Global Analysis of Tomato Gene Expression During *Potato spindle tuber viroid* Infection Reveals a Complex Array of Changes Affecting Hormone Signaling

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Viroids like *Potato spindle tuber viroid* (PSTVd) are the smallest known agents of infectious disease—small, highly structured, circular RNA molecules that lack detectable messenger RNA activity, yet are able to replicate autonomously in susceptible plant species. While viruses supply at least some of the genetic information required for replication, viroids can be considered ‘obligate parasites of the cell’s transcriptional machinery’ (Diener 1987). Viroid infection is often accompanied by a wide variety of symptoms (e.g., stunting, epinasty and leaf distortion, and localized veinal chlorosis and necrosis), and just how these small, noncoding RNAs induce such a strong host response has long been the subject of intense interest.

Viroids are the smallest known agents of infectious disease—small (246 to 401 nt), highly structured, circular RNA molecules that lack detectable messenger (m)RNA activity and yet are able to replicate autonomously in susceptible plant species. While viruses supply at least some of the genetic information required for replication, viroids can be considered ‘obligate parasites of the cell’s transcriptional machinery’ (Diener 1987). Viroid infection is often accompanied by a wide variety of symptoms (e.g., stunting, epinasty and leaf distortion, and localized veinal chlorosis and necrosis), and just how these small, noncoding RNAs induce such a strong host response has long been the subject of intense interest. Several recent views describe the molecular biology of viroid-host interaction (Ding 2009; Flores et al. 2005; Gomez et al. 2009).

The current ICTV classification scheme for viroids divides the 31 recognized species into two families, i.e., the Pospiviroidae family, whose members each contain five conserved structural/functional domains and replicate in the nucleus, and the Avsunviroidae family, whose four members exhibit ribozyme activity and replicate in the chloroplast. *Potato spindle tuber viroid* (PSTVd), the type member of the family Pospiviroidae, was the first viroid to be discovered (Diener 1971). Even before the complete nucleotide sequence of PSTVd had been established (Gross et al. 1978), RNA fingerprinting studies demonstrated the presence of only minor sequence differences between isolates causing mild and severe symptoms in certain sensitive cultivars of tomato (Dickson et al. 1979). The extensive series of sequencing and mutagenesis studies that followed (Sano et al. 1992; Schnolzer et al. 1985; Qi and Ding 2003; Zhong et al. 2008) identified several structural determinants modulating PSTVd pathogenicity and showed that certain of these determinants (e.g., the loop E motif [Zhong et al. 2006]) are located outside the so-called “pathogenicity domain” originally identified by comparison of different viroid species (Keese and Symons 1985). The underlying mechanism or mechanisms responsible for disease induction has remained elusive, but PSTVd replication in ‘Rutgers’ tomato continues to be the most widely used experimental system for studies of viroid pathogenesis.

Although many of the visible symptoms, such as stunting and epinasty, associated with viroid infection are indicative of altered hormone metabolism (Diener 1987), how these metabolic and regulatory changes are connected to visible changes in cell structure is only just beginning to be understood. Early studies of the host response to viroid infection focused on changes at the protein level. Several reports (Camacho Henríquez and Säng er 1984; Gadea et al. 1996) have demonstrated induction of various pathogenesis-related (PR) proteins following infection by either PSTVd or Citrus exocortis viroid (CEVd). PSTVd infection was also shown to stimulate the phosphorylation of a 68- to 70-kDa tomato protein associated with double-stranded RNA-stimulated protein kinase activity (Hiddinga et al. 1988; Langland et al. 1995). Consistent with its potential role in an infection-related signaling cascade, incubation of PKR (its mammalian homolog) with PSTVd strains of varying pathogenicity led to differential activation (Diener et al. 1993). Subsequent efforts to clone the corresponding tomato gene were unsuccessful but, more recently, Ham mond and Zhao (2000, 2009) have characterized a second serine-threonine protein kinase whose expression is up-regu-
lated by PSTVd infection. PKV is a novel member of the AGC VIIIa group of signal-transducing protein kinases that appear to play a key role in regulating gibberellic acid metabolism and signaling.

At the cellular level, viroid infection is accompanied by changes in the structure of cell walls, chloroplasts, and membranous structures in the cytoplasm known as plasmamembranes or paramural bodies, as well as by accumulation of electron-dense deposits (Diener 1987). The onset of stunting is the result of restricted cell growth rather than inhibition of cell division or differentiation in PSTVd-infected tomato; thus, Qi and Ding (2003) demonstrated a positive correlation between stunting and downregulation of LeExp2, an expansin gene encoding a protein known to play an important role in the expansion of young cells via a “loosening” of their cell walls. Perhaps the most intriguing cytopathology associated with viroid infection are disturbances in chloroplast structure, particularly abnormalities in thylakoid membranes and disruption of grana. Although not unexpected for viroids such as Peach latent mosaic viroid that replicate in the chloroplast (Rodio et al. 2007), similar effects have also been reported for several pospiviroid-host combinations in which replication is confined to the nucleus.

Microarray analysis and other transcript profiling techniques have been widely used to study host-pathogen interaction in the context of entire biochemical or developmental pathways (Glazebrook 2005; Whitham et al. 2006; Wise et al. 2007), and over the past several years, it is has become increasingly clear that RNA silencing plays a key role in modulating many of these interactions (Ruiz-Ferrer and Voinnet 2009). Previous attempts to assess the global effects of viroid infection on host gene expression utilizing either cDNA microarrays (Itaya et al. 2002) or differential display (Tessitori et al. 2007) have yielded only a low-resolution picture of viroid-host interaction.

Several groups have reported the presence of small viroid-related RNAs in infected tissue (Ding 2009; Gómez et al. 2009), but their role in disease induction is unclear. Small viroid-related RNAs could function like micro (mi)RNAs or Transacting small interfering RNAs by loading RNA-induced silencing complexes and inactivating endogenous miRNAs (Gómez et al. 2009; Wang et al. 2004). Other data (Di Serio et al. 2010; Schwind et al. 2009) are inconsistent with this view, however, suggesting that viroids may induce symptoms by competing for host enzymes involved in the synthesis of miRNAs and small interfering (si)RNAs, thereby affecting the pathways regulated by these molecules. Attempts to correlate accumulation of specific host miRNAs in viroid-infected plants with levels of their respective target miRNAs have yielded contradictory results. Using Northern blotting, Martin and associates (2007) reported that CEVd infection in tomato had no effect on any of the five miRNA pathways examined. Results of large-scale sequencing studies carried out with PSTVd-infected tomato, in contrast, suggest that levels of certain miRNAs may decrease during infection, thereby disrupting expression of transcription factors required for normal leaf development (Diermann et al. 2010).

In the studies reported here, we have used a combination of microarray analysis, large-scale RNA sequence analysis, and Northern blotting to compare changes in tomato gene expression and miRNA levels associated with PSTVd infection in both sensitive and tolerant cultivars as well as a transformed tomato line expressing small PSTVd siRNAs in the absence of viroid replication. The transcriptional changes detected were extensive, involving several hormone-signaling pathways and varying with host genotype. Little, if any, change was observed in levels of miR159, a host miRNA known to play an important role in gibberellin (GA)-mediated signaling. In the dwarf cultivar ‘Micro-

RESULTS

Effect of host genotype on PSTVd symptom expression.

As described by Diener (1987), visible symptoms of PSTVd infection in tomato include stunting, epinasty, and leaf rugosity and distortion. Several factors, including host genotype, are well-known to influence disease severity (Singh 1973), and Figure 1 compares the symptoms induced by the PSTVd Intermediate strain (Gross et al. 1978) in two different tomato cultivars growing under the warm temperature and moderate light conditions required for strong symptom expression in the greenhouse. A sensitive cultivar like ‘Rutgers’ responds to PSTVd infection with the appearance of strong stunting and epinasty (Fig. 1A). The plants shown in Figure 1A were inoculated on the terminal leaflet of the third true leaf, and symptoms began to appear 11 to 12 days later in leaves 6 to 7. Symptom expression continued to intensify over the next 2 to 3 weeks.

Symptom expression in the tolerant cultivar ‘Moneymaker’ was much less pronounced than that observed in ‘Rutgers’, and PSTVd-infected plants showed little or no stunting (Fig. 1B). Note, however, that certain transformed ‘Moneymaker’ plants did exhibit strong stunting and epinasty. As described by Wang and associates (2004), plants from transgenic ‘Moneymaker’ line IR11-14 are not infected by PSTVd but, rather, contain a noninfectious PSTVd cDNA transgene that supports constitutive synthesis of PSTVd-related small RNAs. It should be noted that these transgene sequences are derived not from the Intermediate strain but from the more severe RG1 strain of PSTVd (Gruner et al. 1995). The timecourse of symptom development in these plants was similar to that observed for PSTVd-infected ‘Rutgers’ plants, but close inspection of their upper leaves revealed a pronounced twisting of the petioles, a symptom not observed on PSTVd-infected plants (Fig. 1D, right). Interestingly, both PSTVd-infected and transgenic ‘Moneymaker’ plants exhibited a marked loss of apical dominance and outgrowth of lateral buds (Fig. 1C to E). A similar loss of apical dominance, usually associated with changes in auxin levels, was not observed among the PSTVd-infected ‘Rutgers’ plants. As shown in Figure 1H, the upper portions of the stems of the transgenic ‘Moneymaker’ IR11-14 plants also exhibited an unusual thickening and curvature not seen in their PSTVd-infected counterparts. Similar symptoms were observed on plants from transgenic line IR11-11.

Changes in gene expression associated with PSTVd infection.

Initial transcript profiling experiments compared changes in gene expression accompanying PSTVd replication and symptom development in ‘Rutgers’ and ‘Moneymaker’ plants. For purposes of comparison, these analyses also included uninfected plants from transgenic ‘Moneymaker’ line IR11-14. Subsequent experiments examined the effects of exogenous hormone application on gene expression in PSTVd-infected ‘MicroTom’ plants. As described by Marti and associates (2006), ‘MicroTom’ is a dwarf hybrid tomato in which both BR biosynthesis and GA signaling are genetically altered. A total of four separate experiments were carried out, and symptom development in the PSTVd-infected ‘Rutgers’ plants included as a positive control in each experiment was quite consistent.

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Fig. 1. Effect of host genotype on *Potato spindle tuber viroid* (PSTVd) symptom expression. **A**, Moderate stunting and epinasty in the sensitive cultivar 'Rutgers' following inoculation with PSTVd-Intermediate strain. Healthy control plant on the left. **B**, Virtual absence of visible symptoms in comparable PSTVd-infected 'Moneymaker' plants (middle). Although uninfected, note the presence of severe stunting and epinasty in a comparable IR11-14 plant containing a less-than-full-length PSTVd cDNA transgene (right). **C** to **E**, Loss of apical dominance in the cultivar Moneymaker associated with either **D**, PSTVd infection or **E**, the presence of a noninfectious PSTVd cDNA transgene. Note the outgrowth of lateral buds in both infected and transgenic plants. **F** to **H**, Comparison of the apical portions of **F**, healthy, **G**, PSTVd-infected, and **H**, transgene-containing 'Moneymaker' plants. Note the thickening and curvature visible only in the stem of the transgenic plant.
Approximately four weeks postinoculation (i.e., two weeks after symptoms had first begun to appear in leaves 6 and 7), the rapidly growing ‘Rutgers’ and ‘Moneymaker’ plants contained a total of 12 to 13 leaves, and the first flower buds had just begun to appear. Leaf 11 was less than half-expanded at this time, but epinasty was visible in its basal leaflets on the PSTVd-infected ‘Rutgers’ plants, and dot blot analysis of tissue samples collected from leaves 2 to 12 showed PSTVd titers to be at near-maximum levels in this leaf. Analysis of infected ‘Moneymaker’ plants revealed a very similar pattern of progeny accumulation throughout the first 5 weeks postinoculation (results not shown). As described below, tissue was collected from leaf 11 on four individual plants in each treatment (healthy, infected, or transgenic) for isolation of total cellular RNA. RNA preparations were treated with DNase to eliminate contaminating DNA before microarray analysis. The resulting data sets were analyzed using a one-way analysis of variance (ANOVA) model that compared infected plants with healthy ones, using both probability ($P = 0.05$) and false discovery rate (FDR) ($\alpha = 0.01$) testing methods.

Using as our initial criterion a $P$ value of 0.05, expression of slightly more than half (i.e., 5,354) of the nearly 10,000 genes represented on the Affymetrix Tomato GeneChip appeared to be significantly altered in PSTVd-infected ‘Rutgers’ plants. Com-

**Fig. 2.** Effects of *Potato spindle tuber viroid* (*PSTVd*) infection on gene expression in sensitive and tolerant tomato cultivars. **A and B,** Effects of PSTVd infection in ‘Rutgers’ (sensitive) and ‘Moneymaker’ (tolerant) tomato as estimated using either **A,** standard ($P = 0.05$) or **B,** more stringent ($\alpha = 0.01$) statistical criteria. For purposes of comparison, the effects of constitutively expressing small, PSTVd-related RNAs in the absence of viroid replication are also shown. **C and D,** Comparison of the effects of PSTVd infection in tomato with those of *Cabbage leaf curl virus* (*CaLCuV*) infection in *Arabidopsis* (Ascencio-Ibáñez et al. 2008), using the same statistical criteria. Note the high degree of concordance between the effects of these two quite different pathogens.
parable numbers for PSTVd-infected and transgene-containing ‘Moneymaker’ plants were 4,189 and 3,346, respectively. More stringent comparisons (i.e., FDR (α) = 0.01) yielded fewer differentially expressed genes, i.e., 2,497, 893, and 458 genes, respectively. Twofold or greater effects were observed for only 622, 295, or 431 genes. To confirm the accuracy of these microarray estimates, six representative genes (q values = −3.85 to +6.98) were selected for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis from among those differentially expressed in PSTVd-infected ‘Rutgers’ plants. Results paralleled those obtained by microarray analysis (Supplementary Table S1). Supplementary Tables S2 to S4 contain complete listings of differentially expressed genes in each treatment.

The series of Venn diagrams presented in Figure 2 compares the effects of PSTVd infection on gene expression in sensitive (Rutgers) and tolerant (Moneymaker) cultivars. These effects were also compared with those detected in diseased (but uninfected) plants from transgenic ‘Moneymaker’ line IR11-14 (Fig. 2A and B). In Figure 2C, the effects of PSTVd infection in ‘Rutgers’ and ‘Moneymaker’ tomato are compared with those associated with geminivirus infection in Arabidopsis (Ascencio-Ibáñez et al. 2008). Cabbage leaf curl virus (CaLCuV) is a geminivirus with a bipartite DNA genome that, like PSTVd, replicates in the nucleus of infected cells. Infection by CaLCuV leads to significant changes in the expression of approximately one-quarter of the Arabidopsis transcriptome, a fraction similar to that affected by PSTVd infection in tomato; likewise, the symptoms observed (e.g., stunting and chlorosis) bear a certain resemblance to those seen on PSTVd-infected tomato.

As the statistical criteria were made more stringent, the estimated number of genes differentially expressed in both infected ‘Rutgers’ and ‘Moneymaker’ plants decreased substantially, i.e.,...
from 2,675 (1,314 + 1,361) to 413 (352 + 61) (Fig. 2A and B). Including the results from the transgenic ‘Moneymaker’ plants revealed that disease severity alone was a rather poor predictor of the number of host genes affected, however. For example, fewer genes were differentially expressed in severely diseased transgenic IR11-14 plants than in infected ‘Rutgers’ plants showing less severe symptoms, i.e., 3,346 vs. 5,354 (P = 0.05) or 458 vs. 2,497 (α = 0.01). With few exceptions, the effects of PSTVd infection were quite similar for all three groups of plants (Fig. 2A and B). Effects observed in infected ‘Moneymaker’ plants were generally smaller than those seen in infected ‘Rutgers’, but the direction of the effect (i.e., up- or downregulation) was identical in almost all cases. To gain further possible insight into the effect of PSTVd infection on tomato gene expression, the two lists were compared with a similar gene list for CaLCuV-infected Arabidopsis (Ascencio-Ibáñez et al. 2008). Not all genes on the Arabidopsis ATH1 array are also present on the tomato array, and BLAST searches failed to reveal Arabidopsis homologs for 441 tomato unigenes whose expression was affected by PSTVd infection. Nevertheless, results summarized in Figure 2C and D revealed certain similarities in the responses of the two hosts to infection by these very different pathogens. One-third or more of all genes up- or down-regulated by PSTVd infection in tomato were also significantly affected by CaLCuV infection in Arabidopsis, and comparison of the direction of these effects revealed a striking degree of concordance (77.3 to 82.9%).

**Functional categorization of differentially expressed genes.**

This first series of analyses indicated that expression of a majority of genes on the tomato array was significantly altered in either PSTVd-infected plants or transgenic plants constitutively expressing PSTVd-related small RNAs. To begin to identify the possible functions of these genes, their Gene Ontology (GO) descriptions were used to group them according to three criteria: cellular component, biological process, or molecular function. Groupings were calculated using a parametric analysis of gene set enrichment (PAGE) strategy (Kim and Volsky 2005), and selected results are presented in Figures 3 and 4.

Effects of PSTVd infection on tomato gene expression have been grouped according to cellular component (Fig. 3). Results for the ‘Rutgers’ plants displaying moderate symptoms are shown, with comparable values for ‘Moneymaker’ plants (both PSTVd-infected and transgenic) included for selected GO categories. Looking at the central portion of this figure, a consistent downregulation of chloroplast function across all three treatments is immediately apparent. Although the degree of overrepresentation (as measured by the Z score) was variable, downregulation appeared greatest in the PSTVd-infected plants. For PSTVd-infected ‘Rutgers’ plants, genes encoding proteins

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**Fig. 4.** Association of differentially expressed tomato genes with specific biological functions. Groups of overrepresented genes, as defined by the Gene Ontology (GO) project, were identified by parametric analysis of gene set enrichment for both Potato spindle tuber viroid (PSTVd)-infected and transgenic plants. Complete results for only the PSTVd-infected ‘Rutgers’ plants are shown, with dark outlines indicating groupings also present in the other two treatments. Arrows within the boxes representing individual GO categories denote the relative degree of overrepresentation for, from left to right, ‘Rutgers’_PSTVd, ‘Moneymaker’_PSTVd, and ‘Moneymaker’ transgenic plants, as well as the direction of the effect (up- or downregulation). Note that names of certain GO categories have been modified to save space and that categories toward the bottom of the figure are more specific and less inclusive than those nearer the top.
associated with the nucleus, plasma membrane, ribosomes, and cell wall and apoplast (all up-regulated) were also overrepresented. Note that effects on organelles other than the chloroplast were confined to this one cultivar and were not observed with either the PSTVd-infected or transgenic ‘Moneymaker’ plants.

When these analyses were repeated using biological function rather than cellular component to compare effects, additional differences among treatments became apparent. Genes in many different categories related to both metabolic activities (Fig. 4, left side) and response to stimuli (Fig. 4, right side) were present in the data set from PSTVd-infected ‘Rutgers’ plants. For both PSTVd-infected and transgenic ‘Moneymaker’ plants, however, the only overrepresented genes were involved in response to stimuli. GO categories near the top of this figure are more general than those toward the bottom, and there is considerable overlap between certain categories. For this reason (and also to simplify the figure), the numbers of genes in each category are not shown. In addition to the five GO categories overrepresented in all three treatments (including response to biotic stimulus and defense response), note that seven other categories were overrepresented in at least two treatments.

As discussed by Whitham and associates (2006), RNA virus infections induce a characteristic series of changes in host gene expression, resembling the response to certain abiotic stresses or defense responses directed against other types of pathogens. These responses involve a number of genes whose expression is controlled by the salicylic (SA), jasmonic acid (JA), and ethylene (ET) signaling pathways. Not all these genes are represented on the Affymetrix tomato microarray, but the expression is controlled by the salicylic (SA), jasmonic acid (JA), and ethylene (ET) signaling pathways. Not all these genes are represented on the Affymetrix tomato microarray, but the response of both sensitive and tolerant tomato cultivars to PSTVd infection was clearly similar to that observed for RNA and DNA viruses.

**Effects on hormone biosynthesis and catabolism.**

The stunting and epinasty visible on the PSTVd-infected ‘Rutgers’ plants (Fig. 1) strongly suggest that viroid infection leads to significant disruption of GA and ET metabolism or signaling or both. To examine this possibility in more detail, we searched gene lists from both PSTVd-infected and transgenic plants for the presence of genes involved in hormone metabolism or signaling. A total of 19 genes involved in the biosynthesis or catabolism of different plant hormones were identified (Table 1). In addition to 1-aminocyclopropane-1-carboxylate oxidase (ethylene biosynthesis) and two enzymes involved in GA biosynthesis, significant changes in the levels of transcripts encoding several enzymes involved in abscisic acid (ABA), BR, cytokinin (CK), and JA metabolism were also detected in PSTVd-infected plants. Auxin metabolism, in contrast, appeared to be relatively unaffected.

In order to compare the effects of PSTVd infection on hormone metabolism with those of CaLCuV, these searches were carried out using The Arabidopsis Information Resource ID numbers rather than corresponding SGN unigene numbers. Data presented in Table 1 indicate that changes in ET, GA, and CK metabolism were limited to PSTVd-infected or transgene-containing tomato. Changes in BR and ABA metabolism may contribute to disease development in PSTVd-infected ‘Rutgers’, but similar changes were largely absent from the more severely affected transgenic IR11-14 plants, in which a major role for GA (and possibly JA) appeared more likely. The isoleucine conjugate of JA synthesized by JAR1 is considered to be a bioactive form of this molecule (Bari and Jones 2009). GA, ABA, and BR are products of a single terpenoid biosynthetic pathway; thus, changes in the levels of transcripts encoding key enzymes in this pathway could have far-reaching effects.

### Table 1. Effects of *Potato spindle tuber viroid* (PSTVd) infection on tomato genes involved in hormone biosynthesis

<table>
<thead>
<tr>
<th>TAIR ID no.</th>
<th>Gene description</th>
<th>PSTVd (‘Rutgers’)</th>
<th>PSTVd (‘Moneymaker’</th>
<th>siRNA (‘Moneymaker’)</th>
<th>CaLCuV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abscisic acid (ABA)</td>
<td>9-cis-Epoxycarotenoid dioxygenase (S)</td>
<td>-0.852</td>
<td>-0.573</td>
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<td>AT2G27150</td>
<td>Aldehyde oxidase (S)</td>
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<td>-0.432</td>
<td>-0.412</td>
<td>+0.189</td>
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<tr>
<td>AT4G19230</td>
<td>ABA 8’-hydroxylase (D)</td>
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<td>-0.880</td>
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<td>...</td>
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<tr>
<td>AT4G34431</td>
<td>UDP-glucosyl transferase family (D)</td>
<td>+3.320</td>
<td>+1.369</td>
<td>+1.235</td>
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<td>Auxin</td>
<td>Short-chain dehydrogenase (S)</td>
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<td>-0.239</td>
<td>+1.619</td>
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<tr>
<td>Brassinosteroid</td>
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<td>AT3G05660</td>
<td>Sterol-C5(6)-desaturase homolog (S)</td>
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<td>+0.272</td>
<td>+0.444</td>
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<tr>
<td>AT4G19820</td>
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<td>AT1G50430</td>
<td>Sterol 8-7 reductase (S)</td>
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<td>Cytokinin</td>
<td>UDP-glucosyl transferase (zeatin) (D)</td>
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<td>AT4G11340</td>
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<td>AT4G05010</td>
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<td>+1.981</td>
<td>+1.367</td>
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<td>Gibberellin (GA)</td>
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<td>GA 20-oxidase-1 (S)</td>
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<tr>
<td>Jasmonic acid</td>
<td>Lipoxigenase (S)</td>
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<td>AT2G60050</td>
<td>12-Octophytoleate reductase 3</td>
<td>+0.593</td>
<td>+0.412</td>
<td>+0.337</td>
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<tr>
<td>AT2G46370</td>
<td>JAR1 (jasmonic acid-amino acid conjugating enzyme)</td>
<td>...</td>
<td>-0.297</td>
<td>-0.825</td>
<td>...</td>
</tr>
</tbody>
</table>

a TAIR = The Arabidopsis Information Resource.
b Genes involved in both hormone synthesis (S) and inactivation or degradation (D) are shown.
c Expression changes expressed as log2. - = effect not statistically significant.
d CaLCuV = Cabbage leaf curl virus (*Arabidopsis thaliana*).
Effects of PSTVd infection on hormone signaling.

Three hormones—SA, JA, and ET—are known to play major roles in regulating plant defense responses against pathogens as well as abiotic stress (Bari and Jones 2009). To gain insight into the relative importance of these and other hormones in mediating viroid-host interaction, we mapped our microarray data onto the various hormone signaling networks, using Pathway Studio software from Ariadne Genomics (Rockville, MD, U.S.A.). Once again, for purposes of comparison, these analyses also included published data for CaLCuV-infected Arabidopsis (Ascencio-Ibáñez et al. 2008).

SA and JA. An important regulatory component of SA signaling is NPR1, a protein whose interaction with TGA transcription factors is responsible for induction of PR protein synthesis. Levels of NPR1 mRNA increased in PSTVd-infected ‘Rutgers’ plants but not in comparable ‘Moneymaker’ plants showing only mild symptoms (Fig 5A). Evidence for induction of PR-1, in contrast, was observed in the almost asymptomatic ‘Moneymaker’ infected plants but not in the severely diseased ‘Rutgers’ or transgenic plants. Major components of the JA signaling pathway include coronatine insensitive 1 (COII), jasmonate resistant 1 (JAR1), and jasmonate insensitive MYC2 (JIN1/MYC2). JAR1 encodes a JA amino-acid synthetase involved in the conjugation of isoleucine to JA, thereby producing the biologically active form of the hormone. COII (an F-box protein involved in SCF-mediated protein degradation) is required for most JA-mediated responses, and JIN1/MYC2 is a transcription factor that regulates expression of certain JA-responsive genes. Our analyses detected changes in only one of these genes (i.e., JAR1), and decreased levels of JAR1 mRNA were detected in both the infected and transgenic ‘Moneymaker’ plants (Fig. 5A). In PSTVd-infected ‘Rutgers’, two components of a mitogen-activated protein (MAP) kinase cascade were strongly up-regulated. One of these components, MAP kinase 4 (MPK4), acts as both a positive regulator of JA signaling and a down-regulator of SA signaling.

ET. Following pathogen inoculation, JA and ET signaling operate synergistically to activate expression of defense-related genes. The ET signaling pathway contains four main modules: i) a phosphotransfer relay located in the endoplasmic reticulum that serves as hormone receptor, ii) an EIN2-based unit, iii) a ubiquitin-mediated protein degradation component, and iv) a transcriptional cascade containing EIN3. Promoters of ET-responsive genes, like basic chitinase, contain GCC boxes that bind transcription factors produced by this cascade. As shown in Figure 5B, transcription of several components of the ET signaling pathway were strongly up-regulated in both PSTVd-infected and transgenic tomatoes expressing PSTVd-related small RNAs. At the beginning of the pathway, EIN4 (a member of ET receptor subfamily 2) was up-regulated in all three types of plants; at end of the pathway, a similar effect was observed for EIN3 and ERF1. Of several EIN3-dependent transcription factors in the pathway, two (ERF1 and ERF2) were strongly up-regulated. Also noteworthy were i) the up-regulation of CTR1 in infected ‘Rutgers’ and ‘Moneymaker’ plants and ii) the strong and uniform upregulation of basic chitinase expression observed in all three cases. CTR1 is a protein kinase that negatively regulates ET signaling, and ERF1 acts as a positive regulator of both ET and JA signaling.

GA and ABA. The key components of GA signaling are one or more DELLA proteins that negatively regulate the expression of several genes that, in turn, up-regulate the ABA pathway. GA and ABA signaling are thus antagonistic (Hartweck 2008). DELLA proteins promote transcription of GID1 (the GA receptor) and also modulate SA and JA-dependent defense responses (Navarro et al. 2008). Tomato contains a single DELLA-encoding gene, GAI (gibberellic acid insensitive) (Bassel et al. 2004) whose transcription was strongly (10.8-fold) repressed in the severely stunted transgenic ‘Moneymaker’ plants (Fig. 5C). We will return to the possible role of GAI in symptom induction when we examine possible infection-related changes in host miRNA populations (discussed below). The Affymetrix tomato array contains probes for only a single GA receptor (GID1C), whose transcript levels rose modestly in PSTVd-infected ‘Moneymaker’ plants. While transcription of ASK2 (involved in ubiquitin-mediated DELLA degradation) was up-regulated in both infected and transgenic plants, upregulation of the tomato homolog of ATSK1 (a SHAGGY-like kinase) was detected only in infected ‘Rutgers’.

ABA signaling is involved in the regulation of many aspects of plant growth and development, playing both a positive and a negative role in modulating host defense against pathogens (Bari and Jones 2009). Effects on the ABA signaling pathway were confined largely to infected ‘Rutgers’ plants showing visible disease symptoms (Fig. 5E). We note, for example, a modest upregulation of ERA1 (β subunit of farnesyl transferase and a negative regulator of ABA signaling) and PLDA1 (phospholipase D α-1 and a positive regulator of ABA-mediated stomatal movement). Two components of guard cell ABA signaling (inositol polyphosphate kinases IPK2a and IPK2b) were down-regulated, one of them (IPK2b) strongly so in infected ‘Rutgers’ plants. As shown in more detail in Table 1, levels of transcripts for several enzymes involved in ABA synthesis, including AAO3 (an aldehyde oxidase catalyzing the final step in ABA synthesis), were also down-regulated.

Brassinosteroids and cytokinins. The BR signaling pathway begins with a membrane-bound receptor-like kinase known as BRI1 (Fig. 5D). Downstream components include the SHAGGY-like protein kinase BIN2, protein phosphatase BSU1, and transcription factors BZR1 and BES1/BZR2. Transcription of BZR2 was modestly (1.44-fold) up-regulated in PSTVd-infected ‘Moneymaker’ plants, but effects on BIN2 were contradictory. A p450 protein encoded by the DWARF (DWF4) gene catalyzes the rate-limiting step in BR biosynthesis, and transcription of DWF4 was down-regulated in both PSTVd-infected and transgenic plants. In Arabidopsis, BR has been reported to induce the expression of genes involved in both ET and JA biosynthesis (Muessig et al. 2006; Yi et al. 1999).

Cytokinins are key regulators of many developmental processes, but their role in plant defense against pathogen attack is poorly understood. CK signaling is mediated by a multistep two-component histidine and aspartate phosphorylation system (Muller and Shen 2007). PSTVd infection in both ‘Rutgers’ and ‘Moneymaker’ was accompanied by downregulation of ARR3 and ARR9, two A-type cytokinin response regulator proteins located in the nucleus. A third A-type response regulator (ATRR3) was down-regulated only in infected ‘Moneymaker’ plants. In PSTVd-infected ‘Rutgers’, two probe sets related to AHP1 (one of several phosphotransfer proteins involved in transfer of the phosphate moiety from the transmembrane receptor to the response regulator) yielded disparate results. In one case, expression was strongly (twofold) down-regulated; in the other case, expression appeared to be 1.3-fold up-regulated (results not shown).

Auxin. Auxin promotes the degradation of a family of transcriptional repressors known as auxin/indole-3-acetic acid (Aux/IAA) proteins that bind to auxin response factors and inhibit the expression of specific auxin-response genes. A variety of different pathogens, including Tobacco mosaic virus (TMV) (Culver and Padmanabhan 2007), have been shown to manipulate AUX signaling to promote virulence and cause disease. In
Fig. 5. Effects of Potato spindle tuber viroid (PSTVd) infection on hormone signaling pathways. Selected components of the Arabidopsis A, salicylic acid and jasmonic acid, B, ethylene, C, gibberellin, D, abscissic acid, and E, brassinosteroid signaling pathways are shown. Dashed outlines indicate genes absent from the Affymetrix tomato array; bold outlines denote genes whose expression levels were significantly affected by one or more treatments; and DNA-binding transcription factors are shown as ovals resting upon three small boxes. From left to right, a series of three arrows summarizes the direction and extent of these effects in PSTVd-infected 'Rutgers', PSTVd-infected 'Moneymaker', and transgenic 'Moneymaker' plants. Bold arrows indicate effects greater than twofold; hyphens denote changes that were not statistically significant.
contrast to CaLCuV, in which an extensive series of effects on various components of the AUX signaling pathway, including the F-box protein TIR1, were apparent (Ascencio-Ibáñez et al. 2008), the effects of PSTVd infection were much more limited. Three genes, ASK2 (involved in ubiquitin-mediated proteolysis), transcription factor IAA3/SHY2, and IAR1 (auxin homeostasis), were up-regulated in PSTVd-infected ‘Rutgers’ plants. Expression of a single transcription factor (IAA14) was downregulated (results not shown).

Effect of PSTVd infection on host miRNA levels.

As part of an innate immune response by the host, virus and viroid infections induce the production of a variety of pathogen-derived small RNAs (Ding 2010; Ruiz-Ferré and Voinnet 2009). In animal and insect cells, virus infection is also accompanied by specific changes in host miRNA profiles (Parameswaran et al. 2010). As described elsewhere (Wang et al. 2011), large-scale sequence analysis of small RNAs isolated from the plants in these studies identified certain apparently cultivar-specific differences between the various PSTVd siRNA populations. Our search for possible evidence of similar changes in tomato miRNA populations began by reanalyzing this large-scale sequence data, this time focusing on the 36 recognized tomato miRNAs included in miRBase. To resolve certain inconsistencies detected during this reanalysis, levels of two of the more abundant miRNAs were directly compared by Northern blot.

Each large-scale sequence analysis yielded approximately 20 million sequence reads—approximately 10 percent of which, in the case of the PSTVd-infected or transgenic plants, was viroid-specific (Fig. 6A). Examination of the host-specific sequences revealed that our samples contained high levels of five well-characterized tomato miRNAs, i.e., >50 reads per million in at least one treatment. Of these five miRNAs, the responses of miR156 and miR159 to PSTVd infection were of particular interest. miR159 is an important component of the GA signaling pathway in which, together with GAI/DELLA, it regulates expression of GAmyb, a transcription factor. Known targets of miR156-mediated silencing include SOC1 (suppressor of CONSTANS, another component of the GA signaling pathway) as well as a member of a group of SQUAMOSA promoter binding–like proteins that play important roles in regulating plant growth and development (Moxon et al. 2008).

In most (but not all) cases, miRNA levels appeared to be moderately (two- to threefold) higher in PSTVd-infected or transgenic plants. One exception was an apparent 20-fold decrease in miR156 levels calculated for infected ‘Rutgers’ plants, and still more discrepancies arose when relative levels of individual miRNAs were compared with those of their respective target mRNAs as determined by microarray analysis. For example, miR159 levels were calculated to increase approximately threefold in both PSTVd-infected and transgenic ‘Moneymaker’ plants, but GAmyb expression was down-regulated only in the transgenic plants. Also, SOC1 expression was down-regulated in both PSTVd-infected ‘Rutgers’ and PSTVd-infected ‘Moneymaker’ plants, but our calculations indicated a dramatic drop in miR156 levels for the infected ‘Rutgers’ plants.

Because several factors can bias miRNA representation in deep-sequenced small RNA cDNA libraries (Hafner et al. 2011), we also carried out a series of Northern analyses to directly compare the relative concentrations of miR159 and miR166 in preparations of small RNAs highly enriched by elution from glass fiber filters. Blots were hybridized with appropriate mixtures of 32P-labeled oligonucleotide probe-specific U6 small nuclear RNA (used as a loading control) plus either miR159 or miR166, and the results are shown in Figure 6B. Comparison of lanes 1 to 5 indicates that miR159 levels remained essentially unchanged across all three treatments. Note that the amount of RNA loaded in lane 5 was fivefold greater than that loaded in lane 6 (10 vs. 2 μg), demonstrating that differences of as little as two- to threefold would have been detectable. Comparison of lanes 7 and 8 reveals a similar lack of change in miR166 levels following PSTVd infection. Because these two hybridizations contained equal concentrations of the respective miRNA-specific probes, the resulting signal intensities can be used to estimate relative target concentrations. Consistent with the significantly lower levels calculated for miR166 (71 to 198 vs. 801 to 6,558 reads per million), signal intensities in lanes 8 and 9 were much lower than those in lanes 1 to 5. Finally, the absence of signal over the corresponding portion of lane 7 indicates that the probe for U6 small nuclear RNA did not cross-react with tomato miRNA.

Effects of BR application on PSTVd-induced changes in gene expression.

Often proposed as the preferred tomato variety for molecular studies (Matsukura et al. 2008), the dwarf hybrid ‘MicroTom’ contains mutations affecting both BR biosynthesis and GA signaling (Marti et al. 2006). To investigate the possible role of BR signaling in disease induction by PSTVd, a final series of microarray analyses was carried out examining gene expression profiles in healthy and PSTVd-infected ‘MicroTom’ plants. In each case, four different treatments were compared; i) control (minus hormone), ii) epi-brassinolide (BR) alone, iii) GA3, and iv) BR + GA3. Infected ‘MicroTom’ plants exhibited virtually none of the symptoms visible in the normal stature variety Rutgers (Fig. 1), even when treated with exogenous hormone. As before, the resulting data were examined for broad changes in the pattern of gene expression using PAGE, and Figure 7 shows the extensive series of changes observed when BR was applied to PSTVd-infected ‘MicroTom’ plants.

In the untreated ‘MicroTom’ plants, PSTVd infection led to significant changes in the expression of 1,095 genes. Four overrepresented gene groupings were associated with such metabolic processes as biosynthesis of aromatic or N-containing compounds (Fig. 7, lightly shaded boxes). Two additional GO groupings were associated with signaling processes; i.e., response to hormones and other endogenous stimuli. Application of BR led to a doubling in the number of significantly affected genes and, for these hormone-treated plants, the number of overrepresented GO groups (Fig. 7, heavily shaded boxes) increased even more dramatically. This was true for both metabolic (17 groups) and signaling-related (seven groups) processes. Especially striking was the fact that five of the signaling-related GO groups identified were also up-regulated in PSTVd-infected ‘Rutgers’ plants in which the final step in BR biosynthesis is not blocked by mutation. Shared GO groups include response to stimulus (GO000508960), chemical stimulus (GO0042221), biotic stimulus (GO0009607), stress (GO0006950), and organic substances (GO0010033). Overlap among metabolism-associated groups was less extensive, i.e., only small molecule metabolic process (GO0044281), small molecule biosynthetic process (GO0044283), and lipid metabolism (GO00066290). Application of BR (discussed below) also led to changes affecting certain components of the BR signaling pathway (Fig. 8B), i.e., a 1.5 to 1.8-fold decrease in transcripts encoding 26S proteasome component RPN9 and BIN2 as well as a modest upregulation of transcription factor BES1.

DISCUSSION

Much is now known about the signaling networks responsible for perception of individual hormones in plants, and numerous interactions have been demonstrated among different
Several recent reviews (Culver and Padmanabhan 2007; Glazebrook 2005; Wise et al. 2007) discuss the use of transcript profiling techniques to probe the role of hormone signaling in various aspects of host-pathogen interaction, including virus infection. Viroids replicate poorly in Arabidopsis (Daros and Flores 2004; Matousek et al. 2004), and thus, the application of systems-based approaches such as microarray analysis to the study of viroid pathogenicity has lagged behind those of viruses such as TMV (Wang et al. 2009) and CaLCuV (Ascencio-Ibáñez et al. 2008). Instead, the focus has remained on the highly structured pathogen genome (Zhong et al. 2006).

Prominent among the different groups of genes whose expression is altered by virus infection are a number of stress- and defense-related genes (Whitham et al. 2006). Broad-based effects on both chloroplast biogenesis (downregulated) and cytoplasmic ribosome synthesis (upregulated) were reported by Dardick (2007), who compared the effects of three different RNA viruses on gene expression in Nicotiana benthamiana. At the whole-plant level, visible symptoms of infection in these plants ranged from almost undetectable (Prunus necrotic ringspot virus) to severe chlorosis, necrosis, and wilting (Tobacco ringspot virus [ToRSV]). Assuming a FDR of 0.01, PSTVd infection in the sensitive cultivar ‘Rutgers’ led to a statistically significant change in expression for approximately one in four tomato genes, an effect similar to that observed for CaLCuV infection in Arabidopsis (Ascencio-Ibáñez et al. 2008). Approximately

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Rutgers Healthy</th>
<th>PSTVd</th>
<th>Moneymaker Healthy</th>
<th>PSTVd</th>
<th>IR 11-14</th>
</tr>
</thead>
<tbody>
<tr>
<td>156 a,b,c</td>
<td>822</td>
<td>43 (0.05)</td>
<td>2,243</td>
<td>1882 (0.84)</td>
<td>5509 (2.46)</td>
</tr>
<tr>
<td>159</td>
<td>2,228</td>
<td>801 (0.36)</td>
<td>1,930</td>
<td>5110 (2.65)</td>
<td>6558 (3.40)</td>
</tr>
<tr>
<td>162</td>
<td>40</td>
<td>382 (9.55)</td>
<td>47</td>
<td>103 (2.19)</td>
<td>107 (2.28)</td>
</tr>
<tr>
<td>166 a,b</td>
<td>71</td>
<td>184 (2.59)</td>
<td>76</td>
<td>198 (2.54)</td>
<td>183 (2.35)</td>
</tr>
<tr>
<td>Total reads (millions)</td>
<td>21.83</td>
<td>18.85</td>
<td>21.93</td>
<td>21.82</td>
<td>21.17</td>
</tr>
</tbody>
</table>

* Frequencies expressed as counts/million reads. Values in parentheses, relative levels in infected/transgenic plants.

Fig. 6. Comparison of tomato miRNA levels in healthy and Potato spindle tuber viroid (PSTVd)-infected tomato. A, Calculated levels derived by searching the respective large-scale sequence data sets for exact matches to the 36 tomato miRNAs listed in miRBase. Values expressed as counts per million reads, and only the most abundant miRNAs are shown. B, Levels of miR159 (lanes 1 to 6) and miR166 (lanes 8 and 9), as determined by Northern blotting. Aliquots of leaf RNAs enriched for small RNAs (10 μg in lanes 1 to 5 and 7 to 9, 2 μg in lane 6) were fractionated by electrophoresis under denaturing conditions and were transferred to a nylon membrane. Portions of the resulting blot were then hybridized with either a mixture of 32P-labeled oligonucleotide probes specific for U6 small nuclear RNA and miR159 (lanes 1 to 6), U6 small nuclear RNA alone (lane 7), or U6 small nuclear RNA and miR166 (lanes 8 and 9). The source of each sample (H, healthy; I, PSTVd-infected; T, transgenic line IR 11-14) is shown below. Equal concentrations of miRNA-specific probes were used for the hybridizations shown in lanes 1 to 5 and 7 to 9.
threefold fewer genes were significantly affected in the tolerant cultivar ‘Moneymaker’, a decrease similar to those observed by Dardick (2007). Symptom intensity alone was not a reliable indicator of the underlying transcriptional changes, however, for severely diseased transgenic ‘Moneymaker’ plants containing a noninfectious PSTVd cDNA derived from the severe isolate RG1 exhibited fewer transcriptional changes than ‘Rutgers’ plants infected with PSTVd-Intermediate. Although it is tempting to speculate that full-fledged viroid replication, including the production of infectious PSTVd progeny and their subsequent movement throughout the plant, may perturb the expression of many genes unaffected by the constitutive expression of PSTVd siRNA alone, several other explanations cannot be excluded. For example, the nucleotide sequences of the Intermediate and RG1 strains of PSTVd differ at several positions within the pathogenicity domain. These sequence changes could lead to small but critical differences in the respective siRNA populations. Finally, none of the transgenic ‘Moneymaker’ lines originally described by Wang and associates (2004) have been well-characterized genetically. Thus, the viroid-like symptoms exhibited by IR 11-4 plants could be due to the insertional inactivation of one or more unidentified host genes.

Like infections by ToRSV and the other viruses studied by Dardick (2007) or Pepino mosaic virus (Hanssen et al. 2011), PSTVd infection was accompanied by a marked repression of genes associated with chloroplast biogenesis. Various abnormalities in chloroplast structure have been reported for PSTVd-infected tomato as well as a number of other viroid-host combinations (Diener 1987), but because PSTVd replication appears confined to the nucleus, the effects observed on chloroplast biogenesis likely reflect disruption of normal host communication between nucleus and chloroplast (Beck 2005). As discussed by Seay and associates (2010), the plant immune system utilizes the chloroplast as the primary site for regulation of cell-death programs that represent an important part of the plant defense response. Because many important chloroplast structural proteins are encoded by nuclear genes and translated on cytoplasmic (rather than plastid) ribosomes, it is not difficult to imagine how viroid replication in the nucleus could trigger chloroplast-based signaling pathways.

Equally intriguing are the apparent effects of PSTVd infection on protein metabolism, in particular the marked upregulation of ribosome biogenesis observed in PSTVd-infected ‘Rutgers’ plants. Viruses encode many of the proteins involved in their replication and movement from cell to cell, and changes in the host protein synthesis apparatus are often viewed as part of a pathogen strategy to increase mRNA competitiveness. The potential benefit of stimulating host protein metabolism to a noncoding (but autonomously replicating) subviral RNA like PSTVd is less clear. Although ribosome biogenesis appeared unaffected in a tolerant cultivar like ‘Moneymaker’ showing little or no visible sign of disease, ubiquitin-mediated proteolysis was up-regulated.

Several lines of evidence indicate that decreases in active GA levels play a major role in development of the stunting often seen in PSTVd-infected tomato. For example, Hammond and Zhao (2009) have recently shown that overexpression of the viroid-induced protein kinase PKV in transgenic tobacco leads to the appearance of symptoms (e.g., stunting, reduced root formation, and delayed flowering) very similar to those observed in PSTVd-infected tomato. Expression of the corresponding antisense RNA, in contrast, resulted in fertile plants that were taller than the nontransformed controls and flowered prolifically. Northern and qRT-PCR analyses revealed altered transcript levels for several genes involved in GA biosynthesis (GA20ox1 and GA3β) and signaling (NTH15 and RSG) but not other genes associated with the SA or JA pathways. Stunted plants overexpressing PKV responded normally to application of exogenous GA3, indicating that their dwarf phenotype is due to a lower content of one or more active GA and not to a

**Fig. 7.** Effects of epi-brassinolide (BR) on gene expression in *Potato spindle tuber viroid* (PSTVd)-infected ‘MicroTom’ tomato. Gene ontology (GO) groupings overrepresented in either buffer-treated control (light shading and outline) or BR-treated plants (heavy shading and outline) were identified by parametric analysis of gene set enrichment. Arrows within individual boxes denote the relative degree of overrepresentation for control (left) and BR-treated (right) plants. Unshaded boxes with very light, dashed outlines represent comparable groupings of genes involved in stimulus and response from PSTVd-infected ‘Rutgers’ or ‘Moneymaker’ plants. Note that names of certain GO categories have been modified to save space and that categories toward the bottom of the figure are more specific and less inclusive than those nearer the top.
lack of hormone responsiveness. In contrast to the upregulation of \( \text{GA20ox1} \) and \( \text{GA3} \beta \) reported by these authors, our results indicate that both genes are down-regulated in PSTVd-infected tomato. Furthermore, the magnitude of these effects were very similar in ‘Rutgers’ and ‘Moneymaker’ plants—this despite the fact that only the infected ‘Rutgers’ plants were visibly stunted. That changes in either BR biosynthesis, signaling, or both might also contribute to symptom development in viroid-infected plants has not previously been considered.

In *Arabidopsis*, BR has been reported to influence the expression of genes involved in both defense or stress responses and hormone biosynthesis (Bari and Jones 2009), and substantial evidence exists for cross-talk between the BR and GA signaling pathways. In the dwarf tomato cultivar ‘MicroTom’, a mutation in the gene encoding the enzyme that catalyzes the rate-limiting step in BR biosynthesis leads to mRNA missplicing and production of a truncated protein. The nature of a second defect in GA signaling is less well understood (Marti et al. 2006), but Figure 7 reveals striking similarities between the transcriptional changes observed in PSTVd-infected ‘MicroTom’ plants treated with exogenous BR and those observed in untreated ‘Rutgers’ or ‘Moneymaker’ plants. Many of the host responses to PSTVd infection thus appear to be channeled through the GA and BR signaling pathways, and certain transcriptional changes may affect pathway function (Fig. 8).

Figure 8A summarizes the effects of PSTVd infection on components of the GA signaling pathway. The central portion shows the \( \text{GA} \rightarrow \text{DELLA} \rightarrow \text{LEAFY} \) transcription factor cascade described by Achard and associates (2004) in which i) miR159 directs cleavage of an mRNA encoding a transcription factor (\( \text{GAMYB} = \text{MYB33} \)) that regulates flowering and anther development via expression of a second transcription factor (\( \text{LEAFY} \)) and ii) miR159 levels are regulated by both GA and ABA (Reyes and Chua 2007) via opposition of DELLAS function. Arranged around the periphery are several related components: \( \text{GA20ox1}, \text{GA3} \beta, \) and PKV; \( \text{MYB101} \) (a second miR159-regulated, ABA-inducible transcription factor not included on the Affymetrix array); \( \text{CONSTANS} \) (a key regulator of flowering); and \( \text{SOC1} \) (suppressor of \( \text{CONSTANS1} \), regulated by miR156 rather than miR159). Our data indicate that PSTVd infection has little or no effect on either the central \( \text{GA} \rightarrow \text{DELLA} \rightarrow \text{LEAFY} \) cascade or levels of miR159. Even in transgenic ‘Moneymaker’ plants in which DELLAS expression was strongly down-regulated, levels of the downstream transcription factor \( \text{LEAFY} \) appeared unchanged.

Somewhat surprisingly, PKV transcript levels in our PSTVd-infected ‘Rutgers’ or ‘Moneymaker’ plants also appeared unchanged. Hammond and Zhao (2009) have reported that overexpression of PKV in transgenic tobacco leads to downregulation of transcription factor \( \text{NTH15} \), and we observed a similar response for \( \text{NTH15} \) in PSTVd-infected tomato. In contrast, the decrease in \( \text{GA20ox} \) and \( \text{GA3} \beta \) transcript levels observed in PSTVd-infected tomato was just the opposite of the effect in transgenic tobacco. The most dramatic consequence of PSTVd infection in our experiments was the strong upregulation of
CONSTANS, an effect also observed in transgenic ‘Money-
maker’ plants. CONSTANS plays a key role in transmission of the pho-
toperiodic flowering stimulus from the leaves to the shoot apex (Jaeger et al. 2006), and further studies will be ne-
necessary to determine what this strong and consistent upregu-
lation indicates about the effects of PSTVd infection on GA sig-
naling.

Effects of PSTVd infection on BR signaling in normal-stat-
ure (‘Rutgers’ and ‘Moneymaker’) were compared with those in
dwarf (‘MicroTom’) tomato cultivars (Fig. 5B). The enzyme
coded by DWF4 catalyzes the rate-limiting step in BR bio-
synthesis. In contrast to the downregulation of wild-type tran-
scripts observed in normal-stature plants, PSTVd infection had
no obvious effect on expression of the splicing defective
DWF4 transcripts in ‘MicroTom’. Instead, comparison of un-
treated and BR-treated infected ‘MicroTom’ plants revealed
that transcriptional changes were confined to three other genes
in the pathway; BIN2, BES1, and RPN9 (Fig. 8A, highlighted).
BIN2 acts to repress transcription of BES1, and thus, down-
regulation of BIN2 would be expected to result in upregulation
of BES1. RPN9 was first identified in a screen for suppressors
of virus movement and encodes a component of the 26S pro-
tosome (Jin et al. 2006). RPN9 appears to function, at least in
part, through regulation of BR signaling and regulates vascular
development by targeting a subset of regulatory proteins, in-
cluding the BR-signaling protein BZR1 for degradation. Ap-
lication of exogenous hormone to infected ‘MicroTom’ plants
appears to up-regulate key aspects of BR signaling, possibly
allowing passage of certain as-yet-identiﬁed upstream “sig-
als” generated by viroid-host interaction and subsequent ex-
pansion of the host response. Two observations, the limited
number of transcriptional changes observed within the BR sig-
naling pathway itself, together with differences between the
patterns of downstream metabolic changes visible in Figures 3
and 7, indicate that BR signaling in the hormone-treated ‘Mi-
croTom’ plants is not completely normal. Additional experi-
ments involving treatment of normal-stature plants with BR
synthesis inhibitors like brassinazole (Asami et al. 2000) may
be quite helpful to identify the individual contributions of BR-
and GA-mediated signaling to disease induction by PSTVd.

MATERIALS AND METHODS

Plant lines and viroid strains.
Seeds of tomato (Solanum lycopersicum L.) cultivars Rut-
gers, Moneymaker, and MicroTom (Meissner et al. 1997) were
purchased from Totally Tomatoes (Randolph, WI, U.S.A.).
Seeds of IR11-11 and IR11-14, two homozygous lines of trans-
genic ‘Moneymaker’ plants described by Wang and associates
(2004) and carrying a noninfectious PSTVd hairpin construct,
were obtained from M.-B. Wang (CSIRO, Canberra, Australia).
Highly infectious, precisely full-length RNA transcripts de-
erived from the Intermediate and RG1 strains of PSTVd were
synthesized as described by Hu and associates (1997).

Analysis of gene expression profiles.
Approximately 10 days postgermination, young tomato
seedlings at the cotyledon stage and growing under greenhouse
conditions were transferred to individual 10-cm pots contain-
ing ProMixBX growing medium (Griffin Greenhouse & Nur-
sery Supplies, Morgantown, PA, U.S.A.) and were allowed to
develop three to four true leaves. The terminal leaflet on the
tree true leaf was dusted with Carborundum (600 mesh) and
was inoculated with a 10-μl aliquot of either 20 mM Na phos-
phate (pH 7.0) or the same buffer containing approximately 70
ng of ribozyme-derived PSTVd-Intermediate RNA transcripts.
Following inoculation, plants were maintained in the green-
house for approximately five weeks under high temperature
and long day conditions (30°C, 16-h days and 25°C, 8-h nights)
favoring high rates of viroid replication and strong symptom
development. To encourage continued rapid growth, ‘Rutgers’ and
‘Moneymaker’ plants were transferred to larger (one-gal-
lon) pots approximately 10 days postinoculation.

The dwarf hybrid cultivar ‘MicroTom’ contains mutations
affecting both BR synthesis and GA signaling (Martí et al.
2006). Young ‘MicroTom’ seedlings were inoculated on a single
cotyledon with PSTVd-RG1 RNA transcripts and were left in
their original 10-cm pots during the entire course of infec-
tion. Aliquots (10 μl) of 10% ethanol containing 0.1% Tween
20 (minus hormone control) or the same solvent containing 0.1
μg of GA3 per microliter, 0.5 ng of BR per microliter, or a
mixture of both hormones were subsequently placed on the
surface of the uninoculated cotyledon (day 0), leaves 1 and 2
(day 5), leaves 1 to 4 (day 10), or leaves 5 to 7 (day 15). At 19
days postinoculation, young, untreated leaves ≤2.5 to 3.0 cm in
length were harvested and processed for RNA extraction.

Accumulation of PSTVd progeny in inoculated ‘Rutgers’ and
‘Moneymaker’ plants was monitored by dot-blot hybridi-
zation as described by Podleckis and associates (1993). Total
RNA was extracted from freshly collected leaf tissue, using TRI
reagent (Molecular Research Center, Cincinnati) according to
the manufacturer’s instructions. Tissue samples were powdered
in liquid nitrogen before extraction, and equal amounts of the
resulting total RNA preparations (as determined by optical
density at 260 nm) were digested with RQ DNase and were
purified by passage through RNaseasy minicolumns (Qiagen,
Valencia, CA, U.S.A.) before microarray analysis.

Microarray analysis of gene expression.
Microarray hybridization, washing, and scanning operations
were carried out at the Biopolymers and Genomics core facility
operated by the University of Maryland School of Medicine
(Baltimore, MD, U.S.A.), using tomato genome arrays pur-
chased from Affymetrix (Santa Clara, CA, U.S.A.). For each
comparison, four biological replicates derived from individual
mock-inoculated or infected plants were processed independ-
ently. Double-stranded cDNA was synthesized from total leaf
RNA (1 to 2 μg), using One-Cycle Target Labeling and Con-
tral Reagents (Affymetrix), and the resulting cDNAs were then
used as template for synthesis of biotin-labeled cRNA probes
(15 μg) that were fragmented before hybridization. Immedi-
ately following hybridization, arrays were developed using an
automated washing and staining protocol (EukGE-WS2v5) on
an FS-450 ﬂuidics station and were scanned with a GeneChip
Scanner 7000 (Affymetrix).

Tomato GeneChip scanning data were analyzed at probe
level to directly extract signal intensities from CEL ﬁles using the JMP Genomics v.3.2 software package (SAS Institute, Cary,
NC, U.S.A.) A robust multichip average (RMA) background
correction was applied to each array, and the data was then
log2 transformed. Next, the data sets were quantile normalized
and were summarized using the MEDIANPOLISH function.
To identify genes signiﬁcantly affected by PSTVd infection or
the presence of PSTVd-derived small RNAs, the data were
processed using a one-way ANOVA model that compared
infected to healthy plants using either a probability ($P = 0.05$)
or FDR (α = 0.01) testing method. MIAME-compliant raw
CEL and normalized intensity data ﬁles are available from the
Tomato Functional Genomics database under accession num-
ber E048. Raw CEL files containing data collected from
Arabidopsis plants infected with CaLCuV using the Affymetrix
ATH1 GeneChip (Ascencio-Ibáñez et al. 2008) were down-
loaded from ArrayExpress and were reanalyzed using JMP
Genomics v3.2 as described above.
PAGE analysis of the resulting transcriptional profiles (Kim and Volsky 2005) was performed using software tools available on the AgriGo website (Du et al. 2010). Starting from lists of genes whose expression levels were significantly affected by PSTVd infection, this program uses the Central Limit Theorem to identify enriched GO terms from among the corresponding list of gene identifiers. Expression levels are taken into account, and PAGE uses a two-tailed test to calculate a Z score indicating the relative degree of overrepresentation for each enriched GO term. To simplify the rather complicated networks produced by PAGE analysis, arrows connecting individual GO terms have been removed from the results presented in Figures 3, 4, and 7.

qRT-PCR analysis

qRT-PCR analysis was carried out in a Stratagene Mx3000P thermal cycler using the AffinityScript QPCR cDNA synthesis kit and Brilliant II SYBER green QPCR master mix (Stratagene, La Jolla, CA, U.S.A.). Primer pairs designed to yield specific PCR products approximately 100 bp in length with either control (actin and tubulin) or experimental genes were selected using the OligoPerfect software (Invitrogen, Carlsbad, CA, U.S.A.). The initial cDNA synthesis reactions containing 1.5 to 2.0 μg of DNase-treated tomato total RNA and 20 μl of oligo(dT) were incubated at 95°C (5 min), 42°C (5 min), and 55°C (30 min). Then, duplicate PCR reactions (40 cycles) containing 1 μl of cDNA in a total volume of 25 μl were incubated at 95°C (30 s), 55°C (60 s), and 72°C (30 s), and the resulting data was averaged.

Large-scale sequencing of small RNAs.

Ribosomal and other high-molecular-weight (LMW) RNAs were removed from pooled preparations of total RNA isolated from individual leaves of healthy or PSTVd-infected tomato plants by overnight precipitation with 2 M LiCl. Small RNAs approximately 15 to 30 nt in length were isolated from the resulting preparations of LMW RNA by electrophoresis in 12% polyacrylamide gels (19:1) containing 8 M urea, over-night elution in 0.5 M ammonium acetate and 0.1% sodium dodecyl sulfate (SDS), and ethanol precipitation. Purified RNAs were dissolved in RNase-free water and were sent to Hokkaido System Science (Sapporo, Japan) for large-scale sequence analysis using a Genetic Analyzer II (Illumina, San Diego, CA, U.S.A.) and reagents and protocols supplied by the manufacturer.

Adapter sequences were removed from the ends of the resulting raw short-read data based on the presence of an exact 10-nt match with the termini of the respective adapters, and identical short reads were grouped according to read size (15 to 29 nt). In this way, adapter-trimmed short-read data were converted to a nonredundant “short-read-sequence occurrence” format. Sequences of known tomato miRNAs were obtained from the miRBase website, and corresponding sequences were identified among the trimmed sequences in our Illumina data sets using specially-written C scripts. To ensure accuracy, comparisons were carried out in both directions. Potential target sites for tomato miRNAs were identified using software tools available on the Tomato Functional Genomics database website.

Quantification of tomato miRNAs.

To independently assess the relative concentration of host miRNAs in healthy and PSTVd-infected leaf tissue, aliquots (10 μg) of LMW RNA isolated from total leaf RNA using the mirVana miRNA isolation kit (Ambion, Austin, TX, U.S.A.) were fractionated by denaturing electrophoresis carried out in 12% polyacrylamide gels (19:1, wt/wt) containing 1x Tris bo-


dating plant responses to viral infection. Mol. Plant-Microbe Interact. 19:1207-1215.

AUTHOR-RECOMMENDED INTERNET RESOURCES

The AgriGo website: bioinfo.cau.edu.cn/agriGO
ArrayExpress database: www.ebi.ac.uk/arrayexpress
miRBase database: www.mirbase.org
Tomato Functional Genomics database: ted.bti.cornell.edu