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Regulation of a Virus-Induced Lethal Disease in Tomato Revealed by LongSAGE Analysis

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Infection of *Cucumber mosaic virus* (CMV) and D satellite RNA (satRNA) in tomato plants induces rapid plant death, which has caused catastrophic crop losses. We conducted long serial analysis of gene expression (LongSAGE) in control and virus-infected plants to identify the genes that may be involved in the development of this lethal tomato disease. The transcriptomes were compared between mock-inoculated plants and plants infected with CMV, CMV/D satRNA, or CMV/Dm satRNA (a nonnecrogenic mutant of D satRNA with three mutated nucleotides). The analysis revealed both general and specific changes in the tomato transcriptome after infection with these viruses. A massive transcriptional difference of approximately 400 genes was found between the transcriptomes of CMV/D and CMV/Dm satRNA-infected plants. Particularly, the LongSAGE data indicated the activation of ethylene synthesis and signaling by CMV/D satRNA infection. Results from inoculation tests with an ethylene-insensitive mutant and treatments with an ethylene action inhibitor further confirmed the role of ethylene in mediating the epinastic leaf symptoms and the secondary cell death in the stem. Results from Northern blot analysis demonstrated the partial contribution of ethylene in the induced defense responses in CMV/D satRNA-infected plants.

Additional keywords: programmed cell death.

Cucumber mosaic virus (CMV) has the largest host range of any known virus and is an important pathogen in agriculture (Palukaitis and García-Arenal 2003). CMV can harbor molecular parasites, called satellite RNAs (satRNAs), that usually attenuate disease symptoms. The CMV satRNAs do not encode any known proteins; therefore, their biological activities depend on the RNA molecules themselves. D satRNA attenuates CMV symptoms in most of its plant hosts but specifically induces systemic necrosis in tomato plants and causes an epidemic lethal disease in tomato fields (Grieco et al. 1997; Jordá et al. 1992; Kaper and Tousignant 1977). Three nucleotides in D satRNA determine the necrogenecity (Sleat et al. 1994), and the secondary structure of the corresponding sequences has been reported (Rodríguez-Alvarado and Roossinck 1997). Virus infections resulting in plant death are not common but do occur in some other agricultural plants, and cause catastrophic crop losses. Little is known about the molecular mechanisms of virus-induced plant lethality. Thus, it is important to understand the cellular and physiological responses in the infected plants that are susceptible to necrogenic viruses.

Our previous work showed that CMV/D satRNA infection caused a systemic programmed cell death (PCD) in the infected tomato plants (Xu and Roossinck 2000). When tomato seedlings at the two or three-leaf stage were inoculated, PCD first occurred in the infected developing phloem cells around the second node below the meristem. This was rapidly followed by a widespread death of the neighboring infected cells that eventually led to the complete collapse of the plant.

PCD is an ordered cell suicide process. It is a fundamentally important component of developmental programs (Pennell and Lamb 1997). PCD also may result from abiotic or biotic stress and causes necrotic diseases or mediates resistance to pathogens (Greenberg and Yao 2004). Accumulating evidence indicates that PCD regulation is conserved between animals and plants. For example, several plant species have homologues of an animal anti-apoptotic gene, *Bax inhibitor 1* (*BI-1*), that inhibit the cell death induced by environmental stresses or by heteroexpression of the animal pro-apoptotic gene *Bax* (Huckelhoven et al. 2003; Kawai-Yamada et al. 2001). The expression of tomato *BI-1* is increased in CMV/D satRNA-infected plants and highly expressed in the cells neighboring dead tissues (Xu et al. 2003), which may protect the cells from spreading death. In addition, high expression of animal anti-apoptotic genes *Bcl-xL* and *Ced-9* in tomato suppresses CMV/D satRNA-induced PCD (Xu et al. 2004).

Many animal viral pathogens trigger PCD in the infected cells to enhance spread or to evade the host defense system (Roulston et al. 1999). On the other hand, PCD is also a host defensive mechanism for eliminating cells infected by viral pathogens (Roulston et al. 1999). In plants, PCD in the hypersensitive response (HR) may play a similar role in limiting viral infection (Greenberg and Yao 2004). In CMV/D satRNA infection, rapidly spreading PCD leads to complete death of tomato plants, although multiple defense responses are induced in the infected plants (Xu et al. 2003). The regulation of CMV/D satRNA-induced PCD in tomato is unknown. Thus, we took an approach of transcriptome analysis, the long serial analysis of gene expression (LongSAGE), to investigate the global change of gene expression in the tissues processing PCD. SAGE is based on the isolation of unique sequence tags from individual transcripts and concatenation of tags serially into long DNA molecules (Velculescu et al. 1995). Through sequencing and identifying the transcripts from which the tags are isolated, SAGE is applied mostly in identification of transcripts present in specific tissues and the quantitative comparison of transcriptomes. It is especially useful for species where comprehensive genome analysis is not yet available. SAGE has proven to be a relatively low-cost but robust method for

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investigating gene expression at the whole-genome scale in different species across the kingdoms (Fizames et al. 2004; Gorski et al. 2003; Jones et al. 2006; Lee and Lee 2003; Lorenz and Dean 2002; Matsumura et al. 1999; Velculescu et al. 2000; Virlon et al. 1999). The recently developed LongSAGE produces longer tag sequences and improves the identification of transcripts corresponding to the tags (Gowda et al. 2004; Saha et al. 2002). Here, we constructed a new attenuating CMV satRNA, Dm satRNA, with three nucleotide differences from the necrogenic D satRNA, and applied LongSAGE to compare the gene expression among the plants infected with CMV, CMV/D satRNA, or CMV/Dm satRNA at the time point where PCD was initiated in the CMV/D satRNA-infected plants. Ethylene and jasmonic acid (JA) were revealed to be involved in PCD regulation and disease progression and likely are signaling pathways of cell death in CMV/D satRNA-infected plants.

RESULTS

Summary of sequenced LongSAGE libraries.

Tomato plants infected with CMV are stunted with stem thinning and shortened internodes, and show severe mosaic symptoms and shoe-string leaves. These symptoms are attenuated in tomato plants infected with CMV/D satRNA, but the plants develop new symptoms of severe leaf epinasty and systemic necrosis. Based on the mapped necrogenic sequence of D satRNA (Sleat et al. 1994), we constructed a mutant satRNA, Dm, with a three-nucleotide mutation near the 3′ end. Dm satRNA infection attenuated the symptoms caused by CMV infection and did not induce leaf epinasty or cell death (Supplemental Figure S1). In order to study the molecular basis of disease symptom expression, eight LongSAGE libraries were generated from virally infected (CMV, CMV/Dm satRNA, or CMV/D satRNA) or mock-inoculated tomato plants at 9 days postinoculation (dpi) when PCD initiated in the stem of CMV/D satRNA-infected plants. Four libraries were from the stem from the top of the plants to the third node and the other four were from the systemic leaf tissue. One thousand clones per library, amounting to approximately 20,000 tags, were sequenced (Table 1). The total number of unique tags detected in all libraries amounted to 48,651, of which 34% had more than two counts in the sequenced eight libraries. Of all the unique tags, 37% had hits in the TIGR tomato Unigene expressed sequence tag (EST) databases and approximately 11% of the remaining tags without any match in the tomato databases had hits in the TIGR EST databases of other solanaceous plants. A small portion of the tags (5%) were mapped in a reverse orientation with respect to EST and tentative contig sequences that may arise from antisense transcripts or sequence orientation errors indicated in the databases (Patankar et al. 2001). In addition, approximately 5% of the tags have multiple hits, mainly for different members of the same gene family. The complete LongSAGE data are provided in the web supplement to this article (Supplemental Table S1).

Comparison of gene expression among the eight libraries.

Each library from virus-infected plants was compared with the corresponding library from mock-inoculated plants to determine differentially expressed genes. The statistical Z test was applied to detect a significant difference for pairwise tag comparison with *P* value at 0.01 (Ruijter et al. 2002). We found that the infection by these three combinations of viruses caused both general and specific changes in the tomato transcriptome. They all upregulated the expression of five genes and downregulated four in the leaf tissues (Table 2). The function of one of the four downregulated genes is unknown. Two genes with decreased expression levels encode proteins involved in energy conversion and one encodes a dehydrin-like protein. The expression of three genes encoding chlorophyll binding proteins in light harvesting complex (LHC) II, a cell wall protein and a cysteine protease inhibitor, was increased. In the stem tissue, only one common gene, photosystem I (PSI) reaction center subunit X *psaK*, was significantly downregulated by all three virus infections. The change of expression in these genes reflects the common response of tomato plants to all of the CMV infections.

CMV infection alone resulted in a much greater change of gene expression in the leaves than in the stems, as did the infection of CMV/Dm satRNA (Table 1). Although CMV symptoms were attenuated in CMV/Dm satRNA-infected plants compared with CMV alone, the expression of more genes was altered in CMV/Dm satRNA-infected plants. Both CMV and CMV/Dm satRNA infection upregulated the expression of *ferredoxin-NADP reductase* (*FNR*) in the stem and some LHC II type I *CAB* genes in the leaves, and downregulated the expression of *plastidic cysteine synthase 1* in the leaves (Table 2).

The most dramatic change in gene expression was caused by the infection of CMV/D satRNA, where the expression of 209 genes was affected in the stem and 182 genes in the leaves (Table 1) compared with the gene expression in noninfected plants. These genes are involved in various biological processes such as photosynthesis, energy conversion, cell division, growth and structure, cell death, osmotic stress, and defense responses. Among them, some genes were similarly regulated by the infection of CMV without satRNAs (Table 2). Tomato plants infected with CMV/D or CMV/Dm satRNA showed attenuated CMV symptoms. In the systemic leaves of these plants, the expression of some known genes was increased, including different isomers of LHC II type I *CAB* genes, defense- and protection-related *proteinase inhibitor* (*pin*) I, *endochitinase*, *catalase*, stress-responsive *metallothionein-like*, and sulfate metabolism-related *Ntdin* and *sulfite oxidase* (Table 2). The expression of other known genes decreased in the leaves, including carbon assimilation-related *RuBisco subunits* and *activase*, *fructose-bisphosphate aldolase*, ribosomal *L27a* and *S12*, and *α-tubulin* (Table 2). Expression of some unknown genes also was decreased. In addition to the transcriptional changes in the leaves, both satRNAs decreased the expression of some photosynthetic and metabolic genes and the gene-encoding peptidase inhibitor in the stem (Table 2). These results indicate the common response of tomato to both parasitic satRNAs, which might be correlated with their common role in attenuating CMV symptoms. However, the differences in the host responses to the different satRNAs is much more dramatic than the similarities, reflecting differences in their biological

Table 1. Summary of long serial analysis of gene expression libraries[a]

	Stem				Leaf			
Libraries	**Mock**	**CMV/D**	**CMV**	**CMV/Dm**	**Mock**	**CMV/D**	**CMV**	**CMV/Dm**
Total tags	20,104	20,526	20,436	20,624	19,774	19,798	19,495	19,889
Upregulated expression	…	97	6	11	…	97	42	53
Downregulated expression	…	112	3	12	…	85	22	32

[a] *Cucumber mosaic virus* (CMV), CMV D satellite RNA (CMV/D), and a mutant of D satellite RNA with three mutated nucleotides (CMV/Dm).

impacts on the host: Dm satRNA leads to milder mosaic symptoms and D satRNA leads to complete plant death.

Comparison of the transcriptomes of CMV/D satRNA- and CMV/Dm satRNA-infected plants.

We compared the transcriptomes of groups of plants infected with CMV and these two satRNAs. First, the LongSAGE libraries of the leaf and stem tissues from CMV/D satRNA- or CMV/Dm satRNA-infected plants were combined into a new LongSAGE library for comparison. Significant differences in gene expression levels of each tag were detected by a Z test statistical analysis ($P \leq 0.01$), which resulted in 201 genes showing significantly lower expression and 200 genes showing higher expression in CMV/D satRNA- compared with CMV/Dm satRNA-infected plants. Among them, the expression of 69 selected genes was analyzed by quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) where the *ubiquitin*-like gene or the *ubiquitin conjugating enzyme* gene were used as endogenous controls. Results from qRT-PCR confirmed the differential expression of 63 genes (Supplemental Table S3.1). The complete tag and gene list resulting from the LongSAGE analysis is in Supplemental Table S2.1. The top 25 tags for genes showing the greatest differences in expression levels are listed in Table 3.

Compared with CMV/Dm satRNA-infected plants, the transcription of a large number of genes functioning in photosynthesis, energy conversion-related metabolism, and energy generation was greatly reduced in the CMV/D satRNA-infected plants at the time when severe leaf epinasty appeared and PCD was initiated (Fig. 1A). The expression of cytoskeleton proteins, nucleosome proteins, and cell reproduction regulators also decreased (Fig. 1A). In addition, the expression of some genes involved in signal transduction pathways was differentially altered by these two satRNAs. For example, the expression levels of *CaM7* and one member of the *14-3-3* gene family were lower in CMV/D satRNA-infected plants, while a homolog to *CaM13*, two *CaM-like* genes, and two other members of *14-3-3* gene family were expressed at higher levels. The transcripts

Table 2. Genes similarly regulated by *Cucumber mosaic virus* (CMV), CMV D satellite RNA (CMV/D), and a mutant of D satellite RNA with three mutated nucleotides (CMV/Dm) in the systemic leaves and stem

Expression, tissue type	Viruses for comparison	Genes with similarly regulated expression in the plants infected by the compared viruses
Upregulated		
Stem	CMV vs. CMV/Dm	Ferredoxin-NADP reductase
	CMV vs. CMV/D	MAPK3
Leaf	CMV vs. CMV/Dm vs. CMV/D	Cell wall protein precursor
		LHCII type I CAB-1B
		LHCII type I CAB 3C
		LHCII type I CAB 16
		Cysteine protease TDI-65 (CYP1)
	CMV vs. CMV/Dm	LHCII type I CAB-1B (TC153542) and CAB4
		With no hit in blast search
	CMV vs. CMV/D	Similar to 26S ribosomal RNA
		Metallothionein-like protein type 2
	CMV/D vs. CMV/Dm	LHCII type I CAB-1B (TC153574)
		Metallothionein-like protein type 2B
		Ntdin
		Wound-inducible proteinase inhibitor I
		ACT domain-containing protein
		Armadillo/β-catenin repeat family protein
		Catalase isozyme1
		Sulfite oxidase
		Acidic 27-kDa endochitinase precursor
Downregulated		
Stem	CMV vs. CMV/Dm vs. CMV/D	Photosystem I reaction center subunit X psaK
	CMV vs. CMV/D	Chaperone DnaJ-like
	CMV/D vs. CMV/Dm	RuBisCO small subunits
		Oxygen-evolving enhancer protein 3-1
		DIP-1 transcription factor
		Haloacid dehalogenase-like hydrolase
		Chloroplast protein 29 (CP29) polypeptide
		PSI-N, PSI-F
		Carboxypeptidase inhibitor
Leaf	CMV vs. CMV/Dm vs. CMV/D	Dehydrin-like protein
		Carbonic anhydrase
		Transketolase
		No hit in blast search
	CMV vs. CMV/Dm	Plastidic cysteine synthase 1
	CMV vs. CMV/D	TC154324; TC156543
		Chloroplast protein 47
		Glycine dehydrogenase
		Calmodulin-7
	CMV/D vs. CMV/Dm	RuBisCO small subunit 3A/3C
		RuBisCO activase
		Histidinyl dehydrogenase or Rubisco smallsubunit 1 or LHC II type I CAB-3C
		Ribosomal protein L27a
		40S ribosomal protein S12
		α-Tubulin
		Splicing factor
		Fructose-biphosphate aldolase
		TC162814; CV476487

of *mitogen-activated protein kinase* (*MAPK*)*3* and *phospholipase PLDβ1* also were more abundant in CMV/D satRNA-infected plants.

Numerous genes involved in pathogenesis, stress, and defense responses were activated by CMV/D satRNA infection whereas some others were expressed at lower levels compared with CMV/Dm satRNA-infected plants (Fig. 1A and B). In addition to induced or increased expression of various *pathogenesis related* (*PR*) and *heat shock protein* (*HSP*) genes in CMV/D satRNA-infected plants, the expression of some metabolic genes with active roles in anti-microbial attack and disease resistance was upregulated, including *divinyl ether synthase*, *Snakin2*, *phenazine biosynthesis-like protein*, *polyphenol oxidase D*, *CCoAMT*, *N-hydroxycinnamoyl transferase*, fungal *endoglucanase inhibitor*, and *lipid desaturases*. On the other hand, genes encoding lipid transfer proteins, feebly protein, defensin and thionins, leucine-rich repeat proteins, germin-like protein, polygalacturonase inhibitor, and a different member of the polyphenol oxidase gene family were expressed at lower levels compared with CMV/Dm satRNA-infected plants. CMV/D satRNA infection also affected the expression of some genes involved in osmotic stress response and regulation such as *aquaporin 1*, *PIP2*, *PM28B*, *trehalose phosphatase*, *dehydrin TAS14*, and *betaine-aldehyde dehydogenase*.

Previously, we detected a high accumulation of H_2O_2 in CMV/D satRNA-infected plants (Xu et al. 2003). Results from the LongSAGE data show significant changes in the transcription levels of some redox regulators. The transcripts for *dehydroascorbate reductase*, some *peroxidases*, and *thioredoxin* were reduced, but the expression of *GST* (glutathione-*S*-transferase), *catalase*, and some other members of *peroxidases* was increased. There also were increased transcripts for *GABA transaminase* and *nonsymbiotic hemoglobin* (*nsHb*) in the CMV/D satRNA-infected plants. The GABA shunt can prevent the accumulation of reactive oxygen species (ROS) and cell death (Bouché et al. 2003). The nsHb is a key enzymatic system for NO scavenging in plants under hypoxic or other abiotic stress (Perazzolli et al. 2006).

Table 3. Top 25 genes expressed at significantly higher levels and top 25 genes expressed at significantly lower levels in plants infected with *Cucumber mosaic virus* D satellite RNA (CMV/D) and a mutant of D satellite RNA with three mutated nucleotides (CMV/Dm)

TagID	CMV/D[a]	CMV/Dm[a]	Z value	TC_id	Annotation
Higher levels					
TAG_008820	254	26	13.69	AI899543;TC161968	Pathogenesis-related leaf protein 6 precursor (P6) (ethylene-induced protein P1)
TAG_015230	144	2	11.8	TC155130	Pathogenesis-related leaf protein 4 precursor (P4)
TAG_008353	166	24	10.35	TC153698	Homologue to Tragopogon dubius large subunit 26S ribosomal RNA; partial (80%)
TAG_008585	92	6	8.72	TC161922;TC161924	1-Aminocyclopropane-1-carboxylate (ACC) oxidase1; ACC oxidase 4)
TAG_005183	80	2	8.64	TC162690	*Weakly similar to* IBR domain putative, *partial (6%)*
TAG_013755	86	5	8.52	TC162694	Pathogenesis-related protein P2 precursor
TAG_005017	108	15	8.42	TC153736;TC154261; TC153735;TC153622	Homologue to chitinase, partial (96%); basic 30-kDa endochitinase precursor; homologue to endochitinase A precursor (CHN-A) , partial (97%);similar to endochitinase 3 precursor
TAG_004060	63	1	7.77	TC153783	NP24 protein precursor (pathogenesis-related protein PR P23)
TAG_013756	59	0	7.7	TC164904	Ethylene-responsive proteinase inhibitor I precursor
TAG_008519	65	4	7.37	TC153787	Similar to hydroxyproline-rich glycoprotein precursor, partial (58%)
TAG_008337	51	2	6.75	TC161804	Similar to expressed protein, partial (55%)
TAG_000799	354	198	6.73	AI775310;TC153945	Cell wall protein. II. protein.ide.II chlorophyll a/b binding polypeptide of p; cell wall protein precursor, complete
TAG_005438	45	1	6.51	TC162180;TC153756	Homologue to mitochondrial gene for 26S ribosomal RNA, partial (77%); similar to expressed protein, partial (28%)
TAG_013701	42	0	6.5	TC153768	Asparagine synthetase
TAG_007466	55	5	6.48	TC162175	Similar to zinc finger (C3HC4-type RING finger) family protein, partial (83%)
TAG_008600	41	0	6.42	TC161887	Homologue T6D22.2, partial (48%)
TAG_014898	41	0	6.42		
TAG_008550	45	3	6.08	TC153877	Similar to UP|O49881 (O49881) TSI-1 protein, partial (77%)
TAG_007470	42	2	6.05	TC161989	Weakly similar to phenazine biosynthesis PhzC/PhzF family protein, partial
TAG_013319	36	0	6.02	TC155643	Weakly similar to expressed protein, partial (26%)
TAG_008510	43	3	5.92	TC154448	Glutathion-S-transferase
TAG_008509	34	0	5.85	TC162426	Lemir
TAG_003922	47	5	5.85	TC163774	Wound-induced protein, partial (56%)
TAG_008803	33	0	5.76	TC166492	Phosphatidylinositol 4-kinase, partial (18%)
Lower levels					
TAG_001339	11	177	–12.08	TC153844	Similar to thiazole biosynthetic enzyme, chloroplast precursor, partial (87%)
TAG_001122	142	368	–9.98	AW623883;TC161862; TC161852;TC161851; TC161842;TC161823; TC161805;TC161770; TC161767;TC161766	Similar to RuBisCo small chain 3B chloroplast precursor, partial (45%); homologue to small subunit precursor (Brandstadter et al. 1996), partial (21%); homologue to RuBisCo small chain 3A/3C partial (84%); RuBisCo small chain 3A/3C, partial (54%); similar to ABA-responsive protein-like (At5g13200), partial (63%); homologue to RuBisCo small chain 3A/3C partial (81%); RuBisCo small chain 3A/3C; similar to mitochondrial import inner membrane translocase subunit Tim17/Tim22/Tim23 family protein weak similarity to SP|Q99595 mitochondrial import inner membrane, partial (64%); RuBisCO small subunit 3A/3C; RuBisCO small subunit 3B, partial (97%); RuBisCO small subunit 3A/3C

(*continued on following page*)

[a] Number of times tag occurred.

Along with the drastic change in transcription in the CMV/D satRNA-infected plants, the expression of some transcriptional factors also was affected. For example, the expression of RNA polymerase I (pol I) transcription factor *RRN3* was lower in CMV/D satRNA-infected plants, as were transcription factors involved in developmental regulation (e.g., MADS-box gene *Jointless* and *HMG-I/Y*), in repressing defense response (e.g., *SEBF*), and in stress response (e.g., a putative *AGP 2*). In the meantime, another putative MADS box gene, *HoxD4b*; *Nam-like protein 4*, with an unidentified function in plants; and *DP 1*, with a potential role in regulating the cell cycle were upregulated by CMV/D satRNA infection. Three genes encoding stress-responsive transcription factors, a *WRKY-type*, a *TGA2.1*, and a *bZIP*, were induced or highly expressed.

The expression of the genes involved in protein synthesis, processing, and degradation also was altered in CMV/D satRNA-infected plants. The transcription of several essential ribosome subunits for organelle protein synthesis decreased. However, some cytoplasmic ribosomal subunits and initiation factors were expressed at higher levels while the expression of some others was reduced. This suggests a reduction in organelle protein synthesis in CMV/D satRNA-infected plants. Similar to the genes involved in protein synthesis, the expression of some proteases and *pin*s was induced or enhanced while others decreased or were suppressed. Particularly the transcription of *ubiquitin-related protein*s and *subtilisin-like protease*s was highly activated.

Several genes whose homologs have been reported to regulate plant cell death also were upregulated in CMV/D satRNA-infected plants, including *BI-1* (Kawai-Yamada et al. 2001, 2004; Watanabe and Lam 2006), *copine-related protein* (Yang et al. 2006b), *S-adenosylmethionine decarboxylase* (Papadakis and Roubelakis-Angelakis 2005), *GST* (Kampranis et al. 2000), and one HR *elicitor-like protein*. When the *P* value for the statistical analysis was raised to 0.05, the transcripts of *SGT1* (Peart et al. 2002) and *vacuolar processing enzyme* (*VPE*) *III* (Hara-Nishimura et al. 2005) in CMV/D satRNA-infected plants were significantly more abundant than in Dm satRNA-infected-plants.

Overall, one of the most dramatic differences between the transcriptomes of D and Dm satRNA-infected plants is the highly increased expression of the ethylene-induced defense-related and ethylene synthesis-related genes by CMV/D satRNA infection (Table 3; the top 25 upregulated genes). Because ethylene is an important plant hormone with significant roles in multiple biological processes, such as development, stress response, cell growth, and death, the biological role of ethylene in CMV/D satRNA-induced lethal disease was studied further.

The role of ethylene in PCD induced by CMV/D satRNA infection.

The youngest leaves of wild-type and the ethylene-insensitive mutant *Nr* tomato plants were inoculated with CMV/D satRNA at the two- to six-leaf stage. The disease symptoms were attenuated in the infected *Nr* plants (Fig. 2). Leaf epinastic symptoms did not develop or were very mild (Fig. 2B). The appearance of systemic necrosis was delayed by 2 to 3 days when the seedlings were inoculated at the two- to three-leaf stage. When wild-type plants were inoculated at the five- to six-leaf stage, cell death normally started from both the apical part and the stem around the petiole of the inoculated leaf (Xu and Roossinck 2000). In the infected *Nr* plants, the apical ne-

Table 3. (*continued from preceding page*)

TagID	CMV/D[a]	CMV/Dm[a]	Z value	TC_id	Annotation
Lower levels (*Continued*)					
TAG_001049	49	186	–8.91	TC153705;TC153697; TC153689;TC153609; TC153545;TC153542	Homologue soybean 18S ribosomal RNA gene, partial (65%); chlorophyll a-b binding protein 1B (LHCII type I CAB-1B) (LHCP), partial (72%); LHCII type I CAB-1B; LHCII type I CAB-1B; LHCII type I CAB-1B; LHCII type I CAB-1B, partial (97%)
TAG_003260	2	78	–8.48	TC163263	Abscisic acid and environmental stress inducible protein TAS14 (Dehydrin TAS14)
TAG_000335	33	125	–7.29	TC153979	Weakly similar to oxygen-evolving enhancer protein 3-1 (OEE3) partial (90%)
TAG_000593	86	207	–7.04	AI781366;TC162684; TC161905;TC156040; TC154648;TC153838	RuBisCo small subunit precursor; Histone H4; RuBisCo small chain 2; RuBisCO small subunit 2A, partial (64%); RuBisCo small subunit precursor, partial (84%); homologue to 40S ribosomal protein S23 (S12)
TAG_000972	19	94	–7.03	TC161832	Homologue to photosystem I reaction center subunit V (PSI-G), partial (63%)
TAG_001011	20	93	–6.84	TC153919	Homologue to Aquaporin 1
TAG_000791	37	124	–6.83	TC162340	Similar to photosystem II, partial (58%)
TAG_001412	71	179	–6.8	AI778961;TC161953; TC161777;TC153641; TC153640;TC153636	Homologue to histidinol dehydrogenase, partial (19%); RuBisCO small subunit 1 (LESS17); homologue to ribosomal protein L27a, partial (98%); RuBisCO small chain 1, partial (98%); LHCII type I CAB-3C; Nolana humifusa 18S rRNA gene, partial (24%)
TAG_001322	8	62	–6.43		
TAG_000489	60	153	–6.34	TC153692;TC153598; TC153596	LHCII type I CAB-1B, partial (83%); homologue to LHCII type I CAB-3C; LHCII type I CAB-16, partial (62%)
TAG_000904	19	82	–6.25	TC153542	LHCII type I CAB-1B, partial (97%)
TAG_003094	6	54	–6.18	TC155828	Similar to aspartyl protease family protein, partial (47%)
TAG_001610	33	106	–6.17	TC153574	LHCII type I CAB-1B, partial (69%)
TAG_002946	26	92	–6.05	TC153917	Cab-6A
TAG_000437	9	59	–6.04	BG628332;TC162025	Oxidase like protein
TAG_001326	1	39	–5.99	TC154040	Similar to CP12 precursor, partial (75%)
TAG_002982	5	48	–5.89	BI203427;TC153749	Secretory peroxidase, partial (98%)
TAG_000905	21	77	–5.63	TC161773	Plastocyanin, chloroplast precursor
TAG_002084	4	40	–5.41	TC153552	Homologue to 24K germin like protein precursor, partial (93%)
TAG_001687	10	53	–5.4	TC153837	Cab-11
TAG_002925	5	42	–5.38	TC154324	Similar to expressed protein , partial (62%)
TAG_000340	10	51	–5.23	TC153995;TC153994	Photosystem I subunit XI; homologue to PSI subunit XI
TAG_007147	3	35	–5.18	BG628314;TC162149	Metallocarboxypeptidase inhibitor precursor (MCPI)
TAG_005436	0	27	–5.18	TC163207	Weakly similar to proline-rich protein 1, partial (48%)

crosis was delayed by 3 to 6 days and either no visible necrosis or a limited area of necrosis was detected in the stem around the petiole of inoculated leaves (Fig. 2D). Microscopic observation of the cross sections of the stem showed a limited cell death in the vascular cells in the infected *Nr* plants compared with a widespread cell death in the various types of cells in the wild-type plants (Figs. 2E through J).

When an ethylene action inhibitor, silver thiosulfate (STS), was sprayed on the infected wild-type and *Nr* tomato plants at 6 dpi, the leaf epinastic symptom was suppressed in wild-type tomato plants and widespread cell death in the leaves of the infected plants obviously was delayed and limited to the veins (Table 4). A slight delay of the systemic necrosis along the stem up to the top of the plants also was detected. A dramatic delay of apical necrosis in the STS-sprayed *Nr* plants was detected several times. No significant delay of apical necrosis was found in wild-type plants due to less accessibility of surface-sprayed STS into the apex with a more closed structure. In conclusion, results from the analysis of STS treatment and the inoculation assay of *Nr* tomato indicate that ethylene plays a critical role in the leaf epinastic symptom and mediates the rapid secondary cell death in the cells adjacent to the vascular cells.

We investigated the roles of JA and salicylic acid (SA) in the development of systemic necrosis in CMV/D satRNA-infected tomato plants. *NahG* transgenic plants that don't accumulate SA, sense-systemin transgenic plants that constitutively accumulate JA, and *def1* mutant plants that are deficient in JA accumulation were inoculated with CMV/D satRNA. There was no significant difference in symptom development among the infected wild-type tomato plants and all the tested mutant plants except the *def1* tomato mutants (data not shown). When the *def1* mutant plants were inoculated at the two- to three-leaf stage, a 5- to 7-day delay of plant death was detected in the infected mutant plants (Fig. 3A through C). When the plants were inoculated at the five- to six-leaf stage, longer delays in apical necrosis and plant death were observed. However, no difference was found in the development of necrosis in the stem around the petiole of inoculated leaves between the wild-type and mutant plants (Fig. 3D and E).

The role of ethylene in defense response in CMV/D satRNA-infected tomato plants.

Results from LongSAGE analysis show that *ACC oxidase 1* (Table 3) was highly expressed in CMV/D satRNA-infected plants and the gene with the largest increase in expression level

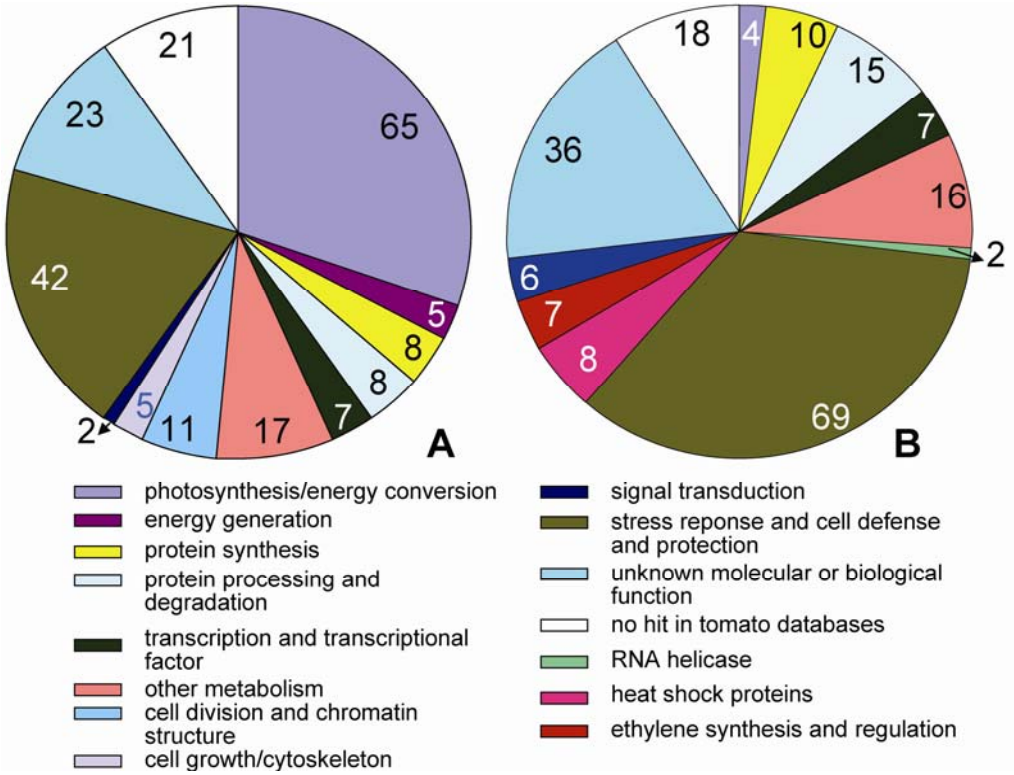

Fig. 1. Distribution of functional groups of genes differentially expressed in *Cucumber mosaic virus* D satellite RNA (CMV/D satRNA) versus a mutant of D satRNA with three mutated nucleotides (CMV/Dm satRNA)-infected plants. **A,** Taken from the analysis of the 201 downregulated genes and **B,** taken from the 200 upregulated genes. Numbers inside the fragments of the pies are the numbers of the different genes or gene family members. The color-coded legend is the same for both pies.

Table 4. Effects of ethylene action inhibitor silver thiosulfate (STS) on disease symptom development[a]

	STS (100 μM)		STS (50 μM)		Water control	
Symptom	**Wild type**	***Nr***	**Wild type**	***Nr***	**Wild type**	***Nr***
Leaf epinasty	Very mild	No	No	No	Severe	Very mild
Leaf necrosis	A few brown leaves, 12 dpi	A few brown leaves, 12–13 dpi	A few green leaves with necrotic veins, 13 dpi	A few green leaves with necrotic veins, 12–13 dpi	Necrotic leaves, 8–9 dpi	Necrotic leaves, 11–12 dpi
Apical necrosis	9 ± 1 dpi	11 ± 1 dpi	10 dpi	17 ± 1 dpi	9 ± 1 dpi	10 ± 1 dpi
Stem necrosis	11 dpi	11 dpi	11dpi	12 dpi	8–9 dpi	11–12 dpi

[a] Number of days postinoculation (dpi) = time when the symptoms appeared.

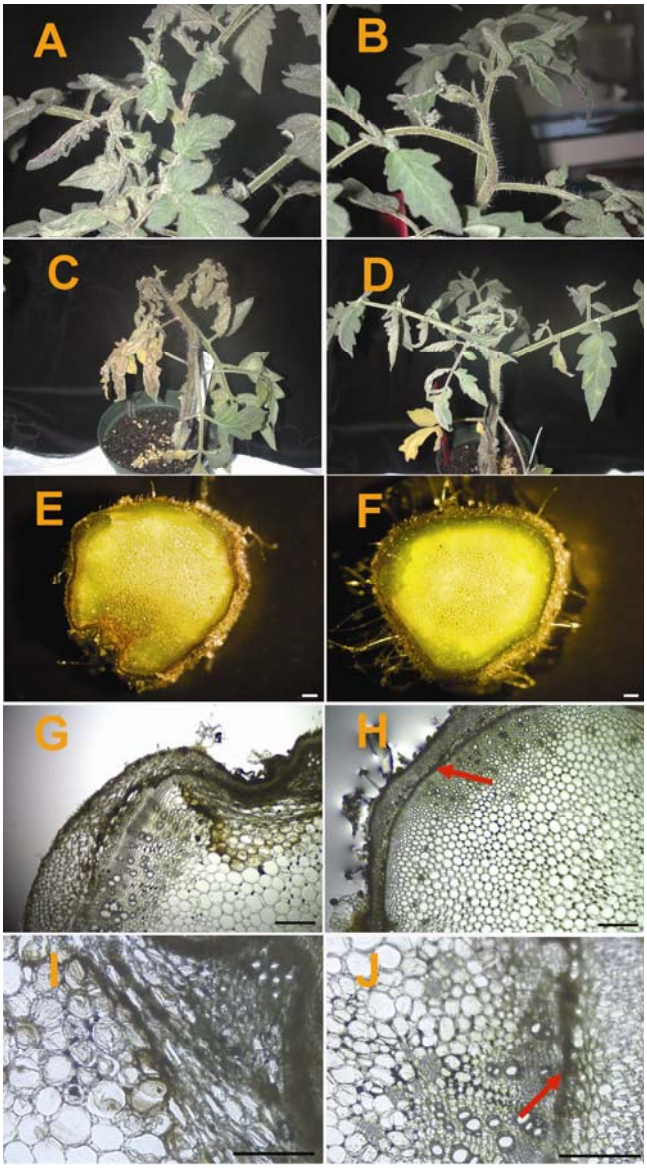

Fig. 2. Symptoms in *Cucumber mosaic virus* D satellite RNA (CMV/D satRNA)-infected wild-type and *Nr* mutant tomato plants. **A, C, E, G,** and **I,** Wild-type plants and **B, D, F, H,** and **J,** *Nr* mutant plants at **A** and **B,** 10 days postinoculation (dpi) and **C** and **D,** 15 dpi. Cross section of the stem at 15 dpi under a dissecting microscope at **E** and **F,** ×5 and **G** and **H,** ×10. **I** and **J,** Magnified portion of **G** and **H** (×20). Bars in **E** through **J**, 100 µm. Arrows in **H** and **J** indicate the limited cell death in the vascular cells in the infected *Nr* plants.

was an ethylene-induced *PR* gene, indicating activated ethylene biosynthesis and ethylene-induced defense responses. Ethylene may directly regulate the expression of defense-related genes or indirectly boost defense responses by activating the expression of the transcription factors, *ethylene responsive factor*s (*ERF*) (Broekaert et al. 2006). The expression of *ERF* in the LongSAGE libraries was too low for reliable statistical analysis. Transcript for the ethylene biosynthesis rate-limiting enzyme *ACC synthase* (*ACS*) was not detected in the libraries. Thus, the expression of *ACS*, three *ERF* homologs (two *DDTFR* genes and *Pti4*), and one defense responsive gene (*PR-2*) was examined by Northern blot with the RNA samples from infected wild-type and mutant *Nr* plants at different time points after inoculation (Fig. 4). The plants were inoculated at the five-leaf stage. Results show an obvious increase of *PR-2* expression in the infected wild-type plants at 10 dpi when epinastic leaf symptoms were developing. *PR-2* expression levels remained high until complete plant death. The expression of *ACS* gradually increased during the symptom development and reached the highest level when cell death occurred, and then decreased. In the infected *Nr* plants, the expression of *PR-2* and *ACS* were increased only slightly at the time when necrosis appeared. The expression of three *ERF* homologs increased in both infected wild-type and *Nr* plants but with different temporal patterns. Two *DDTFR* genes and *Pti4* were expressed at a slightly lower level in the infected *Nr* plants. At the time when cell death occurred in the infected mutant *Nr* plants, *DDTFR* genes were barely detectable whereas *Pti4* transcript accumulated to a high level. Thus, some other factors must be involved in the upregulation of *Pti 4.* In conclusion, results from both LongSAGE and Northern analysis indicate that ethylene synthesis was highly enhanced during disease symptom development and ethylene was partially involved in regulation of general defense responses in CMV/D satRNA-infected plants.

DISCUSSION

Many genes involved in photosynthesis, biosynthesis, cell structure, defense, and other metabolic functions were affected by CMV infection with or without satRNAs.

Here, we took the LongSAGE approach to investigate the global transcriptome changes in diseased tomato plants infected with CMV, CMV/D satRNA, or CMV/Dm satRNA compared with mock-inoculated plants. Both general and specific responses were discovered in CMV-infected plants with or without satRNAs. The expression of many genes involved in various physiological activities such as photosynthesis, biosynthesis, metabolism, cell structure, and defense was altered in CMV-infected plants. Even though there was some similarity in tran-

Fig. 3. Symptoms in *Cucumber mosaic virus* D satellite RNA (CMV/D satRNA)-infected wild-type, systemin-sense transgenic, and *def1* mutant tomato plants inoculated at the two- to three-leaf stage. **A–C,** Plants at 10 days postinoculation (dpi); **D** and **E,** stems from plants at 16 dpi (plants were inoculated at the five- to six-leaf stage); **A** and **D,** wild-type plants; **B,** systemin-sense transgenic plants; **C** and **E,** *def1* mutant plants. The arrows in **D** and **E** indicate the necrosis in the stem.

scriptomic changes among the tomato plants infected with CMV or the satRNAs in this study, the differences were conspicuous and comprehensive, which was consistent with their different induced symptoms.

Transcriptomic changes induced by different viruses and viroids analyzed by amplified fragment length polymorphism, microarray, and macroarray analyses demonstrate the increase or induction of some common genes associated with stress or defense responses in different host plants (Escalettes et al. 2006; Geri et al. 1999; Golem and Culver 2003; Itaya et al. 2002; Senthil et al. 2005; Whitham et al. 2003). A similar response was induced in tomato plants infected with CMV or CMV/satRNAs. In most of the published cases, photosystem II (PSII) and its activity are affected by virus infection but not PSI (Hodgson et al. 1989; Lehto et al. 2003; Pérez-Bueno et al. 2004). Here, the infection of CMV/satRNAs not only decreased the transcripts of PSII *oxygen-evolving enhancer* (*OEC*) *protein 3-1* and *chloroplast protein* (*CP*) *29*, but also PSI components *psaK*, *psaN*, and *psaF*. It is unique that CMV/satRNA infection downregulated several noncore components of PSI. The infection of CMV strain Y decreases OEC components in tobacco plants (Takahashi and Ehara 1992), but it is not known if the transcript level is altered at the same time. Here, significant changes of OEC transcripts were not found in CMV-infected plants, but only in CMV/satRNA-infected plants. This complexity also can be found in the tobacco responses to *Tobacco mosaic virus* (TMV), where two different strains of TMV cause opposite changes in RuBisco concentrations (Balachandran et al. 1994).

CMV/D satRNA infection induces a comprehensive change in the tomato transcriptome.

Results of the LongSAGE analysis confirm our previous work about induced multiple defense and self-protection responses in CMV/D satRNA-infected plants (Xu et al. 2003). All the genes that we investigated previously were positively detected in the sequenced libraries, and the change of their expression after virus infection also was similar to the LongSAGE results. The differential expression of some other genes was further confirmed by qRT-PCR analysis. This indicates the reliability of the LongSAGE analysis in our study. More importantly, the comprehensive change in the transcriptome of CMV/D satRNA-infected plants gives new and systematic information about the affected cellular and physiological activities.

The massive shift of gene expression from cell growth to stress responses is reflected in the altered expression of the transcription factors seen in this LongSAGE and Northern analyses. The transcription factors that bind some specific cis-acting elements associated with stress-response belong to the *AP2/ERF*, *WRKY*, and *TGA* gene families (Jakoby et al. 2002; Kim and Zhang 2004) that all were specifically induced in CMV/D satRNA-infected plants. Their activation contributes to the transcriptional changes of most stress-responsive genes. On the other hand, *SEBF* was suppressed in CMV/D satRNA-infected plants. SEBF represses the expression of *PR-10a* (Boyle and Brisson 2001); thus, suppression of *SEBF* enhances defense response. In addition, *RRN3* of RNA pol I and some MADS box genes and *HMG-I/Y* were downregulated. *RRN3* is an essential RRN gene required for the transcription of rDNA in *Saccharomyces cerevisiae* (Yamamoto et al. 1996). HMB-I/Y regulates the high expression of several housekeeping genes (Webster et al. 2001). The reduction of their transcript abundance reflects the drastic slowing down of plant growth and development.

Compared with the transcriptome in CMV/Dm satRNA-infected plants, the transcripts of the genes encoding CaMs, PLD, and MAPK were increased in CMV/D satRNA-infected plants, indicating enhanced activity of those gene-associated signal transduction pathways. PLD plays multiple regulatory roles in stress responses and PCD (Wang 2005). In addition, several *14-3-3* genes were up- or downregulated. The 14-3-3 proteins bind phosphorylated target proteins and are an integral part of signal transduction (Ferl 2004). Individual 14-3-3 proteins have specific targets in primary metabolism, ion transport, cellular trafficking, and gene expression. Thus, the expressional change of *14-3-3* genes and other components in different signal transduction pathways implicates a radical physiological change in CMV/D satRNA-infected plants and is associated with the dramatic transcriptome change and severe disease symptoms.

The difference between D and Dm satRNA is only three nucleotides, but approximately 400 different genes were differentially expressed between CMV/D and CMV/Dm satRNA-infected plants. This suggests that plant responses to viral infection could vary remarkably with different viruses or virus strains, even the ones with very few nucleotide differences. Thus, generalizing the transcriptional responses to virus infection should be done with caution. In addition, D satRNA doesn't express any proteins in plants; therefore, it affects cellular physiology through the primary or secondary structure of

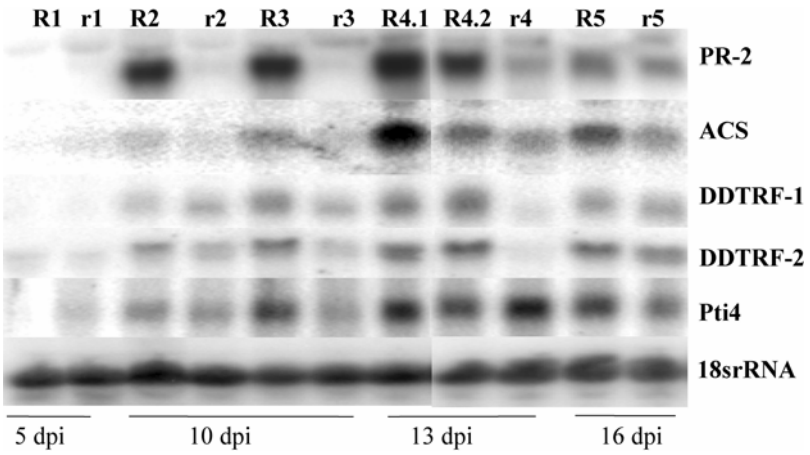

Fig. 4. Northern blot analysis of the temporal expression of *PR-2*, *ACS*, *DDTRF-1*, *DDTRF-2*, *Pti4*, and *18s rRNA* in infected wild-type and *Nr* mutant tomato plants. R = wild-type tomato and r = *Nr* mutant plants. R1 and r1, plants showing no symptoms; R2, plants developing leaf epinasty; R3, plants showing severe epinasty; r2, r3, and r4, plants with no obvious symptoms; R4.1, plants developing systemic necrosis; R4.2 plants showing severe necrosis; r5, plants developing necrosis in the apical part of the plants; and R5, complete necrosis in the apical part.

the RNA, particularly the three nucleotides that determine its necrogenecity, that are in a conserved structural region (Rodríguez-Alvarado and Roossinck 1997). Many recent studies have revealed the direct regulatory roles of nonencoding RNA in transcription and translation. D satRNA may have a similar function in affecting a few transcription factors or signal transduction pathways that result in a massive change of the tomato transcriptome and physiology.

Ethylene plays an important role in the CMV/D satRNA-induced lethal disease.

Ethylene regulates many aspects of plant growth, development, and responses to the environment. The tomato *epi* mutant has a phenotype of severe leaf epinasty due to the overproduction of ethylene (Fujino et al. 1988), similar to one of the symptoms induced by CMV/D satRNA infection. Leaf epinasty also can be caused by the ethylene-independent auxin pathway (Ursin and Bradford 1989). Results from inoculation tests with the ethylene-insensitive mutant demonstrate the positive regulatory role of ethylene in the development of epinastic leaf symptoms. Ethylene signaling also is required for disease symptom development in plants infected with some bacterial and fungal pathogens (Bent et al. 1992; Lund et al. 1998). Ethylene has been correlated with the disease symptoms in viroid-, CMV-, and *Cauliflower mosaic virus*-infected plants (Gadea et al. 1996; Geri et al. 1999; vanLoon et al. 2006). Thus, inhibition or interruption of ethylene signaling could be a good option for disease control.

Ethylene itself doesn't induce plant cell death but is necessary for PCD in several developmental processes and regulates PCD in many stress-inducible processes (Lund et al. 1998; Overmyer et al. 2003; Steffens and Sauter 2005; Young et al. 1997). The present study shows that ethylene mediates the rapid secondary cell death in the stem of CMV/D satRNA-infected tomato plants and ethylene insensitivity reduces necrotic disease symptoms and delays plant death. In TMV-infected tobacco plants, ethylene promotes resistance-associated necrotic lesions although it has no effects on the initiation of cell death (Ohtsubo et al. 1999). Thus, ethylene-mediated host PCD plays an important role in both disease progression and immunity, which share a common switch at the MAPKKK cascade in other plant–pathogen systems (delPozo et al. 2004). Phosphorylation of ACS2 and ACS6 by AtMAPK6 leads to the accumulation of ACS proteins and elevated ethylene production in *Arabidopsis* (Liu and Zhang 2004). The mechanisms underlying the induced ethylene synthesis in CMV/D satRNA-infected plants is unknown, but probably is connected with the highly expressed MAPK3 and MAPK4 homologues as detected by the LongSAGE analysis. On the other hand, MAPK cascades also are regulated by a wide range of signals, including ethylene and ROS (Jonak et al. 2002). Thus, the disease symptom expression must result from a complicated interaction of different signal transduction pathways.

The precise role of ethylene signaling in cell death regulation remains unknown. Inhibition of ethylene synthesis or perception blocks camptothecin-induced H_2O_2 production and PCD, indicating the correlation of ethylene and H_2O_2 stress (deJong et al. 2002). H_2O_2 plays a central role in PCD (Overmyer et al. 2003). Our previous work showed the accumulation of H_2O_2 in the stem of CMV/D satRNA-infected tomato plants when cell death was occurring. However, ethylene alone is insufficient to induce either H_2O_2 accumulation or cell death (deJong et al. 2002), suggesting that additional unknown factors must be involved. Recently, it was found that conditional expression of NbCD1, a class II ERF protein from *Nicotiana benthamiana*, induces cell death (Nasir et al. 2005). On the other hand, overexpression of another ERF member in the ethylene-signaling pathway confers resistance to Bax-, heat-, and H_2O_2-induced plant cell death (Ogawa et al. 2005). We didn't detect the expression of tomato homologs of these two genes, although several other ERF members had increased expression in CMV/D satRNA-infected plants. It is unclear whether they play a role in CMV/D satRNA-induced PCD in tomato.

Despite the important role of ethylene in CMV/D satRNA-induced disease, primary cell death still occurred in the apical part of the plants and vascular cells in the stem in ethylene-insensitive mutants. Very little is known about the initiation mechanisms in virus-induced host PCD or the regulatory and execution pathways. Disease-related PCD induced by virus infection shares similar features with other stress- or developmental-related PCD at cellular and molecular levels (Greenberg and Yao 2004; van Doorn 2005; Xu et al. 2004). The ubiquitin and 26S proteasome pathways play significant roles in negatively or positively regulating HR cell death, including ubiquitin (Abramovitch et al. 2006), ubiquitin ligases (Janjusevic et al. 2006; Yang et al. 2006a; Zeng et al. 2004), proteasome subunits (Kim and Zhang 2004), and SGT1 (Peart et al. 2002). VPE is essential for TMV-induced HR cell death and is involved in various types of plant cell death (Hara-Nishimura et al. 2005). Two serine proteases, the subtilisin-like protease homologs, have caspase-like activities and regulate PCD in *Avena sativa* (Coffeen and Wolpert 2004). Thus, the high expression of these genes in CMV/D satRNA-infected tomato plants may be correlated with the regulation of PCD.

In addition to ethylene, a lack of JA accumulation also delayed the apical death in CMV/D satRNA-infected tomato plants. However, deficiency in JA synthesis had no effect on the primary cell death or the cell death progression in the stem. Reduced accumulation of SA had no effect on any CMV/D satRNA-induced disease symptoms. The interactions among ethylene, JA, and SA signaling pathways are very complicated and sensitive in stress responses, including their effects on cell death and plant-pathogen susceptibility (Dong 1998; Mur et al. 2006). The outcomes of their interactions are plant-pathogen specific in most cases. Here, in CMV/D satRNA-infected plants, LongSAGE data showed an increased expression of the genes involved in ethylene synthesis but very low expression of JA synthesis-related genes. Compared with CMV/Dm satRNA-infected plants, some JA-responsive genes were downregulated. This may indicate a low activity of JA signaling at the time point assayed.

Results from the inoculation tests with ethylene and JA tomato mutants only demonstrate their role in disease symptom development; however, they often are associated with defense responses. Results from Northern blot analyses show that ethylene contributed to the induction of *PR-2* expression in CMV/D satRNA-infected plants. However, *DDTFR* genes and *Pti4* were only partially regulated by ethylene. Other unidentified factors also must be involved in increasing their expression. *Pti4* is inducible by both ethylene and SA (Gu et al. 2000), and Pti4 can mediate cross talk between SA and ethylene/JA pathways and regulate defense-related gene expression via GCC box and non-GCC box genes (Chakravarthy et al. 2003; Huang et al. 2004). Several SA-responsive genes, such as the ones encoding PR proteins (e.g., PR1, PR2, and PR5), also were upregulated in our LongSAGE analysis. Thus, SA may be one other potential regulator in induced defense response, although it has no effects on CMV/D satRNA-induced PCD.

In this study, LongSAGE has been applied successfully in investigating the transcriptome changes in tomato plants infected with CMV with or without satRNAs. Based on the analysis of the tags with hits on the known genes, we found that CMV infection affected the expression of many genes involved in cell structure, defense, photosynthesis, biosynthe-

sis, and other metabolism. Different sets of genes were induced or suppressed due to the infection of tomato by CMV and parasitic satRNA D or its mutant Dm. CMV/D satRNA infection induces severe leaf epinasty and systemic necrosis and leads to rapid plant death. This radical change was accompanied by the massive change of transcription of approximately 400 different genes compared with CMV/Dm satRNA-infected plants. Particularly, the LongSAGE data indicate the activation of ethylene synthesis and signaling, which mediated the epinastic leaf symptoms and the secondary cell death in the stem and contributed to part of the induced defense responses. The huge amount of LongSAGE data and the thorough knowledge of the transcriptome changes in plants infected with CMV with or without satRNAs will lead to new gene discovery and help to further the study of molecular mechanisms underlying complex disease symptom expression and plant PCD.

MATERIALS AND METHODS

Viruses.

Viral RNAs of the Fny strain of CMV and of D satRNA used for inoculation were prepared as previously described (Xu and Roossinck 2000). The construct for producing the infectious mutant of D satRNA, Dm satRNA, was generated by PCR mutagenesis with pDsat4 as the template. The PCR product was cloned into pGEM-Teasy vector (Promega Corp., Madison, WI, U.S.A.). The inserts were sequenced through a 3730 DNA Analyzer (ABI, Foster City, CA, U.S.A.), and the clone with mutations at positions 285 (G to A), 290 (T to G), and 292 (C to T) was selected as the plasmid construct for preparing the template for in vitro transcription of Dm satRNA. The plasmid was linearized with *Sma*I, and Dm satRNA was generated through in vitro transcription with Sp6 RNA polymerase (Ambion, Austin, TX, U.S.A.) as previously described. Zucchini squash (*Cucurbita pepo* cv. Elite) was infected by rub inoculation of the transcripts of the genomic RNAs of Fny CMV and Dm satRNA. Virus was purified, and viral RNAs were extracted by the protocol previously described (Roossinck and White 1998).

Plant materials and treatments.

For constructing the LongSAGE libraries, tomato (*Solanum lycopersicum* L. cv. Rutgers) seedlings at the two-leaf stage were inoculated with 10 µl of buffer only (mock-inoculation control) or the viral RNAs of CMV with or without D or Dm satRNA at a total concentration of 400 ng/µl. Inoculated plants were grown in Conviron chambers set up under identical environmental conditions (28°C, light-dark cycle of 16 and 8 h, respectively).

For the study of phytohormones involved in disease development, the seedlings of different mutants or transgenic plants and their corresponding wild-type cultivars were inoculated at the two- to six-leaf stage. These mutants include *NahG* transgenic tomato (*S. lycopersicum* L. cv. Money maker), sense systemin transgenic tomato and *def1* mutant (*S. lycopersicum* L. cv. Castelmart), and never ripe (*Nr*) mutant (*S. lycopersicum* L. cv. Pearson). The inoculated plants were grown under normal greenhouse condition (28°C, light-dark cycle of 16 and 8 h, respectively). All the wild-type plants of these three different tomato cultivars showed the same temporal and spatial development of systemic necrosis after the infection of CMV/D satRNA.

For the analysis of ethylene action inhibition, STS was prepared at concentrations of 50 and 100 µM and sprayed on the above soil portion of the wild-type and *Nr* plants daily after 5 dpi until the complete apical death of the plants. These plants were grown in the Conviron chambers as above.

For the comparison of cell death patterns in the stems of infected wild-type and mutant tomato plants, the stems were excised and then sectioned by hand with a razor blade. The sections were examined under a Nikon dissecting microscope and Nikon Microphot photomicroscope.

Library construction, LongSAGE, and qRT-PCR analysis.

Systemic leaf tissue and the stem from the top down to the third node were pooled from 50 individual plants. Each sample tissue (2 g) was frozen in liquid nitrogen and subjected to mRNA isolation using the Invitrogen mRNA isolation kit (FastTrack mRNA isolation kit; Invitrogen, Carlsbad, CA, U.S.A.). The polyA RNA samples were subjected to SAGE and LongSAGE protocols (versions 1.0e and 1.0a) essentially as described (Velculescu et al. 1995). The SAGE software (version 4.5) was used to extract the tag sequences. A Smith-Waterman algorithm based on semiglobal alignment was applied to map all LongSAGE tag sequences to corresponding EST unigene of TIGR (release 10.1, June 2004). This was implemented by a Timelogic Hardware Accelerating System. All the unigene sequences were obtained from the databases of five solanaceous species: tomato, *Nicotiana benthamiana*, pepper, potato, and tobacco. If one tag had multiple corresponding EST sequences in several species, tomato was used in preference over other species. Only those unigenes or EST sequences or their reverse complementary strands with 100% exact match with the LongSAGE tags were determined as the corresponding sequences.

To compare the levels of gene expression among different libraries and look for the differentially expressed genes, a Z test statistical method described by Ruijter and associates (2002) was implemented by PHP script to calculate a Z value for each tag sequence by the occurring times between any selected two libraries. The Z value 2.58 ($P > 0.99$) was set as the threshold to screen the tags in the library of interest with significant difference in expression level from the ones in the control library. The tags with Z value larger than 2.58 were picked as upregulated tags and those with Z value less than –2.58 were determined to be downregulated tags. Functional categories of differentially expressed genes were obtained using the gene ontology in TIGR tomato Unigene/EST database as well as manual analysis through related-literature search for updates. The Excel program was used to draw the pie diagrams for the distribution of each functional group.

For real time qRT-PCR analysis, total RNA was extracted from the apical leaf and stem tissues above the third node of the inoculated plants at 9 dpi using TRIzol Reagent with the protocol provided by the manufacturer (Invitrogen). Total RNA was treated with RNase-free DNase treatment (Promega Corp.). First-strand cDNA was synthesized with the Omniscript RT kit (Qiagen, Crawley, U.K.) using oligo(dT) 15 according to the manufacturer's instructions. Primer pairs were designed using Primer Express Software (version 3.0; Applied Biosystems, Foster City, CA, U.S.A.). Real-time PCR was conducted in an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems), using the intercalation dye SYBRGreen I as a fluorescent reporter. Quantification of PCR products was performed via a calibration curve procedure using *ubiquitin conjugating enzyme 8* as an endogenous control. The ratio of gene-specific expression to the expression level of the designated calibrator was defined as relative expression using a standard curve method described in User's Bulletin no. 2 (Applied Biosystems).

Northern blot analysis.

The wild-type and *Nr* tomato plants were inoculated with CMV/D satRNA at the five-leaf stage. The apical part of the infected plants (from the top to the third node of the stem) were harvested at 5, 10, 13, and 16 dpi and frozen in liquid

nitrogen. RNA extraction, electrophoresis, and Hybond N+ membrane transfer were conducted as previously described (Xu et al. 2003).

The cDNA clones containing the partial fragments of PR-2 (cLES4I4), Pti4 (cLED5A19), DDTRF10/A-1 (cLES6I9), DDTRF10/A-2 (cLED35J9), and ACS6 (cLED2N19) were requested through the TIGR database website. These clones were confirmed by sequence analysis. The purification and labeling of the cDNA inserts with P^{32}-dCTP, and hybridization procedures, were performed as previously described (Xu et al. 2003).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

SAGE website: www.sagenet.org
TIGR's Gene Index Project website: www.tigr.org/tdb/tgi/index.shtml
TimeLogic hardware accelerating system website: www.timelogic.com