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e-Xtra*

Genome-Wide Analysis of Differentially Expressed Genes During the Early Stages of Tomato Infection by a Potyvirus

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Plant responses against pathogens cause up- and downward shifts in gene expression. To identify differentially expressed genes in a plant-virus interaction, susceptible tomato plants were inoculated with the potyvirus *Pepper yellow mosaic virus* (PepYMV) and a subtractive library was constructed from inoculated leaves at 72 h after inoculation. Several genes were identified as upregulated, including genes involved in plant defense responses (e.g., pathogenesis-related protein 5), regulation of the cell cycle (e.g., cytokinin-repressed proteins), signal transduction (e.g., CAX-interacting protein 4, SNF1 kinase), transcriptional regulators (e.g., WRKY and SCARECROW transcription factors), stress response proteins (e.g., Hsp90, DNA-J, 20S proteasome alpha subunit B, translationally controlled tumor protein), ubiquitins (e.g., polyubiquitin, ubiquitin activating enzyme 2), among others. Downregulated genes were also identified, which likewise display identity with genes involved in several metabolic pathways. Differential expression of selected genes was validated by macroarray analysis and quantitative real-time polymerase chain reaction. The possible roles played by some of these genes in the viral infection cycle are discussed.

The severity of plant virus diseases can vary broadly in intensity, from very mild symptoms seen in tolerant plants up to very severe symptoms and plant death (Collmer et al. 2000; Hull 2002; Krause-Sakate et al. 2005). As virus and host coevolve, a complex interaction is developed, involving several and distinct mechanisms of virus attack and plant defense. The strategies used by viruses to infect plants can lead to a number of fundamental changes in the plant's physiology, including structural modifications in the host cell (Hull 2002), changes in the plant cell cycle (Hanley-Bowdoin et al. 2004), and suppression of post-transcriptional gene silencing (Merai et al. 2006; Voinnet 2005), among others. On the other hand, plants have developed mechanisms to protect themselves from viral infection, such as post-transcriptional gene silencing (Ding and Voinnet 2007), activation of hypersensitive responses, and onset of systemic acquired resistance (Baker et al. 1997; Durrant and Dong 2004; Seo et al. 2004). These responses are accompanied by changes in gene-expression patterns that include the production of pathogenesis-related (PR) proteins and of several proteins involved in cell signaling (Cooper 2001).

Currently available techniques allow the analysis of an organism's responses to induced biotic and abiotic stresses in a genome-wide scale. In plant-pathogen interaction studies, such analyses have revealed important mechanisms and metabolic pathways involved in the host's responses to infection by fungi, bacteria, viruses, and nematodes (Wise et al. 2007). Studies on plant-virus interactions have focused on the effects of viral infection in the gene expression profile of susceptible hosts (Whitham et al. 2006). Most of these studies have focused on viruses infecting model plants or artificial hosts, such as *Arabidopsis thaliana* and *Nicotiana benthamiana* (Golem and Culver 2003; Senthil et al. 2005; Whitham et al. 2003; Yang et al. 2007). Only recently have such studies been carried out in natural hosts (Gandia et al. 2007; Pompe-Novak et al. 2006).

Potyviruses comprise the largest and, collectively, the most economically important group of plant viruses (Shukla et al. 1994). The potyvirus genome is composed of one linear, positive-sense, single-stranded RNA molecule. The viral RNA is about 10 kb long with a covalently linked 5′ terminal viral protein (VPg) and a 3′ poly-A tail, and contains a single open reading frame (ORF) flanked by 5′ and 3′ nontranslated regions (Berger et al. 2005). The ORF is expressed as a precursor polyprotein of about 340 kDa, which is cleaved into 8 to 10 functional proteins by the activity of three proteases present in the polyprotein (Carrington et al. 1990). The main consequence of this gene expression strategy is that all viral proteins are produced in stoichometrically identical quantities, independently of the amount required for the virus to complete its infection cycle. The excess proteins accumulate in host cell as cytoplasmic and nuclear inclusion bodies (Dougherty and Hiebert 1980). This gene expression strategy is extremely damaging to the host cell, which is reflected in the severe symptoms normally induced by potyviruses in the infected plant (Berger et al. 2005; Shukla et al. 1994). Moreover, potyviruses encode a suppressor of gene silencing named HC-Pro (Anandalakshmi et al. 1998; Brigneti et al. 1998; Kasschau and Carrington 1998). HC-Pro acts in two different pathways, inhibiting small interfering RNA accumulation and promoting an increase in the accumulation of endogenous micro RNAs involved in the control of plant development (Kasschau et al. 2003; Mallory et al. 2002).

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Pepper yellow mosaic virus (PepYMV) is a typical potyvirus recently described infecting sweet pepper (*Capsicum annnum*) (Inoue-Nagata et al. 2002) and tomatoes (*Solanum lycopersicum*) (Cunha et al. 2004). It is closely related to *Potato virus Y* (PVY), to such an extent that PVY and PepYMV isolates cannot be differentiated based on host range and symptomatology (Truta et al. 2004). PepYMV causes a severe yellow mosaic in tomatoes and nowadays, among tomato viruses in Brazil, is second only to the begomovirus complex in terms of economical importance (Cunha et al. 2004). Due to its useful biological properties (high efficiency of sap-transmission, high titer in infected leaves, and readily identifiable symptoms), its close relationship to other potyviruses, particularly PVY, and its economical importance, PepYMV is a suitable option for transcript profiling studies.

In this study, changes in the gene-expression profile during the early (pre–systemic infection) stages of tomato infection by PepYMV were investigated using suppressive subtractive hybridization (SSH). A large number of genes were identified as either up- or downregulated, providing a starting point for subsequent studies on their potential role in plant-potyvirus interactions.

RESULTS

Symptoms in tomato plants infected by PepYMV.

Yellow mosaic and mild leaf distortion were evident in systemically infected (noninoculated) leaves of 'Moneymaker' tomato plants 10 days after PepYMV inoculation (Fig. 1). No evident macroscopic symptoms of viral infection were observed in the inoculated leaves. No symptoms developed in mock-inoculated plants. In the first experiment ("systemic infection library"), all PepYMV-inoculated plants developed symptoms and infection was confirmed by indirect enzyme-linked immunosorbent assay (ELISA) (data not shown). In the second experiment ("local infection library"), all plants with inoculated leaves collected at 72, 96, and 120 h after inoculation (hai) developed symptoms of PepYMV infection, while six and seven plants (out of 12) with leaves collected at 24 and 48 hai, respectively, developed symptoms. ELISA results confirmed that all plants with leaves collected at 72, 96, and 120 hai were infected and that all corresponding mock-inoculated plants remained virus-free (data not shown).

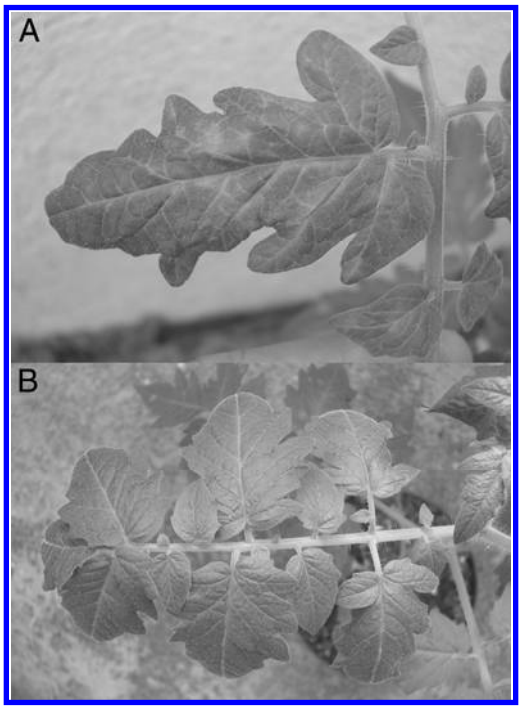

Fig. 1. Symptoms induced by *Pepper yellow mosaic virus* (PepYMV) in tomato plants, cv. MoneyMaker, 10 days postinoculation. **A,** PepYMV-infected plant displaying yellow mosaic and mild leaf crumpling and **B,** mock-inoculated plant.

Since every virus-inoculated plant in the first experiment was found positive for PepYMV infection, purified mRNA from the 14-, 21-, and 28-dai (days after inoculation) treatments were pooled together and were used to construct the subtractive library. In the second experiment, the 72-hai treatment was the first treatment to show all plants infected and, thus, was used to perform the subtraction.

Genes differentially expressed in the early stages of tomato infection by PepYMV.

To identify differentially expressed genes in the tomato-PepYMV interaction, two subtractive libraries were constructed, one using systemically infected leaves collected at 14, 21, and 28 dai (systemic infection library) and another using inoculated leaves collected at 72 hai (local infection library). Subtractions were done in both directions (forward and reverse), resulting in two expressed sequence tag (EST) collections for each library, one corresponding to upregulated genes (forward collection), and another to downregulated genes (reverse collection).

From the systemic infection library, 96 clones were sequenced from the forward collection. Sequence analysis indicated a low complexity in the library, since most sequences showed similarity with rRNA genes (data not shown). Subsequent analyses of the systemic infection library were not carried out.

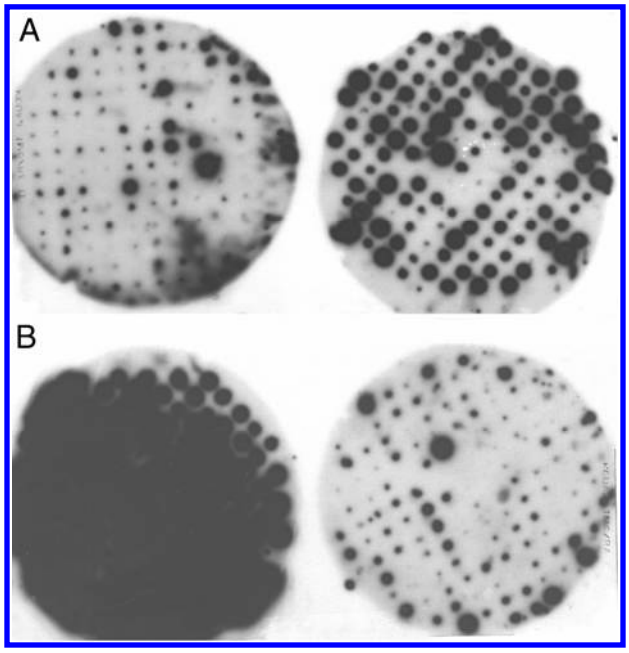

Fig. 2. Validation of the "local infection" subtractive library by differential screening. **A,** Membranes hybridized with the forward probe. **B,** Membranes hybridized with the reverse probe. To the right, clones corresponding to upregulated expressed sequence tags (EST) (forward collection). To the left, clones corresponding to downregulated EST (reverse collection). All blots were hybridized using the same amount of probe DNA and were exposed for the same time, to allow comparisons. The stronger signal in the upper right blot as compared with the upper left blot indicates that the forward collection is enriched for upregulated cDNAs. Likewise, the "overexposed" blot at the bottom left reflects the stronger hybridization signal of the reverse collection to the reverse probe, as compared with the signal of the same collection to the forward probe, as expected if this collection is enriched for downregulated cDNAs.

For the local infection library, a preliminary screening was performed to confirm whether or not the forward and reverse collections were enriched with differentially expressed genes. For this, dot blot arrays of 100 randomly picked clones from each collection were hybridized separately with the forward- and reverse-subtracted probes. Clones from the forward and the reverse collections hybridized strongly with the homologous probes and weakly with the heterologous ones (Fig. 2). These results confirmed that both collections of the local infection library were enriched with differentially expressed genes, i.e., upregulated genes in the forward collection and downregulated genes in the reverse collection.

A total of 1,344 clones were sequenced, 970 from the forward and 374 from the reverse collection. Sequence analysis showed that 6.4% of the EST were present in both collections. These EST represent genes that were equally expressed in both samples (PepYMV-inoculated and mock-inoculated plants) and that were not removed during the subtraction. A total of 777 clones of the forward collection and 104 clones of the reverse collection represent unique genes, with 12.1 and 63.9% redundancy, respectively. Some of these genes are listed in Tables 1 and 2 and the complete list of identified genes is available in Supplementary Tables S2 and S3. The genes listed in Tables 1 and 2 are those for which a putative role during the viral infection cycle could be proposed based on what is known in terms of potyvirus replication, gene expression, and movement. These results indicate that, at 72 hai, there was more induction than repression of gene expression in PepYMV-inoculated tomato leaves. Not a single clone in the forward collection showed similarity with the viral genome, confirming that viral RNA added to the mock-inoculated sample was efficient in subtracting viral genes.

The genes identified were classified into functional categories using the Munich Information Center for Protein Sequences (MIPS) database. Among EST from the forward collection (upregulated genes), 12.9% are involved in signal transduction and 10.3% in protein synthesis (Fig. 3A). Within the 12.9% involved in signal transduction, 4.9% are involved in Ca^{2+}-mediated signal transduction, 5.1% are kinases, 1.4% are classified as transmembrane receptor-like tyrosine kinases, and 2% as transmembrane receptor-like serine/threonine kinases. Within the 10.3% involved in protein synthesis, 1% are genes involved in translation control, 3.7% are members of the ubiquitin/proteosome pathway, 1.1% are proteases activators, 1.8% are kinase activators, 0.8% are enzyme inhibitors, and 1.8% are related to the assembly of protein complexes. A significant portion of the

Table 1. Partial list of upregulated expressed sequence tags in the interaction tomato-*Pepper yellow mosaic virus* (PepYMV) at 72 h postinoculation

BLASTx putative identification	GenBank no.	*E* value	Validation
Cell defense[a]			
Glutathione-conjugate transporter MRP4 (*Arabidopsis thaliana*)	NP_182301	1E-10	n.d.[b]
Glutation-*S*-transferase (*Solanum lycopersicum*)	CAB61885	5E-05	n.d.
Leucine-rich repeat protein (*S. lycopersicum*)	CAA64565	9E-06	n.d.
Leucine-rich repeat-like resistance protein (*Gossypium hirsutum*)	AAK70805	2E-05	n.d.
Membrane protein Mlo14 (*A. thaliana*)	NP_564257	2E-05	n.d.
PR4b (*Capsicum chinense*)	BAD11073	4E-52	n.d.
PR5 (*S. lycopersicum*)	AY257487	2E-16	Macroarray
Cell cycle			
Cytokinin-repressed protein CR9 (*Cucumis sativus*)	BAA06153	1E-04	qRT-PCR[c]
Cytokinin-repressed protein (*Pinus pinaster*)	CAC84488	9E-27	qRT-PCR
GTP-binding nuclear protein RAN2 (*S. lycopersicum*)	P38547	4E-10	n.d.
Kinesin-like protein (*A. thaliana*)	CAB89042	9E-33	n.d.
PR STH-2. (*S. tuberosum*)	P17642	1E-08	Macroarray
Cell fate			
NAC2-like protein (*A. thaliana*)	CAB62457	2E-07	n.d.
Rapid alkalinization factor 4 (*S. chacoense*)	AAR00328.1	1E-60	Macroarray
Signal transduction			
CAX-interacting protein 4 (*A. thaliana*)	AAO17572	1E-51	qRT-PCR
Ethylene receptor homolog (*S. tuberosum*)	AAD12777	9E-05	n.d.
Fen kinase (*S. lycopersicum*)	AAF76314	5E-33	n.d.
GTP binding (*A. thaliana*)	NP_191788	9E-69	n.d.
Putative serine/threonine protein phosphatase type one (*A. thaliana*)	NP_187209	1E-32	Macroarray
SNF1 kinase complex anchoring protein (*S. lycopersicum*)	AAO89082	3E-09	qRT-PCR
Wall-associated kinase 1 (*A. thaliana*)	NM_101479	3E-09	Macroarray
Transcription			
Ribonuclease/ transcriptional repressor (*A. thaliana*)	NM_179584	6E-28	n.d.
WRKY transcription factor 22 (*A. thaliana*)	AF442392	5E-26	Macroarray
Chromatin-remodeling factor CHD3 (*Oryza sativa*)	AAL47211	2E-09	n.d.
F-box family protein-like (*O. sativa*)	XP_468361	3E-34	n.d.
Homeobox 1 protein (*S. lycopersicum*)	AAD09582	5E-30	n.d.
KNAT4; transcription factor (*A. thaliana*)	NP_196667	1E-26	n.d.
Nucleoid DNA-binding-like protein (*A. thaliana*)	CAB81805	4E-49	n.d.
PKL (PICKLE) (*A. thaliana*)	NP_565587	3E-50	Macroarray
Probable SCARECROW gene regulator (*A. thaliana*)	BAB08619	2E-36	Macroarray
Stress response			
ATP-dependent Clp protease-like protein (*A. thaliana*)	BAB09167	9E-46	Macroarray
Calnexin-like protein (*S. lycopersicum*)	BAD99512	4E-15	Macroarray
Catalase 2 (*S. lycopersicum*)	AAD41256	4E-16	Macroarray
CPase I B (*Hordeum vulgare*)	1314177B	6E-09	Macroarray

(*Continued on following page*)

[a] Functional category according to the Munich Information Center for Protein Sequences database.
[b] n.d. = not done.
[c] Genes validated by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) were also validated by macroarray.

EST (9.1%) was classified as involved in the biogenesis of cellular components. Within these, 3.8% are genes involved in membrane biogenesis and 2.8% were classified as cell defense-related genes, including genes involved in oxidative stress responses (0.8%) and cell detoxification involving cytochrome P450 (1%). Among the EST in the reverse collection (downregulated genes), 17.3% are genes involved in metabolic processes, 9.1% in stress responses, 8.4% correspond to genes involved in protein synthesis, 7.2% were classified as involved in defense responses, and 1% correspond to transposable elements (Fig. 3B). A global analysis of both collections suggests a complex interaction pattern in which virus and host attempt to regulate, each to its own favor, different steps of the various cell processes.

Validation of differential expression.

In order to validate some of the genes identified as differentially expressed in the local infection library, 358 clones of the forward collection and 70 from the reverse collection were arrayed in duplicates and were analyzed by macroarray for their expression profile during infection by PepYMV in inoculated leaves at 72 hai (Fig. 4). Only clones showing an evident contrast between the hybridization signals with the probes prepared from mock-inoculated and PepYMV-infected cDNAs were considered as differentially expressed. The differential expression of 53 genes was validated in this manner (Tables 1 and 2). Clones for which differential expression was not confirmed could correspond to differentially expressed transcripts that accumulate at lower levels in the infected tissues and, therefore, were not detected as such under our assay conditions.

Quantitative analysis by real-time polymerase chain reaction (PCR).

Differential expression of a number of the genes previously validated by macroarray analysis was further confirmed by quantitative real-time PCR (qRT-PCR). Of the four housekeeping genes tested as internal controls, *APT1* (adenine phosphoribosil-transferase 1) displayed the smallest variation among all treatments (data not shown) and, therefore, was chosen as the normalizer in all qRT-PCR analyses.

Seven upregulated genes and three downregulated genes were selected for quantitative analysis (Table 3). The upregulated genes were analyzed using RNA preparations from inoculated leaves at different timepoints after inoculation (0, 24, 48, 72, and 96 hai). The downregulated genes were analyzed at a single timepoint (72 hai). All analyses were carried out as three independent biological replications (RNA extracted from three different sets of PepYMV- and mock-inoculated plants).

For the genes corresponding to cystein proteinase, threonine endopeptidase, translationally controlled tumor protein (TCTP),

Table 1. (*Continued from previous page*)

BLASTx putative identification	GenBank no.	*E* value	Validation
Stress response (*continued*)			
DNA-J-like protein (*S. lycopersicum*)	AAF28382	8E-20	Macroarray
GAL83 protein (*S. tuberosum*)	CAB52141	3E-11	n.d.
Heat shock cognate 70 kDa protein 1 (*S. lycopersicum*)	P24629	3E-10	n.d.
HSP90 (*S. lycopersicum*)	AY368907	9E-25	Macroarray
Induced stolon tip protein (*Capsicum annuum*)	AAR83854	9E-15	n.d.
Multiple stress-associated zinc-finger (*O. sativa*)	AAN15744	3E-04	n.d.
MutT domain protein-like (*A. thaliana*)	BT000563	6E-30	n.d.
NOD26-like membrane integral protein (*Zea mays*)	AAK26753	3E-49	n.d.
OHP2 (one-helix protein 2) (*A.thaliana*)	NP_564432	9E-35	n.d.
P23-like protein (*S. tuberosum*)	ABA40472	3E-55	n.d.
Putative peroxidase (*A. thaliana*)	NP_194904	9E-61	Macroarray
Stress induced protein (*Glycine max*)	X60044	1E-51	Macroarray
Stress-responsive one-helix (*O. sativa*)	BAD52885	6E-37	Macroarray
Threonine endopeptidase (*A. thaliana*)	NP_178042	6E-11	qRT-PCR
Translationally controlled tumor protein (*S. lycopersicum*)	AAT65968	7E-04	qRT-PCR
Biogenesis of cellular compounds			
Membrane protein 37K precursor chloroplast inner envelope (*Nicotiana tabacum*)	CN745821	3E-16	n.d.
Outer envelope membrane protein homolog (*A. thaliana*)	NP_568378	3E-09	n.d.
Protein synthesis			
20S proteasome alpha subunit B (*A. thaliana*)	NP_178042	4E-52	Macroarray
Putative mRNA capping enzyme (*A. thaliana*)	AAD56326	1E-54	Macroarray
Translational inhibitor protein p14.5 (*A. thaliana*)	AF375446	2E-16	Macroarray
Metabolism			
BOU (a bout de souffle) binding (*A. thaliana*)	NP_568670	4E-47	Macroarray
Calcium-binding protein CAST (*S. tuberosum*)	Q09011	1E-34	Macroarray
Calmodulin-related protein (*A. thaliana*)	AAM67124	2E-12	n.d.
Cysteine proteinase (*S. lycopersicum*)	CAA88629	1E-17	qRT-PCR
Glycine dehydrogenase (decarboxylating) (*A. thaliana*)	NP_180178	2E-04	n.d.
Lipase-like protein (*A. thaliana*)	CAB85518	5E-54	n.d.
Ly200 protein (*C. annuum*)	AAR83884	1E-48	n.d.
Mitochondrial succinate dehydrogenase iron-sulphur subunit (*A.thaliana*)	CAC19857	4E-07	n.d.
mRNA-binding protein precursor (*S. lycopersicum*)	AAD21574	6E-05	n.d.
NTCP23-like cysteine proteinase (*N. tabacum*)	AAK07729	6E-50	Macroarray
Polyubiquitin 10 (*A. thaliana*)	NP_849301	3E-05	n.d.
Polyubiquitin (*O. sativa*)	AAX40652	3E-43	Macroarray
Porin-like protein (*A. thaliana*)	BAB08784	1E-36	Macroarray
PSI-N; calmodulin binding (*A. thaliana*)	NP_201209	3E-37	Macroarray
Putative senescence-associated protein (*O. sativa*)	AAL79714	1E-48	Macroarray
Ubiquitin activating enzyme 2 (*A. thaliana*)	BAB08968	1E-48	Macroarray
Unknown proteins			
Endoribonuclease (*A. thaliana*)	NP_188674	1E-09	Macroarray
Unknown (*S. lycopersicum*)	AAF75750	2E-16	Macroarray

CAX-interacting protein 4, and SNF1 kinase complex anchoring protein, a significant increase in mRNA accumulation was observed at 72 hai, and it was maintained until 96 hai (Table 3). Interestingly, except for TCTP, no significant increase was observed for the earlier timepoints (24 and 48 hai) (Table 3). TCTP was already upregulated at 48 hai although not at 24 hai (Table 3). For the genes corresponding to the two cytokinin-repressed proteins, only a minor increase in mRNA accumulation was observed at 72 hai (Table 3). For CR9 however, this increase was also observed for all other timepoints (24, 48, and 96 hai). Among the downregulated genes, a significant decrease in mRNA accumulation was observed for those encoding for cell-wall glycine-rich protein and wound-induced protein CBP1 precursor (Table 3). Only a minor reduction was observed for the transcript of the zinc finger protein. These changes were verified consistently in the three biological replications of the qRT-PCR assay.

DISCUSSION

In this work, we attempted to carry out a global analysis of gene expression during tomato infection by PepYMV, a plant RNA virus of the genus *Potyvirus* in the family *Potyviridae*. Two subtractive libraries were constructed, one from systemically infected leaves and one from locally infected leaves. Surprisingly, the preliminary analysis of the systemic infection library indicated that almost all clones corresponded to rRNA genes. It is possible that the subtraction reaction was not successful (a second subtraction was not attempted). It is unlikely that shifts in gene expression at those later stages of the infection are of a smaller magnitude and cannot be appropriately detected by SSH, since at least one study using this technique in a plant-potyvirus interaction has identified differentially expressed genes later in infection (Pompe-Novak et al. 2006). Significantly, in the study by Pompe-Novak and associates (2006), mRNAs were not pooled from different timepoints. It is conceivable that pooling mRNA from systemically infected leaves at 7, 14, and 21 dpi hinders the detection of differentially expressed genes; genes that could be upregulated at one timepoint could be downregulated at another. It would be interesting to verify whether differentially expressed genes would be identified for the tomato-PepYMV interaction in systemically infected leaves at each one of the three timepoints (7, 14, and 21 dpi) independently.

Differences in gene expression were much more evident in the local infection library; a total of 881 genes were identified

Table 2. Partial list of downregulated expressed sequence tags in the interaction tomato-*Pepper yellow mosaic virus* (PepYMV) at 72 h postinoculation

BLASTx putative identification	GenBank no.	*E* value	Validation
Cell defense[a]			
Disease resistance protein BS2 (*Capsicum chacoense*)	BM173590	6E-53	Macroarray
Hypersensitive-induced response protein (*Arabidopsis thaliana*)	AAM63689	4E-14	Macroarray
Mlo protein-like (*A. thaliana*)	NP_201398	9E-17	n.d.[b]
Pathogenesis-related protein 3 (*Phaseolus vulgaris*)	DW359656	2E-40	n.d.
Programmed cell death protein (*A. thaliana*)	NP_001032143	8E-15	Macroarray
Resistance complex protein I2C-3 (*Solanum lycopersicum*)	AF004880	1E-14	n.d.
Cell cycle			
Cyclin (*A. thaliana*)	Z36397	2E-32	n.d.
ZW10 (*A. thaliana*)	NM_128850	2E-17	n.d.
Cell fate			
Gip1-like protein (*Petunia x hybrida*)	CAD10105	2E-16	n.d.
Proteinase homolog F19B15.70 (*A. thaliana*)	AB04405	9E-17	Macroarray
RPS6-like protein (*A. thaliana*)	BAB67768	6E-15	Macroarray
Cellular localization			
Probable transporter (*A. thaliana*)	NM_114644	6E-29	n.d.
Signal transduction			
Receptor-like protein kinase (*Nicotiana tabacum*)	AB073628	2E-16	n.d.
Transcription			
bZIP transcription factor BZI-2 (*N. tabacum*)	AY045572	6E-15	n.d.
Putative zinc finger protein (*Zea mays*)	AAS00453.1	5,00E-64	qRT-PCR[c]
Stress response			
26S proteasome regulatory particle non-ATPase subunit5 (*Oryza sativa*)	AB070259	6E-30	n.d.
Wound-inducible carboxypeptidase (*S. lycopersicum*)	AAF44708	9E-74	qRT- PCR
Biogenesis of cellular components			
Cell wall protein (*S. lycopersicum*)	CAA54561	2E-02	qRT-PCR
Pectinesterase (*A. thaliana*)	AAL49785	4E-10	Macroarray
Metabolism			
Cystein protease inibitor (*A. thaliana*)	NM_129651	1E-28	Macroarray
Cytochrome oxidase subunit III (*A. thaliana*)	DT003457	3E-16	Macroarray
NIFS-like protein CpNifsp precursor (*A. thaliana*)	AF419347	1E-16	n.d.
Permease 1—common ice plant (*A. thaliana*)	AAM20536	3E-15	n.d.
Phytoene dehydrogenase-like (*A. thaliana*)	AB023033	9E-17	Macroarray
Chlorophyll a/b binding protein (*S. tuberosum*)	BI176415	6E-30	n.d.
Probable sugar transporter (*A. thaliana*)	AAD20917	2E-14	n.d.
Pyruvate/2-oxoglutarate dehydrogenase	CAB75899	6E-29	Macroarray
Thioredoxin domain containing 1 (*Rattus norvegicus*)	AAH94308	3E-33	Macroarray
Ubiquinol—cytochrome-c reductase (*S. tuberosum*)	CAA55862	2E-26	n.d.
Non-classified proteins			
Hypothetical protein (*S. lycopersicum*)	BAD95796	2E-21	Macroarray
PINHEAD/ZWILLE protein (*A. thaliana*)	AF174272	5E-04	Macroarray

[a] Functional category according to the Munich Information Center for Protein Sequences database.
[b] n.d. = not done.
[c] Genes validated by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) were also validated by macroarray.

as either up- or downregulated. Differential expression of 53 genes was confirmed by macroarray analysis, and 10 were further confirmed by real-time PCR. Together with the analysis of putative gene functions, these results highlight the complex interaction taking place between virus and host, with several steps of the same metabolic pathways being potentially induced or repressed during infection.

A comparative analysis of the genes identified in our case with those previously identified in other virus-plant interactions indicates that similar (or even identical) genes are differentially expressed in different interactions. However, the specific requirements for a given host factor by different viruses can be distinct. Thus, different virus-plant interactions can shift the expression profile of a common set of genes, but the outcome of these shifts can be different in terms of the ability of the virus to infect the host.

Several genes encoding for putative heat-shock proteins (HSP) were identified as upregulated, something which was also observed for other virus-plant interactions (Escaler et al. 2000; Gandia et al. 2007; Pompe-Novak et al. 2006; Senthil et al. 2005; Smith et al. 2004; Whitham et al. 2003; Yang et al. 2007) and was shown to be essential for adenovirus replication in LMH cells (Glotzer et al. 2000). The induction of HSP could be a host reaction to the synthesis of a large quantity of exogenous proteins. Alternatively, since many HSP have a role

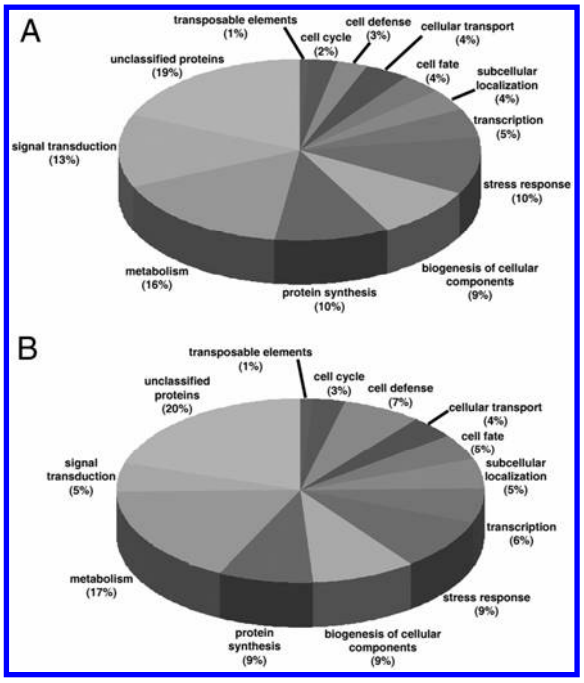

Fig. 3. Classification of expressed sequence tags (EST) identified in the subtractive library. **A,** Upregulated and **B,** downregulated EST.

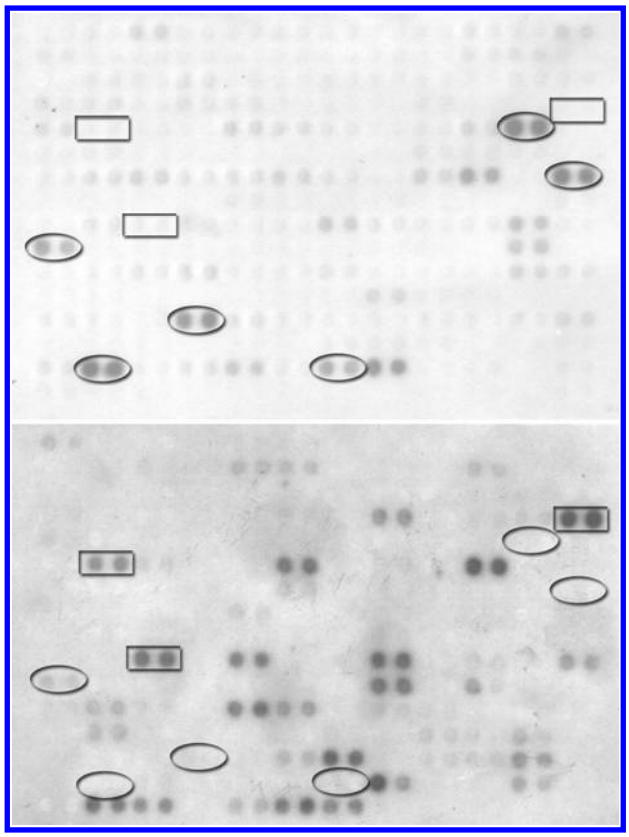

Fig. 4. Macroarray analysis using plasmid DNA from clones identified in the subtractive library. Replica membranes were hybridized to probes prepared from mock-inoculated (upper panel) or *Pepper yellow mosaic virus* (PepYMV)-inoculated plants (bottom panel) at 72 h postinoculation. Clones corresponding to induced genes, indicated by rectangles, yield a much higher intensity signal when hybridized to the probe prepared from infected plants as compared with the probe prepared from mock-inoculated plants. Clones corresponding to repressed genes, identified by circles, yield the opposite result.

Table 3. Changes in gene expression estimated by quantitative real-time reverse transcription-polymerase chain reaction

Gene[b]	GenBank no.[c]	Differential expression	Fold change at[a]				
			0 hai[d]	24 hai	48 hai	72 hai	96 hai
Cysteine proteinase	CAA88629	Induced	–0.033 ± 0.044	+0.100 ± 0.020	+0.030 ± 0.028	+1.493 ± 0.021	+1.313 ± 0.033
Threonine endopeptidase	NP_178042	Induced	–0.043 ± 0.037	–0.173 ± 0.019	–0.110 ± 0.023	+1.273 ± 0.038	+1.299 ± 0.020
Translationally controlled tumor protein	AAT65968	Induced	+0.024 ± 0.022	+0.550 ± 0.014	+2.950 ± 0.051	+1.970 ± 0.030	+1.828 ± 0.069
CAX-interacting protein 4	AAO17572	Induced	–0.169 ± 0.055	+0.446 ± 0.038	+0.730 ± 0.047	+1.480 ± 0.053	+1.592 ± 0.045
Cytokinin-repressed protein	CAC84488	Induced	+0.013 ± 0.037	+0.098 ± 0.023	–0.048 ± 0.089	+0.720 ± 0.035	+0.321 ± 0.032
SNF1 kinase complex anchoring protein	AAO89082	Induced	–0.011 ± 0.028	–0.281 ± 0.032	+0.665 ± 0.047	+1.300 ± 0.040	+1.290 ± 0.020
Cytokinin-repressed protein CR9	BAA06153	Induced	+0.043 ± 0.025	+0.639 ± 0.055	+0.651 ± 0.017	+0.683 ± 0.021	+0.699 ± 0.037
Cell wall glycine-rich protein	CAA54561	Repressed	n.d.	n.d.	n.d.	-1.013 ± 0.015	n.d.
Wound-induced protein CBP1 precursor	AAF44708	Repressed	n.d.	n.d.	n.d.	–2.460 ± 0.031	n.d.
Putative zinc finger protein	AAS00453.1	Repressed	n.d.	n.d.	n.d.	–0.280 ± 0.026	n.d.

[a] Induced or repressed.
[b] Putative annotation acording to BLASTx analysis.
[c] Access number of the sequence yielding the lowest *E* value.
[d] Average and standard deviation of three biological replications. hai = h after inoculation; n.d. = not done.

as molecular chaperones, it is possible that potyviruses may use HSP to assist in the correct folding of their own proteins. Some plant viruses encode HSP homologs involved in particle assembly and viral cell-to-cell movement (Agranovsky et al. 1998; Alzhanova et al. 2001; Prokhnevsky et al. 2002), indicating that HSP may be necessary for these processes to occur efficiently. It is relevant to note that potyviruses do not encode a HSP homolog or a dedicated movement protein (Revers et al. 1999).

Additional virus movement-related genes identified as upregulated include those encoding for calreticulin and DNA-J-like proteins. It has been demonstrated that elevated levels of calreticulin interfere with cell-to-cell movement of *Tobacco mosaic virus* (TMV), blocking movement protein targeting to the plasmodesmata (Chen et al. 2005). DNA-J-like proteins were identified as susceptibility factors, possibly by recruiting HSP70 chaperones to assist in viral cell-to-cell movement (Hofius et al. 2007). Interestingly, replication of *Brome mosaic virus* (BMV) in yeast mutants for a DNA-J homolog was inhibited before or during negative strand synthesis (Tomita et al. 2003), suggesting that different viruses may use the same host factors to assist in different points of the infection cycle.

A large number of genes involved in defense responses were identified as downregulated, suggesting that the virus targets different host-defense pathways. Some of the genes identified in this category code for analogs of resistance genes previously identified in tomato, such as the *Ve* gene, involved in the signal transduction pathway for resistance to the fungus *Verticillium dahliae* (Kawchuk et al. 2001); a gene with similarity to one of the members of the I2C complex, involved in resistance to *Fusarium oxysporum* (Ori et al. 1997); and the gene encoding the BS2 protein, which confers resistance to *Xanthomonas campestris* pv. *vesicatoria* (Tai et al. 1999). It is possible that these genes control common regulatory checkpoints leading to general defense responses.

A comparison of the percentages of EST classified as involved in defense responses between the upregulated and downregulated collections (2.8 and 7.2%, respectively) indicates that, at least in inoculated leaves at 72 hai, the virus is apparently more efficient in inhibiting defense responses than the host is in activating them.

Among the many up- and downregulated genes, we have paid special attention to those encoding proteins involved in the ubiquitin/proteasome pathway (upregulated), those encoding protease inhibitors (downregulated), those involved in the RNA-silencing response (up- and downregulated), and the one encoding the TCTP (upregulated).

In plants, protein degradation regulated by the ubiquitin/26S proteasome complex controls several cellular processes, such as embryogenesis, hormone-based signaling, senescence, and pathogen defense responses (Smalle and Vierstra 2004). Considering the great accumulation of viral proteins that takes place in potyvirus-infected cells, it is reasonable to assume that this pathway could be used by the host as a defense mechanism against viral infection. The induction of proteins involved in the ubiquitin/26S proteasome pathway was also verified for other virus-host interactions, such as pea–*Pea seed borne mosaic virus* (PSbMV) (Aranda et al. 1996), *Arabidopsis*–TMV (Schenk et al. 2003), tomato–*Potato spindle tuber viroid* (Itaya et al. 2001), *Saccharomyces cerevisae*–BMV (Kushner et al. 2003), and *Nicotiana benthamiana–Impatiens necrotic spot virus* (Senthil et al. 2005). Furthermore, a direct interaction between the proteasome and the HC-Pro protein encoded by the potyvirus *Lettuce mosaic virus* has been demonstrated (Ballut et al. 2005), suggesting a role for this pathway in the viral infection process. Potyviruses replicate in association with the endomembrane system, and the viral replication complex becomes inactivated after synthesis of an RNA strand due to loss of the VPg protein (Revers et al. 1999). It is possible that potyviruses use the ubiquitin/proteasome pathway to remove these inactive replication complexes and allow the assembly of new, functional ones. The significant identification (3.8%) of genes involved in membrane synthesis supports this hypothesis.

Potyviruses express their proteins by polyprotein processing, using the activity of three proteases embedded in the polyprotein (Carrington et al. 1990). It is reasonable to assume that the host will attempt to inhibit this process by expressing protease inhibitors. Downregulation of these genes would be a counterdefensive strategy of the virus.

Genes involved in the RNA silencing pathway were identified both in the up- and downregulated collections, which is not surprising considering the pivotal role of this pathway in virus-plant interactions. Downregulated genes include those encoding proteins with double-stranded (ds)RNA binding domains and PAZ/PIWI domains, both of which are conserved in proteins involved in the RNA silencing pathway. An upregulated gene encoding a calmodulin-like protein was also identified. The potyvirus RNA silencing suppressor HC-Pro was shown to interact with a calmodulin-like protein, rgs-Cam (Anandalakshmi et al. 2000).

The gene encoding the TCTP was identified as one of the most strongly induced genes. TCTP is a highly conserved protein in eukaryotes, involved in several cellular processes such as cell growth, cell cycle progression, cell protection against stresses, and apoptosis (Bommer and Thiele 2004). However, although it has been associated with these processes, its precise role in each one of them has not been clearly established.

Among its many potential roles, it has been proposed that TCTP could be involved in dsRNA-mediated defense responses. It was demonstrated that TCTP messenger (m)RNA is highly structured and capable of activating the dsRNA-dependent protein kinase PKR (Bommer et al. 2002), which in turn induces interferon-mediated antiviral responses and a decrease in protein synthesis in mammalian cells (Schneider and Mohr 2003; Valchanova et al. 2006). As a counter defensive strategy, many RNA viruses express proteins that bind dsRNA and inhibit dsRNA-mediated defense responses (Child et al. 2004, 2006). Although the interferon response is absent in plants, RNA silencing is triggered by dsRNA, and therefore, plant viruses also express proteins involved in dsRNA-mediated responses. Indeed, it has recently been suggested that dsRNA binding could be a general strategy used by plant viruses to suppress RNA silencing (Merai et al. 2006). It is possible that the induction of TCTP expression in infected plants is part of a dsRNA-induced defense response.

The ability of plants to mount an efficient defense response depends on their capacity to recognize the pathogen early in the infection process. Therefore, an increased knowledge of the genetic, biochemical, and molecular mechanisms involved in the early events of the virus-host interaction could lead to the establishment of more efficient control strategies. The analysis of differentially expressed genes carried out in this work allowed the formulation of several hypotheses related to the infection process and the corresponding defense mechanisms activated by the host (which, in our system, is susceptible to the virus). Functional analyses will be necessary to confirm these hypotheses and to define in a more specific way the role of these differentially expressed genes in the virus-host interaction.

MATERIALS AND METHODS

Viral isolate, plant material, and inoculation.

The PepYMV isolate used in this study was obtained from naturally infected chili pepper (Truta et al. 2004) and has been

stored at –20°C in the form of *Nicotiana debneyi* dried leaf material. The isolate was reactivated and maintained in *Nicotiana debneyi* and in *S. lycopersicum* 'Moneymaker' plants grown under greenhouse conditions. Standard sap inoculations were carried out using 0.05 M phosphate buffer, pH 7.2, containing 0.1% (wt/vol) sodium sulfite and Carborundum 600 mesh as an abrasive.

S. lycopersicum 'Moneymaker' seedlings were maintained in a growth chamber set for 26°C and a 16-h photoperiod. The first pair of true leaves was sap-inoculated as described above, using buffered extract prepared from PepYMV-infected tomato leaves. Two experiments were carried out. For the first experiment (systemic infection library), the two youngest, fully expanded, noninoculated leaves were collected immediately before and at 7, 14, 21, and 28 dai. For the second experiment (local infection library), inoculated leaves were collected immediately before and at 24, 48, 72, 96, and 120 hai. In both experiments, control plants were mock-inoculated with buffered extract prepared from healthy tomato leaves and leaves were collected at the same times. For each collecting time, a composite sample was prepared from 12 individual plants in order to average out gene expression differences due to leaf-to-leaf and plant-to-plant variations. Leaves in each of these composite samples were frozen in liquid nitrogen immediately after removal from the plants and were stored at –80°C until RNA extraction.

Viral detection in inoculated plants.

All inoculated and noninoculated tomato plants were tested for PepYMV infection by indirect ELISA (Clark et al. 1986), using a polyclonal antiserum prepared in our laboratory (Truta et al. 2004) to ensure that mock-inoculated plants remained virus-free and that every inoculated plant was indeed infected.

Preparation of subtracted cDNA libraries.

Total RNA was extracted from leaves using the Concert plant RNA reagent (Invitrogen, Carlsbad, CA, U.S.A.), according to the manufacturer's instructions. mRNA was isolated from 500 μg of total RNA using the NucleoTrap nucleic acid purification kit (BD Biosciences, San Jose, CA, U.S.A.), following the manufacturer's instructions.

Subtracted libraries were constructed using the PCR-Select cDNA suppressive subtraction kit (Clontech, Mountain View, CA, U.S.A.). For the systemic infection library, mRNAs from virus-infected leaves collected at 14, 21, and 28 dpi were pooled and subtracted against mRNAs pooled from mock-inoculated leaves collected at the same times. For the local infection library, mRNAs from virus-infected leaves collected at 72 hai were subtracted against mRNAs from mock-inoculated leaves collected at the same time. For both libraries, subtraction reactions were performed in the forward and reverse directions. The forward subtraction used mRNA from virus-infected leaves as tester and excess mRNA from mock-inoculated leaves as driver, thus cloning upregulated cDNAs. The reverse subtration is carried out inversely, cloning downregulated cDNAs. In the forward subtration, viral cDNA was added along with driver cDNA to prevent cloning of viral cDNAs. Subtracted cDNAs were cloned into the pGEM-T-Easy vector (Promega, Madison, WI, U.S.A.) and were completely sequenced.

Differential screening.

Subtracted clones obtained from the forward and reverse collections were randomly selected and were blotted onto nylon membranes (Hybond N^+, GE Healthcare, Piscataway, NJ, U.S.A.) according to standard techniques (Sambrook and Russel 2001). Each membrane was prepared in duplicate. The probes used were the forward- and reverse-subtracted cDNAs labeled with [α^{32}P]-dATP, using the Prime-it II random primer labeling kit (Stratagene, La Jolla, CA, U.S.A.) following the manufacturer's instructions. Hybridization was carried out at high stringency, according to standard techniques (Sambrook and Russel 2001).

Sequence analysis.

The clones obtained were sequenced in a MegaBACE 100 automated sequencer at Universidade Estadual de Santa Cruz's Bioinformatics laboratory, (Ilhéus, BA, Brazil), using the DYEnamic ET terminator cycle sequencing kit (GE Healthcare) according to the manufacturer's instructions.

The EST obtained were subjected to a database search using BLASTx (Altschul et al. 1990). Only similarities with expected value (*E*) smaller or equal to 10^{-3} were considered significant, if no lower scores were obtained. Functional categorization was done according to the MIPS Functional Catalogue database.

Macroarray analysis.

Recombinant plasmid DNA from the forward and reverse collections (200 ng each) were denatured with 0.5 M NaOH and were arrayed in duplicates onto nylon membranes (Hybond N^+). Each membrane was also prepared in duplicate. Two complex probes were prepared, one from cDNA from PepYMV-inoculated leaves at 72 hpi and the other from cDNA of mock-inoculated leaves at 72 hpi. For this, total RNA was extracted as described and was used as a template to synthesize double-stranded cDNA (Sambrook and Russel 2001). This complementary (c)DNA was labeled with [α^{32}P]-dATP, and hybridization was carried out at high stringency, as described. Three independent experiments were performed, in which cDNA was prepared from different sets of PepYMV- or mock-inoculated plants (biological replications).

qRT-PCR.

For quantitative analysis of gene expression, total RNA was extracted as described from PepYMV-infected and mock-inoculated tomato leaves at 0, 24, 48, 72, and 96 hpi. Total RNA (2 μg) was DNAse-treated according to standard techniques (Sambrook and Russel 2001) and was used for cDNA synthesis. Real-time PCR reactions were carried out in a final volume of 25 μl, using a SYBR Green master mix and an ABI7500 thermal cycler (Applied Biosystems, Foster City, CA, U.S.A.), following manufacturer's instructions. Each sample was analyzed in triplicate. Three biological replications of the experiment were carried out, i.e., cDNA was synthesized from three different sets of PepYMV- and mock-inoculated tomato plants. The three biological replications were carried out over a period of 15 months, in which the first replication was done in the first month and the second and third replications were done in the sixth and 15th months, respectively.

To determine the optimal internal reference for normalization of gene expression, four housekeeping genes supposed to have their transcriptional activity unaltered during viral infection were tested: actin (accession number BT013524), β-6-tubulin (BT013153), elongation factor 1α (BT013246), and *APT1* (BT012816).

Primers for all target sequences (Supplementary Table S1) were designed using Primer Express software (Applied Biosystems). The specificity of the amplicons was checked by melting-curve analysis and by acrylamide-gel electrophoresis. Expression levels were normalized to the *APT1* gene, and relative quantification was performed using the comparative cycle threshold method ($\Delta\Delta C_T$) (Livak and Schmittgen 2001).

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AUTHOR-RECOMMENDED INTERNET RESOURCE

MIPS Functional Catalogue database: mips.gsf.de/projects/funcat