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Virus-induced gene silencing reveals signal transduction components required for the *Pvr9*-mediated hypersensitive response in *Nicotiana benthamiana*

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ABSTRACT

Resistance to pathogens mediated by plant resistance (R) proteins requires different signaling transduction components and pathways. Our previous studies revealed that a potyvirus resistance gene in pepper, *Pvr9*, confers a hypersensitive response (HR) to pepper mottle virus in *Nicotiana benthamiana*. Our results show that the *Pvr9*-mediated HR against pepper mottle virus infection requires *HSP90, SGT1, NDR1*, but not *EDS1*. These results suggest that the *Pvr9*-mediated HR is possibly related to the SA pathway but not the ET, JA, ROS or NO pathways.

1. Introduction

R gene-mediated resistance is a mechanism that plants use to defend against pathogen infection (Kang et al., 2005). The most common *R* genes encode for nucleotide-binding site-leucine-rich repeat (NBS-LRR) proteins, which are classified into two groups: the TIR-NBS-LRR proteins, which contain an N-terminal domain with Toll/Interleukin-1 receptor homology, and the CC-NBS-LRR proteins, which are characterized by an N-terminal coiled-coil motif (van Ooijen et al., 2007). This grouping, however, is not always precise because there are hundreds of NBS-LRR genes that differ somewhat in structure. The interaction between an R protein and its elicitor can generate either extreme resistance, which confines the pathogen to a single cell, or a hypersensitive response (HR), which limits the pathogen to a few cells (Carr et al., 2010).

An HR requires the involvement of signaling proteins downstream of *R* protein-elicitor recognition. The roles of *HSP90*, *SGT1*, *EDS1*, and *NDR1* as the signaling components in *R* gene-mediated HR have been studied. The molecular chaperone *HSP90*, in collaboration with its co-chaperone, has a crucial function in activating proteins involved in signal transduction in eukaryote cells (Pearl and Prodromou, 2006; Richter and Buchner, 2001). In plants, the HSP90 protein is involved in the signaling of resistance mediated by *Rx*, *N*, and *RPS2* (Botër et al., 2007; Takabatake et al., 2007; Takahashi et al., 2003). As related to plant resistance, a common co-chaperone of HSP90 is SGT1. SGT1 is a conserved eukaryotic protein that functions in the cell cycle, kinetochore assembly, and protein degradation (Kitagawa et al., 1999). More importantly, *SGT1* is an essential component of *R* gene-mediated resistance (Azevedo et al., 2002; Botër et al., 2007). Two other genes, *EDS1* and *NDR1*, are independently required for at least two *R* gene-mediated signaling pathways; the dependence on *EDS1* or *NDR1* has been attributed to R protein structure rather than to the pathogen (Aarts et al., 1998).

Plant transduction signals involved in *R* gene-mediated resistance include the following endogenous plant signaling molecules: salicylic acid (SA), ethylene (ET), jasmonic acid (JA), reactive oxygen species (ROS), and nitric oxide (NO) (Locato et al., 2016; Neill et al., 2002; Pieterse et al., 2009). Regulators of these signaling molecules and their pathways have been identified in plants. NPR1 is a key regulator of systemic acquired resistance and is essential for transduction of the SA signal (Pieterse and Van Loon, 2004). EIN2 and COI1 are regulators of ET and JA pathways, respectively (Glazebrook et al., 2003). Rbohs are required for H_2O_2 accumulation, and the silencing of RbohB eliminates the oxidative burst induced in *Nicotiana benthamiana* by the elicitor INF1 of the potato pathogen *Phytophthora infestans* (Asai et al., 2008; Yoshioka

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et al., 2003). NOA1 is involved in NO production and defense response in IFN1-treated N. benthamiana (Kato et al., 2008).

Virus-induced gene silencing (VIGS) is a widely used technique that blocks expression of plant genes in a sequence-specific manner (Baulcombe, 1999). VIGS is commonly used to study the functions of genes related to plant defense responses. VIGS, for example, was used to determine the role of *SGT1* in *R* gene-mediated resistance in *N. benthamiana* (Peart et al., 2002). Similarly, VIGS was used to identify the role of *EDS1* and *NPR1* in *N*-mediated resistance in *N. benthamiana* (Liu et al., 2002). However, applications of VIGS also have some disadvantages (Burch-Smith et al., 2004). Inoculation of the plant with the virus can result in reduced plant growth or plant death (Broderick and Jones, 2014). Therefore, VIGS should be modified to reduce the virulence of the virus.

We previously identified a new *R* gene from pepper, named *Pvr9*, that confers HR to pepper mottle virus (PepMoV) in *N. benthamiana*; we also previously identified a viral elicitor, potyvirus NIb, of Pvr9 (Tran et al., 2015). The pathway of the HR mediated by *Pvr9*, however, is poorly understood. In this study, we used an optimized VIGS technique to investigate the signal transductions controlling *Pvr9*-mediated HR.

2. Results and discussion

2.1. Optimization of insert length for tobacco rattle virus (TRV) based VIGS in N. benthamiana

In a study of optimization of VIGS using TRV in *N. benthamiana*, efficient and maximum silencing levels were obtained with insert sizes between 192 and 1304 bp and about 350 bp, respectively (Liu and Page, 2008). To determine the effectiveness of the TRV-induced VIGS under our plant growing conditions, we inserted 330 bp fragment of *NbPDS* (DQ469932) from *N. benthamiana* into the pTRV2 vector (AF406991). The VIGS construct had systemically lethal effects in the infiltrated plants: plants died within a few days after agroinfiltration of the pTRV empty vector (data not shown); partial necrosis and growth retardation were evident 2 weeks after agroinfiltration of 330-bp fragment of *NbPDS* despite the 1:10 dilution of the *Agrobacterium* suspension (Fig. 1, panel B). This made it difficult to identify cell death-related genes.

We noted that the replicating TRV containing shorter-length insert is more virulent. In a study of the application of VIGS in petunia, Broderick and Jones (2014) introduced a fragment of GFP to pTRV2 and thus reduced the viral virulence. With a similar strategy, we introduced the untranslatable 714-bp fragment of GFP into pTRV2 as a reference for silencing evaluation and further functional analysis. The 730-bp fragment of *NbPDS* was used as a positive control for VIGS evaluation. Based on evaluation of symptoms 2 weeks after the delivery of the VIGS clone, the virulence of TRV clones was eliminated by these inserts, and the photo-bleaching effect of the pTRV::PDS (730 bp) clone was almost complete in the non-inoculated systemically infected leaves (Fig. 1, panels C and D). The expression level of PDS in the silenced plant was reduced by approximately 80% in comparison to the

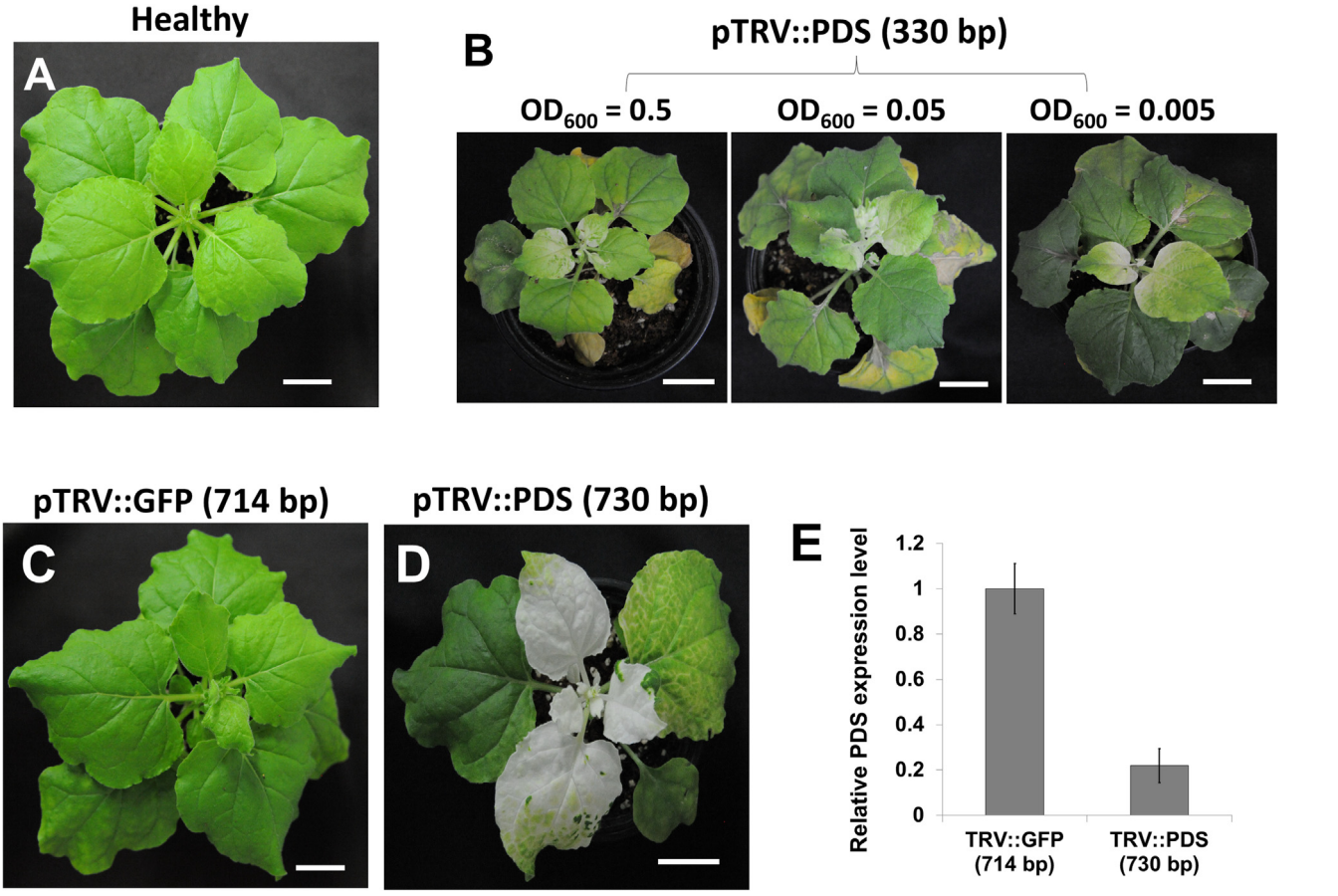

Fig. 1. Optimization of insert length for the TRV-based VIGS in *N. benthamiana*. (A) A 4-week-old healthy plant. (B) Systemic necrosis symptoms in plants that were agroinfiltrated with TRV::PDS (330 bp of PDS) at different *Agrobacterium* concentrations (OD_{600}). (C, D) Plants that were agroinfiltrated with TRV::GFP and TRV::PDS (730 bp of PDS). (E) Expression level of *PDS* in PDS-silenced plants (in D) relative to that in control plants (in C). Scale bars = 3 cm (in A–D).