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Proteomic analysis of *Arabidopsis* constitutive expresser of pathogenesis-related gene1 (*Cpr30/cpr1-2*) mutant

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Abstract

We have previously identified a gain-of-resistance mutant named *constitutive expresser of pathogenesis-related gene1* (*cpr30*/*cpr1-2*). The mutant has dwarf morphology and gives rise to constitutive resistance to the bacterial pathogen *Pseudomonas syringae* in *Arabidopsis*. To elucidate the pattern of protein expression regulated by CPR1, we carried out a proteomic analysis of the *cpr1-2* mutant compared with wild type (WT). Proteins of both 4-week-old WT and *cpr1-2* plants were extracted by TCA-acetone-phenol and subjected to two-dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption/ionization-time-of–flight (MALDI-TOF) mass spectrometry. The results showed that the expression levels of some proteins were significantly affected in the *cpr1-2* mutant, including pathogen defense-related proteins, redox-related proteins, energy metabolism proteins, signal transduction proteins, and proteins involved in protein folding and homeostasis. A majority of the identified proteins were related to reactive oxygen species (ROS) and some were central components of the unfolded protein response (UPR), suggesting that the mutation of CPR1 evoked oxidative stress and UPR signaling pathway activation, which may have contributed to the gain-of-resistance phenotype of *cpr1-2*. Some novel components of the differentially expressed proteins, such as GSR1, GB3, ClpC1, and ClpP5 that are rarely reported to be related to pathogen defense, were up-regulated in *cpr1-2*, indicating new functions of these proteins or CPR1.

Keywords: proteomics; CPR1; negative regulator; disease resistance.

Abbreviations： HR_hypersensitive response; MALDI-TOF_matrix-assisted laser desorption/ionization-time-of–flight; PR_pathogenesis-related; ROS_reactive oxygen species; SAR_systemic acquired resistance; 2-DE_two-dimensional gel electrophoresis; UPR_unfolded protein response.

Introduction

Plants have developed a complex and efficient defense mechanism to cope with pathogen attack. In incompatible interactions between plants and pathogenic microorganisms, the signal of pathogen infection can be recognized by host plants using their resistance (R) gene products and activating a series of defense mechanisms including changes in ion fluxes, a burst of reactive oxygen species (ROS), induction of defense-related genes, phytohormone accumulation, callose deposition, and eventually hypersensitive response (HR)-like cell death (Hoeberichts and Woltering, 2003; Zipfel and Robatzek, 2010). The HR, one of the most efficient and immediate resistance reactions against pathogen attack in plants, is a form of programmed cell death (PCD) that can restrict the growth and spread of a pathogen (Lam, 2004). Concomitant with the appearance of the HR, another important response, known as systemic acquired resistance (SAR), is induced in uninfected tissues. The SAR, which is characterized by elevated levels of salicylic acid (SA) and expression of pathogenesis-related (PR) proteins, confers long-lasting resistance throughout the plant to prevent subsequent infection by a broad range of pathogens (Durrant and Dong, 2004). Although many new components involved in plant immunity have been previously elucidated, some nodes of the intact pathway of plant defense remain undiscovered.

Impressive achievements in genome and transcriptome analyses have contributed greatly to our understanding of the defense responses in plants. For example, Ascencio-Ibanez et al. (2008) explored the altered gene expression profile of *Arabidopsis thaliana* infected with cabbage leaf curl virus (CaLCuV), and identified several CaLCuV-responsive genes encoding a diverse array of functional categories. However, transcriptome information alone is inadequate to deduce the gene function because mRNA levels are not always correlated well with protein levels due to post-transcriptional and post-translational regulation. Because proteins are directly related to actual function, proteomic studies can provide a more direct and accurate assessment of biochemical processes. Several proteomic studies of plant-pathogen interactions have been performed yet. Specific changes in the *Arabidopsis* proteome in response to basal- and R-gene mediated resistance have been compared (Jones et al., 2004). Proteomic approaches have also been utilized to investigate the disease resistance in other model plants including alfalfa, cucumber and rice (Colditz et al., 2004; Deepak et al., 2008; Mahmood et al., 2009).

One practical approach to study the plant resistance mechanism is the utilization of gain-of-resistance mutants because they display spontaneous lesions that resemble HR-like cell death in the absence of pathogens. Most of these mutants exhibit enhanced resistance to pathogen infection and constitutive activation of a defense mechanism (Lorrain et al., 2003). Several genes responsible for the gain-of-resistance phenotype have been isolated and characterized including membrane-associated proteins (Büschges et al., 1997; Lorrain et al., 2004), ion channel family members (Balagué et al., 2003), zinc-finger proteins (Dietrich et al., 1997), heat stress transcription factors (Yamanouchi et al., 2002), and ubiquitin/proteasome pathway enzymes (Zeng et al., 2004). It has been speculated that these genes are key players in defense pathways. Therefore, comprehensive study of gain-of-resistance mutants would provide valuable information for us to understand the plant immune mechanism.

The *Arabidopsis* mutant *cpr1-2*, like other gain-of-resistance mutants, exhibits HR-like cell death, SAR-like constitutive defense responses, and enhanced resistance to pathogens. The *cpr1-2* mutant was first identified and designated as *cpr30* (Gou et al., 2009). Later, it was found to be allelic to *cpr1* (Cheng et al., 2011); thus, the *cpr30* was renamed as *cpr1-2* and the gene responsible for the mutant was referred as *CPR1* in the following research. As an F-box protein, CPR1 interacts with ASK (SKP1) proteins to form SKP1-CULLIN-F-BOX (SCF) protein complex, and negatively regulates plant defense (Gou et al., 2009). More recent studies have shown that CPR1 controls the stability of two notable plant resistance proteins, SNC1 and RPS2, by directly targeting them for ubiquitination and 26S proteasome-dependent degradation (Cheng et al., 2011; Gou et al., 2011). To further identify CPR1-regulated proteins and investigate the resistance mechanism of *cpr1-2*, we compared the protein profiles of wild type (WT) and *cpr1-2* mutant using two-dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption/ionization-time-of–flight (MALDI-TOF) mass spectrometry.

Results and Discussion

Comparison of protein profiles between wild-type and cpr1-2 mutant

As described in previous studies (Gou et al., 2009), the *cpr1-2* mutant had a severely dwarfed stature and exhibited an HR-like morphology in the absence of pathogens (Fig 1). To investigate the regulatory role of CPR1 in defense responses, both 4-week-old WT and *cpr1-2* plants were harvested, and proteomic analyses of the total proteins were carried out. The proteins were separated in a 24-cm gel within a 4-7 pI range and a 10-120 kDa size range. The representative Coomassie brilliant blue (CBB)-stained 2-DE gel profiles of WT and *cpr1-2* plants are shown in Fig 2A. More than 1200 protein spots were detected in the gels, and approximately 950 protein spots were matched between the three WT gels and three mutant gels. Analysis of the 2-DE patterns revealed that the expression levels of 52 proteins in *cpr1-2* were significantly changed by more than 2-fold ($P<0.05$), including 51 up-regulated spots and 1 down-regulated spot (Fig 2B). Some of the spots, such as 1225 and 1268, were present in the mutant gels while being absent in the control gels. Other spots showed quantitative changes in intensity. For example, spot 1385 had higher abundance in *cpr1-2* plants and very low abundance in WT plants (Fig 2C).

Identification of differentially expressed proteins

The 52 protein spots showing statistically significant increase or decrease were excised from gels, digested with trypsin and identified by MALDI-TOF mass spectrometry. Thirty-three spots, the majority of which were strongly up-regulated with sufficient abundance, had high and credible identification scores. The remaining proteins could not be identified due to their low concentration. Thirty-one proteins were identified from the 33 spots, whereas spots 516 and 542 represented the same protein, ribulose-1,5-bisphosphate carboxylase/ oxygenase (Rubisco) large subunit, and beta-1,3-glucanase 2 which were identified in spots 1145 and 1368. The identified proteins were classified into five groups based on their putative functions: pathogen-defense, redox homeostasis, energy metabolism, signal transduction, and protein folding and homeostasis (Table 1). These proteins may function together to maintain the continuous disease resistant reaction.

Pathogen defense-related

The *cpr1-2* mutant has a spontaneous HR phenotype and displays remarkable resistance against pathogen infection. As expected, three PR proteins, PR2 (spot 1161), PR5 (spot 1385) and a PR3 member (the chitinase class 4-like protein, spot 1225), were found to be significantly up-regulated in the *cpr1-2* mutant. PR2 and PR5 are markers for SA-activated SAR (Pieterse and Van Loon, 2004). The chitinase class 4-like protein catalyzes the hydrolysis of chitin and functions as a fungal cell wall degrading enzyme (Xu et al., 2007).

Arginase was identified in spot 1050. Arginase hydrolyzes arginine to urea and ornithine and is a key enzyme in plant polyamine biosynthesis, and its expression can be induced by wounding, jasmonic acid treatment and bacterial pathogen attack (Chen et al., 2004). Spot 812 was identified as SGT1a. SGT1a is required for the stabilization of many R proteins and is an essential component that is involved in R gene-mediated disease resistance. Mutant analysis in *Arabidopsis* showed the SGT1a functions in pathogen infection. Overexpression of this gene could enhance pathogen resistance. Furthermore, SGT1a could interact with RAR1 and SKP1 (Azevedo et al., 2006); the former is a rate-limiting positive regulator of multiple R gene-triggered responses (Muskett et al., 2002); whereas the latter is a component of the SKP1/Cullin1/F-box protein (SCF) ubiquitin ligase complex, suggesting that SGT1a functions are in disease resistance through a proteasome-mediated proteolytic pathway.

Redox homeostasis

Many gain-of-resistance mutants with phenotypes mimicking *cpr1-2* have acquired their enhanced resistance to pathogen invasion partially through the accumulation of ROS (Lorrain et al., 2003). The generation of ROS is an important early signaling event during plant-pathogen interaction, and it often triggers oxidative stress that disturbs cellular redox homeostasis. Six proteins involved in redox homeostasis were identified to be up-regulated in the *cpr1-2* mutant, including four GSTs, NUDT7, and SDR3.

The *Arabidopsis* Glutathione S-transferase (GST) family is comprised of a large number of enzymes that catalyze the conjugation of a number of electrophilic compounds to the tripeptide glutathione (GSH). The GST family functions as an inevitable enzymatic antioxidant system because it protects

Table 1. Differentially expressed proteins between WT and *cpr1-2* mutant seedlings.

Spot no.	Protein name	NCBI accession no.	Molecular weight (kDa)	Isoelectric point	Mascot score	No. matching peptides	Protein coverage (%)	Fold increase ($p<0.05$)
Pathogen defense-related								
1161	PR2	15230262	37316	4.85	110	9	36	34.5071
1385	PR5	15222089	26148	4.75	144	9	45	38.9853
1368	Beta-1,3 glucanase -2	166916096	17935	4.74	91	5	46	∞ *
1225	Chitinase	15224308	30668	5.78	88	7	31	∞
1050	Arginase	79325029	29295	6.62	91	8	47	2.0533
812	SGT1a	17017308	39253	4.98	65	4	18	2.27984
Redox-related								
1326	GSTF2	2554769	23983	5.93	309	22	90	6.07185
1348	GSTF6	15218640	23471	5.80	188	12	68	6.87657
1336	GSTF7	15218639	23583	6.14	153	12	56	4.03006
1354	GSTF8	20197312	24119	6.09	188	16	80	7.68229
1097	NUDT7	15235458	32092	5.32	204	16	55	7.36295
1270	SDR3	15226503	27058	6.53	61	5	29	∞
Energy metabolism								
516	Rubisco large subunit	7525041	53435	5.88	207	19	41	∞
202	Aco2	4586021	98720	5.79	180	19	32	3.39045
204	Aco3	118572817	108427	6.71	139	13	17	4.54606
491	F6N23	6382043	66980	6.62	204	21	47	3.36872
899	GSR1	15240288	39318	5.28	132	11	40	4.91545
1310	CA1	30678350	37426	5.74	113	10	48	0.43695
Signal transduction								
564	CRT1	79320099	49283	5.58	110	14	31	2.72835
658	CRT2	2155517	48157	4.36	109	12	26	4.12104
1110	Calmodulin	23197656	26813	4.84	110	10	50	∞
1268	GB3	21617896	24405	5.82	74	10	43	∞
Protein folding and homeostasis								
477	PDIL1-1	15219086	55857	4.81	248	17	45	6.03676
455	PDIL1-2	15223975	56614	4.9	267	17	46	5.69277
255	PDIL1-3	22331799	64400	4.74	135	19	35	4.81655
974	PDIL2-1	79324941	35598	5.72	68	4	24	2.78945
318	BIP	15241844	73584	5.08	203	19	28	3.61292
328	HSC70-2	15241847	71748	5.03	224	19	32	∞
237	ClpC1	18423214	103616	6.36	204	26	31	3.86138
1376	ClpP5	18378982	32335	8.35	74	6	22	2.41935
720	RAD23d	15240922	40065	4.58	69	5	10	2.45482

* The protein spot only present in the mutant gels and absent in the WT gels.

Fig 1. Phenotype of wild type (WT) and *cpr1-2* mutant. Four-week-old WT plants (Col-0) and *cpr1-2* mutants grown in soil. Compared with WT, the mutant was severely defected for growth, exhibiting dwarf stature and backward curly leaves.

plant cells from oxidative damage by ROS scavenging (Marrs, 1996). The expression of GSTs can be induced by a wide range of stimuli, such as chilling (Seppänen et al., 2000), hypoxic stress and dehydration (Moons, 2005), wounding (Vollenweider et al., 2000) and pathogen attack (Mauch et al., 1993). In the present study, we identified four GSTs that were induced in *cpr1-2*: GSTF2 (spot 1326), GSTF6 (spot 1348), GSTF7 (spot 1336) and GSTF8 (spot 1354). These GSTs all belonged to the plant-specific class phi, and they were previously found to be up-regulated by pathogen attack (Jones et al., 2004). Furthermore, the promoter of GSTF8 contained an Ocs element (Chen and Singh, 1999) – an element that is activated during the plant defense response.

NUDT7 (spot 1097) encodes a protein with nudix hydrolase activity that is an NADH pyrophosphatase and ADP-ribose pyrophosphatase. It plays an important role in maintaining cellular redox homeostasis, mainly through modulating the levels of NADH and ADP-ribose (Ge et al., 2007). Overexpression of *NUDT7* confers enhanced tolerance to oxidative stress. The *NUDT7* transcript levels were rapidly and transiently induced during both abiotic stresses, such as ozone and osmoticum, and biotic stresses imposed by avirulent pathogens. NUDT7 was demonstrated to be a negative regulator of the basal defense response based on the observation that the *nudt7* mutant displays an enhanced resistance to bacterial pathogens (Jambunathan et al., 2010). These data implicate *NUDT7* as a key gene linking cellular redox changes and downstream signaling. Similar to *NUDT7*, *SDR3* (spot 1270) encodes a short-chain dehydrogenase/ reductase that catalyzes NAD(P)(H)-dependent oxidation/ reduction reactions. It was recently demonstrated that SDR3 functions as a positive factor in plant defense responses, partially through the regulation of AtPR-1 gene expression (Hwang et al., 2011). Hwang et al. (2011) also found that, although its homolog SDR1 plays a critical role in abscisic acid (ABA) biosynthesis, the SDR3 functions via other unknown pathways rather than ABA signaling.

Energy metabolism

High energy costs are associated with the activation and maintenance of the pathogen resistance mechanism. Thus, it is not unexpected that several proteins involved in the photosynthetic metabolism and citrate cycle pathways are identified. Spot 516 was identified as a CO_2- (and O_2)-fixing enzyme, ribulose 1,5-bisphosphate carboxylase/oxygenase – a very abundant protein that controls the crucial step of partitioning ribulose 1,5-bisphosphate between the assimilatory photosynthetic metabolism and its carbon-releasing counterpart, the photorespiratory pathway (Albuquerque et al., 2001). Spot 202 and spot 204 were identified as aconitases Aco2 and Aco3 that catalyze the conversion of citrate to isocitrate through a cis-aconitate intermediate. The two proteins have roles in regulating resistance to oxidative stress and cell death in *Arabidopsis* and *Nicotiana benthamiana*. The fact that aconitase affects the transcript level of a superoxide dismutase CSD2 may account for this regulation of resistance to oxidative stress and cell death (Wolfgang et al., 2007).

An NAD-dependent malic enzyme (NAD-ME2, spot 491) was induced in *cpr1-2*. The NAD-ME2 decarboxylates malate produces pyruvate and CO_2 in the presence of a divalent metal ion, using NAD as a cofactor. This enzyme plays a key role in the partitioning of carbon flux by regulating the levels of organic acids and reduced cofactors (Chang et al., 2003). Apart from being involved in the photosynthetic production of carbohydrates and photorespiration, non-photosynthetic roles for NAD-ME2 have also been proposed, including biosynthesis of proteins and lipids, cellular pH regulation, generation of H_2O_2 and defense responses (Tronconi et al., 2010). Another dramatically induced protein spot was identified as a glutamine synthetase (GSR1, spot 899) that catalyzes the first step in the conversion of inorganic nitrogen (ammonium) into an organic form (glutamine). The GSR1 functions in nitrogen assimilation and is responsible for the re-assimilation of the ammonia generated by photorespiration and the depletion of nitrogen during senescence (Miflin et al., 2002).

The abundance of protein spot 1310 was decreased in *cpr1-2*, which was identified as carbonic anhydrase 1 (CA1). The CA1 is a Zn-containing metalloenzyme that catalyzes the reversible hydration of CO_2 (Smith et al., 2000) and is engaged in a broad range of biochemical processes, involving carboxylation or decarboxylation reactions, such as photosynthesis and respiration. In addition to its function as a carbonic anhydrase, CA1 was also identified as a salicylic acid-binding protein (SABP3), and the expression level of CA1 was induced in *Arabidopsis* infected with *Alternaria*

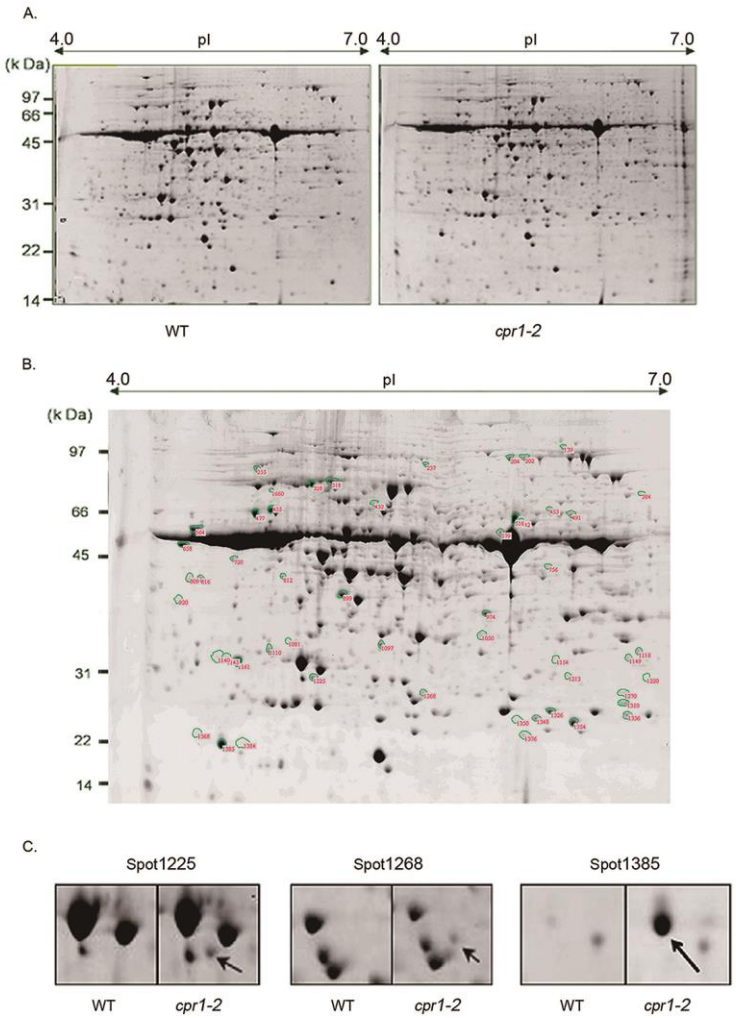

Fig 2. Two-dimensional electrophoresis protein profiles of WT and cpr1-2 mutant. (A) Comparison of protein patterns derived from WT and cpr1-2 plants. The gels were stained with CBB. (B) Protein spots differentially expressed in cpr1-2 plants. Image analysis was performed using Image Master 6.0, and the spots that showed significant differences were circled and numbered. (C) Examples of protein spots significantly different between WT and cpr1-2 mutant plants.

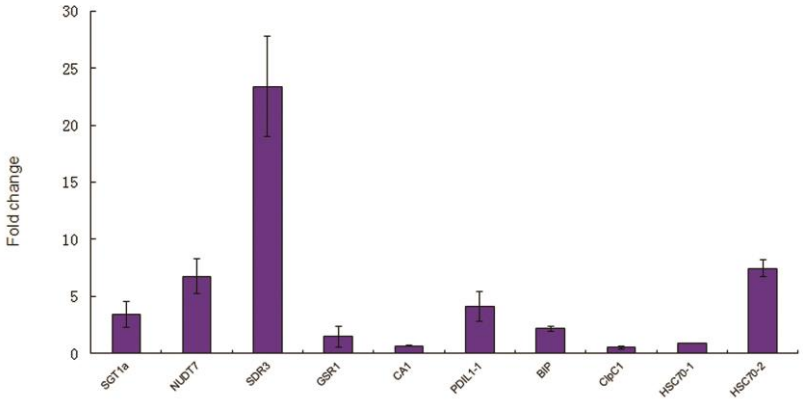

Fig 3. Expression changes at mRNA levels of several genes with significant difference at protein levels between WT and cpr1-2 mutant. Real-time RT-PCRs were performed to examine the relative mRNA levels of 4-week-old WT and cpr1-2 plants. The results indicated that the protein and mRNA levels were not well correlated. Error bars represent standard deviations from three replicates.

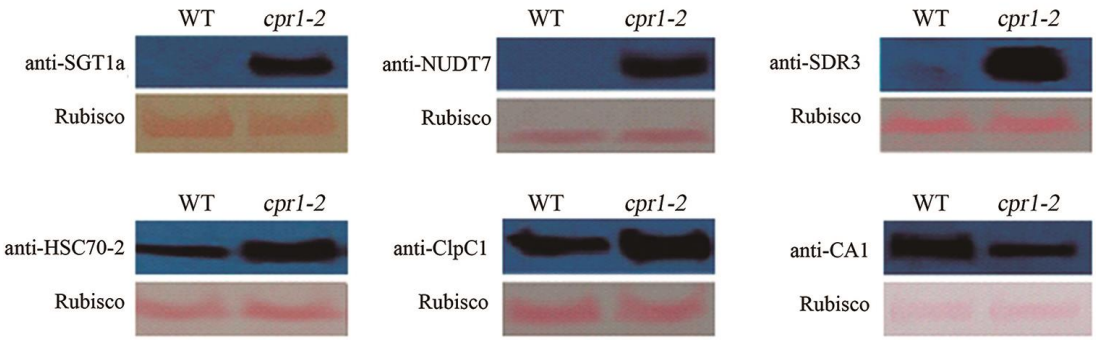

Fig 4. Western blot analysis of several differentially-expressed proteins in WT and cpr1-2 mutant. Four-week-old plants were harvested for western blot analysis. The results indicated that the expression of these six proteins was greatly affected in the cpr1-2 mutant, further confirmed the accuracy of 2-DE data. The ribulose bisphosphate carboxylase oxygenase (rubisco) stained by Ponceau S was used as protein loading control.

Table 2. Primers of selected genes for real-time PCR

Gene	Sense primer (5′- 3′)	Antisense primer (5′- 3′)
SGT1a	GGAATCGAATGGGACAGTGC	ACCAGGTTTCAAGATTTGTCTACAT
NUDT7	ATCGCGTGACCAAGCCTCTG	AGCACGTAAGCGTTGGGAGA
SDR3	CGTTGCACCATACGCGGTG	CTCCTTCAAATGGGCTTAACGACGC
GSR1	AATCTCTGCACCCACCCCTC	CCAGGTAGAGTCCTGGCTTTGC
CA1	TGGAGTGAAGAGATGGGAACCGA	TGACCCTTTGCGAGCTCACC
PDIL1-1	CTCGTGAAGCTGAGGGTATTG	TGTGCGAAATCTAACTCAGAG
BIP	TCACTTGGGAGGTGAGGACTTT	CTCACATTCCCTTCGGAGCTTA
ClpC1	TCATGGCTATGGCCACAAGGG	GGCACATTCATTGCCTGTCGC
HSC70-1	TAGCCGCCTTATTCGTCTTC	ATACCGATAGCTGGTCCTTCTC
HSC70-2	AAGAAACAGGAGAAGGATGAGAA	TGGTTCTGTTTCCTTGATCATTA
TUB2	GCCAATCCGGTGCTGGTAACA	CATACCAGATCCAGTTCCTCCTCCC

brassicicola (Mukherjee et al., 2010), suggesting that it may play a role in plant immunity with the activator SA. Other explanations for the decreased CA1 expression levels in *cpr1-2* require further investigation.

Signal transduction

Signal transduction factors are required for the activation of defense responses. Several proteins involved in signal transduction were identified (Table 1), i.e., two calcium ion-binding proteins (CRT1, spot 564; CRT2, spot 658), a calmodulin (CaM, spot 1110) and a small Rac-like GTP-binding protein (GB3, spot 1268). Calcium is an important second messenger involved in the signaling network of plants, and calcium signaling orchestrates a number of cellular physiological processes and stress responses, including pathogen infection. Calreticulin (CRT) is a ubiquitous endoplasmic reticulum-resident molecular chaperone involved in multiple cellular processes, such as protein folding and calcium homeostasis. Calmodulin is a conserved calcium receptor and plays an important role in sensing and transducing changes in cellular calcium concentration. Some CaMs are reported to be related to plant defense because their RNA and protein levels are elevated by pathogen infection (Luan et al., 2002). Small GTP-binding proteins regulate diverse processes in eukaryotic cells such as signaling, cell proliferation, cytoskeletal organization and intercellular membrane trafficking (Vernoud et al., 2003).

Protein folding and homeostasis

Many proteins, especially PR2 and PR5, had significantly increased expression levels that were associated with CPR1 mutation (Table 1). Several proteins were detected to promote the proper folding and organelle partitioning of proteins and/or to prevent the aggregation of nascent or damaged proteins. Most of which were molecular chaperones located in the endoplasmic reticulum, including a series of protein disulfide isomerase-like proteins (PDIL1-1, spot 477; PDIL1-2, spot 455; PDIL1-3, spot 255; PDIL2-1, spot 974), a binding protein (BIP, spot 318) and a 70 kDa heat-shock cognate protein (HSC70-2, spot 328). PDILs proteins contain thioredoxin (TRX) domains and act as catalysts of disulfide bond formation (Gruber et al., 2009); hence, stabilizing the tertiary and quaternary structures of protein folding. BIP and HSC70-2 are members of the HSC70/DnaK family, and are involved in assisting the folding of *de novo* synthesized polypeptides and the import/translocation of precursor proteins. These proteins are responsible for protein folding, assembly, translocation and degradation in a broad array of normal cellular processes (Vitale and Boston, 2008). They also function in the stabilization of proteins and membranes, and can assist in protein refolding under stress conditions.

RAD23d, a member of the Radiation Sensitive23 (RAD23) family, was identified in spot 720. Raasi and Wolf (2007) reported that besides the RAD23d role in nucleotide-excision repair, it is also involved in the endoplasmic reticulum-

associated degradation (ERAD) sub-pathway, because it removes incorrectly folded and misassembled secretory proteins via its association with the ubiquitin (Ub)/26S proteasome system.

Notably, BIP, PDILs, HSC70-2, as well as CRT1 and CRT2, all reside in the endoplasmic reticulum (ER). Induction of these ER-resident chaperones is a molecular signature of the unfolded protein response (UPR) (Zhang et al., 2006). Environmental stresses, such as oxidation and pathogen invasion often cause the accumulation of unfolded proteins in the ER. The UPR is the strategy employed by plants to restore normal ER function and prevents the potential cytotoxic impact of malformed proteins, mainly through promoting the folding and degradation of the newly synthesized proteins (Ye et al., 2011). The UPR signal plays an important role in plant immunity, possibly because ER chaperones are necessary for the secretion of PRs or primary target genes of NPR1 (Wang et al., 2005). Expression of BIP, PDILs, HSC70-2, CRT1 and CRT2 is induced by pathogen infection (Edith et al., 1999), and the mutants of these chaperones negate plant pathogen resistance (Wang et al., 2005). It has been proposed that the UPR is not only a feedback mechanism of plant immunity but also an early signal transduction pathway for plants to synthesize defense-related proteins.

Two proteases, ClpC1 (spot 237) and ClpP5 (spot 1376), were also identified as being induced in *cpr1-2*. The ClpC1 is an HSP100 chaperone, while ClpP5 is a proteolytic subunit, both of which belong to the ATP-dependent Clp protein family. The Clp proteases are critical regulatory factors for many metabolic cellular processes and are vital for degrading proteins damaged during environmental stresses. The mRNA and protein levels of Clp proteases are increased by intense light, cold and ozone (Zheng et al., 2002). Therefore, Clp proteases likely play a positive role in the stress response of plants.

Correlation analysis of mRNA and proteins levels

The protein and mRNA levels of post-transcriptional and post-translational modifications are not always correlated. We compared the mRNA abundance of some of the affected proteins via quantitative RT-PCR to determine whether there are any differences between mRNA and their protein levels. Good correlation between mRNA and protein levels was observed in some cases, but not in all others. As shown in Fig 3, the mRNA levels of *SGT1a, NUDT7, SDR3, PDIL1-1, BIP, HSC70-2* were up-regulated in the *cpr1-2* mutant, which were well correlated with the proteomics data. However, GSR1 and ClpC1, which were up-regulated at the protein level, did not have significant changes at the mRNA level, and CA1, the down-regulated protein, also did not change significantly at the mRNA level, indicating that some post-transcriptional events occurred. Furthermore, because *HSC70-1* is closely related to *HSC70-2*, we examined the mRNA level of *HSC70-1*. We observed only a small change (0.83-fold) in the *HSC70-1* mRNA level, which was in contrast to the large change observed in the *HSC70-2* mRNA level (7.41-fold), suggesting that HSC70-2, as opposed to HSC70-1, is more directly involved in the CPR1-regulated resistance pathway.

Confirmation of 2-DE data

Based on our 2-DE data, we decided to further investigate the expression levels of the following six proteins via western blot, including SGT1a, NUDT7, SDR3, HSC70-2, ClpC1 and CA1. As shown in Fig 4, western blot analysis also indicated that the expression of these proteins was greatly affected in the *cpr1-2* mutant indeed: SGT1a, NUDT7, SDR3, HSC70-2, ClpC1 were up-regulated and CA1 was down-regulated, implying that they may have important roles in defense responses. In addition, the western blot results also demonstrated that 2-DE analysis yields relatively accurate measurement of protein expression.

Materials and Methods

Plant materials and culture

The materials were sampled from *A. thaliana* ecotype Columbia-0 (WT) and *cpr1-2* mutant (Gou et al., 2009). Seeds were sterilized and sown on Murashige and Skoog medium supplemented with 0.8% agar and 3% sucrose. Petri dishes were incubated in a growth chamber (22 ºC, 16 h of daylight; light intensity 100 μE m^{-2} s^{-1}) for up to two weeks. *A. thaliana* lines were propagated under greenhouse conditions, as previously described (Gou et al., 2009).

Protein extraction and quantification

Four-week-old WT and *cpr1-2* plants were harvested for protein extraction. Three grams(g) of the material was homogenized to a fine power with a mortar and pestle in liquid nitrogen, and then transferred to 50 mL tubes containing 30 mL 10% (w/v) trichloroacetic acid/0.07% β-mercaptoethanol in acetone. The mixture was vortexed for two minutes and then incubated overnight at -20 ºC. Afterwards, tubes were centrifuged at 13,000 g for 40 minutes at 4 ºC and the supernatants were discarded. The pellets were washed with 30 mL ice-cold acetone containing 0.07% β-mercaptoethanol for at least three times and then dried at 4 ºC. The crude extract was resuspended in a lysis buffer containing 7 M urea, 2 M thiourea, 2% w/v CHAPS, 65 mM DTT, and 1 μL bromophenol blue (BPB). The mixture was centrifuged at low temperature for 20 min at 13,000 g, with the supernatants collected into fresh tubes. The protein extracts were quantified using a 2-D Quant kit (GE Healthcare) with bovine serum albumin as the standard. Approximately 1 mg was used for 2-DE.

Two-dimensional gel electrophoresis (2-DE)

One mg protein dissolved in 450 μL rehydration solution [8 M urea, 2% w/v CHAPS, 0.002 g/mL dithiothreitol (DTT) and 1% immobiline drystrip gel (IPG) buffer] and then was loaded onto an Immobiline™ DryStrip (GE Healthcare, pH 4–7, 24 cm) for the first dimension of separation that performed on an Ettan IPGphor IEF system (GE Healthcare Bio-Sciences AB). After a total of 90,000 VHS IEF, the strips were incubated in an equilibration buffer (6 M urea, 30% w/v glycerol, 2% w/v SDS, 50 mM Tris-HCl pH 8.0 and 1% bromophenol blue) first with 0.01 g/mL DTT (GE Healthcare) for 15 min, and then with 0.025 g/mL iodoacetamide (GE Healthcare) for another 15 min. The second electrophoretic dimension was performed on 12.5% SDS-PAGE using the Ettan Dalt six apparatus (GE Healthcare Bio-Sciences AB) and electrophoresed at 0.5 W/gel for one hour followed by 20 W/gel at 20 ºC until the bromophenol blue dye front had run off the bottom of the gels. The gels were stained by Coomassie blue R250.

Image scanning and statistical analysis

The 2-DE gel images of three different experiments were analyzed using ImageMaster 2D Platinum 6.0 (GE Healthcare). Image spots were initially automatically detected, matched and then manually edited as necessary. The intensity volume of each spot was processed by background subtraction and total spot volume normalization. Each gel analyzed was matched to the reference gel, and matched spots were grouped into subclasses. The normalization method, provided by ImageMaster 2D Platinum 6.0 software, divides each spot volume value by the sum of the total spot volume values to obtain individual relative spot volumes. The differences in expression between WT and mutant were analyzed by Student's *t*-test with $P<0.05$ considered significant.

In-gel digestion and mass spectrometry

The differentially expressed protein spots was excised manually and in-gel digestion of these protein spots was performed by incubation at 37 ºC overnight according to the method previously described (Giribaldi et al., 2007). Samples were loaded onto an AnchorChip target plate (Bruker Daltonics) and mass spectrometry analysis was performed using an AutoFlexII TOF/TOF mass spectrometer (Bruker Daltonics) in a positive ion reflection mode with a 20 kV accelerating voltage and 150 ns delayed extraction time (Su et al., 2011).

The peptide fingerprint was analyzed by Flex Analysis 3.0 software (Bruker Daltonics) and identified by searching the mass profiles of the NCBInr database using the search engine MASCOT (http://www.matrixscience.com) with the following parameters: trypsin as cleaving enzyme, peptide mass tolerance 30 ppm, and one missed cleavage allowed. Carbamidomethylation of Cys and oxidation of Met were set as fixed modifications.

Real-time quantitative RT-PCR

Four-week-old WT and *cpr1-2* plants were used for RNA extraction using the Trizol reagent (Invitrogen). Reverse transcription was carried out on 1 μg RNA using the First Strand cDNA Synthesis kit (Invitrogen) following the manufacturer's protocol. PCRs were performed using the SYBR Green Mix (Takara) with the ABI PRISM 7300 Sequence Detection System (Applied Biosystems). Amplification was monitored in real-time and computes for each reaction the threshold cycle (Ct), defined as the PCR cycle at which exponential growth of PCR products begins. The expression level was normalized to the *Tubulin2* (*TUB2*) control. Specific primers for each gene selected are shown in Table 2.

Western blot assay

Protein extraction, separation, and immunoblot assay were performed as previously described (Xu et al., 2008). The primary antibodies were made by Abmart Company, and a horseradish peroxidase–conjugated goat anti-rabbit antibody also from Abmart company was used as the secondary antibody. The protein membranes were visualized using an enhanced chemiluminescence kit (Roche) and exposed to x-ray films.

Conclusion

In the present study, we investigated the CPR1-regulated proteins by comparing the proteomic profiles of WT with cpr1-2 mutant plants using 2-DE gels coupled with MALDI-TOF-MS. Fifty-two differentially expressed proteins were revealed, and 33 of them were further identified by MS analysis. These proteins were involved in several physiological processes that function cooperatively during the plant defense response and are responsible for the resistance phenotype of *cpr1-2*. A majority of the unregulated proteins were involved in responses to pathogen invasion, confirming that CPR1 is a negative regulator of the plant defense response. Furthermore, a large percentage of the identified proteins were implicated in ROS generation, modulation and detoxification, and proteins with central roles in the UPR, suggesting that oxidative stress occurred and the UPR was activated in *cpr1-2*. Some proteins, such as GSR1, GB3, ClpC1 and ClpP5, were up-regulated in *cpr1-2*, which are rarely reported to be related to the plant defense and their functions are still unclear. The identification of novel CPR1-regulated proteins not only provides new insights to elucidate molecular mechanisms involved in a lesion mimic mutant but also contributes to our understanding of how the ubiquitin pathway relates to plant immunity.

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