plants G_N-S::GFP number 9.517 and nontransgenic-C were thrips inoculated with TSWV and confirmed by ELISA to be positive for TSWV and G_N-S::GFP presence 15 days after inoculation. For the acquisition of virus, L1 thrips 0 to 18 h old were placed on plant tissue that was contained in petri plates with 15% water agar and sealed with parafilm. The plates were kept at an incubator (25°C, with a photoperiod of 12 h of light and 12 h of darkness) for a 24-h AAP. The larval thrips were moved to plastic cups with green bean pods for another 24-h period to allow virus replication inside the thrips and clearing of TSWV-infected plant tissue from the gut lumen. From each cup (replicate), 15 thrips were collected into a 1.5-ml microfuge tube and homogenized with a micropestle in 150 µl of ice-cold GEB. Samples were vigorously vortexed (30 s), incubated on ice for 30 min, and tested by DAS-ELISA (Agdia Inc.) for TSWV presence. Additionally, plant tissue (a leaf disc, diameter approximately 13 mm) was collected after the AAP from each replicate plate and tested for TSWV infection by DAS-ELISA in order to compare the relative virus titer in plant tissue used for AAP. Control thrips not exposed to TSWV-infected tissue were also sampled. Thrips cups were maintained in an incubator and fresh green beans added until adult eclosion (8 to 9 days). ELISA absorbance values for thrips (15 pooled individuals) or for plant tissue used for AAP were compared by treatment (TSWV-infected versus healthy) by the Mann-Whitney test in GraphPad Prism (v.5.04; GraphPad Software, La Jolla, CA, U.S.A.).

Determination of TSWV acquisition by qRT-PCR.

To determine the normalized abundance of TSWV N transcripts in larval or adult thrips after an AAP on transgenic and nontransgenic TSWV-infected tissues, four thrips were collected per replication 24 h after an AAP (L1 thrips 42 to 50 h

old) or adult thrips immediately after a 48-h inoculation access period for three independent experiments. The single thrips were placed into individual RNase-free 1.7-ml microcentrifuge tubes, flash frozen in liquid nitrogen, and stored at -80°C . RNA was extracted from individual thrips, real-time qRT-PCR was done, and TSWV N normalized abundance was calculated, as described previously (Rotenberg et al. 2009). The normalized abundance of TSWV N RNAs to actin expression (internal reference) values were $\log_{10}$ transformed and compared by experiment between treatments (TSWV-exposed versus healthy-tissue-exposed) by the Mann-Whitney test in GraphPad Prism. The difference in TSWV abundance between thrips exposed to nontransgenic or transgenic plants was estimated for larval or adult thrips by dividing the nontransgenic by the transgenic median normalized TSWV abundance value calculated for all biological replicates.

Inoculation access period.

Adult thrips (24 h after eclosion, 10 individuals per replicate) from each thrips cohort (three independent experiments) exposed to transgenic and nontransgenic TSWV-infected tissue were individually tested for transmission of TSWV in a 48-h period, as previously described (Rotenberg et al. 2009). Each experiment included three biological replicates for transgenic and for nontransgenic tissue, for a total of 60 thrips per experiment. Four thrips per replicate per experiment were randomly sampled for qRT-PCR after the inoculation access period. Normalized TSWV N RNA values ($\log_{10}$ transformed) were compared by experiment between treatments (TSWV-exposed versus healthy-tissue-exposed) by the Mann-Whitney test in GraphPad Prism. The efficiencies of TSWV transmission by groups of 10 adult thrips per replicate per experiment were compared between treatments (fed as larvae on transgenic plant tissue versus fed on nontransgenic plant tissue) by the Mann-Whitney test in GraphPad Prism.

Correlation analyses for virus titer

in larval thrips and virus titer or transmission in adults.

Correlation analyses in Figure 7 were done with the Spearman's rank correlation coefficient in GraphPad Prism (v.6.00; GraphPad Software) and the figures were generated with SigmaPlot 12.0. For Figure 7A, the value of normalized abundance of TSWV N RNAs (median out of four individual thrips) tested per cohort of thrips exposed to TSWV-infected transgenic and nontransgenic plants was scatter graphed (XY) and analyzed by Spearman rank correlation coefficient to test the correlation between TSWV abundance in larval thrips and the corresponding virus abundance in adult thrips originating from the same AAP cohort. In Figure 7B, the relative titer of virus (determined by ELISA) in a pooled sample of 15 larval thrips was scatter graphed against the percentage of transmission of adult thrips (sample of 10 thrips per cohort, tested individually for transmission) originating from the corresponding AAP cohort as the 15 larval thrips.

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