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Effect of defective interfering RNAs on the vertical transmission of *Tomato black ring virus*

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Abstract: Viruses are thought to be the ultimate parasites, using host resources for multiplication. Interestingly, many viruses also have their own 'parasites', such as defective interfering RNAs (DI RNAs). One of the plant viruses whose infection can be accompanied by subviral RNAs is the *Tomato black ring virus* (TBRV). DI RNAs associated with the TBRV genome were generated *de novo* as a result of prolonged passages in one host. DI RNAs modulate the TBRV accumulation and the severity of the symptoms induced on the infected plants. In this study, we have addressed the question of whether DI RNAs can also affect TBRV vertical transmission through seeds. The experiments were conducted using the TBRV-Pi isolate and *Chenopodium quinoa* plants. *C. quinoa* plants were infected with TBRV-Pi with and without DI RNAs. Overall, 4 003 seeds were tested, and the analysis showed that the presence of DI RNAs made the TBRV-Pi seed transmission 44.76% more efficient. Moreover, for the first time, we showed that DI RNAs are being transferred from generation to generation.

Keywords: TBRV; *Chenopodium quinoa*; DI RNAs; DAS-ELISA; seed transmission

Infection by RNA viruses can be accompanied by subviral RNAs, such as defective RNA (D RNA), defective interfering RNA (DI RNA) and satellite RNA (satRNA) (Simon et al. 2004; Pathak & Nagy 2009). These subviral RNAs are relatively short, non-infectious and their replication, encapsidation and spread depend on a helper virus. The presence of subviral RNAs might have a great impact on the viral replication, accumulation and symptoms observed on the infected plants (Graves et al. 1996; Pathak & Nagy 2009; Hasiów-Jaroszewska et al. 2018). Additional RNA molecules have been found in many representatives of the *Nepovirus* genus, which infect a wide range of economically important plants, contributing to losses in the quality and yield worldwide. The *Tomato black ring virus* (TBRV) is a member type of the genus *Nepovirus* within the *Secoviridae* family. TBRV is distributed worldwide and, since 1957, there have been reports of significant damage caused by TBRV infections to several

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important hosts: strawberry, potato, celery, and artichoke (Gallitelli et al. 2004). TBRV effectively infects quinoa (*Chenopodium quinoa* Willdenow), which is a crop traditionally grown in the Andes highlands (Galwey 1989). Moreover, quinoa has high levels of tolerance to various adverse abiotic factors, such as drought, frost and salinity (Galwey 1989; Jacobsen et al. 1998), making it a crop with a great potential for global agriculture as problems with drought and salinization are growing.

TBRV is transmitted by plant-parasitic nematodes of the genera *Xiphinema* and *Longidorus* (Harrison et al. 1961; Brunt et al. 1966). The virus can be also transmitted through seeds of different plant species, which makes it possible for the virus to spread over wide areas (Lister & Murant 1967; Pospieszny et al. 2020). Recently, Pospieszny et al. (2020) have shown that TBRV is effectively transmitted with tomato seeds. Indeed, the efficiency of the seed transmission is dependent on the combination of the tomato cultivar and the virus isolate, ranging from 1.69 to 14.57%. Infected seeds play a major role in TBRV epidemiology: (*i*) providing an initial source of inoculum on the field, (*ii*) serving as a major route for long-distance dissemination, (*iii*) enabling the virus to survive in the environment by means of vertical transmission. The TBRV genome consists of two single-stranded RNAs of positive polarity. Both RNAs contain a small protein VPg (viral protein genome-linked) at their 5' ends and have polyadenylated 3' ends. RNA1 is used for the viral replication and polyproteins' processing and RNA2 is used for the encapsidation and movement in the plant (Digiaro et al. 2015). The TBRV infection can be accompanied by subviral RNAs, such as satellite or defective RNAs. SatRNAs share little sequence similarity with the viral genomic RNAs, whereas D RNAs are derived from the genome of the helper virus by further deletions or rearrangements. D RNAs are synthesised by the viral RNA-dependent RNA polymerase (RdRp) that also replicates the parental virus genomes. D RNAs are often generated *de novo* as a result of prolonged passages in one host and contain parts of the non-coding regions of their helper virus' genome and preserve a portion of the open reading frame(s) (Graves et al. 1996; Pathak & Nagy 2009; Hasiów-Jaroszewska et al. 2012). They contain all the *cis*-acting elements necessary for their continued replication by the viral RdRp and can accumulate *de novo* to very high levels. They often interfere with the replication of the parent viral RNAs and prevent the over-accumulation of viral products (Paudel & Sanfaçon 2018). D RNAs interfering with the multiplication of their helper viruses are called defective interfering RNAs (DI RNAs). In the infected plants, the DI RNA could represent up to 60% of the virus specific RNA, but the percentage of encapsidated DI RNA could be as low as 3–4%, and the majority of DI RNAs is not transmitted by vectors. Most DI RNA can efficiently accumulate in inoculated tissue, but they do not always move systemically, as exemplified by the *Cucumber mosaic virus* (CMV) DI RNA, which disseminates over long distances in tobacco plants, but not in tomato plants. The presence of D RNAs was also confirmed for some TBRV isolates. Short D RNAs (approximately 400–500 nt) originated from TBRV-genomic RNA 1 during prolonged passages in one host (Jończyk et al. 2004; Hasiów-Jaroszewska et al. 2012; Rymelska et al. 2013). It has been recently shown that TBRV DI RNA strongly affected the development of the symptoms and virus accumulation in infected plants (Hasiów-Jaroszewska et al. 2018). The magnitude of the interference effect depends on the interplay between the TBRV isolate and the host species.

Considering the importance of the seed transmission in TBRV epidemiology and the interference of subviral RNAs with the virus replication, here, we aimed at analysing the impact of DI RNAs on the TBRV transmission through *C. quinoa* seeds. TBRV is transmitted through *C. quinoa* in the range from 30 to more than 60% depending on the isolate used (Pospieszny, unpublished data). This high range of seed transmission indicated that the TBRV-*C. quinoa* model will be useful for this type of experimental studies.

MATERIAL AND METHODS

Seed transmission. In this research, a TBRV-Pi isolate was used which originally did not have any additional RNAs. DI RNAs arose from the TBRV-Pi genome after 15 passages (105–110 days) in *C. quinoa* plants under greenhouse conditions (Hasiów-Jaroszewska et al. 2018). The *C. quinoa* plants were mechanically inoculated (at the five-leaf stage) with the TBRV isolate with and without the DI RNA. The fragments of the plants were ground with a 0.05 M phosphate buffer (8.7 g/L of KH_2PO_4; pH 7.2) at a ratio of 1 : 5 (w/v). The mechanical inoculation consisted of the gentle rubbing on lightly Carborundum-dusted leaves (300 mesh grit powder). The mock plants were inoculated with the buffer. The plants

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were grown until seed maturity in an insect-free greenhouse at 23 – 26 °C and exposed to a 14-h light period. The plants were also monitored weekly for the development of symptoms. The collected seeds were disinfected by incubating them in 10% (w/v) trisodium phosphate for two hours, rinsed with water for 30 min and then dried. This treatment was carried out to minimise the possibility that any viral infection that had occurred was not simply the result of the presence of the virus on the seed coat, but rather as the result of an embryonic infection (Ling 2008). The seeds were stored in paper bags at 5 °C for four weeks. In order to determine the seed transmission rates of the *C. quinoa* seeds, the seeds collected from infected plants were sown in 24-well trays containing a sterilised substrate (2 : 1; peat : sand), with a single seed per well. The seedlings were grown in a greenhouse at temperatures ranging from 22 to 25 °C with a 14-h photoperiod. The plants were also monitored for the development of symptoms. Five to six weeks after sowing, each plant was individually tested using the double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and bioassayed in the *C. quinoa* plant. The leaf tissue collected from each seedling was homogenised in a plastic bag (BioReba, Switzerland) with an extraction buffer (1 : 10 w/v) (Loewe, Germany) using a Homex 6 homogeniser (BioReba AG, Switzerland). The samples were tested in duplicate wells per sample. The DAS-ELISA test was performed in accordance with the manufacturer's instructions, using a TBRV-specific antiserum supplied by DSMZ (DSMZ, Germany) and 100 µL of the plant extract. The healthy and TBRV-infected *C. quinoa* leaf samples were included in each ELISA plate to serve as negative and positive controls, respectively. The absorbance values (A_{405}) were measured using an immune plate reader (BioTek Instruments, USA). The recorded values in the infected samples were more than twice the values of the healthy control plants. To confirm that the ELISA-negative samples contained no viable virus particles, a bioassay on the *C. quinoa* plant was conducted. The mechanical inoculation consisted of the gentle rubbing on the lightly Carborundum-dusted (300 mesh grit powder) *C. quinoa* leaves. The plants were maintained in an insect free greenhouse at 23 – 25 °C and exposed to a 14-hour light period. The symptoms were evaluated three weeks after the inoculation and the presence of the TBRV in the infected plants was verified by ELISA as described above. The plants which grew from the infected seeds and which tested positive for the presence of TBRV served as sources for the next generation of seeds, and the above-mentioned experiments were performed in the next season (2018). Moreover, the plants from the second generation were verified for the presence of DI RNAs. The purified viral preparations were obtained from 50 g of the plant tissue in a sucrose gradient as described previously (Rymelska et al. 2013). The viral RNA was isolated from the purified virus preparation using proteinase K digestion and phenol/chloroform extraction and subsequent ethanol precipitations as described by Sambrook et al. (1989). The occurrence and length of the extracted RNA were analysed by electrophoresis in a 1% agarose gel with Midori Green and viewed in UV light. The molecules shorter than the TBRV-genomic RNAs were extracted from the gel and purified using a Zymoclean™ Gel RNA Recovery Kit (ZymoResearch, USA), according to the manufacturer's instructions. The small RNAs were amplified using a Transcriptor One-Step RT-PCR Kit (Roche, Germany), according to the manufacturer's instructions. In order to amplify the short RNAs, a set of the primers complementary to the conserved 5' and 3' UTRs of both the TBRV RNA1 and RNA2 was used (Hasiów-Jaroszewska et al. 2012; Rymelska et al. 2013). The obtained RT-PCR products were directly sequenced by an external company (Genomed, Warsaw, Poland) and the sequences were compared with other TBRV D RNAs described to date.

Statistical analysis. The data obtained from the seed transmission experiments were fitted to a logistic regression model using a factorial generalised linear model (GLM) with binomial distribution and a logit link function (based on the lowest Bayesian information criterion among alternative models). The model incorporates the experimental blocks (2017 and 2018) and the presence/absence of DI RNAs as orthogonal factors. The statistical significance of each main factor and their interaction was assessed using a likelihood-ratio test that asymptotically follows the χ^2 probability distribution.

RESULTS

The plants infected with two TBRV variants displayed different type of symptoms. Generally, the plants mechanically inoculated with TBRV-Pi without the DI RNA as well as those which grew from the infected seeds (originated from plants infected with TBRV-Pi without the DI RNA) – displayed

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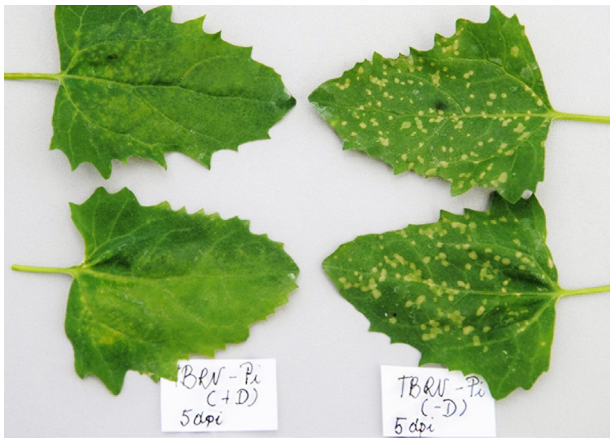

Figure 1. Local symptoms on the *C. quinoa* leaves caused by the mechanical inoculation of the TBRV-Pi with DI RNA (left) and TBRV-Pi without DI RNA (right)

DI RNA – defective interfering RNA; TBRV-Pi indicates + DI RNA; TBRV-Pi indicates – DI RNA

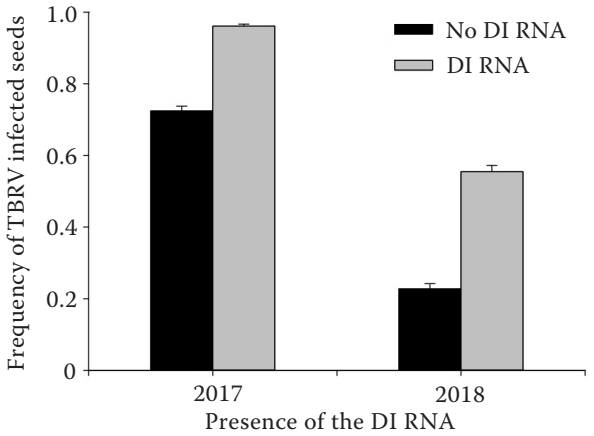

Figure 2. Estimated frequencies of the TBRV seed transmission in two experimental blocks and in the presence or absence of the DI RNAs

The means and standard deviations (SD) were estimated from the logistic regression model described in the methods section (omnibus goodness-of-fit test: χ^2 = 1 364.7233, 3 *df*, $P < 0.0001$); the error bars represent ± 1 SD

symptoms of severe chlorosis, leaf malformations and growth reduction. On the contrary, the plants infected with TBRV-Pi with the DI RNAs and those which grew from the collected seeds (originated from plants infected with TBRV-Pi with the DI RNA) displayed only mild symptoms of infection (Figure 1). It confirms that the DI RNAs play a role in the symptom attenuation. Overall, in 2017 and in 2018, 1 190 and 1 221 and 770 and 822 seeds collected from the plants infected with TBRV with and without the DI RNAs were analysed, respectively. The results from the ELISA test (Table 1) show that the presence of the DI RNAs has a significant impact on the ratio of the seed transmission. Firstly, a significant difference exists between both experimental blocks (Figure 2; χ^2 = 999.4191, 1 *df*, $P < 0.0001$), with a 57.63% higher average frequency of the seed transmission in the first generation (2017) than in the second one (2018). Secondly, the presence of the DI RNAs in the infected plants significantly increases the rate of the seed transmission (Figure 2; χ^2 = 463.6381, 1 *df*, $P < 0.0001$). On average, plants containing the DI RNAs transmit the TBRV via the seeds 44.76% more effectively than the plants without the DI RNAs. Thirdly, the effect of the presence of the DI RNAs on the seed transmission is not consistent among the years, but significantly higher in the second experiment (2018) (Figure 2; χ^2 = 17.7723, 1 *df*, $P < 0.0001$): in the first block, the presence of DI RNAs increased the seed transmission efficiency 1.33-fold, and the magnitude of the increase in the second block was more than twice this value (2.45-fold). Interestingly, in the symptomatic *C. quinoa* plants that grew from seeds infected with TBRV-Pi with the DI RNAs, the presence of the DI RNAs was confirmed. The additional band of about 500 nt in length was observed in an agarose gel (Figure 3). The RNA was purified and subjected to RT-PCR. The sequencing of the RT-PCR products confirmed the presence of the DI RNAs identical with those previously described

Table 1. Effect of the DI RNAs on the rate of the TBRV transmission through the seeds of *C. quinoa*

Presence of DI RNA in plants	Number of seeds infected	Number of seeds tested	Rate of seed-transmission (%)
First generation (2017)			
+ DI RNA	1 144	1 190	95.8
– DI RNA	885	1 221	72.5
Second generation (2018)			
+ DI RNA	427	770	55.5
– DI RNA	187	822	22.7

DI RNA – defective interfering RNA

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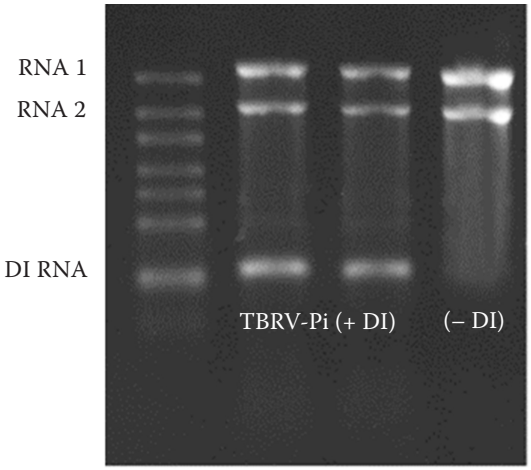

Figure 3. Electrophoretic separation of the RNAs obtained from the purified viral preparations from the plants which grew from seeds infected with the TBRV-Pi with DI RNA and TBRV-Pi without DI RNA

DI RNA – defective interfering RNA

for TBRV-Pi. The observed DI RNAs contained a small fragment of a 5' non-coding region (20 nt), portion of the C-terminal end of the RNA dependent RNA polymerase (RdRp) and the almost entire 3' non-coding region of the genomic RNA 1 (Hasiów-Jaroszewska et al. 2018). This finding suggests that the DI RNAs are transferred (through the seeds) from generation to generation. The results for both generations regarding the transmission rate are slightly different, however, they indicated that, in both cases, the presence of the DI RNA leads to an increase in the seed transmission.

DISCUSSION

In a previous study by Hasiów-Jaroszewska et al. (2018), a comparison was made of the accumulation level of two TBRV isolates: TBRV-Pi and TBRV-S1 (collected from lettuce) in the presence and absence of the D RNAs and in the following plant species: *Solanum lycopersicum* Linnaeus, *Lactuca sativa* Linnaeus, *C. quinoa*, and *Nicotiana tabacum* Linnaeus. The RT-qPCR experiments and a further statistical analysis were performed to determine whether the presence of the D RNAs had an impact on the parental virus accumulation (Hasiów-Jaroszewska et al. 2018). For both TRBV-Pi and TRBV-Sl, the maximum accumulation was observed in *C. quinoa* and the lowest one was observed in *S. lycopersicum*. Nevertheless, the presence of the D RNA reduces the accumulation of the TBRV by approx. 26%, on average. It has been shown that the D RNAs, which derived from the TBRV genome, interfere with the parental virus replication (Hasiów-Jaroszewska et al. 2018). Here, it has been shown that TBRV can be very effective in the transmission through *C. quinoa* seeds and the presence of the DI RNAs has a significant impact on this phenomenon. The plants infected with TBRV without the DI RNAs often displayed chlorotic or necrotic symptoms, whereas the symptoms were often attenuated on those infected with TBRV plus the DI RNAs. This confirmed significant role of DI RNAs on the symptom development. The development of necrotic symptoms is generally thought to play a role in restricting the virus movement. The slow movement of the virus may not only limit the virus accumulation in floral and macrogametophyte tissues, but could also impede the viral establishment in the embryo meristem, generally considered essential for the seed transmission (Hanada & Harrison 1977). Probably, the attenuation of the symptoms by DI RNAs favoured a wider transmission range and better adaptation of the virus to the ever-changing environment. Three major mechanisms of interference by the DI RNAs have been described: (*i*) competition for viral and host resources, which impairs the virus replication and attenuates symptoms; (*ii*) the DI RNAs-triggered a post-transcriptional gene silencing (PTGS gene silencing response); (*iii*) modulation of the functions of the viral factors (Holland et al. 1978; Szittya et al. 2002; Lukhovitskaya et al. 2013). New models for the DI RNA-mediated reduction in helper virus levels and symptom attenuation include the DI RNA enhancement of PTGS, which is an antiviral defence mechanism in plants (Simon et al. 2004).

Competition for limited viral and host resources drive the evolution of the D RNAs as faster rates than that of their parental virus. Due to the fact that DI RNAs have to compete with the helper virus for the viral RdRp, they continuously evolve under high selection pressure. The rate of the DI RNAs evolution is likely to be faster than their helper viruses (Pathak & Nagy 2009). The formation and accumulation of the DI RNA is regulated by the number of factors that depended on the virus isolate, host and the interaction between the virus and the host, given a specific environmental condition (Inoue-Nagata et al. 1997). The primary determinant of the seed transmission was found to be in the TBRV-genomic RNA1 (Hanada & Harrison 1977). The property of the seed-borne transmission has been found to be

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linked to the RNA1 of two nepoviruses, *Raspberry ringspot virus* (RpRSV) and TBRV, which exclude the role of the capsid, coded by the RNA2 (Hanada & Harrison 1977). Similarly, the property of the seed-borne transmission has been found to be linked to RNA1 CMV (Hampton & Francki 1992) and the *Pea early-browning virus* (PEBV) (Wang et al. 1997). The RNA 1 of the TBRV codes five functional proteins: The P1A protein contains a conserved motif characteristic for a proteinase-cofactor (PCo). The P1B protein contains sequence motifs characteristic of a nucleoside triphosphate-binding (NTB) helicase, the P1C protein is the viral genome linked protein (VPg), the P1D protein is the proteinase (Pro) and the P1E protein has sequence motifs characteristic of aRdRp (Pol). A common characteristic of the major determinants of seed-borne transmission is the presence of cysteine-rich motifs that regulate the transmission process by affecting the replication and movement of the virus in the reproductive tissue of the respective hosts, and thereby influence the infection of the embryo (Johansen et al. 1994). DI RNAs probably modulate the function of the protein coded by RNA1 by interfering with the viral gene products. However, further studies are necessary to analyse the TBRV – DI RNAs interactions.

In summary, this study has shown that TBRV can be effectively transmitted through *C. quinoa* seeds and the presence of DI RNAs has a significant impact on this phenomenon. Moreover, we have proven that DI RNAs can be transmitted through seeds from generation to generation.

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