SHORT COMMUNICATION

THE SMALL GENOMIC SEGMENT OF **TOMATO SPOTTED WILT VIRUS ISOLATE BR20** IS NECESSARY BUT NOT SUFFICIENT TO INDUCE LETHAL NECROSIS IN **NICOTIANA BENTHAMIANA** AND LOCAL NECROTIC LESIONS IN **NICOTIANA TABACUM** cv. WHITE BURLEY

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**SUMMARY**

A consistently different phenotype was observed in *Nicotiana benthamiana* and *Nicotiana tabacum* cv. White Burley plants infected by the Brazilian *Tomato spotted wilt virus* (TSWV) isolate Br20 and the two Italian isolates p272 and p105/43.14. Based on the phenotype of experimentally obtained reassortants carrying the small (S) genomic segment of p272 or p105/43.14 and the medium (M) and large (L) segments of Br20, we concluded that the small segment of Br20 is required for eliciting lethal necrosis in *N. benthamiana* and local necrotic lesions in *N. tabacum*. To evaluate whether the Br20 nucleocapsid (N) or the non-structural (NSs) protein encoded by the S segment were able to cause the distinctive symptoms, either alone or in combination, the three allelic variants (Br20, p272 and p105/43.14) of each gene were transiently expressed *in planta* through agroinfiltration. All the alleles of the N and NSs proteins accumulated abundantly in infiltrated *N. benthamiana* and *N. tabacum* leaves. No specific necrotic reaction was observed in any of the single or combined agro-infiltrations of the Br20 alleles, suggesting that the S-encoded proteins of isolate Br20 are not sufficient to elicit the specific symptomatic response and that the co-involvement of other regions of the genome might be necessary.

Key words: Tospoviruses, Tomato spotted wilt virus, symptom severity, reassortment, agroinfiltration.

*Tomato spotted wilt virus* (TSWV), the type member of the genus *Tospovirus*, family *Bunyaviridae*, causes major economic losses to a wide range of plants (Germann et al., 1992; Parrella et al., 2003). The viral genome consists of three single-stranded RNA segments of negative or ambisense polarity. The large (L) segment (8.9 kb) encodes the RNA dependent RNA polymerase in the complementary sense (de Haan et al., 1991). The medium (M) segment (4.8 kb) encodes the glycoproteins precursor in the complementary sense and the non-structural NSm protein in the viral sense (Kormenlink et al., 1992). The small (S) segment (2.9 kb) encodes the nucleocapsid (N) protein and the non-structural protein (NSs) in the complementary and viral sense respectively (de Haan et al., 1990; Kormenlink et al., 1991). In particular, the N protein is the main component of the viral nucleocapsid. Its involvement is crucial to the viral cycle, regulating functions in early events of transcription and replication (Steinecke et al., 1998). The same protein also interacts with the viral polymerase, with the envelope glycoproteins, and with the tubular structures formed by the NSm protein (Uhrig et al., 1999). Moreover, it binds ssRNA *in vitro* (Richmond et al., 1998) and interacts with the Gc protein *in vivo* (Snippe et al., 2007).

The NSs protein is expressed in infectious thrips (Wijkamp et al., 1993) and accumulates in the salivary glands, suggesting a possible role in vector transmission (Goldbach et al., 1996). This protein has recently been identified as a silencing suppressor (Bucher et al., 2003; Takeda et al., 2002) and evidence for its involvement in specific virus-host interactions has been given (Margaria et al., 2007). The amount of NSs was hypothesized to be associated with the severity of symptom expression (Kormenlink et al., 1991).

We observed a consistently different phenotype on plants infected by the Brazilian TSWV isolate Br20 in comparison with two previously characterized northern Italian isolates, p272 and p105/43.14, capable of infecting *Tsw* resistant pepper (Margaria et al., 2007). Symptom differences were consistent in the environmental conditions tested and previously described (Roggero et al., 2002). However, inoculum obtained from the source host *Emilia sonchifolia* prevented interference from defective RNAs (Inoue-Nagata et al., 1997). Br20 causes a rapid lethal necrosis in *N. benthamiana*, about 10 days post-inoculation, and local necrotic lesions in *N. tabacum* cv. White Burley, in contrast to the Italian isolates, which both cause a mild mosaic without necrosis and crumpling of the young leaves in *N. benthamiana* and chlorotic symptoms in *N. tabacum* (Fig. 1 and data not shown for isolate p272).
In a previous work, reassortant isolates carrying the L and M segments of Br20 and the S segment of p272 (reassortant isolate IFA201) or p105/43.14 (reassortant isolate IFA231) were obtained (Margaria et al., 2007). Interestingly, these isolates elicited the mild symptoms typical of the Italian parental strain in *N. benthamiana* and *N. tabacum*, but no necrotic response (Fig. 1 and data not shown for reassortant isolate IFA201). These findings suggested that the S RNA was involved in determining the differences in symptomatology, and that the S segment of Br20 was necessary for the induction of lethal necrosis in *N. benthamiana* and local necrotic lesions in *N. tabacum*.

Since the full S segment of the three parental isolates had previously been sequenced (Margaria et al., 2007) (Br20 accession No. DQ915948; p272 accession No. DQ376181; p105/43.14 accession No. DQ376182), a comparison of the Italian and Brazilian isolates showed that several nucleotide (nt) mutations occurred in the coding and non-coding regions of these sequences. With respect to the proteins, one silent nt mutation was observed between the two Italian isolates in the N gene. Several nt and four amino acid (aa) differences were present in the Br20 N protein-encoding ORF in comparison with the comparable Italian alleles. Moreover, several mutations at the nt and aa level were always present between the Italian and Brazilian NSs sequences. These differences allowed us to speculate on a possible role of these proteins in eliciting the specific Br20-associated necrotic response.

As a further step, we wanted to verify if a specific protein encoded by the Br20 S genomic segment was sufficient to elicit the specific necrotic symptoms. To this aim, the entire S segments from the three virus isolates were cloned in both orientations, and tested, either alone or in combination, for their capability to induce the necrotic response in agroinfiltrated plants. For cloning purposes, viral nucleocapsids were purified from infected *N. benthamiana* leaves and nucleic acids extracted with phenol-chloroform procedure and Na-Acetate/Ethanol precipitation (de Haan et al., 1990). The S RNA of each isolate was purified from 1% TAE gel (Qiogene, USA) and used as a template to obtain cDNA using the J13 primer containing the eight terminal nucleotides conserved in all tospoviral RNA termini as described (Margaria et al., 2007). A BamHI restriction site was inserted at the 5' and 3' ends to allow the ligation reaction of the purified PCR product (Qiagen, USA) to the binary plasmid pBin61 (Bendahmane et al., 2002), in the presence of 3 unit of the T4 DNA ligase (Promega, USA). Six recombinant plasmids were obtained, carrying the S segment of Br20, p272 or p105/43.14 in both orientations, to allow the expression of the two proteins encoded by each isolate under the 35S promoter and the Nos terminator (Tab. 1).

Each clone was sequenced to confirm that mutations were not introduced into either of the two ORFs during the amplification reaction by the thermo-stable DNA polymerase (data not shown). Liquid cultures of transformed C58C1 *Agrobacterium tumefaciens* cells were used to infiltrate 15-20-day-old plants of *N. benthamiana* and *N. tabacum* cv. White Burley (Bendahmane et al., 1999). To avoid experimental variation, one half of each leaf was inoculated with the Br20 derived construct and the other half with the analogous construct derived from an Italian isolate or with the vector itself. In order to have the simultaneous expression of the two proteins encoded by the same isolate and check for possible interactions between the two viral proteins that could cause the induction of necrosis, a mixture was also infiltrated of two *A. tumefaciens* cultures transformed with the two clones derived from the same S RNA in

| Table 1. Constructs and derived recombinant proteins used in this study. v-mRNA= viral mRNA; vc-mRNA= viral complementary mRNA. |
|-----------------|-----------------|-----------------|
| pBin61\(^a\) derived Constructs | Encoded mRNA | Encoded protein |
| pBin61-Br20N   | TSWV\(^b\) vc-mRNA | N\(^c\)          |
| pBin61-Br20NSs | TSWV\(^b\) v-mRNA | NSs\(^d\)        |
| pBin61-p272N   | TSWV vc-mRNA    | N               |
| pBin61-p272NSs | TSWV v-mRNA     | NSs             |
| pBin61-p105/43.14N | TSWV vc-mRNA | N               |
| pBin61-p105/43.14NSs | TSWV v-mRNA | NSs             |
| pBin61-TBSV\(^e\) p19 | p19\(^f\) mRNA | p19             |
| pBin61-GFP\(^g\) | GFP mRNA       | GFP             |

\(^a\) pBin61 is the binary plasmid used for agroinfiltration Bendhamane et al., 2002
\(^b\) TSWV= Tomato spotted wilt virus
\(^c\) N= nucleocapsid protein from TSWV
\(^d\) NSs= non structural protein from S segment of TSWV
\(^e\) TBSV= Tomato bushy stunt virus
\(^f\) p19= protein from TBSV known to cause severe necrosis
\(^g\) GFP= green fluorescent protein
Both orientations (not shown).

As a positive control for a viral protein causing lethal necrosis and local necrotic lesions, we agroinfiltrated the pBin61 vector carrying the p19 gene of *Tomato bushy stunt virus* (TBSV) (Scholthof et al., 1995). Vector pBin61 alone or a pBin61-GFP vector expressing the Green fluorescent protein (Margaria et al., 2007) served as negative control.

Western blot analysis was used to assess the *in planta* expression of each of the viral proteins encoded by the S segment and symptoms were monitored to detect any specific phenotypic reaction associated with the expression of a specific allele. Plants were kept under greenhouse conditions (Roggero et al., 2002) and the expression of proteins was examined after three days by Western blot analysis using the primary antibodies A514 (Heinze et al., 1998) and AE353 (Roggero et al., 1998) to detect the N and NSs protein, respectively. Symptom development on the agroinfiltrated plants was monitored daily.

All plants tested positive for protein expression by Western blot analysis three days after agroinfiltration. Both the N and the NSs proteins of Br20 and the Italian isolates accumulated abundantly in the plant hosts after single (Fig. 2 and data not shown for isolate p105/43.14) or combined (not shown) infiltration with the respective clones. The Br20 N protein accumulated at a lower level compared to the alleles of the Italian strains, while no difference was observed in the accumulation level of the NSs proteins.

No specific symptom development associated with the expression of either of the two viral proteins, whether alone or in combination, was observed (Fig. 3 and Table 2). Furthermore, there was no specific response in the negative control, except for chlorotic spots induced on *N. tabacum* 7 days post inoculation.

As expected, we observed a strong necrotic reaction when the pBin61 plasmid encoding the TBSV p19 protein was agroinfiltrated and a green fluorescence in the
plants infiltrated with the pBin61-GFP construct (Fig. 3 and data not shown). We also attempted agroinfiltration of isolate Br20 S-encoded protein in leaves infected with the mild Italian strains, but no necrosis developed (data not shown).

Several determinants have been associated with virus-induced specific symptoms in host plants, i.e. whole ORFs (de Assis Filho et al., 2002; Kagiwada et al., 2005; Szilassi et al., 1999; Tribodet et al., 2005), non coding sequences (Rodriguez-Cerezo et al., 1991; van der Vossen et al., 1996; Zhang et al., 1994), or silent mutations (Hirata et al., 2003).

A limited number of studies have been published on the biology of TSWV-host plant interaction (Best, 1961; Hoffmann et al., 2001; Jahn et al., 2000; Mandal et al., 2006; Margaria et al., 2007; Pang et al., 1993; Qiu et al., 1998, 1999). Pseudorecombination was used to investigate the biological properties of the different segments of the multipartite TSWV genome, by obtaining reassortants from parental strains with different biological properties, and analyzing their symptom expression in different host plants (Best, 1961; Pang et al., 1993; Qiu et al., 1998). For instance, Qiu et al. (1998) showed that the lesion diameter in Cucumis sativus cv. National Pickling maps to segment M and infection to N. tabacum cv. White Burley to segment L of TSWV RNA. In similar experiments, M and the S RNAs of the Hawaiian isolate TSWV-A proved to be associated with the ability to overcome the resistance conferred by the Sw5 and Tsw genes to tomato and pepper, respectively (Hoffmann et al., 2001; Jahn et al., 2000). Influence of the intergenic region (IGR) of segment S in the biological properties of TSWV was studied by Qiu et al. (1998), who reported an involvement of the IGR length in the competitiveness of the reassortment process. Similar results are available for the M segment (Hoffmann et al., 2001). Reassortment was also used by Okuda et al. (2003) to show that the S segment of the taxonomically related Watermelon silver mottle virus was associated with differences in the symptom in Tetragonia expansa.

The influence of the S RNA segment on biological properties was also determined for members of another genus (Phlebovirus) in the family Bunyaviridae that infects animals and humans (Bridgen et al., 2001; Perrone et al., 2007; Vialat et al., 2000).

Previous results have shown that the determinants for biological properties and specific symptomatic expression in TSWV map to different genomic segments ac-

Table 2. Presence (+) or absence (−) of necrotic symptoms on N. benthamiana and N. tabacum cv. White Burley infiltrated with different pBin61 constructs. Necrosis was observed only in plants expressing the TBSV p19 protein positive control.

<table>
<thead>
<tr>
<th>Encoded protein</th>
<th>TBSVp19</th>
<th>GFP</th>
<th>Br20</th>
<th>p272</th>
<th>p105/43.14</th>
<th>Br20</th>
<th>p272</th>
<th>p105/43.14</th>
</tr>
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<tbody>
<tr>
<td>N. benthamiana</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N. tabacum</td>
<td>+</td>
<td>-</td>
<td>-</td>
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</table>
According to the host plant and the property considered. However, very little is known about specific determinants responsible for the induction of specific symptoms.

In this work, experimentally derived reassortants allowed us to prove that the S RNA of Br20 is necessary for the necrotic response of *N. benthamiana* and *N. tabacum* cv. White Burley. Using *A. tumefaciens* as a delivery and expression system, we obtained the *in planta* transcription and expression of the alleles of both viral proteins encoded by S RNA. Previous data showed transient heterologous expression to be a suitable tool to investigate pathogen/host interactions and to study single gene effects on plant response without virus interference (Anna-mali *et al.*, 2005; Marillonet *et al.*, 2005; Scholthof *et al.*, 1996; Takeda *et al.*, 2002; Voinnet *et al.*, 2000). In addition, the transient approach is a quick way to achieve expression of recombinant proteins, while avoiding the lethal necrosis induced by necrosis-inducing proteins in the early stage of development of stable transgenic plants. Co-agroinfiltration of different constructs is also a common tool used to study protein–protein interaction at the single cell level, and a number of reports confirm the *in vivo* delivery in the same cell of multiple constructs (Canto *et al.*, 2007; Li and Chye, 2004).

No specific necrotic symptoms related to the N and NSs viral proteins encoded by segment S of Br20 were observed, indicating that they are not sufficient, whether alone or in combination, for inducing the necrotic response. Kormenlink *et al.* (1991) suggested that a correlation exists between the amount of NSs and

![Symptoms induced by agroinfiltration](image-url)

**Fig. 3.** Symptoms induced by agroinfiltration of different pBin61 derived constructs on *N. tabacum*. No specific reaction was observed when the pBin61 vector encoding the N (A) or NSs (B) protein of Br20 or p272 was agroinfiltrated. The same situation was observed for the p105/43.14 constructs (not shown). C) Necrotic reaction induced by the expression vector carrying the p19 protein gene of *Tomato bushy stunt virus*. D) Chlorotic spots induced by the pBin61 expression vector 7 days post infiltration.
the severity of symptoms, for severe symptoms were elicited by isolates expressing higher amounts of the NSs protein than the mild strains. Our data detected no difference in the level of accumulation of the NSs protein between Br20 and the TSWV Italian isolates. The absence of symptom expression in the combined infiltrations seems also to exclude the possibility that the interaction between the two S RNA-encoded proteins induces the specific response. Moreover, also the corresponding miRNAs, either alone or in combination, are not sufficient for necrotic symptoms development.

Therefore, we suggest that the non coding sequences of S RNA or a synergic interaction between elements of S RNA and elements of the M and/or L RNA may be involved in the necrotic response. Previous works showing that the N protein interacts with proteins encoded by other genomic segments (Snippe et al., 2007; Uhrig et al., 1999) support this hypothesis. No interaction between the NSs protein and those encoded by other genomic segments has been reported so far, nor between the proteins encoded by the S RNA and plant factors.

Lack of a reverse genetic system for TSWV hampers the possibility to further explore the complexity of the necrotic response in N. benthamiana and N. tabacum.

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REFERENCES


