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The N protein of *Tomato spotted wilt virus* (TSWV) is associated with the induction of programmed cell death (PCD) in *Capsicum chinense* plants, a hypersensitive host to TSWV infection

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ABSTRACT

In sweet pepper, the *Tsw* gene, originally described in *Capsicum chinense*, has been widely used as an efficient gene for inducing a hypersensitivity response (HR) derived *Tomato spotted wilt virus* (TSWV) resistance. Since previously reported studies suggested that the TSWV-S RNA mutation(s) are associated with the breakdown of *Tsw* mediated TSWV resistance in peppers, the TSWV genes N (structural nucleocapsid protein) and NS_S (non-structural silencing suppressor protein) were cloned into a *Potato virus X* (PVX)-based expression vector, and inoculated into the TSWV-resistant *C. chinense* genotype, PI 159236, to identify the *Tsw*-HR viral elicitor. Typical HR-like chlorotic and necrotic lesions followed by leaf abscission were observed only in *C. chinense* plants inoculated with the PVX-N construct. Cytopathological analyses of these plants identified fragmented genomic DNA, indicative of programmed cell death (PCD), in mesophyll cell nuclei surrounding PVX-N-induced necrotic lesions. The other constructs induced only PVX-like symptoms without HR-like lesions and there were no microscopic signs of PCD. The mechanism of TSWV N-gene HR induction is apparently species specific as the N gene of a related tospovirus, *Tomato chlorotic spot virus*, was not a HR elicitor and did not cause PCD in infected cells.

1. Introduction

A broad range of defense responses are induced in resistant plants deploying resistance gene (R-gene)-mediated perception of pathogen elicitors (also called avirulence or Avr products; Keen, 1990; Staskawicz et al., 1995; Soosaar et al., 2005). Following successful infections, inhibition of pathogen spread occurs by rapid cell death in host tissues at and around pathogen entry points. This so-called hypersensitivity response (HR) is based on a typical gene-for-gene based resistance reaction (Flor, 1942). Macroscopically, the manifestation of HR appears as local necrotic lesions in the inoculated leaf, subsequently followed by foliar abscission. Programmed cell death (PCD) is thought to be involved in HR (Heath, 2000; Shirasu and Schulze-Lefert, 2000). PCD is a genetically controlled process that can be triggered in multicellular organisms as a response to pathogen invasion and stress signals (Raff, 1998; Danon et al., 2000; Chichkova et al., 2004; Eckardt, 2006). In plants, several tissues or whole organs undergo PCD either as part of their normal development (senescence) or in response to pathogens and environmental stresses (Greenberg, 1996). Although the PCD mechanism in plants has not been completely elucidated, several morphological and biochemical similarities between PCD in plants and animals have been described in different experimental conditions. These include condensation of the nucleus and the cytoplasm, cleavage of DNA into short ~180-bp fragments (DNA fragmentation), cytochrome *c* release from mitochondria and disruption of mitochondrial membrane potential (Morel and Dangl, 1997; Danon et al., 2000; Balk and Leaver, 2001; Lam et al., 2001; Hoeberichts and Woltering, 2003; Eckardt, 2006).

Hypersensitivity and PCD responses to plant virus infections occur in cells at the site of viral replication and manifest as discrete necrotic lesions in otherwise phenotypically normal tissues. If successful, systemic viral movement will be halted by a HR and infections will be confined to lesions and their adjacent cells. In the last decade, several plant R-genes have been cloned and their respective viral elicitor proteins identified. For the majority of

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virus/host interactions in which these so-called avirulence determinants have been characterized, HR-like mechanism are triggered by particular viral proteins (Whitham et al., 1994; Bendahmane et al., 1999, 2000; Spassova et al., 2001; Vidal et al., 2002; Takahashi et al., 2002; Lanfermeijer et al., 2003).

Tomato spotted wilt virus (TSWV), the type species of *Tospovirus* genus within the *Bunyaviridae* family, has a wide host range and is transmitted by thrips in a propagative–circulative manner (Goldbach and Peters, 1996; Prins and Goldbach, 1998). In pepper (*Capsicum chinense*), several lines expressing a hypersensitivity-like response to TSWV have been reported. These resistant lines include PI 152225, PI 159236 (Black et al., 1991; Boiteux and de Ávila, 1994), CNPH 275 or "Panca" (Boiteux et al., 1993), PI 15 (Jorda et al., 1994), C00943 (Hobbs et al., 1994) and 7204 (Nuez et al., 1994). Inheritance studies performed with PI 152225, PI 159236 and CNPH 275 showed that the resistance in these genotypes was monogenic, dominant and located at the same locus, which was subsequently named Tsw (Boiteux, 1995; Black et al., 1996; Moury et al., 1997; Jahn et al., 2000). It is noteworthy that these resistant lines are not resistant to the closely related tospoviruses, *Tomato chlorotic spot virus* (TCSV) and *Groundnut ringspot virus* (GRSV) (Boiteux and de Ávila, 1994). Roggero et al. (2002) reported a new TSWV isolate, which was able to break the *Tsw* mediated HR resistance of *C. chinense* PI 152225 leading to a systemic infection. Recently, other *Tsw*-resistance-breaking isolates have been also reported in Spain and Australia (Thomas-Carrol and Jones, 2003; Margaria et al., 2004).

The TSWV genome consists of three negative and/or ambisense ssRNA segments called L (large), M (medium) and S (small). The L RNA encodes the viral replicase (De Haan et al., 1991). The M RNA encodes two proteins, the Gn-Gc precursor and the NS_M movement protein gene. The S RNA encodes a non-structural protein (NS_S), the TSWV gene silencing suppressor (Takeda et al., 2002; Bucher et al., 2003), and the nucleocapsid protein (N) in an ambisense coding strategy. Whereas reassortant studies have indicated that unmapped TSWV M RNA mutation(s) are responsible for the breakdown of TSWV resistance in tomato that is mediated by the Sw-5 gene (Hoffmann et al., 2001), S RNA mutation(s) are associated with the breakdown of *Tsw* mediated TSWV resistance in peppers (Jahn et al., 2000). Margaria et al. (2007) have indirectly implicated the NSS gene as the avirulence factor involved in *Tsw* mediated resistance. They did not, however, demonstrate that NS_S plays a direct role on the induction of local necrotic lesions typical of HR in sweet pepper genotypes carrying the *Tsw* gene.

In this report we investigated the possible association of PCD with the HR induced by inoculation of TSWV on pepper. We investigated the ability of two TSWV genes (N and NS_S) and one TCSV gene (N) to trigger a HR in a *C. chinense* genotype (PI 159236) carrying the *Tsw* gene. Since no reverse genetics approach is available for tospoviruses, the ability of these three genes to induce a HR was investigated by firstly expressing them from a *Potato virus X* (PVX) vector (Chapman et al., 1992) and then evaluating the occurrence HR by both symptomatology and TUNEL (TdT-mediated dUTP Nick-End Labeling)-based cytopathological analyses. We provide evidence that the N protein, but not the NS_S protein of TSWV, triggers the HR in *C. chinense*, and that *Tsw* mediated HR probably involves the activation of PCD pathways.

2. Methods

2.1. Virus sources and RNA purification

Tomato spotted wilt virus (TSWV BR-01) (De Avila et al., 1993) was maintained in *Nicotiana rustica* by mechanical inoculation using 0.05 M sodium phosphate buffer containing 0.01 M sodium sulfite, pH 7.0, under greenhouse conditions. Nucleocapsids of TSWV were partially purified from systemically infected *N. rustica* plants, as described previously (De Avila et al., 1993). Viral RNA was extracted from nucleocapsid preparations using Trizol (Invitrogen), following manufacturer's recommendations. Routine DNA and RNA manipulations were performed according to Sambrook et al. (1989).

2.2. Amplification of N and NS_S genes by RT-PCR

First-strand cDNAs of N and NS_S genes of TSWV were synthesized from extracted RNA using corresponding anti-sense primers for each gene. The retro-transcription reactions were employed with MMLV-RT (Invitrogen) according to manufacturer's instructions using purified RNA at 37 °C for 1 h. The N and NS_S cDNAs of TSWV were PCR-amplified using forward (F) and reverse (R) gene-specific primers containing ClaI and SalI restriction enzyme sites for further cloning in the binary vector pGR107 (Jones et al., 1999). A total of 50 μl amplification reactions were carried out in a Mastercycler (Eppendorf) thermocycler using 2 μl of cDNA, 5 μl of 10X Platinum *Taq* polymerase High Fidelity buffer (Invitrogen), 100 ng of gene specific forward and reverse primers, and 0.25 U of Platinum *Taq* polymerase High Fidelity (Invitrogen). The following primers were used to amplify the cDNA fragments. For N-gene amplification F-NTSWV1 (5′-CCC ATC GAT ATG TCT AAG GTT AAG CTC-3′; ClaI site underlined) and R-NTSWV2 (5′-CCC GTC GAC TTC AAG CAA GTT CTG CGA G-3′; SalI site underlined); and for NS_S-gene amplification F-NS_STSWV1 (5′-CCC CCA TGG ATC GAT GTC TTC AAG TGT T-3′; ClaI site underlined) and R-NS_STSWV2 (5′-CCC GTC GAC TTA TTT TGA TCC TGA ACG-3′; SalI site underlined) were used. Amplification reactions (50 μl) were denatured for 5 min at 94 °C prior to 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min.

2.3. Construction of recombinant Agrobacterium tumefaciens binary PVX vectors

PCR products obtained for N and NS_S genes of TSWV were digested with ClaI and SalI restriction enzymes, size-fractionated on 1.0% agarose gels and purified (Sephaglass Band Prep Kit, Amersham Bioscience) prior to ligation to the binary vector pGR107 (Jones et al., 1999), digested with ClaI and SalI. Correct cloning was confirmed by sequencing of selected clones. Recombinant binary plasmids (Fig. 1) were maintained and propagated in *Escherichia coli*, strain XL-1 Blue, grown in the presence of 50 μg/ml kanamycin. Plasmid DNA of the binary expression constructs was purified from *E. coli* cultures by alkaline lysis and then heat-shock transferred into *A. tumefaciens* strain GV3101. The cells were grown for 2 days at 28 °C in LB agar plates supplemented with kanamycin as selective agent. The transformation was checked by performing a PCR using specific primers for each gene as described previously. pGR107 transcribes an infectious PVX RNA using the CaMV 35S promoter after introduction into plant cells using *A. tumefaciens* GV3101 stab inoculation ('agro-inoculation').

2.4. Agro-inoculation of PVX clones into target plants

Transformed *Agrobacterium* cells with PVX-N and PVX-NS_S constructs, and pPVX (wild-type vector) as a control, were used to inoculate leaves of *C. chinense* PI 159236, *Nicotiana benthamiana* and *Datura stramonium* plants at the two to five-leaf developmental stage. Inoculation was done by puncturing the leaves with a sterile toothpick tip that touched the *Agrobacterium* colonies (Lu et al., 2003). The development of symptoms was monitored daily up to 35 days after inoculation. As additional control, the wild-type TSWV BR-01 was individually inoculated to *N. benthamiana* and *C. chinense* PI 159236 plants.