Identification of genes required for Cf-dependent hypersensitive cell death by combined proteomic and RNA interfering analyses

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Abstract

Identification of hypersensitive cell death (HCD) regulators is essential to dissect the molecular mechanisms underlying plant disease resistance. In this study, combined proteomic and RNA interfering (RNAi) analyses were employed to identify genes required for the HCD conferred by the tomato resistance gene Cf-4 and the Cladosporium fulvum avirulence gene Avr4. Forty-nine proteins differentially expressed in the tomato seedlings mounting and those not mounting Cf-4/Avr4-dependent HCD were identified through proteomic analysis. Among them were a variety of defence-related proteins including a cysteine protease, Pip1, an operative target of another C. fulvum effector, Avr2. Additionally, glutathione-mediated antioxidation is a major response to Cf-4/Avr4-dependent HCD. Functional analysis through tobacco rattle virus-induced gene silencing and transient RNAi assays of the chosen 16 differentially expressed proteins revealed that seven genes, which encode Pip1 homologue NbPip1, a SIKP type MAP kinase Nbf4, an asparagine synthetase NbAsn, a trypsin inhibitor LeMir-like protein NbMir, a small GTP-binding protein, a late embryogenesis-like protein, and an ASR4-like protein, were required for Cf-4/Avr4-dependent HCD. Furthermore, the former four genes were essential for Cf-9/Avr9-dependent HCD; NbPip1, NbAsn, and NbMir, but not Nbf4, affected a nonadaptive bacterial pathogen Xanthomonas oryzae pv. oryzae-induced HCD in Nicotiana benthamiana. These data demonstrate that Pip1 and LeMir may play a general role in HCD and plant immunity and that the application of combined proteomic and RNA interfering analyses is an efficient strategy to identify genes required for HCD, disease resistance, and probably other biological processes in plants.

Key words: Cf, Cladosporium fulvum, defence, gene silencing, hypersensitive cell death, proteomics, regulation, resistance, RNAi.

Introduction

Plants have evolved two lines of defence to counter-attack pathogens’ infection: pathogen-associated molecular pattern-triggered immunity and effector-triggered immunity (ETI) (Jones and Dangl, 2006; De Wit, 2007). ETI is initiated by recognition of pathogen effector proteins, including avirulence (Avr) proteins by plant resistance (R) proteins, and is hallmarked by development of hypersensitive cell death (HCD).

The pathosystem of tomato (Solanum lycopersicum Mill.) and its leaf mould fungal pathogen Cladosporium fulvum is a model system to study ETI conferred by receptor-like protein type R proteins (Joosten and De Wit, 1999; Rivas
and Thomas, 2005; Wang et al., 2006b; Stergiopoulos and De Wit, 2009). A set of *C. fulvum* effector genes and tomato *R* genes (Cf), including four complementary R/Avr gene pairs, have been cloned from this pathosystem. These are *Cf-9/Avr9* (Van den Ackerveken et al., 1992; Jones et al., 1994), *Cf-4/Avr4* (Joosten et al., 1994; Thomas et al., 1997), *Cf-2/Avr2* (Dixon et al., 1996; Luderer et al., 2002), and *Cf-4E/Avr4E* (Takken et al., 1998; Westerink et al., 2004). All known *Avr* products are secreted to the extracellular space and carry an even number of cysteine residues, but show no other sequence similarity (Van den Ackerveken et al., 1992; Joosten et al., 1994; Luderer et al., 2002; Westerink et al., 2004). However, all CfS are homologous, extracellular, membrane-anchored glycoproteins that mainly consist of leucine-rich repeats (LRR) (Jones et al., 2000). A thioredoxin, CITRX, negatively regulates HCD resulting from Avr9 and Avr4 recognition by Cf-4 and Cf-9, respectively, is distinct in cell death pattern and intensity (Cai and Cf-4, respectively, is distinct in cell death pattern and intensity (Cai et al., 2000) and sensitivity to environmental conditions (Thomas et al., 2000; Van der Hoorn et al., 2000; Cai et al., 2001; De Jong et al., 2002; Wang et al., 2005). In comparison with the *Avr9/Cf-9*-dependent HCD, *Avr4/Cf-4*-dependent HCD is more rapid, with necrosis appearing primarily in the veins (Thomas et al., 2000; Van der Hoorn et al., 2000; Cai et al., 2001), and less sensitive to high temperature and high relative humidity (De Jong et al., 2002; Wang et al., 2005).

Significant progress has been made to understand the defence signal transduction initiated with Cf/Avr recognition. An oxidative burst, calcium-dependent kinases, MAP kinases, and a K⁺ efflux are involved in regulation of *Cf-9/Avr9*-initiated defence responses (Piedras et al., 1998; Blatt et al., 1999; Romeis et al., 1999, 2000, 2001; De Jong et al., 2000). A thioredoxin, CITRX, negatively regulates *Cf-9*-dependent cell death (Rivas et al., 2004). Transcript profiling reveals that expression of several hundreds of genes alters upon Cf/Avr recognition, which include 290 ACRE (*Avr9/Cf-9* rapidly elicited) (Durrant et al., 2000), 442 ART (*Avr4*-responsive tomato) (Gabriëls et al., 2006), and 367 ACE (*Avr4/Cf elicited) (Hong et al., 2007; Zhu et al., 2008) genes. Recently, further studies employing virus-induced gene silencing (VIGS) identified several genes that are essential for *Cf*-dependent HCD and/or resistance, which include a CC-NB-LRR type resistance protein analogue gene *NRCl* (Gabriëls et al., 2007), protein kinase genes *ACIK1* (Rowland et al., 2005) and LeMPKs (Stulemeijer et al., 2007), two E3 ubiquitin ligase genes *CMGP1* (Gonzalez-Lamothe et al., 2006) and *PUB17* (Yang et al., 2006), and a F-box protein gene *ACIF1* (Van den Burg et al., 2008), demonstrating that protein phosphorylation and the ubiquitin-proteasome pathway are pivotal in regulation of *Cf*-dependent HCD and resistance. Additionally, Avr2 inhibits cysteine protease Rcr3 and thereby initiates *Cf-2*-dependent defence signalling (Kruger et al., 2002; Rooney et al., 2005). Avr2 also inhibits another cysteine protease, *Phytophthora*-inhibited protease 1 (Pip1), which has been suggested to be an operative target of Avr2 in a recently developed ‘decoy’ model (Shabab et al., 2008; van der Hoorn and Kamoun, 2008; van Esse et al., 2008).

However, the exact role of Pip1 in defence response to *C. fulvum* is still largely unknown. Additionally, 12 proteins were differentially phosphorylated in the tomato seedlings mounting *Cf-4*-dependent HCD and the control HCD seedlings (Stulemeijer et al., 2009); however, role of these phosphoproteins in *Cf-4*-dependent HCD and resistance is still unclear.

To further understand the molecular mechanism underlying Cf-conferred HCD and resistance to *C. fulvum*, this study identified a set of novel genes required for *Cf-4*-dependent HCD employing combined proteomic and RNA interfering (RNAi) analyses.

**Materials and methods**

**Plant growth and sampling**

Tomato (*Solanum lycopersicon*) lines of Moneymaker (MM) carrying the *C. fulvum* resistance gene *Cf-4* (MM-*Cf-4*) or carrying no known *Cf* gene but transformed with the *C. fulvum* avirulence gene *Avr4* (MM-*Avr4*), and the F1 progeny of crossing between MM-*Cf-4* and MM-*Avr4* (MM-*Avr4/Cf-4*) (Cai et al., 2001) were used in this study. Seeds were surface sterilized, germinated, sown in trays, and grown in plant growth chambers (RXZ-450D, Saifu Instrument Manufacturer, Ningbo, China) with a light/dark regime of 16/8 h, 70% humidity at 22°C, as described previously (Wang et al., 2005). Cotyledons of the MM-*Avr4/Cf-4* seedlings were collected when pin-point hypersensitive necrosis was just visible by the naked eye on the lower sides of the cotyledons. Meanwhile, cotyledons of the MM-*Cf-4* seedlings were also collected as the control. The cotyledon samples were immediately frozen in liquid nitrogen and stored at −80°C prior to protein extraction.

**Preparation of protein samples for two-dimensional polyacrylamide gel electrophoresis**

Protein was extracted from sampled cotyledons by trichloracetic acid (TCA) method as described by Damerval et al. (1986) with some modifications. One gram of tomato cotyledons were finely ground in liquid nitrogen and solubilized in lysis buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 65 mM DTT, 0.002% β-mercaptoethanol). Proteins were precipitated at −20°C and centrifuged at 40,000 g at 4°C for 1 h. The pellets were washed three times with cold acetone containing 0.07% (v/v) β-mercaptoethanol and centrifuged at 40,000 g at 4°C for 1 h. The precipitates were lyophilized and solubilized in lysis buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 65 mM DTT, 0.4% (w/v) each carrier ampholyte pH 5–7 and pH 3–10). Insoluble debris was removed by centrifugation at 40,000 g at 4°C for 1 h and supernatants were collected, separated into aliquots, and stored at −80°C. Protein concentration was determined by Bradford assay using bovine serum albumin as a standard.

**Two-dimensional polyacrylamide gel electrophoresis and image analysis**

IPG strips (Immobiline DryStrip pH 3–7 NL, 24 cm, GE Healthcare) were rehydrated at 25°C for 12 h with 450 μl rehydration buffer (7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 65 mM DTT, 0.2% (w/v) each carrier ampholyte pH 5–7 and pH 3–10, 0.002% (w/v) bromophenol blue) containing 300 μg protein for analytical...
gels or 1 mg protein for preparative gels. The rehydrated strips were electrofocused with Etta n IPGphor II IEF unit following the protocol provided by the manufacturer (Amersham Biosciences, GE Healthcare) with the addition of the following two steps at the beginning to make the salt removal more efficient: 100 V step and hold for 1 h; 250 V step and hold for 1 h. After focusing, the IPG strips were incubated first in equilibration buffer (6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS, 75 mM TRIS-HCl, pH 8.8) containing 1% (w/v) DTT and then in the same equilibration buffer but containing 2.5% (w/v) iodoacetamide. The second-dimension separation was performed using the Etta n Dansitx electrophoresis system (Amersham Biosciences) with 12% polyacrylamide gels at 1 W/gel for 1 h followed by 13 W/gel until the dye front reached the bottom of the gels. Six gels (three each for samples of MM-Cf-4 and MM-Cf-4/Avr4) were run simultaneously each time as three technical replicates and the two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) analysis was repeated three times for three independent sets of biological samples as three biological replicates. Analysis gels were visualized by silver staining compatible with MS analysis (Yan et al., 2000) and scanned with the ImageMaster 2D Platinum v. 6.0 (Amersham Biosciences). Spot detection and normalization, gel matching, and statistical data analysis (P-value <0.05) were conducted with ImageMaster 2D Platinum v. 6.0 (Amersham Biosciences) following the manufacturer’s instructions. Proteins that displayed two-fold or greater changes in the spot’s relative volume (spot volume/total spot volume × 100) were collected as differentially expressed proteins. In-gel tryptic digestion, MS analysis, and database search

Approximately 1 mg protein from each sample (MM-Cf-4/Avr4 and MM-Cf-4) was separated by 2-D PAGE and the gels were stained using Coomassie Brilliant Blue (CBB). Protein spots that showed different abundance in gels loaded with the MM-Cf-4/Avr4 (HCD⁺) and MM-Cf-4 (HCD⁻) samples were excised from CBB-stained gels and destained with 100 mM NH₄HCO₃ in 30% acetonitrile (ACN). Subsequently, the destaining buffer was removed and the gel pieces were lyophilized and rehydrated in 30 μl of 50 mM NH₄HCO₃ containing 50 μg trypsin (Promega, USA). After overnight digestion at 37 °C, the peptides were excised three times with 0.1% trifluoroacetic acid (TFA) in 60% ACN. Extracts were pooled together and lyophilized. The resulting peptide mixtures were kept at −80 °C for MS analysis. A protein-free gel piece was treated similarly and used for a control to identify the proteolysis products.

MS analysis of protein spots was performed at the Research Centre for Proteome Analysis, Chinese Academy of Sciences, Shanghai, China. The peptide mixtures were redisolved with an equal volume of cyano-4-hydroxycinnamic acid (10 mg/ml, Sigma), saturated with 50% acetonitrile in 0.05% TFA and analysed with an AutoFlex MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Proteins were first analysed by MALDI-TOF MS. The samples that were unable to be identified by MALDI-TOF MS analysis were subjected to MALDI-TOF/TOF MS analysis with ‘LIFT’ technology (Suckau et al., 2003). The lists of peaks for both the MALDI-TOF and the MALDI-TOF/TOF mass spectra were generated by flexAnalysis 2.4. Peaks with intensity ≥500 and S/N >6 were automatically picked up. The known contaminant ions (keratin and tryptic autodigest peptides) were excluded. The peptide calibration standard (Bruker) was used for external calibration, and the matrix and autolytic peaks of trypsin were used for internal calibration to ensure the accuracy of protein identification.

MS and MS/MS spectra were directly analysed by database searching using MASCOT 2.1 (MatrixScience, London, UK) with BioTools 3.0 (Bruker Daltonics, Bremen, Germany). Mass spectra were searched against the NCBI nonredundant protein (NCBI nr) database within the taxonomy Viridiplantae (green plants). Protein-matching searches that resulted in a score of over 68 were considered significant (P < 0.05). Individual ion scores of over 40 indicated identity or extensive homology (P < 0.05) existing in the compared proteins. A retrieved protein with the highest score in each Mascot search was accepted as the target protein for the spot subjected to identification. If peptides matched to multiple members of a protein family with the same or very similar scores, the S. lycopersicum protein with the highest score was the first choice; otherwise, a protein with pl and molecular weight mostly coinciding with the value predicted from 2-D PAGE profiles was considered as the identified protein. The search parameters were set as follows: enzyme, trypsin; fixed modifications, carbamidomethyl cysteine; variable modifications, methionine oxidation; mass values, monoisotopic; protein mass, unrestricted; peptide charge state, 1+; maximum missed cleavages, 1; peptide mass tolerance, ±0.1 ppm; fragment mass tolerance, ±0.8 Da.

Cloning of Nicotiana benthamiana and tomato cDNA fragments corresponded to the differentially expressed proteins

cDNAs corresponding to the differentially expressed proteins were cloned from Nicotiana benthamiana and tomato through reverse-transcription (RT) PCR. The tomato cDNAs and their N. benthamiana homologs corresponding to the differentially expressed proteins were obtained through tBLASTn search against the DFCI The Gene Indices database (http://compbio.dfci.harvard.edu/tgi/) and the SOL Tobacco Gene Indices database (http://www.pnpg.org/tgi/) using amino acid sequences of the differentially expressed proteins as queries. Primers were designed according to the retrieved cDNA sequences for the parts that had the highest identity to the tomato target proteins and had restriction enzyme sites added to the 5'-end (Supplementary Table S1, available at JXB online). Total RNA was isolated from normal tomato and N. benthamiana plants using TRIZOL reagent. cDNA generation from total RNA and subsequent PCR were performed using PrimeScript RT-PCR kit (TaKaRa Biotechnology, China). Products of RT-PCR were ligated into pUC-mT vector (Sangon, China) and sequenced. The obtained sequences were analysed for homology to the tomato differentially expressed proteins using DNAstar software (DNASTAR, USA) and deposited in the NCBI database.

Tobacco rattle virus-induced gene silencing analysis

The target cDNA fragments were ligated into the tobacco rattle virus (TRY) VIGS vector pYL156 (provided by Dr SP Dinesh-Kumar, Yale University) using the appropriate enzymes (Supplementary Table S1). The recombinant vector as well as pTRV1 was electrotransfected into cells of Agrobacterium tumefaciens strain GV3101. VIGS in N. benthamiana was performed as described previously (Wang et al., 2006a; Xu et al., 2007). Three weeks after agroinfiltration, the plants were subjected to HCD analysis. For each gene, over 10 plants were used for each VIGS analysis experiment and the VIGS experiments were conducted in duplicate.

HCD analysis

For Cf-4/Avr4- and Cf-9/Avr9-dependent HCD assessment, agrobacteria expressing an Avr and a Cf, respectively, were cultured as described (Wang et al., 2006a). The pellets were collected by centrifugation and suspended to give an OD₆₀₀ of 4.0. After recovery, suspensions of agrobacteria expressing an Avr and its complementary Cf were mixed in a 1:1 ratio to obtain the final agro-inocula for infiltration. The agro-inocula were infiltrated into the top three completely developed leaves of each N. benthamiana, Nicotiana tabacum, and tomato plants with a sterilized syringe without a needle. The agro-inoculated plants were grown in plant growth chambers at 25 °C with a light/dark 16/8 h regime. Three days later, the HCD in infiltrated area was investigated.
Phenotypes of HCD were briefly grouped into three grades based on the intensity and emergence percentage of tissue death in the agroinfiltrated area: full and nearly full HCD; partial HCD; and no HCD (Supplementary Fig. S1). A full HCD meant that tissue over the whole agroinfiltrated area completely died and collapsed (Supplementary Fig. S1A and B); a nearly full HCD signified that most tissue of the agroinfiltrated area (over 75%) coherently died and collapsed (Supplementary Fig. S1C); a partial HCD indicated that only partial tissue of the agroinfiltrated area (less than 75%) died and the dead tissues often scattered into several patches frequently with yellowish margins (Supplementary Fig. S1D–F); and no HCD referred to none of the tissue of the agroinfiltrated area dying (Supplementary Fig. S1G–I).

To analyse Xanthomonas oryzae pv. oryzae (Xoo)-induced HCD, Xoo was cultured in Wakimoto’s medium plates at 28 °C for 2 days. Colonies were collected with sterilized water and suspended in semi-quantitative RT-PCR analysis as described (Cai et al., 2007). Total RNA was extracted with TRIZOL reagents (Invitrogen, USA). cDNA generation from total RNA and subsequent PCR were performed with the primers listed in Supplementary Table S2 using a PrimeScript RT-PCR kit (TaKaRa Biotechnology).

Reverse-transcription PCR analysis

Transcript abundance of the genes for VIGS and transient RNAi analysis in N. benthamiana and N. tabacum plants was examined by semi-quantitative RT-PCR analysis as described (Cai et al., 2007). Total RNA was extracted with TRIZOL reagents (Invitrogen, USA). cDNA generation from total RNA and subsequent PCR were performed with the primers listed in Supplementary Table S2 using a PrimeScript RT-PCR kit (TaKaRa Biotechnology).

Transient RNAi analysis

A 300bp fragment of Pip1 was amplified with primers PIP1-F3 (CAGAGCTC(SacI)GGTACC(KpnI)GACGAGGTCTCGGTTG-TGAAA) and PIP1-R3 (CAGGAATCC(BamHI)CTCGAG(X-hol)AGCGTAGGGAACGACGCAAAC) using as template the plasmids carrying Pip1 cloned earlier in this study. This fragment was cloned into pBS-IN flanking the intron sequence of Phaseolus vulgaris nitrite reductase gene (U10419) first in the reverse direction with Xhol/KpnI and then in the forward direction with SacI/BamHI. The released hairpin sequence cassette was then cloned into binary vector pC1305-35S after the CaMV 35S promoter with Km promoter to obtain the Pip1 RNAi construct pC1305-35S::Pip1-RNAi. Agrobacteria transformed with this construct were infiltrated into all sectors of left halves of leaves of Sumsun 35S::PIP1-RNAi. Agrobacteria transformed with empty vector, as control, were infiltrated into the right half leaves. The NN tobacco plants while agrobacteria transformed with empty vector, as control, were infiltrated into all sectors of left halves of leaves of Sumsun 35S::PIP1-RNAi. Agrobacteria transformed with this construct were infiltrated into all sectors of left halves of leaves of Sumsun tobacco plants while agrobacteria transformed with empty vector, as control, were infiltrated into the right half leaves. The plants were grown at 25 °C in plant growth chambers for 2-D PAGE experiments, 2345 protein spots were detected in silver-stained 2-D gels loaded with 300 μg protein per gel. After 2-D PAGE using IPG strips (24 cm, pH 3–7 NL), the gels were silver stained. Protein spots that expressed differentially over two-fold in the cotyledons of the HCD+ and HCD− seedlings are numbered and indicated with arrows in the gels in which the spots displayed with higher abundance. Spot numbers are as given in Supplementary Table S3.

Results

Two-dimensional polyacrylamide gel electrophoresis profiles of proteins from Cf-4/Avr4 tomato seedlings mounting a hypersensitive cell death (HCD).

To investigate the change on protein expression during development of Cf-4/Avr4-dependent HCD, the proteomes of the cotyledons of tomato seedlings carrying the gene pair Cf-4/Avr4 and thus mounting a HCD (HCD+) seedlings and those carrying only the Cf-4 gene and thus developing no HCD (HCD− seedlings) were comparatively analysed. Typical protein profiles for the two type seedlings are shown in Fig. 1. According to the statistical data of the 2-D PAGE experiments, 2345 ± 75 protein spots for the HCD+ seedlings and 2387 ± 68 for the HCD− seedlings were detected in silver-stained 2-D gels loaded with 300 μg protein per gel. In total, 66 protein spots were expressed differentially at least two-fold between the HCD+ and HCD− seedlings (Fig. 1). Among them, 59 were up-regulated (Fig. 1A), while seven were down-regulated (Fig. 1B) in the HCD+ seedlings. As expected, this result indicates that expression of proteins significantly and globally altered during development of Cf-4/Avr4-dependent HCD.
To identify the differentially expressed proteins, preparative 2-D PAGE followed by CBB gel staining instead of silver staining was performed so that the loading volume could be increased from 300 μg to 1 mg per gel and thus the protein spots could be more easily identified successfully. The differentially expressed protein spots were excised from the gels and then subjected to in-gel trypsin digestion and subsequent MS identification analysis. Forty-nine proteins were successfully identified (Supplementary Table S3). The MS spectra of these identified proteins are listed in Supplementary Fig. S2. The close-up spot-to-spot comparison of differential expression of the identified 49 proteins in the HCD+ and HCD− seedlings are shown in Fig. 2.

Among the identified protein spots, one was a predicted protein with unknown function, while the other 48 were annotated to be involved in defense response (40.8%), signal transduction (8.2%), metabolism (24.5%), transcriptional regulation (6.1%), protein degradation (2.0%), growth and development (4.1%), miscellaneous functions (8.2%), and photosynthesis (4.1%) (Fig. 3, Supplementary Table S3). The majority of the identified proteins (46/49) were up-regulated in the HCD+ seedlings.

Nearly half of the 46 up-regulated proteins were defense-related proteins, among which were four glutathione S-transferases belonging to different classes, such as phi and tau, and two phospholipid hydroperoxide glutathione peroxidases (PHGPx). Glutathione S-transferases are involved in oxidative-stress metabolism and detoxification and PHGPx are antioxidant selenoenzymes that directly reduce membrane-bound lipid hydroperoxides (Dixon et al., 2009 and references therein). This indicates that glutathione-mediated antioxidation is one of the major responses upon the occurrence of Cf-4/Avr4-dependent HCD and/or that glutathione-mediated metabolism and maintenance of reductive state of membrane-bound lipids is important in regulation of Cf-4/Avr4-dependent HCD. The importance of glutathione in disease resistance has been shown with a pad2 mutant (Parisy et al., 2007). The cysteine protease Phytophthora-inhibited protease 1 (Pip1, spot 59) and a trypsin-inhibitor family protein LeMir (Lycopersicon Esculentum Miraculin, spot b60), were not detected in the HCD− seedlings but significantly accumulated in seedlings mounting Cf-4/Avr4-dependent HCD (Fig. 2, Supplementary Table S3). Additional up-regulated defence-related proteins were a set of well-known pathogenesis-related (PR) proteins including four PR10, three PR7 (P69), a PR5 and a PR9, and two chitinases, as well as a β-1,3-glucanase. Some of them were up-regulated nearly or over 10 times in the HCD+ seedlings over the HCD− seedlings. For instance, a subtilisin-like protease P69 (spot 23), a peroxidase (spot 31), a PR10 protein STH-2 (spot 14), and a basic 30 kDa endochitinase (spot 15) were up-regulated 39, 18.6, 9.8, and 9.4 times, respectively, in the HCD+ seedlings compared with the HCD− seedlings (Fig. 2, Supplementary Table S3).

There were four signalling-related proteins that were up-regulated in the HCD+ seedlings. These included a SIPK type MAP kinase (LeMPK1), a Ras-related GTP-binding protein, and two ACC oxidases. Other up-regulated proteins...
in the HCD\(^+\) seedlings included an APF1-like transcription factor, a S-adenosylmethionine-dependent methyltransferase, several molecular chaperons such as a protein disulfide isomerase and two luminal-binding proteins, and a functionally unknown protein.

Three down-regulated spots corresponded to a chloroplast thiazole biosynthetic protein, a thioredoxin, and an ASR (Abscissic acid, Stress, Ripening) protein (ASR4). A thioredoxin, CITRX, has been reported to specifically negatively regulate \(Cf-9\)-dependent HCD (Rivas et al., 2004). It will be intriguing to investigate the role of the thioredoxin identified in this study in \(Cf\)-dependent HCD and resistance.

Taken together, the proteomic data clearly show that \(Cf-4/Avr4\) plants are reprogrammed at the translational level to activate defence responses.

Comparison of expression profiles for \(Cf-4/Avr4\)-dependent HCD in protein and transcript levels

Previously, the present study laboratory and others have identified the transcript profiles for \(Cf-4/Avr4\)-dependent HCD through cDNA-amplified fragment length polymorphism (AFLP) analysis and have cloned 278 differentially expressed \(ACE\) fragments that correspond to 128 type proteins (Hong et al., 2007; Zhu et al., 2008) and 343 \(ART\) (\(Avr4\)-responsive tomato) fragments (Gabriëls et al., 2006). However, as far as is known, this is the first report on the proteomic analysis of expression profiles for \(Cf/Avr\)-dependent HCD. To better understand regulation of \(Cf-4/Avr4\)-dependent HCD at different levels and to validate the proteomic analysis in this study, the expression profiles for HCD at the protein level (this study) and the transcript level (previous studies: Gabriëls et al., 2006; Hong et al., 2007; Zhu et al., 2008) were compared. As shown in Table 1, genes encoding 22 out of the 49 differentially expressed spots identified in this study, which corresponded to 13 type proteins, were detected to be differentially expressed at the transcript level as well. These proteins were involved in defence response, signal transduction, metabolism, protein synthesis, miscellaneous functions, and photosynthesis (Table 1). This partial overlap between the two expression profiles validates the proteomic analysis in this study and demonstrates that \(Cf-4/Avr4\)-dependent HCD is regulated at both the transcriptional and the translational levels. Proteomic analysis could thus provide additional information related to the regulation of \(Cf-4/Avr4\)-dependent HCD and resistance.

Identification of genes required for \(Cf-4/Avr4\)-dependent HCD through VIGS analysis

To evaluate the function of the differentially expressed proteins and thereby to identify the genes required for \(Cf-4/Avr4\)-dependent HCD, VIGS analysis, a rapid reverse-genetics gene function analysis technique, was conducted in \(N. benthamiana\) for genes corresponding to the differentially expressed proteins. \(N. benthamiana\) instead of the host plant tomato was chosen for VIGS analysis because the former is the model plant for VIGS analysis, with the highest and most reproducible silencing efficiency, and the agroinfiltration-based \(Cf-4/Avr4\)-dependent HCD detection analysis is easier and more repeatable in this plant, as reported earlier (Gabriëls et al., 2006). From the total of 49 differentially expressed proteins, 16, most possibly related to HCD and defence regulation according to sequence annotation, were selected for further functional analysis (Supplementary Table S1). Fragments of \(N. benthamiana\) homologues of the genes encoding the tomato differentially expressed proteins were cloned employing RT-PCR with primers designed according to the \(N. benthamiana\) homologue cDNAs retrieved

### Table 1. The Differentially expressed proteins which were consistently detected in both this proteomic analysis and the previous cDNA-amplified fragment length polymorphism (AFLP) transcript profiling analysis (Hong et al., 2007; Zhu et al., 2008)

| Differentially expressed proteins | Corresponding protein spot number in this proteomic analysis | Corresponding transcript number in previous cDNA-AFLP transcript profiling analysis |
|----------------------------------|-----------------------------------------------------------|--------------------------------------------------------------------------------|
| Glutathione S-transferase         | 2, 10, 22, 40                                             | 95, 148, 198                                                                   |
| Pathogenesis-related protein STH-2 | 3, 14                                                    | 140                                                                              |
| Pathogenesis-related protein 10  | 13, 17                                                   | 78, 79, 114                                                                    |
| Subtilisin-like protease          | 23, b44                                                   | 150, 189                                                                        |
| Acidic 26 kDa endochitinase       | 15, 25                                                   | 152, 153, 158, 192, 237, 283, 285a                                             |
| Ribulose-1,5-bisphosphate carboxylase | 63, b50                                              | 237, 238, 259, 328, 330, 357, 358a                                             |
| 1-Aminocyclopropane-1-carboxylate oxidase 1 (ACC oxidase 1) | 5, b34 | 162, 164 |
| Asparagine synthetase             | b48                                                      | 180                                                                              |
| Quinone-oxidoreductase            | 4                                                        | 10                                                                               |
| Glucan endo-1,3-beta-glucosidase A | 26                                                       | 116, 186                                                                        |
| Peroxidase                       | 31                                                       | 203                                                                              |
| Inorganic pyrophosphatase         | 39                                                       | 4                                                                                |
| Esterase                         | 20                                                       | 117, 208                                                                        |
from tBLASTn search against either the DFCI The Gene Indices database or the Tobacco Gene Indices database (Supplementary Table S1). Bioinformatic analysis of the sequencing data revealed that most of the cloned N. benthamiana fragments had sequence identity higher than 70% at the amino acid level compared with the counterparts of the tomato differentially expressed proteins (Supplementary Table S1, Supplementary Fig. S3). These 16 N. benthamiana fragments were subcloned into VIGS vector pYL156 for TRV-induced gene silencing analysis.

For most of the selected genes, VIGS-treated plants grew and developed normally, similarly to the control plants treated with agrobacteria transformed with the empty silencing vector (EV). However, VIGS-induction treatment (agroinfiltration) for six out of the 16 genes resulted in abnormal growth and development of the treated plants (Fig. 4). These genes encoded a proteasome 20S beta 1.1 subunit (NbPb, 19), a luminal-binding protein (NbBiP, 58), a SIPK type MAP kinase (Nb4f, b12), an asparagine synthetase (NbAsn, b48), an ASR protein (NbASR, b67), and a chloroplast thiazole biosynthetic protein (NbTHI, b73) (Table 2). VIGS-induction treatment for both NbPb and NbBiP caused severe necrosis and finally death of the whole plant, although the dynamics of plant death were different. In NbPb-silencing-treated plants, the newly developed leaves were crinkly and showed clear necrotic symptoms 12 days after VIGS treatment (dat) (Fig. 4A, B). One week later, the whole plant died (Fig. 4C). VIGS-induction treatment for NbBiP led to a more rapid and severe necrosis in comparison with NbPb. Necrosis occurred in the infiltrated areas and the top newly developed leaves as early as 7 dat in NbBiP-silencing-treated plants. The whole plants died at 14 dat (Fig. 4D). This result demonstrates that the proteasome 20S beta 1.1 subunit and BiP genes are somehow associated with plant cell death. Silencing induction treatment for both Nb4f and NbAsn led to leaf roll and inflorescence malformation. However, phenotype development resulting from the Nb4f-VIGS treatment was much weaker and slower. No obvious phenotype was observed within 4 weeks after VIGS treatment (wat). Around 5 wat, leaf edges of the VIGS-treated plants rolled to the lower sides and twisted (Fig. 4E), while calyxes and corolla crinkled (Fig. 4F). For NbAsn, however, the growth of the VIGS-treated plants was significantly retarded at 3 wat: the sizes of newly developed leaves were much smaller and the development of shoots and inflorescences was significantly delayed (Fig. 4G). Furthermore, interestingly, unlike Nb4f-silencing-treated plants, the top newly developed leaves of the NbAsn-silencing-treated plants rolled to the upper sides tightly (Fig. 4G). At 6 wat, inflorescence was distorted, with corolla

Fig. 4. Influence of virus-induced gene silencing (VIGS)-inducing treatment for six genes on growth and development of N. benthamiana plants. Genes subjected to VIGS analyses encoded (A–C) a proteasome 20S beta 1.1 subunit (NbPb, 19), (D) a luminal-binding protein (NbBiP, 58), (E, F) a SIPK type MAP kinase (Nb4f, b12), (G, H) an asparagine synthetase (NbAsn, b48), (I) an ASR (Abscisic acid, Stress, Ripening) protein (NbASR, b67), and (J) a chloroplast thiazole biosynthetic protein (NbTHI, b73). Plants were infiltrated with cell suspensions of Agrobacterium
twisted and broken (Fig. 4H). Additionally, \( \text{NbASR} \)-silencing-treated plants were dwarfish with leaves turning in thicker (Fig. 4I), while \( \text{NbTHI} \)-silencing-treated plants showed leaf chlorosis before 3 wat and later bleaching (Fig. 4J). These data reveal that the SIKP type MAP kinase \( \text{NbF4} \), ASN, and ASR might be related with plant growth and development.

With exclusion of the two lethal genes \( \text{NbPb} \) and \( \text{NbBiP} \), the remaining 14 genes were subjected to evaluation of their role in \( Cf-4/Avr4 \)-dependent HCD by comparison of HCD in the silenced plants and nonsilenced EV plants at 3 wat. In EV plants, HCD strongly formed in the agroinfiltrated areas, most belonging to the full HCD category. In VIGS-treated plants, however, severity of HCD varied depending on the gene subjected to silencing analysis. As shown in Table 3 and Fig. 5, \( Cf-4/Avr4 \)-dependent HCD in VIGS-treated plants for four genes, which encode a SIKP type MAP kinase (\( \text{NbF4}, \text{b12} \)), a Pip1-like protein (\( \text{NbPip1}, \text{b59} \)), an ASN (\( \text{NbAsn}, \text{b48} \)), and a LeMir-like protein (\( \text{NbMir}, \text{b60} \)), was completely abolished (no HCD) in over 50% of the agroinfiltrated leaves and was much weaker and in a smaller area (partial HCD) in the remaining agroinfiltrated leaves than in the EV plants, demonstrating that these four genes are essential to \( Cf-4/Avr4 \)-dependent HCD. HCD in plants treated by VIGS for three genes, which encode a small GTP-binding protein (\( \text{NbRas}, \text{b16} \)), a late embryogenesis (Lea)-like protein (\( \text{NbLea}, \text{b46} \)), and an ASR4-like protein (\( \text{NbASR}, \text{b67} \)), was also significantly compromised but to a relatively weaker extent compared with the above four genes. HCD in these plants was abolished (no HCD) in over 30% of the agroinfiltrated leaves, indicating that these genes were also required for \( Cf-4/Avr4 \)-dependent full HCD.

RT-PCR analysis to verify gene silencing

To verify gene silencing in the VIGS-treated plants, RT-PCR analysis was conducted with the primers listed in Supplementary Table S2. Transcripts of the target genes \( \text{NbF4}, \text{NbPip1}, \text{NbAsn}, \) and \( \text{NbMir} \) accumulated to much lower levels in the VIGS-treated plants compared with the EV plants (Fig. 6), indicating that the observed phenotypes in these plants are caused by the targeted gene silencing.
silencing of NbPip1, NbAsn, and NbMir, but not Nbf4, attenuated Xoo-induced HCD.

### Transient RNAi analysis for function of Pip1 in Cf-4-, Cf-9-dependent, and Xoo-induced HCD in tobacco

To confirm the VIGS results, transient RNAi analysis was further executed to investigate the function of HCD-affecting genes represented by Pip1 in Cf-4-, Cf-9-dependent, and Xoo-induced HCD in tobacco. A Pip1 RNAi construct was made for this transient RNAi analysis, which carried a hairpin sequence cassette that comprised of two copies of a 300 bp fragment of Pip1 flanking the intron sequence of the P. vulgaris nitrite reductase gene in the opposite direction.

On the control halves of the leaves, all three types of HCD occurred strongly at 3 days post infiltration, while on the Pip1-RNAi-treated halves, no obvious HCD developed (Fig. 10). This confirmed what was observed earlier in VIGS experiments and once more demonstrated that Pip1 is required for Cf-4-, Cf-9-dependent, and Xoo-induced HCD.

### Discussion

**Genes required for Cf-4/Avr4-dependent HCD and disease resistance**

The tomato–C. fulvum pathosystem is one of the model systems for studying gene-for-gene resistance (Joosten and De Wit, 1999; Rivas and Thomas, 2005; Wang et al., 2006b; Stergiopoulos and De Wit, 2009). Great effort has been made in several laboratories over the world to understand the molecular mechanisms of the Cf-dependent defence response. Several important regulators of Cf-dependent HCD and resistance have been identified. However, the signal transduction pathways leading to Cf-dependent HCD and resistance are still far from clear.
The present study employed a combined proteomic and RNAi analysis system and successfully identified a set of genes required for Cf-4/Avr4-dependent HCD, among which are a Pip1-like protein (59), a SIPK type MAP kinase (b12), an ASN (b48), and a LeMir-like protein (b60). Pip1 is a papain-like cysteine protease. Expression of Pip1 is induced by butylhydroxytoluene (BTH) treatment and infection of pathogens (Tian et al., 2007; Shabab et al., 2008). Pip1 was found to map at the same genetic locus with another cysteine protease gene Rcr3, which mediates the recognition of Avr2 by Cf-2, initiating Cf-2-dependent defence signalling (Kruger et al., 2002; Rooney et al., 2005; Shabab et al., 2008; van Esse et al., 2008). Avr2 can inhibit both Pip1 and Rcr3, and Pip1 predominates over Rcr3, in apoplasts of BTH-treated tomato leaves (Shabab et al., 2008). Thus, it has been suggested that Rcr3 may act as a decoy to trap the fungus into a recognition event of Avr2 by Cf-2, but rather Pip1 is the operative target of Avr2 (Shabab et al., 2008; van der Hoorn and Kamoun, 2008). However, the other role of Pip1 independent of Avr2, such as in other Cf/Avr-dependent, or even Cf/Avr-independent, HCD and defence, is unclear. This study found that Pip1 protein (spot 59) is not detected in seedlings that do not
develop \( \text{Cf-4} / \text{Avr4} \)-dependent HCD, but accumulate abundantly in seedlings mounting the HCD (Figs. 1 and 2). Employing VIGS analysis, the \( \text{N. benthamiana} \) homologue of the tomato \( \text{Pip1} \) (\( \text{NbPip1} \)) was found to be required not only for \( \text{Cf-4} / \text{Avr4} \)- and \( \text{Cf-9} / \text{Avr9} \)-dependent host plant defence to fungal pathogen \( \text{C. fulvum} \) but also for \( \text{Cf/Avr} \)-independent nonhost plant defence to the bacterial pathogen \( \text{Xoo} \) (Figs. 5-10, Table 3). This phenomenon that a protease may mediate defence to distinct types of pathogens has been reported for two papain-like cysteine proteases \( \text{Rcr3} \) and \( \text{C14} \). \( \text{Rcr3} \) is the target of two types of protease inhibitors, EPICs of the oomycete pathogen \( \text{Phytophthora infestans} \) and \( \text{Avr2} \) of fungal pathogen \( \text{C. fulvum} \), and plays a role in defence to both pathogens (Song et al., 2009). \( \text{C14} \) is a target of even more protease inhibitors from fungi, oomycetes, and bacteria. Among them are EPICs and a RXLR effector, \( \text{AvrBlb2} \), of \( \text{P. infestans} \), \( \text{Avr2} \) of \( \text{C. fulvum} \), and \( \text{RIP1} \) of \( \text{Pseudomonas syringae} \) (discussion in Kaschani et al., 2010, and references therein). Thus \( \text{C14} \) has been suggested to play a general role in immunity to fungal, oomycete, and bacterial diseases (Kaschani et al., 2010). Taken together, \( \text{Pip1} \), like \( \text{C14} \), may play a role in plant defence against a wide range of pathogens. Plant and pathogen interactors of \( \text{Pip1} \) remain to be further identified.

Previously, several MAP kinases have been found to be involved in regulation of \( \text{Cf-9/Avr9-initiated defence} \)

**Fig. 8.** Virus-induced gene silencing analysis for function of four genes in \( \text{Cf-9/Avr9-dependent hypersensitive cell death (HCD)} \) in \( \text{N. benthamiana} \) plants: (A) empty vector control (EV), (B) a \( \text{Phytophthora-inhibited protease 1 (Pip1)-like protein (59)} \), (C) a SIPK type of MAP kinase (b12), (D, E) an asparagine synthetase (b48), and (F) a LeMir-like protein (b60). The analysis is similar to that for Fig. 5 except that \( \text{Cf-9/Avr9-dependent HCD instead of Cf-4/Avr4-dependent HCD was checked}. \) Spot numbers at bottom right are as given in Supplementary Table S3.

**Fig. 9.** Virus-induced gene silencing analysis for function of four genes in \( \text{X. oryzae pv. oryzae (Xoo)-induced hypersensitive cell death (HCD)} \) in \( \text{N. benthamiana} \) plants: (A) empty vector control (EV), (B) a \( \text{Phytophthora-inhibited protease 1 (Pip1)-like protein (59)} \), (C) a SIPK type of MAP kinase (b12), (D, E) an asparagine synthetase (b48), and (F) a LeMir-like protein (b60). The analysis is similar to that for Fig. 5 except that Xoo-induced HCD instead of \( \text{Cf-4/Avr4-dependent HCD was checked}. \) Spot numbers at bottom right are as given in Supplementary Table S3.

**Fig. 10.** RNAi analysis for function of \( \text{Pip1} \) in \( \text{Cf-4/Avr4- and Cf-9/Avr9-dependent hypersensitive cell death (HCD)} \) and \( \text{X. oryzae pv. oryzae}-induced HCD in tobacco plants. A \( \text{Pip1} \) RNAi construct pC1305-35S::\text{Pip1-RNAi} was made, which carried a hairpin sequence cassette that comprised of two copies of a 300bp fragment of \( \text{Pip1} \) flanking the intron sequence of \( \text{P. vulgaris nitrite reductase gene (U10419)} \) in the opposite direction. \text{Agrobacterium} transformed with this construct was infiltrated into all sectors of left half leaves while \text{Agrobacterium} transformed with empty vector (EV) as control, into right half leaves of Sumsun NN tobacco plants. Two days after agroinfiltration, HCD in each sector of the RNAi-treated leaves was evaluated as described above.

of the tomato \( \text{Pip1 (NbPip1)} \) was found to be required not only for \( \text{Cf-4/Avr4- and Cf-9/Avr9-dependent host plant defence to fungal pathogen \text{C. fulvum but also for \text{Cf/Avr-independent nonhost plant defence to the bacterial pathogen \text{Xoo (Figs. 5-10, Table 3)}} \). This phenomenon that a protease may mediate defence to distinct types of pathogens has been reported for two papain-like cysteine proteases \( \text{Rcr3} \) and \( \text{C14} \). \( \text{Rcr3} \) is the target of two types of protease inhibitors, EPICs of the oomycete pathogen \text{Phytophthora infestans} and \( \text{Avr2} \) of fungal pathogen \text{C. fulvum}, and plays a role in defence to both pathogens (Song et al., 2009). \( \text{C14} \) is a target of even more protease inhibitors from fungi, oomycetes, and bacteria. Among them are EPICs and a RXLR effector, \( \text{AvrBlb2} \), of \text{P. infestans} , \text{Avr2} of \text{C. fulvum}, and \( \text{RIP1} \) of \text{Pseudomonas syringae} (discussion in Kaschani et al., 2010, and references therein). Thus \( \text{C14} \) has been suggested to play a general role in immunity to fungal, oomycete, and bacterial diseases (Kaschani et al., 2010). Taken together, \( \text{Pip1} \), like \( \text{C14} \), may play a role in plant defence against a wide range of pathogens. Plant and pathogen interactors of \( \text{Pip1} \) remain to be further identified.
responses. Two MAP kinases, WIPK (wounding-induced protein kinase) and SIPK (salicylic acid-induced protein kinase), are rapidly and transiently activated after elicitation in Cf-9 transgenic tobacco cell suspensions by Avr9 elicitor (Romeis et al., 1999). Recently, studies employing VIGS revealed that tomato MAP kinases LeMPK1/2/3 have different but also overlapping roles in Cf-4/Avr4-dependent HCD and resistance. LeMPK3, a tomato parologue of WIPK, is essential to both Cf-4/Avr4-dependent HCD and resistance, while LeMPK1 and LeMPK2, two tomato paralogues of SIPK, are only required for Cf-4/Avr4-dependent resistance and HCD, respectively (Stulemeijer et al., 2007). This study identified a differentially expressed protein spot, b12, as being the MAP kinase LeMPK1, which accumulates to a very high level in seedlings mounting Cf-4/Avr4-dependent HCD compared with those not showing HCD (Figs. 1 and 2). To further understand the role of b12, this study cloned a cDNA fragment from N. benthamiana. This 557 bp fragment has over 97% amino acid sequence identity to the counterparts of a set of plant SIPKs, including NbNTF4, NbSIPK, Ntf4, Ntf4-1, Ntf4-2, NtSIPK, and LeMPK1/2 (Supplementary Fig. S3A). Silencing of the gene(s) corresponding to this fragment abolished not only Cf-4/Avr4- but also Cf-9/Avr9-dependent HCD in N. benthamiana (Figs. 5–8; Table 3). This result, together with the previously reported data, clearly demonstrates that SIPKs are pivotal in regulation of Cf-dependent HCD and resistance. However, unlike NbPip1, silencing of NbPip1 (b12) did not alter Xoo-induced HCD (Fig. 9), implying that the role of NbPip1 (b12) is not so wide as NbPip1.

LeMir is named after its sequence similarity (54% amino acid identity) to miraculin, a protein converting a sour taste into a sweet taste by altering human taste perception (Brenner et al., 1998). However, according to the BLAST database similarity search, LeMir is most homologous to a tobacco tumour-related protein (mRNA, U66263; protein, AAC49969) with 82% identity at the amino acid level. Both LeMir and its two homologues belong to the soybean trypsin-inhibitor family on the basis of the sequences; however, their proteinase-inhibitory activity remains to be confirmed (Brenner et al., 1998). LeMir is accumulated in and secreted from roots in response to nematode infection, suggesting a role of LeMir in plant defence against nematode infection (Brenner et al., 1998). Furthermore, over-expression of the tobacco homologue of LeMir results in formation of HCD-like lesions (Karrer et al., 1998), which is in accordance with the current results. This study found that LeMir is translationally up-regulated in HCD+ tomato seedlings (Figs. 1 and 2) and that silencing of a LeMir homologue (Supplementary Table S1, Supplementary Fig. S3C) significantly compromised Cf-4/Avr4- and Cf-9/Avr9-dependent HCD and Xoo-induced HCD in N. benthamiana (Figs. 5–9; Table 3). Collectively, these data show that LeMir plays an important role in the regulation of a variety of types of HCD. However, whether this function is related to its possible proteinase-inhibitory activity requires further confirmation.

Asparagine synthetase (Asn) catalyses the ATP-dependent conversion of aspartate into asparagine, which is a central intermediate in nitrogen metabolism and contributes to nitrogen transport and storage in many higher plants. Thus Asn is a key regulator of nitrogen metabolism and flow. Role of Asn in plant disease resistance is unknown except that there is a clue that an Asn gene is transcriptionally up-regulated in tomato leaves infected by the bacterial pathogen Pseudomonas syringae pv. tomato (Olea et al., 2004). The current study found that Asn protein increasingly accumulates in HCD+ tomato seedlings compared with HCD− seedlings (Figs. 1 and 2) and that silencing of a LeAsn homologue in N. benthamiana, which has 79.3% amino acid sequence identity to LeAsn (Supplementary Table S1, Supplementary Fig. S3D), significantly compromised Cf-4/Avr4- and Cf-9/Avr9-dependent HCD and Xoo-induced HCD (Figs. 5–9, Table 3), indicating that maintenance of Asn-mediated nitrogen metabolism and transport pathway is important for the establishment of these types of HCD. Additionally, the growth of NbAsn-silenced plants was significantly retarded, with smaller and narrower leaves, and later the plants developed shoots and inflorescences (Fig. 4). These results are in agreement with those obtained from plants over-expressing an Asn gene, which shows more numerous and wider leaves and precocious bolting and flowering compared with control plants (Giannino et al., 2008). Therefore, the Asn genes merit exploitation in the breeding of crops with simultaneously high disease resistance and other good agronomic traits such as high yields and a short vegetative stage.

Other proteins possibly required for Cf-4/Avr4-dependent HCD included a small GTP-binding protein (b16), a late embryogenesis (Lea)-like protein (b46), and an ASR4-like protein (b67).

Combined differential expression profiling and RNAi analysis system is an efficient strategy to identify genes required for HCD and resistance and probably other biological processes

This study employed a strategy involving combined proteomic and RNAi analyses to identify genes required for HCD and disease resistance in plants. This strategy is one example of combining differential expression profiling and RNAi assays to identify important genes involved in biological processes. RNAi assays include a common technique that requires the construction of transgenic plants with a binary vector harbouring a hairpin-resulting sequence cassette and another more rapid and straightforward technique, VIGS, which avoids plant transformation (Burch-Smith et al., 2004; Xu et al., 2008).

The combined differential expression profiling and RNAi analysis system has been used to identify factors required for Cf/Avr-dependent HCD and disease resistance. Using cDNA-AFLP as a differential gene expression profiling tool and VIGS as a RNAi tool, a set of essential regulators of Cf-9/Avr9- and Cf-4/Avr4-dependent HCD and disease resistance were successfully identified, the former identifying...
the protein kinase gene ACIKI (Durrant et al., 2000; Rowland et al., 2005), two E3 ubiquitin ligase genes CMPGI (Gonzalez-Lamothe et al., 2006) and PUB17 (Yang et al., 2006), and a F-box protein gene ACIIF1 (Van den Burg et al., 2008) and the latter identifying a CC-NB-LRR type resistance protein analogue gene NRCl (Gabriëls et al., 2006, 2007). This study executed differential expression profiling at the translational level. Finally seven regulators of Cf-4/Avr4-dependent HCD were screened out (Table 3, Figs. 5–10).

The expression profiles for Cf-4/Avr4-dependent HCD at the protein level (this study) and the transcript level (Gabriëls et al., 2006; Hong et al., 2007; Zhu et al., 2008) are not identical. This could be because some genes are highly transcribed but are somehow translated less or not at all. In addition, this difference could be due to the experimental design: i.e., for some differentially expressed protein spots detected by 2-D PAGE analysis, the corresponding transcripts might not be identified by the previous cDNA-AFLP analysis because of the restriction enzymes (only one) used for this analysis (Hong et al., 2007; Zhu et al., 2008). Similarly, for some differentially expressed transcripts detected by cDNA-AFLP analysis, the corresponding proteins might not have been identified by 2-D PAGE analysis in this study because of the pH range of IPG (pH 3–7) and staining method (CBB) used for this analysis. Taken together, the combination of proteomic analysis and VIGS assay to identify genes required for Cf/Avr-dependent HCD in this study is still valuable, has been proved to be efficient, and is complementary to the previously employed combined cDNA-AFLP analysis and VIGS assay to identify new important components of Cf/Avr-dependent HCD.

Collectively, the current and others’ data demonstrate that differential expression profiling and RNAi analysis system is an efficient strategy to identify novel essential HCD and disease resistance regulators. Additionally, the major techniques included in the strategy are differential expression profiling analysis and RNAi (including VIGS) assay, which have been more and more extensively employed to dissect many other plant biological processes such as insect resistance, stress, development, and metabolism. In fact, this study found two genes, which encode a proteasome 20S beta 1.1 subunit and a BiP, that might be involved in regulation of plant cell death, and three genes, which encode a SIPK type MAP kinase, an ASN, and an ASR-like protein, that might be related with plant growth and development (Fig. 4, Table 2). Therefore, the combined differential expression profiling. The RNAi analysis system is potentially a versatile strategy to dissect a variety of plant biological processes.

Supplementary material

Supplementary data are available at JXB online.

Supplementary Table S1. Cloned tomato and N. benthamiana cDNA fragments corresponding to the differentially expressed proteins selected for VIGS functional analysis.

Supplementary Table S2. PCR primers used for amplification of the VIGS target gene fragments.

Supplementary Table S3. List of the protein spots differentially expressed between Cf-4/Avr4 (HCD+) and Cf-4 (HCD−) tomato seedlings and identified by MALDI-TOF or MALDI-TOF/TOF MS analyses.

Supplementary Fig. S1. HCD phenotypes.

Supplementary Fig. S2. The MS spectra data for the differentially expressed protein spots.

Supplementary Fig. S3. Alignments of amino acid sequences predicted from the cloned N. benthamiana fragments with the counterparts of the reported homologous sequences from tomato, N. tabacum and N. benthamiana.

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