

Symposium

The 6th Molecular Plant-Microbe Interactions

December 5, 2003, Daejeon, Korea

Relationship Between Plant Viral Encoded Suppressor to Post-transcriptional Gene Silencing and Elicitor to R Gene-specific Host Resistance

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(Received on November 1, 2003; Accepted on November 29, 2003)

Many important horticultural and field crops are susceptible to virus infections or may possess a degree of resistance to some viruses, but become infected by others. Plant viruses enter cells through the presence of wounds, and replicate intracellularly small genomes that encode genes required for replication, cell-to-cell movement and encapsidation. There are numerous evidences from specific virus-host interactions to require the involvement of host factors and steps during viral replication cycle. However, viruses should deal with host defense responses either by general or specific mechanisms, targeting viral components or genome itself. On the other hand, the host plants have also adapted to defend themselves against viral attack by operating different lines of resistance responses. The defense-related interactions provide new insights into the complex molecular strategies for hosts for defense and counter-defense employed by viruses.

Defense strategy of host plant by R gene-mediated resistance

Induced resistance depends on the recognition of a pathogen by the plant, generating a cascade of events, which eventually leads to the expression of defense mechanism. The recognition between virus and host is very specific, sometimes host plants can distinguish strains of virus. One such response involves recognition of pathogen-encoded ligands (elicitors) by plant defense resistance (*R*) gene encoded receptor (Dangl and Jones, 2001), suggesting that viral *Avr-R* recognition likely occurs inside plant cells. This results in a strain-specific induction of resistance mechanism described by the gene-for-gene hypothesis

(Flor, 1971), which is often manifested as a hypersensitive response (HR) at the entry of infection and systemic acquired resistance (SAR) in the whole plant (Hunt and Ryals, 1996; Sticher et al., 1997). Generally, the speed of recognition and ensuing induction of resistance are key determinants in the success of resistance. Disease will occur if the virus is faster than the induced response, if no elicitors are produced or if suppressors prevent the plant defense. In view of evolutionary point, virus overcomes cultivar-specific resistance genes, suggesting that virus evolve at a much faster rate than their resistant host.

Significant progress in the understanding of host-pathogen interactions has been achieved in the last decade by the cloning of a number of plant *R* genes from different plant species (Dangle and Jones, 2001), including tobacco *N* gene (Whitham et al., 1994) against *Tobacco mosaic virus* (TMV), potato *Rx1* and *Rx2* genes (Bendahmane et al., 1999) against *Potato virus X* (PVX), and *Arabidopsis HRT* gene against *Turnip crinkle virus* (TCV) (Cooley et al., 2000). Such *R* genes belong to the NBS-LRR family of resistance gene, respectively, encoding a protein with a specific amino terminal domain. *N* gene product contains a domain similar to the toll-interleukin-1 receptor (TIR) (Whitham et al., 1994; Baker et al., 1997), while *HRT* and *Rx* genes contain coiled-coil sequences (CC domains) between N-termini and NBS domains (Bendahmane et al., 1999; Cooley et al., 2000). To induce resistance in a host plant cultivar carrying *R* gene, a strain specific elicitor encoded by or produced by the action of corresponding *Avr* gene should be present in a particular strain of a virus. Three major types of viral genes, encoding the coat protein (CP) (Bendahmane et al., 1995; Berzal-Herranz et al., 1995; Taraporewala and Culver, 1996), RNA replicase (Kim and Palukaitis, 1997; Padgett et al., 1997), movement protein (Meshi et al., 1989; Weber et al., 1993) or N1a protease (Mestre et al., 2000) have been demonstrated to be

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avirulence determinants. The 126 kDa replicase protein (Padgett et al., 1997; Abbink et al., 1998) of TMV is the viral *Avr* protein for which the cloning of a matching *R* gene, *N* has been reported. TCV CP has been found to be the *Avr* gene product for the corresponding *R* gene, *HRT* present in TCV-resistant Dijon-17 plants (Zhao et al., 2000).

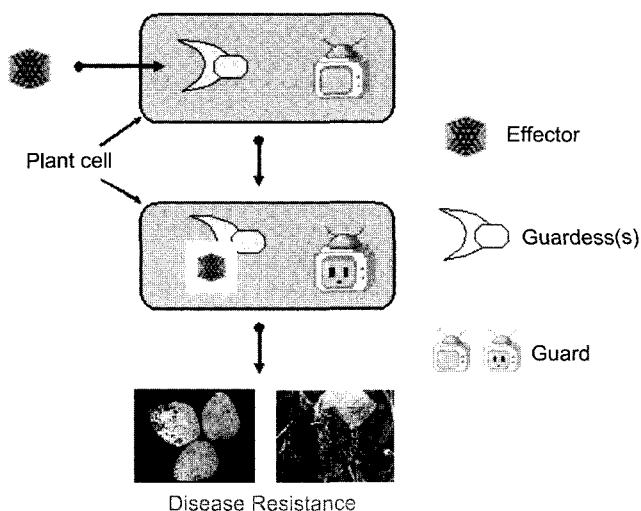
How *R* and *Avr* gene products activate plant defense responses is not clearly understood. Since no single *R* protein mediates direct interaction with corresponding *Avr*, leading to the speculation that multi-protein complexes are probably involved in virus recognition. There are several reasons why the gene-for-gene model is not fit with some evidences. First, numerous *R* genes have been identified, corresponding to multiple and sometimes unrelated *avr* genes (Rossi et al., 1998; Cooley et al., 2000; Mackey et al., 2002). Secondly, the analysis of complete genome sequences of *A. thaliana* has revealed that there are at most 200 putative *R* genes, the number is insufficient to account for gene-for-gene resistance against all potential pathogens. Thirdly, it is not reasonable for viruses to encode a gene or its product recognizable by a host *R* gene or its product that ultimately would lead to their removal.

Recently, an interesting explanation of plant *R* gene recognition called a “guard role” for *R* gene products, namely, the ‘guard hypothesis’ was proposed (van der Biezen and Jones 1998) to explain the interactions between the major classes of molecules identified in resistance responses. The hypothesis describes that *R* gene products “guard” the targets of microbial virulence factors, detect interaction of pathogen’s virulence factors with its host intracellular target, and subsequently induce defense responses. This hypothesis is supported from the observation that many avirulence gene products constitute a subset of pathogen virulence factors involved in the mediation of disease (White et al., 2000). More recently, Dangl and Jones (2001) reiterated the presence of direct interactions

among these components and predicted two “mechanistic scenarios”. Most recently, it is proposed that *R* proteins monitor the activities of multiple effectors, by detecting physiological changes of ‘guardee’ caused by ‘effectors’ in the cell, not by direct binding of effectors (Shao et al., 2002; Schneider, 2002). Figure 1 shows an update model of ‘guard hypothesis’ modified according to Schneider (2002). Typical evidence supporting this model is *Pto* and *Prf*, are both required for AvrPto-triggered resistance in tomato to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (Salmeron et al., 1996). AvrPto is the elicitor (effector), small hydrophobic protein that is delivered into the plant cell by the bacterial type III secretion system (Jin and He, 2001). It interacts with Pto protein, one of the host factors, which encodes serine/threonine kinase referred to the ‘guardee’, because it is protected by “guard” molecule *Prf*. Another similar evidence is *HRT* resistance pathway of TCV-*Arabidopsis* system, where *Arabidopsis* protein, TIP, was found to interact specifically with TCV CP (Ren et al., 2000). TIP belong to a member of NAC family of proteins, suggesting that it may be a transcriptional activator, but lacks both NBS and LRR motifs like Pto protein. On the other hand, the product of *HRT* contains both NBS and LRR motifs like *Prf*.

Defense strategy of host plant by post-transcriptional gene silencing

Transgenic expression of viral genes has been shown to provide high level of resistance due to activation of an intrinsic sequence-specific plant defense mechanism. It is believed to have evolved as a defense mechanism against foreign genetic elements, such as viruses, viroids and transposable elements (Vance and Vaucheret, 2001). If transcription of the target gene is blocked, it can be transcriptional gene silencing (TGS), but if the target gene is transcribed but its mRNA is degraded in a sequence-specific manner before it is translated, it can be post-transcriptional gene silencing (PTGS). Taken together called gene silencing or RNA silencing, which is characterized by a decrease in the steady state levels of mRNA of a specific target gene. In plants, virus-induced gene silencing (VIGS) is a PTGS phenomenon in which a hosts target endogenous gene or transgene is silenced upon infection by a plant virus carrying a sequence with homology to the target gene. VIGS can be induced by RNA viruses (Jones et al., 1999; Pelissier et al., 1999; Wassenegger, 2000; Waterhouse et al., 2001) and DNA viruses (Atkinson et al., 1998; Kjemtrup et al., 1998; Peele et al., 2001; Turnage et al., 2002), suggesting that plant virus replicating in the cytoplasm can function as both the initiator and the target of PTGS (Jones et al., 1998; Baulcombe, 1999; Guo et al., 1999). Similar



mechanisms were later discovered in other organisms, including quelling in filamentous fungus *Neurospora crassa* (Cogoni and Macino, 1999) or RNA interference (RNAi) in *Caenorhabditis elegans* (Fire et al., 1998) and *Drosophila melanogaster* (Kennerdell and Carthew, 1998). Therefore, PTGS is a conserved eukaryotic RNA surveillance system that leads to the elimination of the targeted RNA and eliminates the functions encoded by the targeted RNA (Carrington et al., 2001; Vance and Vaucheret, 2001; Waterhouse et al., 2001; Baulcombe, 2002).

RNA virus-induced VIGS of transgenes can be separated into a virus-dependent initiation stage and a virus-independent but transgene-dependent maintenance stage process (Ruiz et al., 1998; Voinnet et al., 1998; Dalmay et al., 2000). The most potent initiator of PTGS is considered to be double-stranded RNA (dsRNA) (Fire et al., 1998; Kennerdell and Carthew 1998; Waterhouse et al., 1998; Chuang and Meyerowitz, 2000), although single-stranded RNA (ssRNA), both sense and antisense orientations, or even DNA trigger RNA silencing (Voinnet et al., 1998; Voinnet et al., 1999; De Serio et al., 2001). The dsRNA trigger for PTGS can be: (1) the dsRNA replication intermediate in the case of silencing induced by plant RNA viruses and viroids; (2) complementary sense and antisense transcripts from inverted repeats or from antisense transgene RNA interacting with endogene sense mRNA; (3) the product of RNA-dependent RNA polymerase (RdRP) on 'aberrant' transcripts (Vance and Vaucheret, 2001). DsRNA form converted from ssRNA by a host-encoding RdRP (Cogoni and Macino, 1999; Dalmay et al., 2000b; Mourrain et al., 2000; Smardon et al., 2000), which are then degraded by an RNase III-like RNase called Dicer, originally found in cultured *Drosophila* S2 cells (Hammond et al., 2000; Bernstein et al., 2001; Knight and Bass, 2001). Therefore, in the initiation stage the invading RNA triggers a pathway that results in its being degraded into a small RNA species called small interfering RNAs (siRNA), having size of 21-25 nucleotides (nt) that function as a guide for further degradation in the maintenance stage (Hamilton and Baulcombe, 1999; Zamore et al., 2000). The siRNAs lead to sequence-specific cleavage of the homologous host-encoded mRNA by RNA-induced silencing complex (RISC), thought to be a multicomponent nuclease (Zamore et al., 2000; Hammond et al., 2001).

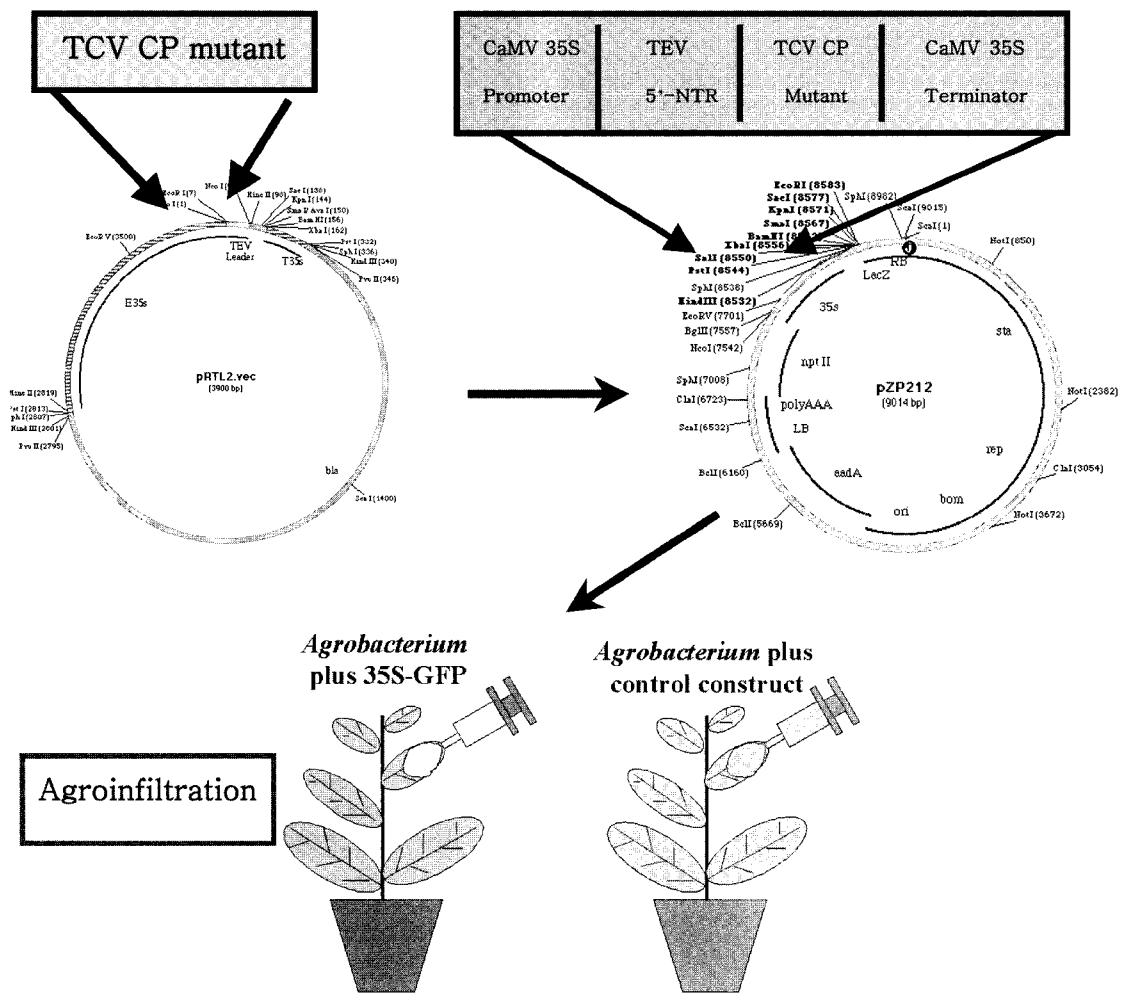
PTGS is independent of trigger RNAs at the maintenance stage, but involves an RdRP activity that amplifies the siRNAs and hence the efficiency of silencing (Lipardi et al., 2001; Sijen et al., 2001; Vaistij et al., 2002). The maintenance stage is highly dependent on a gene silencing signal, thought to be siRNA, that is able to move systemically in plants to virus-free tissues (Jones et al., 1999; Peele et al., 2001; Waterhouse

et al., 2001). Another key feature of PTGS in plants is the association with intracellular signaling between the cytoplasm and the nucleus as well as the systemic spread of the silencing signal between cells and in the vascular system, even if the initiator molecule remains localized or is removed. This signal can move locally through plasmodesmata and systemically via the vascular system (Fagard and Vaucheret, 2000; Mlotshwa et al., 2002). Therefore, a system to amplify the silencing signal must also exist. On the other hand, as in viral exclusion from the shoot apex, the PTGS signal seems to be incapable of entering into and activating PTGS within the apex (Beclin et al., 1998; Voinnet et al., 1998). Most plant viruses cannot invade the shoot apex (Matthews, 1991), suggesting an operating mechanism that protects this region of the plant from viral infection. An RNA signal surveillance system acts to allow the selective entry of RNA into the shoot apex by signal regulation, protecting the shoot apex (Foster et al., 2002). Another evidence for intracellular signaling is that RNA degradation is typically correlated with methylation of homogeneous DNA in the nucleus, as well as to distant parts of the plant. TGS is accompanied by hypermethylation of the promoter region of silenced genes, while PTGS is often associated with hypermethylated DNA in the coding region. The *de novo* methylation of transgenes in PTGS is RNA-directed DNA methylation (RdDM) (Ruiz et al., 1998; Jones et al., 1999; Matzke et al., 2001). It is unclear whether the signal for RdDM is aberrant RNA, dsRNA, or siRNA, although all cases of virus-induced RdDM reported to date involve RNA viruses and viroids that have a dsRNA replication intermediate. DNA methylation is necessary for the initiation of silencing in some case (English et al., 1996; Elmayan et al., 1998; Jones et al., 1998; Kovarik et al., 2000; Morel et al., 2000; Mourrain et al., 2000; Dalmay et al., 2001; Rodman et al., 2002), but not in others (Scheid et al., 1998; Jones et al., 1999; Sonoda and Nishiguchi, 2000; Wang and Waterhouse, 2000; Mallory et al., 2001). Although methylation is associated with VIGS of transgenes, it has been shown to be absent in the silencing of endogenous genes (Jones et al., 1999), suggesting that there may be multiple mechanisms for gene silencing. It is not clear that the transgene methylation and systemic silencing are directed by the same signal molecule(s). In addition to siRNAs, a class of larger small RNAs (24-27 nts) has recently been reported to accumulate in plants silenced by agroinfiltration and in transgenic tobacco lines suppressed for silencing by HC-Pro (Mallory et al., 2002). In contrast to the results from transient assays, grafting experiments revealed no consistent correlation between capacity for systemic silencing and accumulation of any particular class of small RNA (Mallory et al., 2003).

Counter-defense strategy plant viral-encoded suppressor against post-transcriptional gene silencing

To counter this PTGS, many viruses have developed strategies to suppress PTGS and encoded the genes encoding proteins acting as a suppressor. This was first recognized for the potyvirus protein P1/HC-Pro; helper component-proteinase (Brigneti et al., 1998; Kasschau and Carrington, 1998) and later for a wide range of numerous viruses, leading to the suggestion that this was a property associated with most viruses (Voinnet et al., 1999; Voinnet et al., 2000). HC-Pro prevents the plant from responding to the mobile silencing signal but does not eliminate its ability to produce or send the signal, suggesting that HC-Pro operates downstream of transgene methylation and the mobile signal at a step proceeding accumulation of the small RNAs (Mallory et al., 2002). Therefore, potyviral HC-Pro can suppress silencing in tissues where it was already established and thus seems to disrupt the maintenance step of PTGS. Different PTGS-suppressor proteins appear to act at different points in the PTGS pathway. In the case of PVX,

the p25 movement protein has been shown to interfere with an initiation step, possibly preventing conversion ssRNA to dsRNA (Voinnet et al., 2000). Another case of early initiation step was demonstrated with a strong suppressor encoding CP of TCV that most likely interferes the function of the Dicer-like RNase in plants (Qu et al., 2002). In contrast, HC-Pro of *Tobacco etch virus* (TEV) appears to interfere with the maintenance stage (Llave et al., 2000; Mallory et al., 2001), whereas the 2b protein of *Cucumber mosaic virus* (CMV) inhibits the systemic transport of the silencing signal (Guo and Ding, 2002). In contrast, CMV have no effect in tissues where PTGS is established, but are able to prevent the initiation of gene silencing in newly emerging tissues (Brigneti et al., 1998). The difference in inhibition step requires additional characterization of silencing suppressor, contributing to define the mechanism operating the RNA silencing pathway. To date nearly twenty suppressors have been reported, most of which are viral non-structural protein except TCV CP. None of these proteins show sequence similarity and hence appear to have evolved independently to counter silencing-mediated defense.



The N-terminal 25 amino acids of TCV CP were shown to be important suppressor activity of which forms part of the unexposed R-domain that interacts with RNA within the virus particle (Thomas et al., 2003). P15 is a small cysteine-rich protein with no sequence similarity to previously described PTGS-suppressor proteins, possessing 4 C-terminal heptad repeats that can potentially mediate a coiled-coil interaction and targeting to peroxisomes via a C-terminal SKL motif (Dunoyer et al., 2002).

Relationship R gene-mediated resistance with post-transcriptional gene silencing

The CP of TCV suppresses PTGS (Qu et al., 2002) and also the elicitor of resistance response in the *Arabidopsis* ecotype Di-17 carrying the *HRT* resistance gene by mapping the resistance-eliciting domain to the N-terminal RNA-binding (R) domain. Transcriptional factor, TIP, in the *HRT*-mediated resistance pathway, is sufficient to interacting with N-terminal 25 amino acid of TCV CP (Ren et al., 2000). In order to determine the relationship between eliciting activity in *HRT*-based resistance and suppressing activity of CP, CP mutants were constructed and agroinfiltrated into transgenic *Nicotiana benthamiana* containing GFP (Fig. 2). However, a small deletion within R domain of TCV CP eliminated four amino acid residues critical for eliciting the *HRT*-based resistance retains the capability to suppress RNA silencing. Bigger in-frame deletions throughout the whole CP all lost the silencing-suppression activity (91252), concluding from these results that the silencing suppression of TCV CP is not correlated with *HRT*-mediated resistance and most likely requires the intact CP (Choi et al., 2003).

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