Identification and Expression Profiling of Tomato Genes Differentially Regulated During a Resistance Response to *Xanthomonas campestris* pv. *vesicatoria*

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The gram-negative bacterium *Xanthomonas campestris* pv. *vesicatoria* is the causal agent of spot disease in tomato and pepper. Plants of the tomato line Hawaii 7981 are resistant to race T3 of *X. campestris* pv. *vesicatoria* expressing the type III effector protein AvrXv3 and develop a typical hypersensitive response upon bacterial challenge. A combination of suppression subtractive hybridization and microarray analysis identified a large set of cDNAs that are induced or repressed during the resistance response of Hawaii 7981 plants to *X. campestris* pv. *vesicatoria* T3 bacteria. Sequence analysis of the isolated cDNAs revealed that they correspond to 426 nonredundant genes, which were designated as *XRE* (*Xanthomonas*-regulated) genes and were classified into more than 20 functional classes. The largest functional groups contain genes involved in defense, stress responses, protein synthesis, signaling, and photosynthesis. Analysis of *XRE* expression kinetics during the tomato resistance response to *X. campestris* pv. *vesicatoria* T3 revealed six clusters of genes with coordinate expression. In addition, by using isogenic *X. campestris* pv. *vesicatoria* T2 strains differing only by the *avrXv3* avirulence gene, we found that 77% of the identified *XRE* genes were directly modulated by expression of the AvrXv3 effector protein. Interestingly, 64% of the *XRE* genes were also induced in tomato during an incompatible interaction with an avirulent strain of *Pseudomonas syringae* pv. *tomato*. The identification and expression analysis of *X. campestris* pv. *vesicatoria* T3-modulated genes, which may be involved in the control or in the execution of plant defense responses, set the stage for the dissection of signaling and cellular responses activated in tomato plants during the onset of spot disease resistance.

(Pohronezny and Volin 1983). Typical symptoms of the disease are water-soaked lesions that develop into brown necrotic spots. The causal agent of spot disease is the gram-negative bacterium *Xanthomonas campestris* pv. *vesicatoria*, which can be a contaminant on tomato seeds or survive in plant debris and in association with certain weeds (Jones et al. 1986). *X. campestris* pv. *vesicatoria* infects a wide range of hosts in the Solanaceae family, and strains of *X. campestris* pv. *vesicatoria* are classified into different groups according to their pathogenicity on tomato and pepper (Jones et al. 1998). The tomato group (*X. campestris* pv. *vesicatoria* T) includes strains that are virulent only on tomato, the pepper group (*X. campestris* pv. *vesicatoria* P) includes strains that are pathogenic only on pepper, and strains of the tomato and pepper group (*X. campestris* pv. *vesicatoria* TP) are pathogenic on both plant species.

In susceptible plants, *X. campestris* pv. *vesicatoria* strains are able to multiply and spread throughout the plant, leading to the appearance of typical disease symptoms. In resistant plants, the activation of a rapid and localized cell death at the site of infection, known as the hypersensitive response (HR), limits pathogen growth and minimizes disease symptoms. In many plant-pathogen interactions, the development of a HR is associated with several cellular responses that contribute to resistance (Hammond-Kosack and Jones 1996). These responses include the production of reactive oxygen species (ROS), transient opening of ion channels, cell wall fortifications, production of phytoalexins, and synthesis of pathogenesis-related (PR) proteins. Much remains to be learned about defense responses and signaling pathways activated during the resistance response of tomato to *X. campestris* pv. *vesicatoria*, for which reports are limited to the expression study of PR genes (Ciardi et al. 2000).

Plant resistance or susceptibility is dictated by the genetic backgrounds of both the host plant and the invading pathogen. Several sources of resistance to *X. campestris* pv. *vesicatoria* races have been identified in tomato (*Lycopersicon esculentum*) lines that develop a typical HR upon bacterial attack (Jones et al. 1998). Resistance to *X. campestris* pv. *vesicatoria* strains of the pepper group (*X. campestris* pv. *vesicatoria* P) is a common characteristic of tomato cultivars, and it is specified in a gene-for-gene interaction by the *Bs4* resistance (*R*) gene in the plant and by the *avrBs4* avirulence gene in the pathogen (Ballvora et al. 2001; Bonas et al. 1993; Canteros et al. 1991). The *Bs4* gene was isolated recently and was found to encode a nucleotide-binding-leucine rich repeat protein (Schornack et al. 2004). Interestingly, *Bs4*-mediated elicitation of the HR re-

Bacterial spot disease of tomato occurs in warm, moist regions throughout the world and is of great economic importance because it decreases yield and quality of fruit production

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quires functional *EDS1* and *SGT1* genes that encode known components of resistance signaling (Schornack et al. 2004). Sources of tomato resistance to *X. campestris* pv. *vesicatoria* strains of the tomato group (*X. campestris* pv. *vesicatoria* T) were identified in several lines and were genetically characterized (Jones et al. 1998). The tomato cultivar Hawaii 7998 develops a HR upon infection by *X. campestris* pv. *vesicatoria* T strains of race T1 expressing the *avrRxv* avirulence gene (Whalen et al. 1993). In these plants, at least three genes participate with an additive effect in the activation of the defense response (Yu et al. 1995). Resistance to *X. campestris* pv. *vesicatoria* T strains of race T3 (*X. campestris* pv. *vesicatoria* T3) was found in different tomato lines, and it is mediated by gene-for-gene interactions that involve distinct resistance genes and bacterial effectors; in two *Lycopersicon pimpinellifolium* accessions and in the tomato cultivar Hawaii 7981, resistance is mediated by the plant *R* gene *RxvT3*, which interacts with the *X. campestris* pv. *vesicatoria* T3 avirulence gene *avrXv3* (Astua-Monge et al. 2000a; Scott et al. 1995), and in *Lycopersicon pennellii* LA716, resistance is mediated by the *R* gene *Xv4* in the plant and by the *avrXv4* avirulence gene in the bacterium (Astua-Monge et al. 2000b). Although genetic interactions mediating tomato resistance to *X. campestris* pv. *vesicatoria* T strains are characterized, plant genes that are implicated in associated resistance signaling and defense responses are largely unknown.

The recent development of genomics techniques for the study of gene expression profiles, together with the availability of genome and expressed sequence tag (EST) databases for many plant species, has allowed significant progress in the characterization of plant responses to pathogen attack (Wan et al. 2002). For example, gene expression profiles have been examined in *Arabidopsis* challenged by different avirulent pathogens, such as the bacterium *Pseudomonas syringae* pv. *tomato* (Chen et al. 2002; Scheideler et al. 2002; Tao et al. 2003) and the fungus *Alternaria brassicicola* (Narusaka et al. 2003; Schenk et al. 2000), or after treatment with defense-related signaling molecules, including salicylic acid, methyl jasmonate, ethylene, and ROS (Chen et al. 2002; Maleck et al. 2000; Narusaka et al. 2003; Schenk et al. 2000). Similarly, a comprehensive analysis of gene expression in tomato allowed the identification of genes and potentially molecular processes that are associated with resistance to bacterial speck disease mediated by the *Pto R* gene (Mysore et al. 2002, 2003).

In this study, we combined the use of suppression subtractive hybridization (SSH) and microarray analysis to identify and verify genes that are differentially expressed during the resistance response of the tomato cultivar Hawaii 7981 to *X. campestris* pv. *vesicatoria* T3 strains. By this approach, 426 *X. campestris* pv. *vesicatoria*-regulated (*XRE*) genes were identified and classified into functional groups, and their expression profiles were examined following *X. campestris* pv. *vesicatoria* T3 inoculation. *XRE* genes were also analyzed in terms of their inducibility or repression upon expression of the *AvrXv3* effector or infection by *Pseudomonas syringae* pv. *tomato*.

RESULTS

Generation of cDNA subtraction libraries enriched for tomato transcripts modulated by *X. campestris* pv. *vesicatoria* T3 avirulent infection.

As a first step toward the identification of plant genes involved in the tomato resistance response to *X. campestris* pv. *vesicatoria*, we constructed cDNA libraries enriched for *X. campestris* pv. *vesicatoria* T3-modulated genes, using SSH (Diatchenko et al. 1999). Leaf tissues used for preparation of the libraries were derived from plants of the tomato cultivar

Hawaii 7981, which is resistant to *X. campestris* pv. *vesicatoria* T3 strains as a result of a gene-for-gene interaction between the *RxvT3* resistance gene (Scott et al. 1995) and the *avrXv3* avirulence gene (Astua-Monge et al. 2000a). Hawaii 7981 plants were infiltrated by vacuum with a suspension of *X. campestris* pv. *vesicatoria* T3 bacteria at a titer of 10^8 CFU/ml or were mock-inoculated. As typically observed at this inoculum level, Hawaii 7981 leaves developed a HR within 24 h after infection. For each treatment, leaf samples were harvested 4 and 8 h after inoculation, were pooled, and were used for the extraction of mRNA and cDNA synthesis. Reciprocal subtractions of cDNAs derived from *X. campestris* pv. *vesicatoria* T3-treated and mock-inoculated plants yielded cDNA libraries that are enriched in *X. campestris* pv. *vesicatoria* T3-induced (forward library) or *X. campestris* pv. *vesicatoria* T3-repressed (reverse library) genes.

To test subtraction efficiency of the SSH libraries, a reverse Northern analysis was carried out for groups of 63 and 42 cDNA clones from forward and reverse libraries, respectively. Randomly selected SSH cDNAs and control genes were polymerase chain reaction (PCR)-amplified, were separated by electrophoresis, and were transferred onto two identical blots. The *PR* gene encoding (1-3)- β -glucanase was used as a positive control, whereas a gene encoding tomato actin was included as a reference for normalization. After probing the blots with radiolabeled cDNAs derived from *X. campestris* pv. *vesicatoria* T3-infected or mock-inoculated Hawaii 7981 leaves, differential expression was estimated for each individual gene, based on relative hybridization intensities. A typical reverse Northern for cDNAs of the forward library and quantification of their fold induction are presented in Figure 1A and B, respectively. According to this analysis, we found that 21 and 40% of the clones tested for forward and reverse libraries, respectively, were differentially expressed by at least twofold in *X. campestris* pv. *vesicatoria* T3-infected plants as compared with mock-inoculated plants.

Ten and five cDNA clones from forward and reverse libraries, respectively, that showed a significant differential expression in the reverse Northern analysis were subjected to sequence analysis. cDNAs from the forward library were found to be significantly similar to plant genes encoding known proteins involved in stress responses, disease resistance signaling, or vesicular transport. cDNAs from the reverse library were found to encode proteins involved in photosynthesis, senescence, or protein degradation. Four genes derived from the forward library were tested further by Northern blot analysis to confirm their inducibility during the tomato resistance response to *X. campestris* pv. *vesicatoria* T3 infection. As shown in Figure 2, the selected genes were significantly induced upon treatment with *X. campestris* pv. *vesicatoria* T3 but not by mock inoculation. Accumulation of their mRNA was evident 4 h after treatment and reached a peak at 12 or 18 h postinoculation. Taken together, these results confirmed that the SSH libraries were enriched for *X. campestris* pv. *vesicatoria* T3-induced and repressed genes.

Identification of tomato genes differentially expressed during the tomato-*X. campestris* pv. *vesicatoria* T3 incompatible interaction.

To identify tomato genes differentially expressed during the tomato resistance response to *X. campestris* pv. *vesicatoria* T3, we examined expression profiles of genes represented in the forward and reverse SSH libraries. A total of 1,152 randomly selected cDNA clones (576 cDNAs from each library) were printed in triplicate on microarray slides. The array also included cDNAs encoding known plant defense-related genes, as positive controls and cDNAs of unrelated organisms as nega-

tive controls. Leaf samples of *X. campestris* pv. *vesicatoria* T3-infected or mock-inoculated Hawaii 7981 plants were harvested at different timepoints after inoculation (0, 4, 8, 12, and 18 h). Total RNA was extracted from these samples and was used for the preparation of fluorescent probes, which were then hybridized to the microarray slide. Expression profiles of the genes present on the slide were obtained from the analysis of expression ratio intensities measured for *X. campestris* pv. *vesicatoria* T3-treated samples relative to mock-inoculated samples. The cutoff of fold induction used to consider genes as differentially regulated was >1.68 . Although the commonly used threshold value is twofold (Cheong et al. 2002; Scheideler et al. 2002), several studies have shown that a lower cutoff ranging from 1.5 to 2 can be used reliably (Mysore et al. 2002; Zik and Irish 2003), particularly if several replicates are included in the experiment. Using a threshold of 1.68-fold change, we found that among the 1,152 spotted SSH cDNAs, 661 (57%) were differentially expressed with statistical significance ($P < 0.01$) in at least one of the five analyzed timepoints. These cDNA clones were subjected to sequence analysis and were found to correspond to 426 nonredundant genes. They included 128 up-regulated genes and 298 down-regulated genes and were referred to as *XRE* (*Xanthomonas*-responsive) genes. The number of differentially expressed *XRE* genes at each timepoint is reported in Figure 3. Globally, a significant up-regulation of gene expression was detected as early as 4 h after treatment, reached a peak at 12 h, and slightly decreased at 18 h,

Fig. 1. *Xanthomonas campestris* pv. *vesicatoria* T3-inducibility of cDNA clones from the forward suppression subtractive hybridization (SSH) library. **A**, Reverse Northern analysis of SSH clones. Equal amounts of polymerase chain reaction-amplified cDNA inserts were separated on agarose gels and were blotted onto nylon filters. Lanes 1 to 20, inserts of randomly selected clones from the forward SSH library; lane 21, cDNA of basic (1,3)- β -glucanase as positive control; lane 22, cDNA of actin as control for normalization. The duplicated filters were hybridized with 32 P-labeled cDNA derived from Hawaii 7981 plants that were inoculated with *X. campestris* pv. *vesicatoria* T3 (lower panel) or were mock-inoculated (upper panel) and were sampled at 4 and 8 h postinoculation. **B**, Fold induction of gene expression upon *X. campestris* pv. *vesicatoria* T3 treatment as compared with mock inoculation for the SSH clones tested as shown in A. Fold induction was calculated based on densitometry quantification of each band in the membranes shown in A and normalization according to the actin reference. The dashed line indicates a twofold change in gene expression.

shortly before the appearance of the HR, which was clearly visible 24 h after inoculation. Down-regulation of gene expression was evident only 8 h postinoculation, reaching a peak at 12 h and decreasing significantly at 18 h. Taken together, the microarray analysis of SSH cDNAs led to the identification of a large group of *X. campestris* pv. *vesicatoria* T3-modulated genes and set the stage for the analysis of their expression kinetics.

Identification of coordinately regulated *XRE* genes.

To identify common expression kinetics among *XRE* genes, we built a dataset that included expression ratio intensities of the *XRE* genes at different timepoints after *X. campestris* pv. *vesicatoria* T3 infection and mock inoculation. The *k*-means algorithm was applied in a nonhierarchical cluster analysis to the *XRE* dataset and yielded six highly homogenous clusters (Fig. 4). Cluster A consists of 36 down-regulated genes that are affected relatively early by pathogen challenge. The average expression level of this group starts decreasing 8 h after infection, reaches a minimum at 12 h, and slightly increases 18 h postinoculation. Highly represented in this cluster are genes related to stress responses (e.g., catalase isozyme 1 and peroxidase) and respiration (e.g., NADH dehydrogenase and alternative oxidase 1a). Cluster B includes 51 genes that are mostly down-regulated and whose expression steadily decreases during the first 12 h postinoculation and returns to the average level after 18 h. This cluster contains a considerable number of genes related to defense (e.g., encoding phenylalanine ammonia lyase [PAL] and monooxygenase) and protein degradation (e.g., encoding ATP-dependent Clp protease proteolytic subunit and putative ubiquitin-conjugating enzyme) and the largest percentage of unknown genes (35%) among all clusters. Cluster C is the second largest with 106 members showing a relatively constant expression level throughout the first 8 h after bacterial infection and a sharp decrease after 12 h. Highly represented in this cluster are genes encoding stress-related (e.g., glutathione *S*-transferase and alcohol-dehydrogenase) and signaling-related proteins (e.g., several protein kinases and a RelA-SpoT like protein). Cluster D contains 78 members that are predominantly down-regulated and show an unaltered expression throughout the first 8 h after inoculation, followed

Fig. 2. Northern blot analysis of selected suppression subtractive hybridization (SSH) clones. Total RNA was extracted from *Xanthomonas campestris* pv. *vesicatoria* T3-infected or mock-inoculated Hawaii 7981 leaf tissues sampled at the indicated timepoints. RNA samples were subjected to Northern blot analysis, with probes derived from the following SSH clones: *SSH2A3*, *SSH5D10*, *SSH5A12*, *SSH5B8*, and ribosomal RNA (*rRNA*) as a control for equal loading.

by a continuous decrease in expression towards the onset of the HR. Significant in this cluster is the presence of genes encoding proteins related to photosynthesis (e.g., photosystem I reaction center subunit II and ribulose biphosphate carboxylase small subunit), stress responses (e.g., ascorbate peroxidase and HSP90-like protein), and transport (e.g., potassium transporter and tonoplast intrinsic protein). Cluster E, the largest of the six, is composed of 122 up-regulated genes that show a gradual increase in expression levels up to a maximum at 12 h postinoculation, followed by a slight decrease in the subsequent hours. The predominant functional classes in this cluster include genes encoding products related to defense responses (e.g., PR proteins and NPR1), signaling (e.g., mitogen-activated protein [MAP] kinases and a kinase-associated protein phosphatase), protein biosynthesis (e.g., 40S and 60S ribosomal proteins and elongation factor 1- α), and transport (e.g., nitrate and hexose transporters). Cluster F is the smallest cluster with 33 mostly down-regulated members that are characterized by a drastic and transient decrease in expression levels at 12 h after bacterial infection. In this cluster, the most represented functional groups encode proteins related to protein biosynthesis (e.g., elongation factor 1- γ and 60S ribosomal protein L34), photosynthesis (e.g., chlorophyll a/b binding protein 1B and far-red impaired response protein), defense (e.g., Mlo protein homolog and nitrate-induced NOI protein), and sugar metabolism (e.g., UDP-glucose 4-epimerase and enolase).

Functional classification of *XRE* genes.

To explore potential cellular functions for the *XRE* genes, their cDNA sequences were searched against the tomato EST database deposited at The Institute for Genomic Research and against the National Center for Biotechnology Information database using BLASTX searches. The genes were annotated and classified into 20 primary functional groups based on their homology to genes in the database (Table 1). A significant number of *XRE* genes (24% of the entire group) encoded proteins with insufficient similarity to proteins of known function to be classified with confidence and were, therefore, grouped in the category of unknown function. The functional groups with the highest number of genes were those with likely involvement in stress responses, defense, protein synthesis, signaling, and photosynthesis. Among the up-regulated genes, the most represented categories were defense, signaling, protein synthesis, and transport, whereas among the down-regulated genes the functional groups with the highest percentage of genes were stress responses, protein synthesis, defense, signaling, and photosynthesis.

Identification of *XRE* genes directly modulated by expression of the *X. campestris* pv. *vesicatoria* T3 effector AvrXv3.

The microarray analysis described so far allowed us to identify genes of *X. campestris* pv. *vesicatoria* T3-resistant tomato plants that are differentially expressed upon inoculation with avirulent *X. campestris* pv. *vesicatoria* T3 bacteria, as compared with mock-inoculated plants. We next assumed that the expression of the identified *XRE* genes is in part directly modulated by the interaction between the *RxvT3* tomato resistance gene and the *avrXv3* avirulence gene, whereas a group of *XRE* genes is affected by *X. campestris* pv. *vesicatoria* T3 infection through mechanisms unrelated to the onset of resistance. To discriminate between these groups of genes, we first engineered isogenic *X. campestris* pv. *vesicatoria* strains differing only by the *avrXv3* gene. The coding region of *avrXv3* was introduced into the broad-host-range vector pDSK519 under the control of the *lacZ* promoter. This plasmid was then delivered into *X. campestris* pv. *vesicatoria* bacteria of the T2 race,

which are virulent towards Hawaii 7981 plants. To test the capability of the *X. campestris* pv. *vesicatoria* T2 strain carrying the *avrXv3* gene (*X. campestris* pv. *vesicatoria* T2[*avrXv3*]) to induce a HR in *X. campestris* pv. *vesicatoria* T3-resistant plants, Hawaii 7981 leaves were inoculated by vacuum infiltration with a suspension of *X. campestris* pv. *vesicatoria* T2 or *X. campestris* pv. *vesicatoria* T2(*avrXv3*) bacteria at a titer of 10^8 CFU/ml. As shown in Figure 5A, after 24 h a typical HR was observed in leaves inoculated with the *X. campestris* pv. *vesicatoria* T2(*avrXv3*) strain whereas, at this time, no effect was yet detected in *X. campestris* pv. *vesicatoria* T2-infected leaves. To determine the effect of AvrXv3 expression on bacterial growth, Hawaii 7981 plants were infected with *X. campestris* pv. *vesicatoria* T2 and *X. campestris* pv. *vesicatoria* T2(*avrXv3*) strains at a titer of 10^5 CFU/ml and were sampled during a one-week period. Starting four days after inoculation, a significant inhibition of bacterial growth was observed for *X. campestris* pv. *vesicatoria* T2(*avrXv3*) strains as compared with *X. campestris* pv. *vesicatoria* T2 (Fig. 5B). Together, these results indicate that expression of the AvrXv3 effector confers avirulence to this *X. campestris* pv. *vesicatoria* strain of the T2 race during its interaction with Hawaii 7981 plants.

The isogenic bacterial strains *X. campestris* pv. *vesicatoria* T2 and T2(*avrXv3*) were used to identify tomato genes that are specifically modulated by the expression of the AvrXv3 effector during the resistance response of Hawaii 7981. Plants were inoculated with either one of the two bacterial strains, and leaf tissues were harvested after 8 and 12 h. We focused on these two timepoints because we assumed that the earliest significant changes in gene expression would take place after 8 h

Fig. 3. Differential expression of *Xanthomonas*-regulated genes upon *Xanthomonas campestris* pv. *vesicatoria* T3 infection as compared with mock inoculation. Total RNA was extracted from Hawaii 7981 *X. campestris* pv. *vesicatoria* T3-infected or mock-inoculated leaf tissue sampled at the indicated timepoints. RNA samples were used to prepare fluorescently labeled cDNA probes for microarray analysis of 1,152 cDNA clones deriving from suppression subtractive hybridization libraries enriched for *X. campestris* pv. *vesicatoria* T3-modulated transcripts. For each timepoint, four experimental replicates were carried out. Genes were regarded as differentially regulated if, in at least one timepoint, their fold change upon *X. campestris* pv. *vesicatoria* T3 inoculation was at least 1.68 and if they withstood a statistical Student *t* test with a *P* < 0.01. Based on these criteria, filled and empty bars represent the number of nonredundant genes that, at different times postinoculation, were found to be up- or down-regulated, respectively.

whereas the most dramatic variation would occur at 12 h. This assumption is based on the kinetics of gene expression observed during the interaction of *X. campestris* pv. *vesicatoria* T3 with H7981 plants and on the evidence that resistant plants develop HR in response to *X. campestris* pv. *vesicatoria* T2(*avrXv3*) strains with similar timing as upon inoculation with *X. campestris* pv. *vesicatoria* T3. Total RNA was extracted from samples and was used for microarray analysis of the 426 *XRE* genes. By applying a 1.68-fold induction threshold, a total of 62 and 325 *XRE* genes were found to be differentially expressed with statistical significance ($P < 0.01$) at 8 and 12 h after infection, respectively (Fig. 6A). These represented a total of 327 nonredundant genes, including a group of 243 down-regulated and 84 up-regulated genes. Therefore, we deduced that, during *X. campestris* pv. *vesicatoria* T3 infection of resistant plants, about 77% of the identified *XRE* genes were directly regulated by the expression of the *AvrXv3* effector, whereas 23% of them were modulated through mechanisms unrelated to *AvrXv3* and to the onset of resistance.

Analysis of *XRE* gene expression during tomato resistance to *P. syringae* pv. *tomato*.

As a first step toward the identification of genes that participate exclusively in the resistance response of tomato to *X. campestris* pv. *vesicatoria* T3 strains and not in other plant-bacterium interactions, we tested differential expression of *XRE* genes during tomato resistance to the bacterial pathogen *P. syringae* pv. *tomato*. For this analysis, we used the tomato cultivar Rio Grande-PtoR, which expresses the *Pto* resistance gene, and *P. syringae* pv. *tomato* T1 strains expressing the *avrPto* avirulence gene (*P. syringae* pv. *tomato* T1A) (Pedley and Martin 2003). Rio Grande-PtoR plants were infiltrated by vacuum with

a suspension of *P. syringae* pv. *tomato* T1A bacteria at a titer of 10^8 CFU/ml or were mock-inoculated. A typical HR was observed 6 h after treatment only in *P. syringae* pv. *tomato* T1A-infected plants. For the preliminary assessment of gene expression kinetics characteristic of this incompatible interaction, we performed a single microarray hybridization, using samples harvested at different timepoints after inoculation. Tissue samples were collected at 2, 3, 4, and 5 h postinoculation and were used for microarray analysis of the 1,152 cDNAs derived from SSH libraries enriched for *X. campestris* pv. *vesicatoria* T3-modulated transcripts. As shown in Figure 7, differential gene expression was detected starting 2 h after treatment and reached a maximum after 5 h, both in terms of the number of genes and their fold change in expression. Based on these kinetics, an extensive analysis of gene expression was carried out with biological replicates at the 3- and 5-h timepoints, which are comprehensive of early and late gene expression variations. Considering a fold change of 1.68 as cutoff for differential expression, we found that, among the 426 *XRE* genes, 127 and 232 genes were differentially expressed with statistical significance ($P < 0.01$) after 3 and 5 h, respectively, representing a total of 250 nonredundant genes (Fig. 8A). By this analysis therefore, we found that a total of 176 *XRE* genes were only affected during resistance to *X. campestris* pv. *vesicatoria* and not to *P. syringae* pv. *tomato*. To confirm lack of *P. syringae* pv. *tomato* regulation for these genes, we examined their presence in groups of genes that were defined as differentially expressed in published gene expression studies of tomato resistance to *P. syringae* pv. *tomato* (Mysore et al. 2002, 2003). By this comparison, 21 additional *XRE* genes were found to be modulated also during resistance to *P. syringae* pv. *tomato*, restricting the number of *X. campestris* pv. *vesicatoria*-specific *XRE* genes to 155 (Fig. 8B). Interestingly, differential regulation of 94 among them was dependent on expression of the *AvrXv3* effector, as previously found by using the *X. campestris* pv. *vesicatoria* T2 and T2(*avrXv3*) isogenic strains. Together, this analysis identified 94 *XRE* genes that may represent marker genes for the tomato resistance response to *X. campestris* pv. *vesicatoria* T3 expressing the *AvrXv3* effector protein.

Fig. 4. Cluster analysis of gene expression profiles for 426 *XRE* (*Xanthomonas*-regulated) genes. *XRE* genes were grouped in clusters by applying the nonhierarchical *k*-means algorithm to a dataset of expression ratio intensities measured at different timepoints after inoculation of Hawaii 7981 plants with *Xanthomonas campestris* pv. *vesicatoria* T3 relative to control samples. For clusters A through F, a graph representing the mean expression of cluster members and its standard deviation at each timepoint is presented. Expression of each gene was normalized to have mean = 0 and standard deviation = 1 across all timepoints. Dotted lines represent the mean expression level during the entire time course. The number of genes in each cluster (*n*) is indicated.

Table 1. Classification of *XRE* (*Xanthomonas*-regulated) genes into functional categories based on sequence similarity to known genes

Functional category	Up-regulated	Down-regulated	Total	%
Stress responses	6	26	32	7.5
Defense	11	19	30	7.0
Protein synthesis	9	21	30	7.0
Signaling	9	19	28	6.6
Photosynthesis	5	19	24	5.6
Transport	9	11	20	4.7
Glucose metabolism	4	14	18	4.2
Nucleic acid-related	7	11	18	4.2
Protein degradation	3	12	15	3.5
Transcription	2	12	14	3.3
Vesicular transport	3	3	6	1.4
Amino acid metabolism	2	6	8	1.9
Cell wall-related	2	5	7	1.6
Auxin-related	6	3	9	2.1
Respiration/electron transfer	0	6	6	1.4
DNA repair	3	3	6	1.4
Ethylene-related	0	2	2	0.5
Fatty acid metabolism	0	2	2	0.5
Uncategorized ^a	19	29	48	11.3
Unknown ^b	28	75	103	24.2
Total	128	298	426	100.0

^a Genes that encode proteins of other miscellaneous functions.

^b Genes that encode proteins of unknown functions or do not have any homology to an annotated gene in the database.

DISCUSSION

In this report, by a combination of suppression subtractive hybridization (SSH) and microarray techniques, we identify a large number of plant genes differentially regulated during the resistance response of tomato to *X. campestris* pv. *vesicatoria* race T3. As a first step for the elucidation of cellular events taking place during the incompatible interaction between the resistant tomato line Hawaii 7981 and *X. campestris* pv. *vesicatoria* T3, SSH libraries were prepared from leaf tissues of resistant plants infected with bacterial suspensions or mock-inoculated. Based on reverse Northern analysis, 21 and 40% of the cDNA clones from forward and reverse libraries, respectively, were estimated as differentially expressed, indicating enrichment of the SSH libraries for up- and down-regulated genes. By microarray analysis of 1,152 randomly selected SSH clones, 661 of them (57%) were found to be differentially regulated. This higher percentage, as compared with that estimated by reverse Northern, is probably related to the better accuracy and sensitivity of the microarray analysis. The 661 clones represented 426 nonredundant *XRE* genes encompassing approximately 1.2% of the total number of genes (35,000) that are estimated to be present in the tomato genome (Van der Hoeven et al. 2002). Similarly, dramatic alterations in gene expression have been detected during several plant-pathogen incompatible interactions involving up to 2 to 3% of the expressed plant genome (Mysore et al. 2002; Tao et al. 2003; Wan et al. 2002). The *XRE* group included a significantly larger number of down-regulated genes (298) as compared with up-regulated (128) genes. A similar difference in the relative abundance of differentially up- and down-regulated genes was not observed in comprehensive analysis of gene expression performed for other plant-pathogen incompatible interactions (Mysore et al. 2002; Scheideler et al. 2002). In our study, the large proportion of down-regulated genes may be ascribed to the difference in enrichment efficiency observed for the reverse and forward SSH libraries.

Resistance of tomato line Hawaii 7981 to *X. campestris* pv. *vesicatoria* T3 is mediated by a typical gene-for-gene interaction between the *RxvT3* *R* gene, yet to be identified, and the *avrXv3* avirulence gene (Astua-Monge et al. 2000a; Scott et al. 1995). Interestingly, by using isogenic strains of *X. campestris* pv. *vesicatoria* race T2 differing only by the *avrXv3* gene, we found that the vast majority of the *XRE* genes (77%) were directly modulated by expression of the AvrXv3 effector protein. The significant proportion of *XRE* genes that were not affected by AvrXv3 expression (23%) might be modulated by virulence or avirulence factors or pathogen-associated molecular patterns (Parker 2003) expressed by *X. campestris* pv. *vesicatoria* T3.

By monitoring kinetics of *XRE* gene expression at four time-points during an 18-h time course after bacterial inoculation, initial changes in the plant transcriptome were detected between 4 and 8 h postinoculation. We assume that, during this period of time, *X. campestris* pv. *vesicatoria* T3 bacteria first mount an attempted infection through development of the type III secretion system and delivery of effector proteins, and they are then recognized by the plant surveillance system, which induces defense responses. Maximal alteration of *XRE* gene expression was observed at 12 h postinoculation, followed by a decrease in differential expression at 18 h. Cluster analysis of the *XRE* genes revealed six major expression patterns. Interestingly, most of the up-regulated genes were grouped in a single cluster, indicating a highly uniform trend of gene induction. However, down-regulated genes showed different patterns of expression kinetics, suggesting a more complex mode of regulation.

Qualitatively similar but earlier kinetics of differential gene expression were observed during the onset of tomato resis-

tance to *P. syringae* pv. *tomato*, which correlated well with the earlier appearance of the HR (Mysore et al. 2002). The faster response of tomato to *P. syringae* pv. *tomato* infection, as compared with *X. campestris* pv. *vesicatoria* at the same inoculum levels, may reflect differences between the bacteria in their capability to establish a successful infection or plant recognition and signaling mechanisms more efficient against *P. syringae* pv. *tomato* than against *X. campestris* pv. *vesicatoria*. Despite different expression kinetics of tomato responses to *X. campestris* pv. *vesicatoria* and *P. syringae* pv. *tomato*, a large number of *XRE* genes (64%) were differentially expressed during tomato resistance to both pathogens. In this group of *XRE* genes, there are genes representative of several functional classes, such as defense, signaling, and general metabolism, suggesting that, to a significant extent, tomato resistance to *X. campestris* pv. *vesicatoria* and *P. syringae* pv. *tomato* shares both signaling pathways and defense strategies. For example, this group includes the LeMPK3 MAP kinase, which was recently shown to be required for resistance to *P. syringae* pv. *tomato* (Ekengren et al. 2003) and to be induced at the transcript and activity levels during tomato resistance to *X. campestris* pv. *vesicatoria* T3 (Mayrose et al. 2004).

Fig. 5. Hypersensitive response elicitation and inhibition of bacterial growth in Hawaii 7981 leaves infected with *Xanthomonas campestris* pv. *vesicatoria* T2 strains carrying the *avrXv3* gene. **A**, Hawaii 7981 plants were inoculated with bacterial suspensions (10^8 CFU/ml) of *X. campestris* pv. *vesicatoria* T2 (left panel) or *X. campestris* pv. *vesicatoria* T2 carrying *avrXv3* (right panel). Leaves were photographed 24 h after inoculation. **B**, Hawaii 7981 plants were infected as in **A** at a titer of 10^5 CFU/ml. Bacterial populations in the leaves were estimated from samples harvested during a period of seven days. Triangles and circles represent bacterial concentrations of *X. campestris* pv. *vesicatoria* T2 and *X. campestris* pv. *vesicatoria* T2(*avrXv3*) strains, respectively. Data represent the mean $\pm$ standard error ($n = 4$).

Of particular interest is the group of *XRE* genes whose expression is regulated by the effector protein AvrXv3 but is not affected during the tomato-*P. syringae* pv. *tomato* incompatible interaction. These genes may represent specific markers for the AvrXv3-dependent elicitation of the HR. In addition, genes of this group related to signaling functions are good candidates to play a role in signaling pathways specific for *X. campestris* pv. *vesicatoria* resistance. Remarkably, among them we found a receptor-like kinase containing leucine-rich repeats, which resembles in structure the *Xa21 R* gene (Song et al. 1995) and several other protein kinases, including a member of the cell-wall-associated WAK family, which has been shown to be related to plant defense and development (Verica and He 2002). Homologs of a Ser/Thr protein phosphatase 2A β subunit and of a kinase-associated protein phosphatase are also present in this group (Luan 2003).

As expected, classification of *XRE* genes in functional classes revealed a significant overlap with categories of genes differentially expressed in other plant resistance re-

sponses (Maleck et al. 2000; Mysore et al. 2002; Narusaka et al. 2003; Scheideler et al. 2002; Schenk et al. 2000; Tao et al. 2003). The functional classes with the highest number of genes were stress responses, defense, protein synthesis, signaling, and photosynthesis. In agreement with the notion of significant overlapping between responses to biotic and abiotic stress (Cheong et al. 2002; Mysore et al. 2002), a large group of *XRE* genes (7.5%) was found to be related to wounding, heat shock, drought, heavy metal, and oxidative stress. In this group are noteworthy genes encoding tomato homologs of molecular chaperones, including HC70, HSP90, chaperonin 21, and T-complex protein 1. In several plant species, HSP90 is required for disease resistance responses, including those against *Tobacco mosaic virus* and *Potato virus X* and against different *P. syringae* pv. *tomato* strains (Hubert et al. 2003; Liu et al. 2004; Lu et al. 2003; Takahashi et al. 2003). In addition, this chaperone was shown to associate with the N and RPM1 R proteins and with the RAR1 and SGT1 signaling components of disease resistance, which are required for the function of many R proteins and display structural similarity to HSP90 cochaperones (Hubert et al. 2003; Liu et al. 2004; Lu et al. 2003; Takahashi et al. 2003). Remarkable in this group of *XRE* genes is also the presence of genes encoding antioxidant proteins, including catalase, glutathione *S*-transferase, and ascorbate peroxidase. Interestingly, in contrast to other reports of plant-pathogen incompatible interactions, these genes were mostly down-regulated during tomato resistance to *X. campestris* pv. *vesicatoria* T3. Water-stress-related genes (e.g., aquaporin and a tonoplast intrinsic protein) are also found in this group and may be related to the water requirement for pathogen multiplication.

About 7% of all the *XRE* genes were classified as having defense-related functions. Representative of this class are genes encoding PR proteins (e.g., PR-5 and PRB1b), the systemic acquired resistance regulatory protein NPR1, and enzymes involved in the biosynthesis of lignins, fatty acids, and antimicrobial compounds, such as PAL, lipoxigenase, cytochrome P450

Fig. 6. Differential expression of *XRE* (*Xanthomonas*-regulated) genes upon inoculation with *Xanthomonas campestris* pv. *vesicatoria* T2(avrXv3) as compared with *X. campestris* pv. *vesicatoria* T2. **A**, Hawaii 7981 plants were inoculated with suspensions of *X. campestris* pv. *vesicatoria* T2 or *X. campestris* pv. *vesicatoria* T2(avrXv3) bacteria at a titer of 10^8 CFU/ml. Total RNA was extracted from leaf tissues harvested 8 and 12 h after inoculation and was used to prepare fluorescently labeled cDNA probes for microarray analysis of *XRE* genes. Genes were regarded as differentially regulated if their fold change was at least 1.68 and if they withstood a statistical Student *t* test with $P < 0.01$. Filled and empty bars represent the number of genes that at different times postinoculation were found to be up- or down-regulated, respectively. **B**, Pie chart representing the proportion of *XRE* genes that were (striped area) or were not (white area) differentially expressed upon inoculation with *X. campestris* pv. *vesicatoria* T2(avrXv3) as compared with *X. campestris* pv. *vesicatoria* T2.

Fig. 7. Scatter plots representing distribution of suppression subtractive hybridization (SSH) cDNAs expression ratio after inoculation of resistant tomato plants with *Pseudomonas syringae* pv. *tomato*. Rio Grande-PtoR plants were inoculated with *P. syringae* pv. *tomato* T1 strain expressing *avrPto* (*P. syringae* pv. *tomato* T1A; 10^8 CFU/ml) or were mock-inoculated. Total RNA was extracted from leaf tissues harvested 2, 3, 4, and 5 h after inoculation and was used to prepare fluorescently labeled cDNA probes for microarray analysis of 1,152 cDNAs deriving from SSH libraries enriched for *Xanthomonas campestris* pv. *vesicatoria* T3-modulated transcripts. The *x* axis represents the spot intensity of Cy3 (mock-inoculated plants) and the *y* axis represents the spot intensity of Cy5 (*P. syringae* pv. *tomato* T1A-infected plants). Upper and lower diagonal lines represent twofold induction and repression, respectively. The middle diagonal line represents no change in gene expression.

monooxygenase, and divinyl ether synthase. Also intriguing is the presence in this group of two down-regulated and one up-regulated members of the Mlo family and a down-regulated homolog of pepper importin α . The Mlo family in monocots is involved in negative regulation of disease resistance (Stein and Somerville 2002). Interestingly, barley *Mlo* transcript abundance was found to increase in response to different fungal pathogens as well as upon treatment with a wheat powdery mildew-derived elicitor, wounding, and paraquat treatment (Piffanelli et al. 2002). Importin α is part of the nuclear import machinery and was shown to physically interact with the *X. campestris* pv. *vesicatoria* effector AvrBs3 (Szurek et al. 2001). In contrast with our findings in tomato, the importin α transcripts *Caimpa1* and *Caimpa2* in pepper leaves are constitutively expressed and are not affected by *X. campestris* pv. *vesicatoria* infection (Szurek et al. 2001). A number of *XRE* genes were also related to cell-wall metabolism, consistent with cell-wall modifications taking place in response to pathogen attack (Hammond-Kosack and Jones 1996). For instance, this group includes several pectinesterases, extensin-like proteins, and xyloglucan endotransglycosylase.

In many plant-pathogen interactions, activation of defense responses is regulated by signaling networks that originate from the specific recognition of a resistance protein and a pathogen effector (Martin et al. 2003). *XRE* genes with signaling-related functions, which represent possible signal transduction components of *RxvT3/avrXv3*-specified resistance, have been identified in this study. These include homologs of a RelA-SpoT-like protein, several protein phosphatases, serine/threonine kinases, MAP kinases, and receptor-like kinases. As detailed above, part of this group was specific to *X. campestris* pv. *vesicatoria* T3 resistance, and part was modulated also during resistance to *P. syringae* pv. *tomato*. Several *XRE* genes also encode proteins with homology to transcription factors of different families, including EREBP, WRKY, and MADS box-like proteins, which have been previously shown to participate in plant defense responses (Chen et al. 2002; Mysore et al. 2002).

The ascribed functions of large groups of *XRE* genes were related to cellular processes, such as photosynthesis (5.6%), transport (4.7%), glucose metabolism (4.2%), protein synthesis (7%), and degradation (3.5%), indicating that the cell undergoes an overall reorganization during the onset of resistance. Photosynthesis-related genes have been found to be down-regulated in several plant-pathogen incompatible interactions (Mysore et al. 2002; Narusaka et al. 2003; Schenk et al. 2000). Consistent with these reports, a large number of *XRE* genes that were found to be down-regulated encoded components of photosystems I and II, chlorophyll A-B binding (CAB) proteins and ribulose-biphosphate carboxylase small subunit. A few up-regulated *XRE* genes were also photosynthesis-related, supporting the hypothesis of a possible crosstalk between salicylic acid signaling and the pathway regulated by phytochrome A/red light, which leads to induction of *cab* genes (Schenk et al. 2000). Protein degradation plays an important role in disease resistance (Martin et al. 2003). Interestingly, a group of *XRE* genes (3.5%) was found to encode proteins related to the protein degradation machinery, including a ubiquitin-conjugating enzymes, a 26S proteasome subunit, and several proteases. Finally, the largest portion of *XRE* genes (24%) consists of genes encoding proteins of unknown function, whose characterization will certainly broaden our understanding of disease resistance mechanisms.

In conclusion, by using a genomics approach, we identified and profiled a vast group of genes differentially expressed during the resistance response of tomato to *X. campestris* pv. *vesicatoria* T3 bacteria. Future functional characterization of these

genes will assist in the dissection of molecular mechanisms and cellular processes involved in tomato resistance to bacterial spot disease.

MATERIALS AND METHODS

Plant material and bacterial strains.

Tomato (*Lycopersicon esculentum*) cultivars used in this study were Rio Grande-PtoR (carrying the *Pto* resistance gene) (Pedley and Martin 2003) and Hawaii 7981 (carrying the *RxvT3* resistance gene) (Scott et al. 1995). Bacterial strains used were: *P. syringae* pv. *tomato* race T1 expressing *avrPto* (*P. syringae* pv. *tomato* T1A); *X. campestris* pv. *vesicatoria* race T3 strain 97-2 (*X. campestris* pv. *vesicatoria* T3), *X. campestris* pv. *vesicatoria* race T2 strain 5746 (*X. campestris* pv. *vesicatoria* T2) and *X. campestris* pv. *vesicatoria* T2 carrying the *avrXv3* gene. To introduce *avrXv3* into *X. campestris* pv. *vesicatoria* T2 bacteria, the *avrXv3* coding region was PCR-amplified from *X. campestris* pv. *vesicatoria* T3 genomic

Fig. 8. Differential expression of *XRE* (*Xanthomonas*-regulated) genes in resistant tomato plants infected with *Pseudomonas syringae* pv. *tomato*. **A**, Rio Grande-PtoR plants were inoculated with a *P. syringae* pv. *tomato* T1 strain carrying the *avrPto* avirulence gene (*P. syringae* pv. *tomato* T1A) at a titer of 10^8 CFU/ml or were mock-inoculated. Total RNA was extracted from leaf tissues harvested 3 and 5 h after inoculation and was used to prepare fluorescently labeled cDNA probes for microarray analysis of *XRE* genes. *XRE* genes were regarded as differentially regulated if their fold change was at least 1.68 and if they withstood a statistical Student *t* test with $P < 0.01$. Filled and empty bars represent the number of genes that at different times postinoculation were found to be up- or down-regulated, respectively. **B**, Pie chart representing the proportion of *XRE* genes that were (striped area) or were not (white area) differentially expressed during the incompatible interaction between *P. syringae* pv. *tomato* T1A and Rio Grande-PtoR plants.

DNA by using the following primers: 5'-CGCGGATCCAT GACAAGTAGTATCAATCGT-3' and 5'-GACGGAATTCGC GCACCGTTACCCGC-3'. The *avrXv3* PCR product was digested by *EcoRI* and *BamHI* restriction enzymes and was inserted at the corresponding sites into the broad-host-range vector pDSK519 (Keen et al. 1988), which was then used to transform competent *X. campestris* pv. *vesicatoria* T2 cells by electroporation.

Inoculation procedures and in planta bacterial growth.

For pathogen inoculation, six-week-old plants were vacuum-infiltrated with bacterial cultures prepared as follows. *X. campestris* pv. *vesicatoria* strains were grown overnight at 28°C in nutrient yeast glycerol medium (NYG) (Daniels et al. 1984). Bacteria were pelleted by centrifugation, were washed twice with 10 mM MgCl₂, and were diluted in 10 mM MgCl₂ and 0.005% (vol/vol) Silwet-L77. Bacterial cultures of *P. syringae* pv. *tomato* T1A were grown overnight at 28°C in King's B liquid medium (Martin et al. 1993) with the addition of 100 mg of rifampicin per liter and 25 mg of kanamycin per liter and were prepared for inoculation, as described for *X. campestris* pv. *vesicatoria* strains.

To determine in planta bacterial growth, tomato plants were vacuum-infiltrated with bacterial suspensions at a titer of 10⁵ CFU/ml. Leaf samples of inoculated plants were collected at 24-h intervals during seven days. Bacterial growth was measured by grinding four 1-cm² leaf disks in 10 mM MgCl₂, plating serially diluted tissue samples on NYG plates, and counting colony forming units.

Construction of SSH libraries.

Plants of the tomato cultivar Hawaii 7981 were inoculated with *X. campestris* pv. *vesicatoria* T3 bacteria at a titer of 10⁸ CFU/ml or were mock-inoculated. For each treatment, leaf tissues were collected 4 and 8 h after inoculation, were pooled, and were used to extract RNA with the SV total RNA isolation kit (Promega, Madison, WI, U.S.A.). Poly(A)+ RNA was separated from the total RNA using the PolyAT tract mRNA isolation kit (Promega). cDNA synthesis and SSH were carried out using the PCR select subtractive hybridization kit (Clontech, Palo Alto, CA, U.S.A.) according to the manufacturer's instructions, with slight modification. For preparation of the forward library, which is enriched for cDNA fragments of genes whose expression level increased following bacterial inoculation, cDNA prepared from *X. campestris* pv. *vesicatoria* T3-treated plants was used as 'tester' and that from mock-inoculated plants as 'driver.' The reverse subtraction for isolation of cDNA fragments of genes whose expression level decreased following the treatment was carried out with cDNA from mock-inoculated plants as 'tester' and with cDNA from *X. campestris* pv. *vesicatoria* T3-treated plants as 'driver.' The driver/tester ratio was increased to fourfold greater than the suggested ratio. After fractionation of the subtracted cDNAs by 1% agarose gel electrophoresis, fragments greater than 300 bp were inserted into the pTZ57R cloning vector (MBI Fermentas, Vilnius, Lithuania), and the obtained plasmid libraries were transformed into competent *Escherichia coli* DH12S cells by electroporation.

Reverse Northern and Northern blot analysis.

For reverse Northern analysis, cDNA inserts were individually PCR-amplified from the pTZ57R plasmid with the following primers: 5'-TCGAGCGGCCGCCCGGGCAGG-3' and AGCGTGGTCGCGGCCGAGGT-3'. PCR products were separated in agarose gels and were transferred to Hybond N⁺ nylon membranes (Amersham-Biosciences, Piscataway, NJ, U.S.A.). The DNA blots contained cDNA inserts from the SSH library,

and cDNAs of tomato basic (1-3)- β -glucanase and actin (Xiao et al. 2001) as controls. Duplicated filters were hybridized with ³²P-labeled cDNA probes derived from *X. campestris* pv. *vesicatoria* T3-treated or mock-inoculated Hawaii 7981 tomato plants and were synthesized using the RevertAid first strand cDNA synthesis kit (MBI Fermentas). Blots were hybridized and washed according to standard procedures and were exposed to autoradiography for 24 h.

For Northern blot analysis, total RNA (15 μ g from each sample) was denatured at 60°C, was fractionated on a 1% formaldehyde-agarose gel, and was blotted onto a Hybond-N membrane (Amersham-Biosciences). Blots were hybridized with probes labeled with [α -³²P]dCTP (Amersham-Biosciences) by using standard random priming methods. Hybridizations were performed at 60°C for 16 h in a solution containing 50 mM NaH₂PO₄, pH 7.4, 0.75 M NaCl, 2% sodium dodecyl sulfate (SDS), 0.1 mg of salmon sperm DNA per ml, and 5 mM EDTA. After hybridization, blots were washed for 15 min in 2 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% SDS at 25°C, and twice for 15 min in 1 $\times$ SSC and 0.1% SDS at 60°C, and were exposed to autoradiography for 48 h.

Microarray production and experimental design.

Microarray production, hybridization, and data analysis were performed following the MIAME (minimum information about a microarray experiment) guidelines for international standardization and quality control of microarray experiments (Brazma et al. 2001). For each timepoint analyzed, three independent biological repeats were performed with control and experiment cDNAs labeled with Cy3 and Cy5 fluorescent dyes, respectively. In addition, to avoid dye-dependent bias, a fourth experimental repeat was performed for one of the biological samples with reverse labeling (Cy3 versus Cy5). The only exceptions in the number of repeats is the 0-h timepoint shown in Figure 4, for which only two biological replicates were performed. Raw and normalized data of all experiments are available on the Tel Aviv University Department of Plant Sciences website.

For microarray preparation, 1,152 cDNAs derived from the SSH libraries were PCR amplified, as described for the reverse Northern analysis. These included 576 cDNAs from each one of the forward and reverse SSH libraries. In addition, ESTs corresponding to known defense response genes and other control genes were amplified using T3 and T7 primers. PCR products were purified using a Genesis RSP200 liquid handler (TECAN, Maennedorf, Switzerland) and 384-well filter plates (S384PCR10; Millipore, Bedford, MA, U.S.A.) and were vacuum dried and resuspended in 30 μ l of spotting buffer (3 $\times$ SSC, 1.5 M betaine). DNA from each SSH-derived sample was arrayed in triplicate onto γ -amino-propyl-silane coated UltraGAPS slides (Corning, Corning, NY, U.S.A.), using a MicroGrid Pro arrayer (BioRobotics, MA, U.S.A.) with 32 MicroSpot2500 printing pins. After spotting, cDNAs were UV cross-linked, and slides were incubated for 2 h at 80°C. Array fabrication was completed with a 2-min wash in 0.2% SDS, three rinses in double-distilled water, and a final rinse in 90% ethanol. Slides were finally dried by centrifugation.

Probe preparation.

Total RNA was extracted from tomato leaves using the SV total RNA isolation kit (Promega) and was reverse transcribed in the presence of amino-allyl-modified dUTP, and cDNA was labeled indirectly with succinimidyl ester Cy3/Cy5, as follows. For reverse transcription, 100 μ g of total RNA was combined with 10 μ g of Oligo(dT) 12- to 18-mer (Amersham-Biosciences) in a final volume of 10 μ l, was heated at 70°C for 10 min

to denature RNA tertiary structures, and was transferred to 42°C for annealing. A preheated (50°C) 30- μ l mixture containing 1 $\times$ first strand reaction buffer (Invitrogen, Grand Island, NY, U.S.A.), 50 mM MgCl₂, 0.1 M dithiothreitol, 10 mM dNTP mixture (2.5 mM each of dATP, dCTP, dGTP, 1.5 mM dTTP, and 1 mM aminoallyl dUTP), 40 units of RNaseOUT ribonuclease inhibitor (Invitrogen), and 400 units of SuperScript II RNase H (Invitrogen) were added to the RNA, and the reaction was incubated at 45°C for 2 h. After incubation, an additional 400 units of the enzyme were added to the reaction, which was incubated for another 2 h at 45°C. The reaction was stopped by the addition of 10 μ l of 0.5 M EDTA, and the RNA was hydrolyzed by the addition of 10 μ l of 1 M NaOH and incubation at 65°C for 20 min. The reaction mixture was neutralized by the addition of 10 μ l of 1 M HCl, and cDNA was concentrated by ethanol precipitation. Aminoallyl-labeled cDNA was resuspended in 10 μ l of 0.1 M NaHCO₃/Na₂CO₃ buffer, pH 9.3, and aliquots (4.5 μ l in dimethyl sulfoxide) of succinimidyl ester Cy3/Cy5 (Amersham-Biosciences) were added according to the manufacturer's instructions. After 1 h of incubation at room temperature, Cy3/Cy5-labeled cDNA was purified, using a StrataPrep purification kit (Stratagene, La Jolla, CA, U.S.A.).

Slide hybridization.

Slides were incubated for 20 min at 50°C in preheated prehybridization buffer (3 $\times$ SSPE (1 $\times$ SSPE is 0.18 M NaCl, 10 mM NaPO₄, and 2 mM EDTA [pH 7.7]), 48% formamide, 0.4% SDS, 2 $\times$ Denhardt's solution, 180 mg of salmon sperm DNA per ml), were washed consecutively in water, 70% ethanol, and 100% ethanol, and were dried by centrifugation. The probes (Cy3- and Cy5-labeled cDNAs) were mixed, were concentrated by using Microcon filter devices (Millipore), were brought to a volume of 13 μ l in a 2 $\times$ SSPE solution, were denatured at 98°C for 3 min, and were cooled on ice for 30 s. Probes were then added to 17 μ l hybridization buffer (final concentration of 5 $\times$ SSPE, 62.8% formamide, 0.8% SDS, 4 $\times$ Denhardt's solution) containing 15 μ g of poly(A) DNA (Amersham-Biosciences) and 4 μ g of yeast tRNA (Life Technologies, Gaithersburg, MD, U.S.A.), were applied to the slide, and were covered with a Hi-briSlip cover slip (Schleicher & Schuell, Dassel, Germany). Slides were then placed in a hybridization chamber (Corning) and were incubated overnight at 42°C. After hybridization, slides were washed at room temperature for 10 min, twice with 2 $\times$ SSC and 0.1% SDS, twice with 0.2 $\times$ SSC and 0.1% SDS, and twice with 0.2 $\times$ SSC. After a final wash with 0.2 $\times$ SSC at 50°C for 10 min, slides were dried by centrifugation and were scanned at a resolution of 10 μ m per pixel for fluorescence emission at 532 nm for Cy3 and at 635 nm for Cy5 using a 428 Array Scanner controlled by the Jaguar 2.0 software (Affymetrix). Two separate TIFF images were generated for each channel.

Data analysis.

Spot mean intensities were quantified using the ImaGene 5.0 software (Biodiscovery, El Segundo, CA, U.S.A.) and were normalized by the mean of signals using the GeneSight 3.5 software (Biodiscovery). Genes significantly up- or down-regulated were identified by Student *t* statistics. Genes were regarded as differentially regulated if their fold change was at least 1.68 and if they withstood a statistical Student *t* test with *P* < 0.01. For analysis of the microarray data by clustering algorithms, data from experimental replicates for each time-point were averaged. Nonhierarchical *k*-means cluster analysis was performed, using tools included in the Expander 1.2 data clustering software (Sharan et al. 2003). The optimal number of clusters was selected to yield an overall average homogeneity value >0.9.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Microarray Gene Expression Data Society MIAME (minimum information about a microarray experiment) guidelines:
www.mged.org/Workgroups/MIAME/miame.html
 Solanaceae Genomics Network: www.sgn.cornell.edu
 The Institute for Genomic Research (TIGR) database: www.tigr.org
 Tel Aviv University Department of Computer Science gene expression analysis and visualization Expander software for cluster analysis:
www.cs.tau.ac.il/~rshamir/expander
 Tel Aviv University Department of Plant Sciences website:
www.tau.ac.il/lifesci/departments/plant_s/tomato-xre.html