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Molecular Properties of the *Xanthomonas* AvrRxv Effector and Global Transcriptional Changes Determined by Its Expression in Resistant Tomato Plants

Arale Bonshtien,[1] Atar Lev,[1] Avi Gibly,[1] Paul Debbie,[2] Adi Avni,[1] and Guido Sessa[1]

[1]Department of Plant Sciences, Tel-Aviv University, 69978 Tel-Aviv, Israel; [2]Center for Gene Expression Profiling, Boyce Thompson Institute for Plant Research, Ithaca, New York 14853, U.S.A.

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The *Xanthomonas campestris* pv. *vesicatoria* avirulence gene *avrRxv* specifies resistance on the tomato line Hawaii 7998 by interacting with three nondominant plant resistance genes. AvrRxv molecular properties that impinge on its avirulence activity were characterized and transcriptional changes caused by AvrRxv expression in resistant tomato plants were extensively examined. AvrRxv localized predominantly to the cytoplasm and possibly in association with plasma and nuclear membranes in both resistant and susceptible tomato plants. The AvrRxv cysteine protease catalytic core was found to be essential for host recognition, because introduction of mutations in this domain affected the ability of AvrRxv to elicit a hypersensitive response and the inhibition of bacterial growth in resistant plants. In addition, expression profiles were analyzed for approximately 8,600 tomato genes in resistant plants challenged with *X. campestris* pv. *vesicatoria* strains expressing wild-type AvrRxv or a catalytic core AvrRxv mutant. In all, 420 genes were identified as differentially modulated by the expression of a functional AvrRxv, including over 15 functional classes of proteins and a large number of transcription factors and signaling components. Findings of this study allow the development of new hypotheses about the molecular basis of recognition between AvrRxv and the corresponding resistance proteins, and set the stage for the dissection of signaling and cellular responses triggered in tomato plants by this avirulence factor.

Additional keywords: plant disease resistance, type III effectors.

Bacterial spot of pepper and tomato is an economically important disease caused by the pathogen *Xanthomonas campestris* pv. *vesicatoria*. This disease is most severe in warm and humid regions of the world and results in considerable losses in yield and fruit quality. *X. campestris* pv. *vesicatoria* bacteria penetrate the plant through stomata and wounds and colonize intercellular spaces in the aerial parts of the plant, leading to the appearance of typical water-soaked lesions that develop into necrotic spots. Pathogenicity of *X. campestris* pv. *vesicatoria* is dependent on a functional type III secretion system (TTSS), which is highly conserved in plant and animal pathogenic bacteria and directly delivers bacterial effector proteins into host cells (Buttner and Bonas 2003; Staskawicz et al. 2001). As observed in other plant–pathogen systems, loss-of-function mutations in structural genes of the *X. campestris* pv. *vesicatoria* TTSS completely abrogate appearance of disease symptoms in susceptible plants and elicitation of defense responses in resistant plants, highlighting the central role of type III effectors in bacterial pathogenicity and plant disease resistance (Buttner and Bonas 2002).

A large number of type III effectors from different phytopathogenic bacteria have been identified by using genetic and biochemical screens and, more recently, by bioinformatics approaches supported by the availability of whole sequences for several bacterial genomes (Buttner et al. 2003; Collmer et al. 2002; Greenberg and Vinatzer 2003; Salanoubat et al. 2002). A precise function in pathogenicity has been unraveled for only some of the identified effectors, which appear to interfere with basal and induced host defenses, promote pathogen growth, and elicit development of disease symptoms (Abramovitch and Martin 2004; Chang et al. 2004; Hotson and Mudgett 2004). In *X. campestris* pv. *vesicatoria*, type III effectors have been isolated mainly based on their expression pattern and avirulence activity (Buttner et al. 2003). A group of *X. campestris* pv. *vesicatoria* candidate effectors was first isolated in a cDNA amplified fragment length polymorphism survey of genes whose expression is regulated by the HrpG transcriptional activator (Noel et al. 2001). The products of some of these genes then were shown to be secreted by the TTSS and termed *Xanthomonas* outer proteins (Xops) (Noel et al. 2001, 2002, 2003). Additional *X. campestris* pv. *vesicatoria* effectors have been identified as products of avirulence (*avr*) genes that reveal the presence of the attacking pathogen to the surveillance system of resistant plants (Buttner et al. 2003). Recognition of *avr* gene products by corresponding plant resistance (R) proteins leads to the elicitation of defense responses that limit pathogen growth and disease symptoms. Defense responses observed in resistant plants include a rapid and localized cell death at the site of infection, known as the hypersensitive response (HR), production of reactive oxygen and nitrogen species, transient opening of ion channels, cell wall fortifications, production of phytoalexins, and synthesis of pathogenesis-related (PR) proteins (Hammond-Kosack and Jones 1996).

In the tomato line Hawaii 7998, resistance to *X. campestris* pv. *vesicatoria* race T1 strains (race T1) is mediated in part by

Corresponding author: Guido Sessa; Telephone: 972-3-640-9766; Fax: 972-3-640-9380; E-mail: guidos@post.tau.ac.il

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an atypical gene-for-gene interaction between the *X. campestris* pv. *vesicatoria* avirulence gene *avrRxv* and three nondominant plant *R* genes, which participate with an additive effect in elicitation of the HR (Whalen et al. 1993; Yu et al. 1995). *avrRxv* encodes a type III effector that was shown to be constitutively expressed in *X. campestris* pv. *vesicatoria* bacterial cells and secreted through the TTSS (Ciesiolka et al. 1999; Rossier et al. 1999). AvrRxv is a member of the YopJ family of effector proteins, which is named after the *Yersinia pestis* outer protein J and comprises proteins from animal and plant pathogens, as well as from plant symbionts (Hotson and Mudgett 2004; Orth 2002). In *X. campestris* pv. *vesicatoria* strains, four YopJ-like proteins were identified: AvrRxv, AvrBst, AvrXv4, and XopJ (Astua-Monge et al. 2000b; Ciesiolka et al. 1999; Noel et al. 2003; Whalen et al. 1993). Based on their structural characteristics, YopJ-like effectors have been assigned to the C55 peptidase family of the CE clan of cysteine proteases, which share a cysteine nucleophile and a predicted catalytic core composed of three conserved amino acid residues (Hotson et al. 2003; Hotson and Mudgett 2004). Recent findings strongly suggest that targets for the proteolytic activity of at least some of YopJ family members are small-ubiquitin-like modifier (SUMO) conjugated proteins (Orth et al. 2000; Roden et al. 2004). SUMOs are members of a family of ubiquitin-like proteins that are covalently attached to cellular targets by a conjugation mechanism similar to ubiquitylation and regulate various cellular processes, including nuclear transport, signal transduction, stress response, and cell cycle progression (Muller et al. 2001).

In the recent years, development of microarray technologies for the analysis of gene expression profiles, along with the availability of genome and expressed sequence tag (EST) sequence database for many plant species, allowed the identification of cellular processes affected by the expression of specific bacterial effectors in resistant and susceptible plants (Wan et al. 2002). For example, a comprehensive analysis of gene expression in tomato identified genes modulated by the interaction between the Avr proteins AvrPto from *Pseudomonas syringae* pv. *tomato* and AvrXv3 from *X. campestris* pv. *vesicatoria* race T3 with the respective plant R proteins (Gibly et al. 2004; Mysore et al. 2002). Similarly, in *Arabidopsis* spp., changes in gene expression were monitored in incompatible interactions involving the *P. syringae* pv. *tomato* effectors AvrRpt2 and AvrB (Scheideler et al. 2002; Tao et al. 2003). Microarray analysis also was instrumental in the identification of a subset of plant defense responses that are targeted by the AvrPto virulence activity (Hauck et al. 2003).

In this study, molecular properties of the *X. campestris* pv. *vesicatoria* type III effector AvrRxv were examined and its avirulence function was further characterized. Evidence is provided that AvrRxv acts inside the plant cell and that its catalytic core is required for the elicitation of the HR and bacterial avirulence in resistant plants. In addition, an extensive analysis of gene expression profiles allowed the identification of tomato genes that are modulated by the plant recognition of a functional AvrRxv.

RESULTS

Expression of AvrRxv in tomato plants resistant to *X. campestris* pv. *vesicatoria* race T1 induces HR.

Plants of the tomato line Hawaii 7998 are resistant to race T1 strains expressing the AvrRxv avirulence protein (Whalen et al. 1993). An *Agrobacterium*-mediated transient expression assay was used to test whether the expression of AvrRxv inside cells of resistant plants is sufficient to trigger HR. The *avrRxv* coding region was polymerase chain reaction (PCR) amplified from genomic DNA of race T1 bacteria, and introduced into the binary vector pBTEX under control of the *Cauliflower mosaic virus* (CaMV) 35S promoter. The plasmid obtained was transformed into *Agrobacterium tumefaciens* GV2260, which then was infiltrated into mature leaves of tomato plants. Expression of AvrRxv induced an HR in the race T1-resistant line Hawaii 7998 approximately 48 h after inoculation (Fig. 1). No effect was observed in response to AvrRxv expression in the race T1-susceptible line Hawaii 7981, or upon inoculation of plants with an *Agrobacterium* sp. carrying an empty vector. These results demonstrate that expression of AvrRxv is sufficient for elicitation of the HR in race T1-resistant plants. They also suggest that the recognition event between AvrRxv and the three plant genes which are involved in tomato resistance to race T1 (Yu et al. 1995) takes place inside the plant cell.

AvrRxv localization in tomato epidermal cells.

Members of the YopJ/AvrRxv family of cysteine proteases were reported to localize to different subcellular compartments, where they were predicted to interact with diverse host substrates (Deslandes et al. 2003; Roden et al. 2004). A nuclear localization was proposed for AvrRxv based on the presence of potential nuclear localization signals at position 74 (RPRKK) and 363 (RARKIR) in its amino acid sequence (Ciesiolka et al. 1999). To test this prediction and to identify subcellular compartments where AvrRxv may exert its function, subcellular localization of AvrRxv was examined in epidermal cells of tomato leaves. The green fluorescence protein (GFP) and a GFP-AvrRxv fusion were expressed in tomato plants under the control of the CaMV 35S promoter by using an *Agrobacterium*-mediated transient expression assay. Localization of the expressed proteins was visualized by confocal

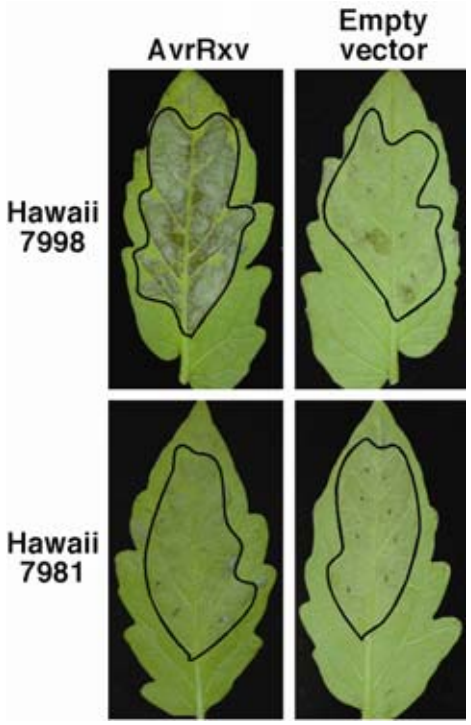

Fig. 1. Hypersensitive response (HR) induced by expression of AvrRxv in tomato plants that are resistant to *Xanthomonas campestris* pv. *vesicatoria* T1 strains. Leaves of tomato lines Hawaii 7998 and Hawaii 7981 were inoculated in the encircled areas with *Agrobacterium tumefaciens* GV2260 strains (optical density at 600 nm = 0.04), which contained either the pBTEX binary vector carrying the *avrRxv* gene or an empty vector. Pictures were taken 72 h after inoculation. Tissue collapse in infiltrated areas reflects elicitation of the HR.

microscopy 24 to 48 h after *Agrobacterium* inoculation in race T1-resistant (Hawaii 7998) and -susceptible (Hawaii 7981) tomato plants. Fluorescence of the GFP control was observed in cytoplasmic strands, in the cell periphery, and in the nucleus (Fig. 2A) where, in previous studies, it was suggested to enter by diffusion due to its small size (28 kDa) (von Arnim et al. 1998). It should be noted that, because of the size of the unstained vacuole, the cytoplasm often appears as a thick band in the cell periphery. The GFP-AvrRxv fusion was detected in cytoplasmic strands, in the periphery of the cell, and around the nucleus in cells of resistant as well as susceptible plants (Fig. 2A). Sections performed by confocal microscopy through and above the nucleus confirmed that GFP-AvrRxv did not accumulate inside the nucleus but around it (Fig. 2B). Functionality of the GFP-AvrRxv fusion in elicitation of the HR was confirmed by the appearance of cell death in inoculated areas of Hawaii 7998 leaves approximately 48 h after inoculation (data not shown). These results suggest that the putative nuclear localization signals identified in the AvrRxv amino acid sequence are not functional in directing the protein to the nucleus and that the AvrRxv effector likely exerts its predicted functions in the cell cytoplasm or in association with nuclear or plasma membranes.

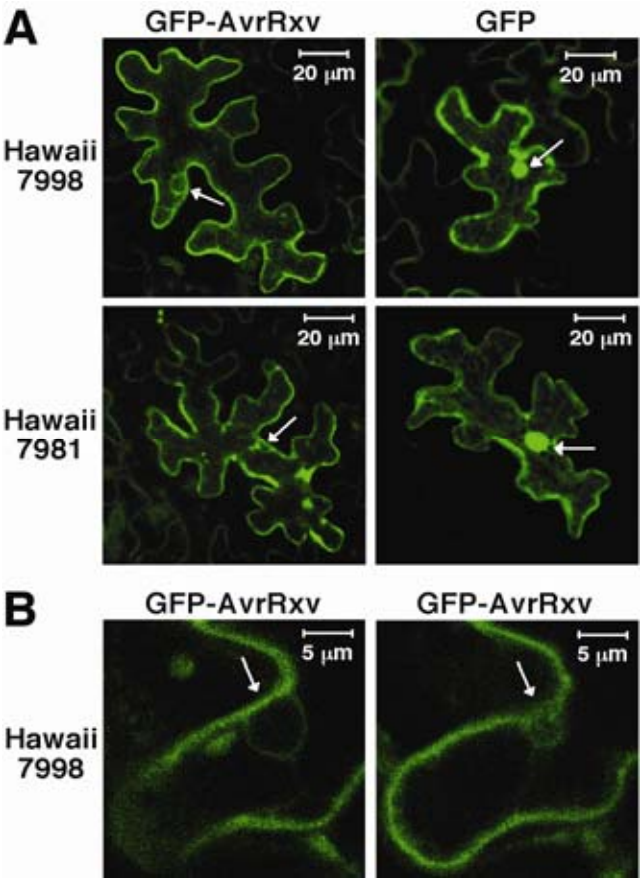

Fig. 2. Subcellular localization of AvrRxv transiently expressed in epidermal cells of tomato leaves. Leaves of the tomato lines Hawaii 7998 and Hawaii 7981 were inoculated with suspensions of *Agrobacterium tumefaciens* GV2260 strains (optical density at 600 nm = 0.04) expressing green fluorescence protein (GFP) or a GFP-AvrRxv fusion as indicated. Distribution of GFP fluorescence in epidermal cells was determined 24 h post inoculation by imaging cells with a confocal laser scanning microscope. Arrows indicate the location of the nucleus in each cell. **A,** Images from a projection stack of multiple confocal sections along the z axis; **B,** images represent a single section above (right) and through (left) the cell nucleus.

Mutations in the AvrRxv protease catalytic core impaired elicitation of the HR and inhibition of bacterial growth.

An intact cysteine protease catalytic core is required for elicitation of the HR mediated by the *X. campestris* pv. *vesicatoria* YopJ-like effectors AvrBst and AvrXv4 in pepper and *Nicotiana benthamiana* plants, respectively (Orth et al. 2000; Roden et al. 2004). Cysteine proteases of the YopJ family, including AvrRxv, are characterized by the presence of three conserved amino acids in their active site that are referred to as the catalytic triad of the enzyme (Orth 2002). In the AvrRxv amino acid sequence, these conserved residues correspond to His 180, Glu 200, and Cys 244. To test the requirement of the AvrRxv catalytic triad for elicitation of the HR in race T1-resistant plants, these three amino acids were independently mutated to alanine in AvrRxv fused to the antihemagglutinin (HA) epitope tag (AvrRxv-HA). As a control, a mutation to alanine also was introduced outside the catalytic core of AvrRxv-HA at Glu 258. The wild-type AvrRxv-HA fusion and its mutant forms were expressed in the tomato line Hawaii 7998 by *Agrobacterium*-mediated transient expression and tested for their capability to induce HR. A typical HR was observed 48 h after *Agrobacterium* inoculation in areas of leaves expressing wild-type AvrRxv-HA or the control AvrRxv(E258A)-HA (Fig. 3A). However, the introduction of mutations in residues of the catalytic triad abolished the HR-inducing activity of AvrRxv-HA. To assess that the AvrRxv-HA mutants, which lost HR-inducing activity, were correctly expressed in planta, their expression levels were tested in *N. benthamiana* leaves. In these plants, *Agrobacterium*-mediated expression is higher than in tomato, probably due to a more efficient infection, and allow protein detection by standard West-

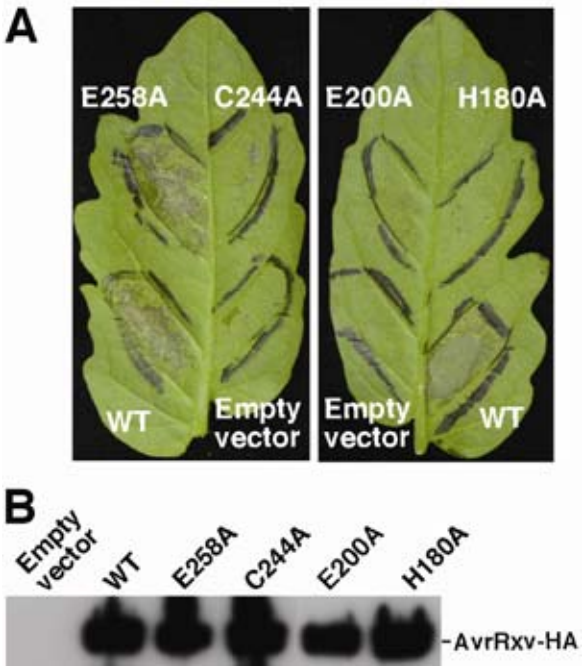

Fig. 3. Effect of mutations in the catalytic core of AvrRxv on its ability to induce the hypersensitive response (HR) in *Xanthomonas campestris* pv. *vesicatoria* race T1-resistant tomato plants. **A,** Leaves of the tomato line Hawaii 7998 were infiltrated into the encircled areas with *Agrobacterium tumefaciens* GV2260 strains (optical density at 600 nm = 0.04) expressing the following proteins: AvrRxv-antihemagglutinin (HA), AvrRxv(H180A)-HA, AvrRxv(E200A)-HA, AvrRxv(C244A)-HA, and AvrRxv(E258A)-HA. Pictures were taken 72 h after inoculation. Tissue collapse in the infiltrated areas reflects elicitation of the HR. **B,** Expression of AvrRxv and its mutant forms in *Nicotiana benthamiana* leaves 48 h after *Agrobacterium* inoculation. Proteins were detected in leaf protein extracts by immunoblot analysis using monoclonal antibodies raised against the HA epitope.

ern blot procedures. As assessed using antibodies raised against the HA epitope, wild-type and mutant forms of AvrRxv were expressed in *N. benthamiana* leaves at similar levels (Fig. 3B).

Next, it was tested whether an AvrRxv intact catalytic triad is required for HR induction when the effector is delivered into tomato cells by *X. campestris* pv. *vesicatoria* bacterial strains. To this aim, the coding regions of *avrRxv-HA* and its mutant form *avrRxv-HA*(C244A) were introduced into the broad host-range plasmid pDSK519 under the control of the *lacZ* promoter and mobilized into *X. campestris* pv. *vesicatoria* bacteria of the T3 race *(*race T3), which lack endogenous AvrRxv and, therefore, are virulent on Hawaii 7998 plants. To test the capability of race T3(AvrRxv-HA) and race T3[AvrRxv-HA(C244A)] strains to induce an HR in race T1-resistant plants, wild-type race T3 and the two race T3 engineered strains were syringe infiltrated at a titer of 10^8 CFU/ml in leaves of Hawaii 7998 plants. A typical HR was observed after approximately 14 h in leaves inoculated with race T3(AvrRxv-HA) (Fig. 4A). At this time, no effect was yet detected in leaves infected with race T3 and race T3[AvrRxv-HA(C244A)], which developed disease symptoms approximately 36 h after inoculation. This result suggests that an intact catalytic core is required for elicitation of the HR mediated by AvrRxv during natural infection of tomato plants by avirulent *X. campestris* pv. *vesicatoria* strains.

To confirm the requirement of an intact catalytic core for AvrRxv avirulence activity, bacterial growth of strains race T3, race T3(AvrRxv-HA), and race T3[AvrRxv-HA(C244A)] was monitored in leaves of race T1-resistant tomato plants. The different strains were vacuum-infiltrated into Hawaii 7998 leaves at a titer of 10^4 CFU/ml, and bacterial populations were counted during a 6-day period. In line with the observation that the race T3 strain expressing AvrRxv-HA elicits an HR in Hawaii 7998 plants, growth of this strain was significantly reduced compared with virulent wild-type race T3 starting 3 days after inoculation (Fig. 4B). However, race T3 strains expressing AvrRxv-HA mutated at Cys 244 showed a growth rate much higher than that of the race T3(AvrRxv-HA) strain and similar to that of wild-type race T3. Together, these results confirm the ability of the AvrRxv effector to confer avirulence to naturally virulent *X. campestris* pv. *vesicatoria* strains and its dependence on an intact cysteine protease catalytic core.

To verify that AvrRxv-HA(C244A) was stably expressed in the race T3 strain and secreted in a type III-dependent manner, *X. campestris* pv. *vesicatoria* race T3 and the *hrp* mutant strain race T3 Δ*hrpA* expressing AvrRxv-HA or AvrRxv-HA(C244A) were grown in *hrp* gene induction medium (Wengelnik et al. 1996). Cell lysate and culture fluid fractions were isolated for the different bacterial strains and analyzed by immunoblotting using antibodies raised against the HA epitope. As shown in Figure 4C and in agreement with previous observations (Rossier et al. 1999), two AvrRxv isoforms were detected in bacterial cells expressing AvrRxv-HA or AvrRxv-HA(C244A). Wild-type and mutant proteins were expressed at similar levels in cell lysates of the different bacterial strains (Fig. 4C). In addition, AvrRxv-HA and AvrRxv-HA(C244A) were detected in the culture fluid of race T3 but not in that of the deletion mutant race T3 Δ*hrpA,* demonstrating that both proteins were secreted in a type III-dependent manner. Thus, the inability of race T3[AvrRxv-HA(C244A)] to elicit an HR in Hawaii 7998 plants probably was due to a loss of AvrRxv function and not to the impairment of its secretion from the bacterium.

Expression of a functional AvrRxv in resistant tomato plants induces global transcriptional changes.

The isogenic bacterial strains race T3(AvrRxv-HA) and race T3[AvrRxv-HA(C244A)] were used to identify genes that are modulated by the expression of a functional AvrRxv effector in resistant tomato plants. Expression profiles of 8,600 tomato genes were examined by hybridizing an array of tomato cDNAs with Cy3- and Cy5-labeled probes derived from leaf tissues of Hawaii 7998 plants infected with race T3(AvrRxv-HA) and race T3[AvrRxv-HA(C244A)] strains, respectively.

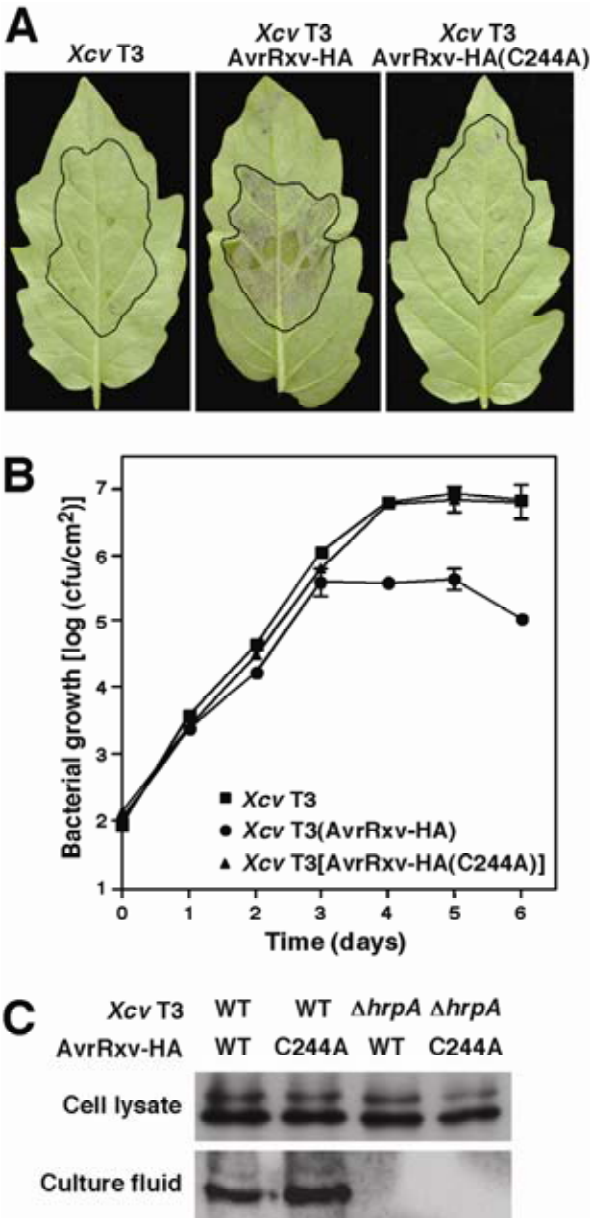

Fig. 4. Effect of a mutation at Cys 244 in the catalytic core of AvrRxv on its avirulence function. **A,** Leaves of the tomato line Hawaii 7998 were syringe-infiltrated into the encircled areas with suspensions (10^8 CFU/ml) of the following bacterial strains: wild-type *Xanthomonas campestris* pv. *vesicatoria* race T3 (left), Race T3 expressing AvrRxv-antihemagglutinin (HA) (center), and race T3 expressing AvrRxv-HA(C244A) (right). Pictures were taken 24 h after inoculation. Tissue collapse in the infiltrated areas reflects elicitation of the hypersensitive response. **B,** Growth curves of race T3 (squares), race T3(AvrRxv-HA) (circles), and race T3(AvrRxv-HA[C244A]) (triangles) bacterial strains in Hawaii 7998 leaves. Plants were vacuum-infiltrated with bacterial suspensions at a titer of 10^4 CFU/ml, and bacterial populations in the leaves were estimated from samples harvested during a period of 6 days. Data represent the mean ± SE (n = 4). **C,** Expression and type III secretion of AvrRxv-HA and AvrRxv-HA(C244A) proteins from race T3 bacteria. Immunoblot analysis of cellular lysate and culture fluid of strains race T3 and race T3 Δ*hrpA* expressing AvrRxv-HA or AvrRxv-HA(C244A) as indicated. Proteins were detected by using monoclonal antibodies raised against the HA epitope.

As described above, plants challenged with race T3(AvrRxv-HA) at a titer of 10^8 CFU/ml developed an HR 14 h after inoculation, whereas plants infected with race T3[AvrRxv-HA(C244A)] developed disease symptoms 36 h after infection. For the preliminary assessment of gene expression kinetics characteristic of these interactions, samples were harvested before infection (0 h) and at different times after inoculation (4, 6, 8, 10, and 12 h), and a microarray hybridization was performed for each time point. Differential gene expression was evident starting 8 h after treatment, reached a maximum at 10 h both in terms of number of genes and their fold change in expression, and decreased after 12 h (Fig. 5).

Based on these kinetics, an extensive analysis of gene expression was carried out with biological replicates of samples harvested 10 h after inoculation when maximal variation in expression is observed. Considering as a cutoff for differential expression a fold change of two, 420 genes were found to be differentially regulated with statistical significance ($P < 0.05$) as a result of expression in the plants of a functional AvrRxv. Among these genes, 377 were upregulated and 43 were downregulated. To explore cellular processes regulated by the avirulence activity of AvrRxv, differentially expressed genes were classified into 15 functional categories based on Solanaceae Genomics Network (SGN) and Gene Ontology Consortium (GO) annotations (Fig. 6A and B). A significant number of genes (31% of the entire group) encoded proteins with insufficient similarity to proteins of known function to be classified with confidence, and therefore were grouped in the category of unknown function. Among the upregulated genes, the categories with the highest percentage of genes were transcription (12%), stress responses (9%), signaling (7%), and defense (6%); whereas, among the downregulated genes, the most represented functional groups were transcription (14%), stress (7%), defense (7%), protein synthesis (7%), and nucleic acid related (7%).

It is remarkable that 12% of the genes that were found in this study to be differentially regulated encode putative transcription factors of different families (Table 1). The families with the highest number of members were AP2/EREBP, WRKY, Myb, and MADS-box. Other transcription factors, such as DREB, bHLH, NAM, homeodomain, Scarecrow, and F-box, also were represented. These transcription factors probably orchestrate the extensive change in gene expression that results from expression of AvrRxv in resistant plants and is dependent on the integrity of the catalytic core of this effector protein.

DISCUSSION

In this study, the *X. campestris* pv. *vesicatoria* AvrRxv effector was characterized in terms of its site of action in the plant cell and structural requirements for its avirulence function. In addition, as a first step toward the elucidation of cellular processes triggered by recognition of AvrRxv in race T1-resistant tomato plants, a set of tomato genes differentially regulated as a result of AvrRxv expression was identified. Expression of AvrRxv in resistant plants was shown to be sufficient for the elicitation of a typical HR, strongly suggesting that recognition of this effector protein occurs inside the plant cell. Once inside the plant cell, AvrRxv appears to localize predominantly to the cytoplasm and possibly in association with nuclear and plasma membranes. A cytoplasmic localization was recently observed for the YopJ-like AvrXv4 effector (Roden et al. 2004). However, an additional YopJ-like effector, PopP2 of *Ralstonia solanacearum,* was shown to accumulate in the cell nucleus of *Arabidopsis* protoplasts (Deslandes et al. 2003). Localization of YopJ family members to different cellular compartments indicates that they probably target different host proteins and have distinct substrate specificities.

Mutations in its putative cysteine protease domain affected AvrRxv avirulence function because elicitation of HR and inhibition of bacterial growth were not observed in resistant plants infected with *X. campestris* pv. *vesicatoria* strains expressing AvrRxv catalytic core mutants. An intact catalytic core was shown to be required for YopJ-mediated inhibition of MAPK and NF-κB signaling in animal cells (Orth et al. 2000), and for AvrBst- and AvrXv4-mediated elicitation of HR in pepper and *N. benthamiana* plants, respectively (Orth et al. 2000; Roden et al. 2004). Together, these findings highlight the central role of the cysteine protease domain and, presumably, proteolytic activity, for the function of YopJ-like proteins inside the host cell. Interestingly, YopJ and AvrXv4 have been proposed to act as SUMO isopeptidase, because they significantly reduce the level of SUMO-conjugated proteins in animal and plant cells, respectively (Orth et al. 2000; Roden et al. 2004). It remains to be established whether AvrRxv also acts

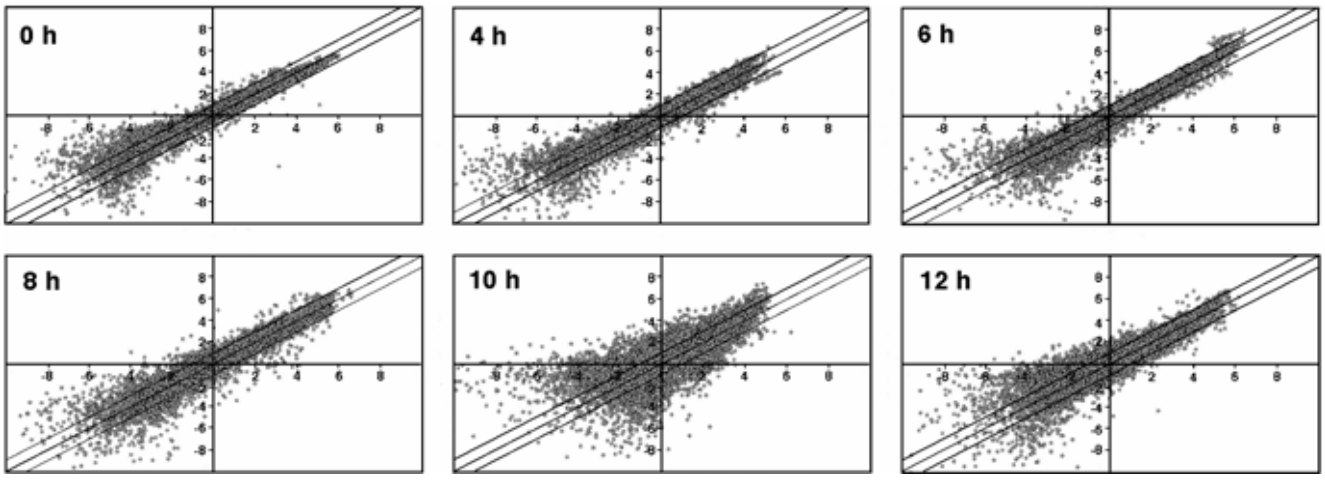

Fig. 5. Scatter plots representing distribution of cDNAs expression ratio after inoculation of *Xanthomonas campestris* pv. *vesicatoria* race T1-resistant tomato plants with race T3(AvrRxv) or race T3[AvrRxv(C244A)] strains. Hawaii 7998 plants were inoculated with bacterial strains at a titer of 10^8 CFU/ml. Total RNA was extracted from leaf tissues harvested prior to inoculation (0 h), or 4, 6, 8, 10, and 12 h post infection, and used to prepare fluorescently labeled cDNA probes for microarray analysis of 8,600 unigenes spotted on microarray slides. The *x* axis represents the spot intensity of Cy3 (race T3[AvrRxv(C244A)]-inoculated plants), and the *y* axis represents the spot intensity of Cy5 (race T3[AvrRxv]-inoculated plants). Upper and lower diagonal lines represent twofold induction and repression, respectively. The middle diagonal line represents no change in gene expression.

as a cysteine protease and if, similarly to YopJ and AvrXv4, it possesses SUMO isopeptidase activity in host cells. Our attempts to detect in vitro protease activity of AvrRxv on a series of substrates, including tomato SUMO (Hanania et al. 1999), were unsuccessful (data not shown). This is consistent with the lack of constitutive activity previously reported for other members of the YopJ family that probably require host factors for activation (Hotson and Mudgett 2004).

The *avrRxv* gene mediates resistance of the tomato line Hawaii 7998 to *X. campestris* pv. *vesicatoria* race T1 strains by an atypical gene-for-gene interaction with three nondominant plant *R* genes which participate with an additive effect in elicitation of the HR (Whalen et al. 1993; Yu et al. 1995). The finding that an intact catalytic core is required for AvrRxv avirulence activity allows the elaboration of new hypotheses about how AvrRxv is recognized by three tomato R proteins. A first possibility is that AvrRxv interacts directly or through an adaptor protein with each of the three R proteins. In this scenario, the putative protease activity of AvrRxv is dispensable for recognition, whereas integrity of the catalytic triad may represent a structural requirement for successful protein–protein interactions. In line with this hypothesis, the YopJ-like PopP2 effector recently was shown to directly interact in a yeast two-hybrid system with the *Arabidopsis* RRS1 R protein, which confers broad-spectrum resistance to several strains of *R. solanacearum* (Deslandes et al. 2003). However, the role of PopP2 catalytic triad and putative protease activity in this physical interaction and in disease resistance is yet to be elucidated. An alternative possibility for AvrRxv recognition by the plant resistance machinery is that tomato R proteins recognize the processed form of a plant protein targeted by the AvrRxv putative protease activity. In this model, the different R proteins could recognize the same or distinct proteins modified by AvrRxv. A similar indirect detection of a pathogen effector via its enzymatic activity has been proposed as the molecular mechanism that mediates resistance of *Arabidopsis* plants expressing the RPS5 R protein to *P. syringae* strains expressing the avirulence protein AvrPphB (Shao et al. 2003). In these plants, the AvrPphB effector, which is a member of the YopT family of cysteine proteases (Shao et al. 2002), proteolytically cleaves the *Arabidopsis* PBS1 protein kinase, and a PBS1 cleavage product is proposed to trigger RPS5-mediated resistance (Shao et al. 2003). Finally, because tomato resistance to race T1 is genetically complex, a combination of distinct recognition mechanisms for different R proteins also is plausible.

In resistant plants, efficient recognition between an avirulence factor and the corresponding R protein triggers the activation of cellular responses that inhibit pathogen growth (Hammond-Kosack and Jones 1996; Martin et al. 2003). To identify tomato genes that are directly modulated by recognition of a functional AvrRxv effector in resistant plants, microarray techniques were employed in combination with isogenic strains of *X. campestris* pv. *vesicatoria* race T3 expressing wild-type AvrRxv-HA or the mutant AvrRxv-HA(C244A), which is not functional in the elicitation of a resistance response. By monitoring kinetics of gene expression for approximately 8,600 tomato genes at different time points after plant infiltration with these two bacterial strains, differential gene expression first was detected at 8 h after inoculation. We assume that, during the first hours of infection, AvrRxv is delivered into the plant cell by the bacterial TTSS and its recognition by plant R proteins triggers activation of signaling pathways leading to the onset of defense responses. Maximal alteration in the plant transcriptome was observed at 10 h after inoculation and a decrease in differential expression was detected at 12 h. Qualitatively similar but slower gene expression kinetics, which well correlated with a slightly delayed HR, previously were observed during the onset of tomato resistance to race T3 mediated by the AvrXv3 effector and the RxvT3 R protein (Gibly et al. 2004). This difference in kinetics may be ascribed to either a more efficient host recognition machinery for AvrRxv than for AvrXv3 or the strength and constitutive character of the *lacZ* promoter used to express AvrRxv in race T3 bacteria compared with the native and *hrp*-regulated *avrXv3* promoter (Astua-Monge et al. 2000a).

Analysis of expression profiles of approximately 8,600 tomato genes performed with leaf tissues harvested 10 h after bacterial infection identified a total of 420 genes differentially regulated by expression of a functional AvrRxv. These include 377 upregulated and 43 downregulated genes. Classification of genes modulated by AvrRxv expression into functional categories revealed a significant overlapping with classes of genes previously shown to participate in other plant resistance responses to bacterial pathogens (Chen et al. 2002; Gibly et al. 2004; Mysore et al. 2002; Scheideler et al. 2002; Tao et al. 2003). The most represented categories were transcription, stress responses, signaling, and defense. In addition to the activation of defense responses, a possible link between transcription and the putative protease ac-

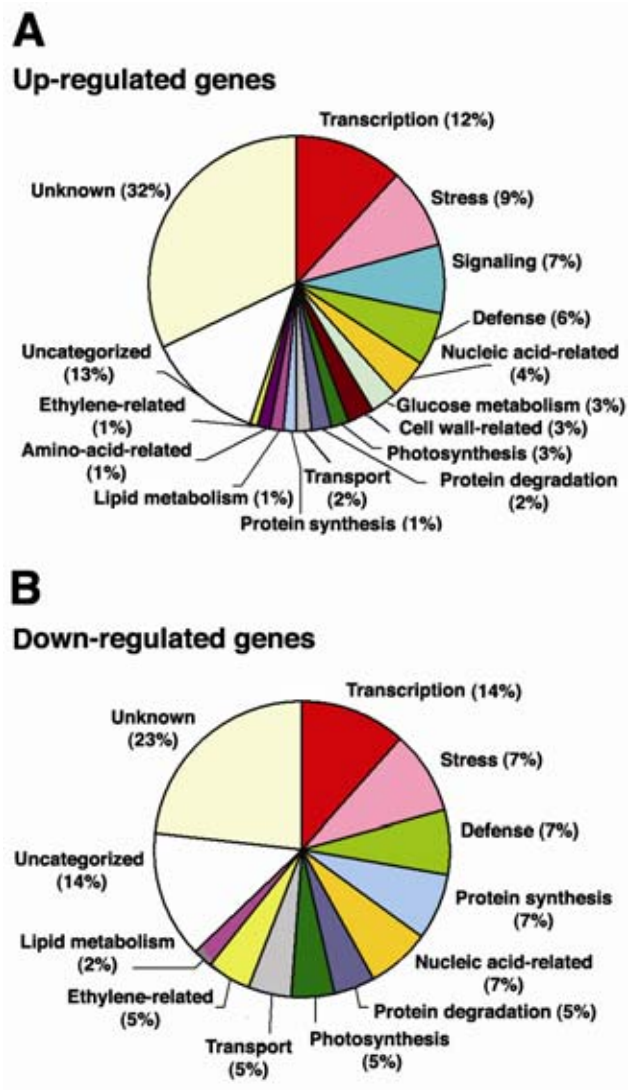

Fig. 6. Classification into functional categories of genes differentially expressed upon inoculation of *Xanthomonas campestris* pv. *vesicatoria* race T1-resistant tomato plants with race T3(AvrRxv) compared with race T3[AvrRxv(C244A)] strains. **A** and **B,** Pie charts show classification and percentage of functional categories for a total of 377 upregulated and 43 downregulated genes, respectively.

tivity of AvrRxv may account for changes in gene expression observed in this study. In fact, although a SUMO protease activity has not been demonstrated for AvrRxv, YopJ-like proteins are predicted to mimic eukaryotic SUMO proteases that, in several instances, have been implicated in transcriptional regulation by interfering with sumoylation of transcription factors (Girdwood et al. 2004; Hotson and Mudgett 2004).

A strikingly high number of differentially regulated transcription factors were identified in this study representing at least 11 different classes (12% of the entire group) (Table I). The largest among them are classes of AP/EREBP-type and WRKY-type transcription factors, particularly well known for their involvement in various biotic stresses (Chen et al. 2002; Eulgem et al. 2000; Riechmann and Meyerowitz 1998). Remarkable in the

Table 1. List of transcription factors differentially regulated during the resistance response of Hawaii 7998 tomato plants to *Xanthomonas campestris* pv. *vesicatoria* T3 strains expressing AvrRxv

Group type, accession number	GenBank best hit	*e* Value	Expression ratio[a]
AP2/EREBP			
AW029674	AP2 domain transcription factor, putative (*Arabidopsis thaliana*)	5.0E-25	3.8
AI779166	AP2 domain transcription factor, putative (*A. thaliana*)	5.0E-20	2.2
BF050264	Floral homeotic protein APETALA2 (*A. thaliana*)	6.0E-35	2.0
AW030386	Pathogenesis-related genes transcriptional activator Pti5 (*Lycopersicon esculentum*)	4.0E-68	2.2
AW038298	Probable AP2 domain transcription factor (*A. thaliana*)	2.0E-07	2.3
AI776626	Putative ethylene response element binding protein EREBP (*A. thaliana*)	1.0E-18	3.2
BG123998	Ethylene response factor 3 (*L. esculentum*)	4.0E-97	2.2
BI421507	Transcription factor JERF2 (*L. esculentum*)	4.0E-95	2.5
WRKY			
AW033040	DNA-binding protein 4 (*Nicotiana tabacum*)	3.0E-58	4.2
AI484414	WIZZ transcription factor (*N. tabacum*)	1.0E-101	3.5
AB061245	WRKY-type DNA binding protein (*Solanum tuberosum*)	9.3E-84	2.1
AW032452	WRKY-type transcription factor (*S. chacoense*)	0	2.2
BI923009	WRKY family transcription factor (*A. thaliana*)	2.0E-18	2.5
Myb			
AI897784	Myb-related protein 305 (*Neogarrhenium filipes*)	1.0E-80	3.5
BE462506	Myb-related protein Ph2 (*Petunia* × *hybrida*)	2.0E-59	2.6
AI489692	Myb-related transcription factor (*L. esculentum)*	4.0E-68	0.4
BE441064	Myb family transcription factor (*A. thaliana*)	7.0E-42	0.4
AW441747	Putative Myb-related DNA-binding protein (*A. thaliana*)	1.6E-31	2.1
MADS-box			
BE431840	MADS-box protein 15 (*Petunia* × *hybrida*)	2.0E-45	2.6
BG124572	MADS-box transcription factor CDM51 (*Chrysanthemum* × *morifolium*)	2.0E-59	4.4
AW037354	Putative MADS-domain transcription factor MpMADS9 (*Magnolia praecocissima*)	0.002	2.7
AW931475	TDR4 MADS-box transcription factor (*L. esculentum*)	1.0E-135	2.1
BHLH			
BF096755	bHLH protein (*A. thaliana*)	3.0E-68	2.0
AI485524	bHLH protein family (*A. thaliana*)	2.0E-17	2.0
AW647683	bHLH protein SPATULA (SPT) (*A. thaliana*)	3.0E-32	2.7
NAM			
AAM34772	NAM-like protein 9 (*Petunia* × *hybrida*)	2.0E-17	2.4
AW220823	No apical meristem (NAM) protein family (*A. thaliana*)	2.0E-92	0.2
BM410468	No apical meristem (NAM) protein family (*A. thaliana*)	2.0E-88	2.3
DREB			
AI896705	C-repeat/DRE binding protein, putative (*A. thaliana*)	7.0E-34	2.1
AI896850	Dehydration-responsive element binding protein 3 (*L. esculentum*)	1.0E-100	2.0
AW035463	Hypothetical protein DREB1A (*A. thaliana*)	6.0E-33	2.4
Homedomain			
AI488166	Homeodomain protein (*A. thaliana*)	9.0E-57	2.3
BF096566	Homeotic protein VAHOX1 (*L. esculentum*)	1.0E-170	0.5
AI899148	Homeodomain protein (*L. esculentum*)	7.0E-98	2.4
Scarecrow			
AW222288	Putative Scarecrow gene regulator (*Oryza sativa*)	4.0E-13	2.6
BI923257	Scarecrow transcription factor family (*A. thaliana*)	1.0E-147	5.7
F-box			
AI895129	F-box containing tubby family protein (*A. thaliana*)	1.0E-173	2.8
AW930724	F-box containing tubby family protein (*A. thaliana*)	2.0E-26	2.4
Zinc finger			
AAF26978	Putative CCHC-type zinc finger protein (*A. thaliana*)	0.08	2.6
AAP85546	Putative RING-H2 zinc finger protein (*O. sativa* [japonica cultivar-group])	1.0E-13	2.8
AI485555	ZPT2-13 C2H2 zinc finger protein (*Petunia* × *hybrida*)	2.0E-42	2.4
Others			
BG125255	AG-motif binding protein-2 (*Nicotiana tabacum*)	1.0E-104	2.1
AI483935	BEL1-related homeotic protein 29 (*S. tuberosum*)	3.0E-34	2.1
AI896377	GT-1-related transcription factor (*A. thaliana*)	4.0E-48	4.6
BI209938	Heat shock transcription factor family (*A. thaliana*)	2.0E-24	2.2
AI898727	Putative RING protein (*Populus* × *canescens*)	5.0E-42	2.1
BI932290	Putative transcription factor (*O. sativa* [japonica cultivar-group])	1.0E-06	2.6
BF051245	SET-domain transcriptional regulator family (*A. thaliana*)	1.0E-140	2.4
AW616081	SPF1 DNA-binding protein (*Ipomoea batatas*)	1.0E-158	2.1

[a] Ratio of gene expression in plants of the tomato line Hawaii 7998 inoculated with *X. campestris* pv. *vesicatoria* T3(AvrRxv) versus in plants inoculated with *X. campestris* pv. *vesicatoria* T3[AvrRxv(C244A)].

AP/EREBP group is the presence of the Pti5 transcription factor, which was isolated based on its interaction with the tomato Pto R protein and is involved in transcriptional activation of PR proteins (Gu et al. 2002). An additional predominant group of transcription factors is represented by MYB-related proteins that have been implicated in several cellular processes, including regulation of secondary metabolism and cell cycle, response to abiotic stress, and pathogen defense (Carre and Kim 2002; Martin and Paz-Ares 1997). Interestingly, an MYB transcription factor recently was shown to be required for tobacco disease resistance to *Tobacco mosaic virus* mediated by the N protein (Liu et al. 2004b). Classes of transcription factors such as MADS-box, homeodomain, NAM-like, and Scarecrow proteins that are associated mainly with the control of different aspects of plant development also were identified (Bolle 2004; Irish 2003; Souer et al. 1996). A role for these transcription factors in plant disease resistance is now emerging based on their differential expression observed during different plant–pathogen interactions (Chen et al. 2002; Gibly et al. 2004; Mysore et al. 2002). Finally, three dehydration and cold-responsive element binding proteins were upregulated (Thomashow 1999), indicating a previously undescribed overlap between disease resistance and cold or water stress.

In resistant plants, signal transduction pathways leading to the activation of defense responses are activated readily by pathogen recognition. A significant percentage of genes modulated by AvrRxv recognition are signaling related (7%). They encode different types of protein kinases, including serine/threonine, receptor-like and MAP kinases, and several protein phosphatases. Remarkable in this group is the presence of proteins that could have pathogen recognition functions in addition to a role in signaling. Among these are three upregulated leucine-rich-repeats transmembrane protein kinases, including a homolog of the rice R protein Xa21, which confers resistance to *X. oryzae* pv. *oryzae*, and a homolog of the Pto kinase, which specifies resistance against *P. syringae* pv. *tomato*. Induction of these and other *R*-like genes identified in this study by a primary infection may represent a strategy to keep the plant surveillance system on alert against subsequent pathogen attacks.

A large number of genes differentially expressed as a result of AvrRxv recognition were classified as stress related (9%). The vast majority of these genes encode antioxidant proteins (e.g., glutathione *S*-transferase, peroxidase, and oxidoreductase) and molecular chaperones (e.g., HSP80 and chaperonin 21), probably reflecting efforts of stressed cells to refold oxidatively damaged proteins and to neutralize the reactive oxygen species released during the oxidative burst that accompanies the onset of disease resistance. It also is possible that the molecular chaperones identified here may participate with R proteins in receptor complexes mediating direct or indirect recognition of the AvrRxv effector. This hypothesis is supported by the recent findings that the HSP90 chaperone associates with the tobacco N and with the *Arabidopsis* RPM1 R proteins and is required for their function in disease resistance (Hubert et al. 2003; Liu et al. 2004a).

As expected, a significant number of differentially regulated genes (7%) were classified as defense related. This group included genes that previously were shown to be induced during other plant–pathogen interactions and encode, for instance, PR proteins (e.g., chitinase, PR P23, and ELI3) and enzymes involved in the biosynthesis of phenylpropanoids and fatty acids (e.g., P450 monooxygenase and phenylalanine ammonia lyase). Transcripts modulated by AvrRxv expression in resistant plants also were related to other cellular processes known to be affected during the onset of disease resistance, such as cell-wall metabolism (3%), glucose metabolism (3%), protein degradation (2%), and transport (2%). Finally, also significant was the high percentage of genes encoding proteins of unknown function (32%), whose role in disease resistance awaits characterization.

MATERIALS AND METHODS

Plant material and bacterial strains.

Tomato (*Lycopersicon esculentum*) cultivars used in this study were Hawaii 7998, which carries the *Rxv*, *Rxv1*, and *Rxv2 R* genes and is resistant to *X. campestris* pv. *vesicatoria* strains of race T1 (Yu et al. 1995); and Hawaii 7981, which carries the *RxvT3 R* gene and is resistant to *X. campestris* pv. *vesicatoria* strains of race T3 (Scott et al. 1995). Bacterial strains used were *X. campestris* pv. *vesicatoria* race T3 strain 97-2, race T3 Δ*hrpA* (a gift from S. Burdman, Hebrew University of Jerusalem), race T1 strain 110C, and *A. tumefaciens* GV2260.

Inoculation procedures and in planta bacterial growth.

For pathogen inoculation, 6-week-old plants were infiltrated by either vacuum or manually using a 1-ml syringe, with bacterial cultures prepared as described here. *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, washed twice with 10 mM $MgCl_2$, and diluted in 10 mM $MgCl_2$ and 0.005% (vol/vol) Silwett-L77. For HR assays, plants were inoculated with bacterial suspensions at a titer of 10^8 CFU/ml. To determine in planta bacterial growth, tomato plants were infiltrated with bacterial suspensions at a titer of 10^4 CFU/ml. Leaf samples of inoculated plants were collected at 24-h intervals during 6 days. Bacterial growth was measured by grinding four 1-cm^2 leaf discs in 10 mM $MgCl_2$, plating serially diluted tissue samples on NYG plates, and counting CFU.

Secretion assay.

Bacterial strains race T3 and the type III deletion mutant race T3 Δ*hrpA* expressing AvrRxv-HA or AvrRxv-HA(C244A) were grown overnight to optical density at 600 nm (OD_{600}) = 0.8 in XVM2 *hrp* gene induction medium (Wengelnik et al. 1996) in the presence of kanamycin at 50 μg/ml. Cellular lysates were obtained by centrifugation of 1 ml of culture at 14,000 × *g* for 10 min and resuspending the pellet in 100 μl of Laemli buffer. To prepare culture fluid fractions, bacterial cells were removed by centrifugation from 50-ml cultures, and supernatants were dried by lyophilization and resuspended in 5 ml of Laemli buffer. Aliquots of 5 μl of cellular lysates and 100 μl of culture fluid fractions were analyzed by immunoblotting and chemiluminescence visualization (Amersham Biosciences, Piscataway, NJ, U.S.A.) using monoclonal HA antibodies (1:1000 dilution; Roche Diagnostics, Mannheim, Germany) and horseradish peroxidase-conjugated secondary antibodies (1:5000 dilution; Sigma-Aldrich, St. Louis).

***Agrobacterium*-mediated transient expression assay.**

A. tumefaciens GV2260 was used for transient expression in tomato plants. Bacteria were grown overnight at 28°C in Luria broth medium containing the appropriate antibiotics, collected by centrifugation, washed, and resuspended to OD_{600} = 0.04 in infiltration medium (10 mM morpholinoethanesulfonic acid [MES], pH 5.5, 10 mM $MgCl_2$, and 200 μM acetosyringone). Bacterial suspensions were manually infiltrated into leaves of 6- to 8-week-old tomato plants using a 1-ml syringe. Protein expression in plants was detected by standard immunoblot analysis as described above.

Plasmid constructions.

For manipulation of the *avrRxv* gene, the *avrRxv* coding region (Whalen et al. 1993) was PCR amplified from race T1

110C genomic DNA by using the following primers: 5′-AGCGGATCCATATGTGCGACTCCATAAGAGTG-3′ and 5′-GCGGGATCCTCAGGATTCTAAGGCGTGACG-3′. The *avrRxv* PCR product was digested with *Bam*HI and inserted at the corresponding site into the pBlueScript SK plasmid (pBS; Stratagene, La Jolla, CA, U.S.A.), creating pBS(*avrRxv*). For *Agrobacterium*-mediated expression, *avrRxv* was excised as a *Xba*I-*Sal*I fragment from pBS(*avrRxv*) and transferred to the pBTEX binary vector (Frederick et al. 1998) under the control of the CaMV 35S promoter.

For *Agrobacterium*-mediated expression of HA-tagged *avrRxv* forms, *avrRxv* was PCR amplified from a pBS plasmid carrying AvrRxv at *Bam*HI and *Spe*I sites using the T7 primer in combination with the oligonucleotide 5′-CGAAGCTTGGA TTCTAAGGCGTGACGGAT-3′. The PCR product was digested with *Hin*dIII and inserted at the corresponding site into a pBS plasmid in frame to the 5′ end of a sequence encoding a double-HA epitope tag. The 3′ end of AvrRxv fused to the double-HA epitope was excised as a *Bgl*II-*Pst*I fragment and subcloned at the corresponding sites in pBS(*avrRxv*). The obtained plasmid was used to perform site-directed mutagenesis of *avrRxv-HA* by using the Quickchange kit (Stratagene) and oligonucleotides as follows: for *avrRxv-HA*(H180A), 5′-GACCCTAGGAGATGGGCTCGCGTCGCGTTCGA-3′ and 5′-TCGAACGCGACGCGAGCCCATCTCCTAGGGTC-3′; for *avrRxv-HA*(E200A), 5′-CGATTATCGCATTGGCGCCTGCG TCTGCTTA-3′ and 5′-TAAGCAGACGCAGGCGCCAATGC GATAATCG-3′; for *avrRxv-HA*(C244A), 5′-AAGTCAATCG GTGGGGCTGTCATATTTTCTCT and 5′-AGAGAAAATAT GACAGCCCCACCGATTGACTT-3′; and for *avrRxv-HA* (E258A), 5′-TGGCGGCATACCAGGCAAGAAGCACCTTT GA-3′ and 5′-TCAAAGGTGCTTCTTGCCTGGTATGCCGC CA-3′. Wild-type and mutant forms of *avrRxv-HA* then were subcloned into the pBTEX binary vector as *Bam*HI-*Sal*I fragments under the control of the CaMV 35S promoter. Resultant pBTEX plasmids were introduced into *A. tumefaciens* GV2260 by electroporation.

To introduce *avrRxv-HA* and its mutant form *avrRxv-HA*(C244A) into race T3 strain 97-2, wild-type and mutant sequences were subcloned as *Bam*HI-*Eco*RI fragments at the corresponding sites into the broad-host-range vector pDSK519 (Keen et al. 1988), which then was used to transform race 97-2 race T3 cells by electroporation.

To express the GFP-AvrRxv fusion protein, the *avrRxv* coding region was excised as a *Bam*HI fragment from pBS(*avrRxv*) and inserted in frame to the 3′ end of the GFP coding region in the pGFP-MRC plasmid (Rodriguez-Concepcion et al. 1999). A *Hin*dIII fragment encoding the GFP-AvrRxv fusion protein then was excised from the pGFP-MRC plasmid and transferred to the pBTEX binary vector under the control of the CaMV 35S promoter. Resultant pBTEX plasmids were introduced into *A. tumefaciens* GV2260 by electroporation.

Microscopy.

Confocal imaging was performed using a Zeiss R510 confocal laser scanning microscope (Zeiss, Thornwood, NY, U.S.A.). Excitation was performed with an argon laser set to 488 nm, and emission was detected with a 525- ± 15-nm bandpath filter. Image analysis was performed with Zeiss AxioVision, Zeiss CLSM-5, and Adobe Photoshop 6.0 (Mountain View, CA, U.S.A.).

Microarray analysis.

Microarray production, hybridization, and data analysis were performed following the minimum information about a microarray experiment (MIAME) guidelines for international standardization and quality control of microarray experiments (Brazma et al. 2001). For qualitative analysis of differential gene expression, a single biological repeat was performed. For detailed and quantitative analysis of gene expression changes, four 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 fifth experimental repeat was carried out for one of the biological samples with reverse labeling (Cy3 versus Cy5). Raw and normalized data of all experiments are available online from the SGN public database.

Microarray slides used in this study contain the TOM1 array fabricated at the Center for Gene Expression Profiling (CGEP, Boyce Thompson Institute, Ithaca, NY, U.S.A.). For slide preparation, 12,860 EST clones, which represent 8,642 independent tomato loci (Van der Hoeven et al. 2002), were PCR 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.), vacuum-dried, and resuspended in 30 µl of spotting buffer (3× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 1.5 M betaine). DNA from each sample was arrayed onto γ-amino-propyl-silane-coated UltraGAPS slides (Corning, Corning, NY, U.S.A.) using a MicroGrid Pro arrayer (BioRobotics, Woburn, MA, U.S.A.) with 32 MicroSpot2500 printing pins. After spotting, DNA was 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% sodium dodecyl sulfate, three rinses in double-distilled water, and a final rinse in 90% ethanol. Slides were finally dried by centrifugation.

Probe preparation and slide hybridizations were performed as previously described (Gibly et al. 2004). For data analysis, spot mean intensities were quantified using the ImaGene 5.0 software (Biodiscovery, El Segundo, CA, U.S.A.) and normalized by the mean of signals using the GeneSight 3.5 software (Biodiscovery). Genes significantly up- or downregulated were identified by Student *t* statistics. Genes were regarded as differentially regulated if their fold change was at least two, and if they withstood a statistical Student *t* test with a *P* value <0.05.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

The Gene Ontology (GO) consortium: www.geneontology.org
MEROPS protease database: merops.sanger.ac.uk
Minimum information about a microarray experiment (MIAME) guidelines: www.mged.org/Workgroups/MIAME/miame.html
The Solanaceae Genomics Network (SGN) public database: www.sgn.cornell.edu
TIGR database: www.tigr.org