Proteome Analysis of Programmed Cell Death and Defense Signaling Using the Rice Lesion Mimic Mutant *cdr2*

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We have previously identified three lesion-mimic mutants, cell death and resistance (*cdr*), in rice. These mutants induce a series of defense responses, including expression of defense-related genes and high accumulation of phytoalexins, indicating that the *cdr* mutants are useful materials to study programmed cell death and defense signaling in rice. Here, we carried out a proteome analysis of the *cdr2* mutant. Total proteins prepared from the wild type and the *cdr2* mutant at three different stages of lesion formation were compared using two-dimensional electrophoresis. We found a total of 37 proteins that were differentially expressed between *cdr2* and wild type. Among them, 28 spots were up-regulated and nine were down-regulated in the *cdr2* mutant. All the protein spots were identified by mass spectrometric analysis. These differentially regulated proteins included defense-related proteins. In addition, 27 proteins were classified as metabolic enzymes, suggesting that the programmed cell death that occurs in the *cdr2* mutant is associated with active metabolic changes. Our study shows that proteome analysis is a useful approach to study programmed cell death and defense signaling in plants.

Plants have evolved defense mechanisms, including the production of phytoalexins, accumulation of antimicrobial proteins, and rearrangement of the cell wall, to protect themselves against microbial attack. One of the major defense systems is the hypersensitive response (HR), which is characterized by rapid induction of local cell death around the infection site of an avirulent pathogen and is thought to confine the pathogen by stopping it from spreading from the invasion site (Dangl et al. 1996; Greenberg 1997; Heath 2000; Lamb and Dixon 1997; Mayer et al. 2001; Pontier et al. 1998). HR cell death requires an active plant metabolism and depends on the activity of the host transcriptional machinery (Hoeberichts and Woltering 2003), including dramatic changes in calcium influx, generation of reactive oxygen species (ROS), expression of pathogenesis-related proteins, production of phytoalexins, and cross-linking of components of the cell wall. HR cell death is also accompanied by morphological changes similar to those in apoptosis in animals, such as nuclear condensation, DNA laddering, and cell shrinkage (Hoeberichts and Woltering 2003; Jacobson et al. 1997).

Many mutants that show spontaneous cell death in the absence of pathogen infection, which are called lesion mimics,

have been identified in *Arabidopsis thaliana* (Balague et al. 2003; Ishikawa et al. 2001; Mach et al. 2001; Nandi et al. 2003; Pilloff et al. 2002), maize (Hu et al. 1998; Simmons et al. 1998), barley (Wolter 1993), and rice (Arase et al. 2000; Takahashi et al. 1999; Yamanouchi et al. 2002; Yin et al. 2000). Many of these mutants exhibit the expression of markers associated with defense responses, including accumulation of auto-fluorescent material, callose deposition, ROS production, increased expression of defense-related genes, elevated salicylic acid level, and activation of systemic acquired resistance. Therefore, defense responses may be constitutively activated in these mutants, indicating that these mutants are useful tools for understanding the comprehensive mechanism of defense responses.

Impressive achievements in genome and cDNA sequencing have yielded a wealth of information for many model organisms, including the plants *Arabidopsis*, and rice. However, sequence information alone is insufficient to deduce gene function. Transcriptome studies using microarray and serial analysis of gene expression technologies are powerful tools to address gene function; however, mRNA expression profiles may represent only putative function, because there is still a questionable correlation between mRNA and protein levels (Futcher et al. 1999; Gygi et al. 1999). In contrast, proteomics provides a more direct assessment of biochemical processes by monitoring the actual proteins performing the enzymatic, regulatory, and structural functions. Recent improvements of high-resolution two-dimensional gel electrophoresis (2-DE) (Gorg et al. 1999; Klose and Kobalz 1995), development of protein and nucleotide databases, and methods for protein identification utilizing modern mass spectrometry, such as matrix-assisted laser desorption ionization time-of-flight mass spectrometry and tandem mass spectrometry (MS/MS), have made the large-scale profiling and identification of proteins a dynamic new area of research in plant biology.

We have previously identified three lesion-mimic mutants (*cdr1*, *cdr2*, and *cdr3*) in rice (Takahashi et al. 1999). These mutants induce activation of defense-related gene expression and high accumulation of phytoalexins. Thus, the *cdr* mutants are good materials to analyze defense-related proteins in rice. Experiments using a protein phosphatase inhibitor, calyculin A, suggested that CDR1 and CDR2 negatively regulate the phosphorylation of proteins involved in the defense-related pathway associated with NADPH oxidase (Takahashi et al. 1999). We recently identified four phosphorylated proteins in *cdr* mutants by using the phospho-proteome method with 2-DE. One of them was prohibitin, which is known to be a chaperone in the assembly of a mitochondrial respiratory chain complex in yeast and mammalian cells (Takahashi et al. 2003).

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Until now, there have been few reports of proteome analysis that focused on the defense signaling pathway in plants (Kim et al. 2003; Peck et al. 2001). Here, we report results of a proteome analysis of the *cdr2* mutant to study programmed cell death and defense signaling in rice. Proteins extracted from the wild type and *cdr2* were compared using 2-DE and identified by the mass spectrometry system. We identified 37 proteins that were differentially expressed in the wild type and *cdr2*. Some of them were likely associated with defense signaling. In addition, many metabolic enzymes were affected by the *cdr2* mutation, indicating that extensive metabolic changes are associated with programmed cell death in rice.

RESULTS AND DISCUSSION

Identification of proteins up- or down-regulated in *cdr2*.

The level of defense resistance to rice blast in *cdr2* is dependent upon lesion formation (Takahashi et al. 1999). The *cdr2* plant exhibits strong resistance when the lesions are fully formed. We used three different stages of leaves, based upon the degree of lesion formation: NL = no lesion, leaves of a 30-day-old *cdr2* plant without any lesions, FL = few lesions, leaves of a 40-day-old *cdr2* plant with few lesions, and ML = many lesions, leaves of a 40-day-old *cdr2* plant with many lesions (Fig. 1).

To identify changes in protein expression during the development of lesions, we used the total protein extracts of *cdr2*-NL, *cdr2*-FL, *cdr2*-ML, and wild type as a control. The proteins extracted using a 7 M urea and 2 M thiourea buffer were precipitated with trichloroacetic acid (TCA) and were analyzed by 2-DE. Gel electrophoresis was performed with pH 4.0 to 7.0 strips and 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel. Approximately 400 protein spots were detected in two-dimensional gels with Coomassie brilliant blue (CBB) staining (Fig. 2A, typical electrophoresis data, wild type and *cdr2*-FL). CBB-stained 2-DE images were acquired with an image scanner and were analyzed to identify proteins differentially expressed between the protein samples. All spots were matched by gel-to-gel comparison, using PDQuest software, and the difference in the relative abundance of each spot protein was analyzed. Some of the spots detected in the gels were not reproducible in the repetitive experiments. Therefore, they were not analyzed in further experiments. As shown in Figure 2B, we identified 37 proteins that were differentially expressed between *cdr2* and wild type. The changes of these protein levels during lesion formation were quantitatively analyzed (Fig. 2C). Among them, 28 spots were up-regulated and nine were down-regulated during the lesion formation of *cdr2* (Fig. 3). These proteins showed >3.0-fold changes in three or more replicate gels. All spots were excised from the gels of wild type, *cdr2*-NL, *cdr2*-FL, and *cdr2*-ML, and the gel pieces were digested with trypsin and subjected to tandem mass spectrometry analysis. These MS/MS data from collision-induced ion fragments were searched against the equivalent theoretical masses derived from the National Center for Biotechnology Information (NCBI) nonredundant protein database, using Mascot software (Perkins et al. 1999). We identified all of them as listed in Table 1. The identified proteins were categorized into three classes based upon their expression pattern and four classes based upon their putative functions (Fig. 3A and B; Table 1). The protein levels of classes I and II were increased and reduced, respectively, along with lesion formation. In class III, the levels of protein were greater in the NL stage and gradually decreased at later stages.

Defense-related proteins.

Five proteins (spots 1, 2, 14, 16, and 21) of class I were abundantly accumulated in the ML stage. Two proteins, pro-

benazole-induced protein (PBZ 1) (spot 1) and caffeic acid 3-*O*-methyltransferase (COMT) (spot 2), have already been reported as defense-related proteins. The *PBZ 1* gene encodes an intracellular pathogenesis-related protein and is activated by treatment by probenazole, which is an effective inducer of host resistance against blast (Midoh and Iwata 1996). A high accumulation of PBZ 1 protein was consistent with our previous observation that *PBZ 1* gene expression is highly induced in lesion-mimic mutants, including *cdr2* (Takahashi et al. 1999). COMT, which is known as an enzyme involved in the phenylpropanoid pathway, is strongly induced after infection of pathogens and fungal-elicitor treatment (Dumas et al. 1988; Jaeck et al. 1992; Pellegrini et al. 1993, 1994). The phenylpropanoid components form a large class of secondary metabolites in plants as part of their antimicrobial defense tools. For example, flavonoids, isoflavonoids, stilbenes, monolignols, and lignins serve as inducible phytoalexins or preformed phytoanticipins in many plant species (Dixon 2001; Dixon et al. 2002), and the phenylpropanoid polymer can act as an inducible physical barrier against pathogen ingress (Mitchell et al. 1999).

We have identified two different ROS scavengers, glutathione *S*-transferase (GST, spot 5) and superoxide dismutase (SOD, spot 4). Interestingly, the levels of these proteins were opposite at the FL and ML stages; the level of GST was high in the FL stage and low at the ML stage, whereas SOD was down-regulated at the FL stage and up-regulated at the ML stage. Although both proteins are known to reduce ROS induced by biotic and abiotic stresses (Alvarez et al. 1998; Edwards et al. 2000), these proteins likely function at different stages of lesion formation. In addition, it is shown that spreading of lesions in the *Arabidopsis lsd 1* mutant is caused by the lack of up-regulation of SOD, which may result in the accumulation of superoxide (Kliebenstein et al. 1999). Thus, down-regulation of SOD at the FL stage suggests that an accumulation of superoxide may be necessary to establish the lesion phenotype.

Molecular chaperones.

We identified four molecular chaperone proteins (spots 6, 7, 8, and 31) that were up-regulated during the appearance of cell death lesion. Heat shock protein (Hsp) 70 and DnaK-type molecular chaperone BiP are members of the Hsp70/DnaK family, and chaperonin-60 and Rubisco subunit binding protein are members of the Hsp60/GroEL family (Fink 1999). These proteins likely play a role in the stabilization and facilitation of refolding of proteins that have been denatured during exposure to various stresses (Bukau and Horwich 1998; Fink 1999). Hsp70 and Hsp60 have been found to functionally cooperate

Fig. 1. Lesion-mimic phenotypes of the *cdr2* mutants. NL = leaves of 30-day-old *cdr2* plant without any lesions, FL = leaves of a 40-day-old *cdr2* plant with few lesions, and ML = leaves of a 40-day-old *cdr2* plant with many lesions.

in vitro and are regulated for the maintenance of proteins in cell (Langer et al. 1992). Additionally, in *Escherichia coli*, the DnaK and GroE systems cooperate for the preservation of aggregation in the folding of ferredoxin-NADP reductase (FNR) (Dionisi et al. 1998). FNR is known to be a member of ubiquitous flavoenzymes involved in oxidoreductive pathways in bacteria (Arakaki et al. 1997). Our data showed that FNR (spot 33) was up-regulated at the ML stage, suggesting that Hsp70 and Hsp60 may cooperate for pre-FNR processing in rice.

Metabolic enzymes.

The differentially expressed proteins contained 27 metabolic enzymes. S-adenosylmethionine (SAM) synthase (spot 3) catalyzes the conversion of L-methionine and ATP into SAM. SAM serves as a precursor of the plant hormone ethylene, which is induced by wounding, drought, and pathogen invasion (Arimura et al. 2002). SAM synthase accumulated during lesion formation in *cdr2*, suggesting that ethylene is involved in cell death. This is consistent with the fact that ethylene regu-

Fig. 2. Two-dimensional electrophoresis maps of wild type and *cdr2* mutants. **A**, Comparison of spot patterns derived from wild type and leaves of 30-day-old *cdr2* plant with few lesions (*cdr2*-FL). Isoelectric focusing was performed with 13-cm IPG strips, pH 4 to 7. After separation with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% acrylamide gel), the gels were stained with CBB. Image analysis was performed using PDQuest. **B**, Standard image constructed on the PDQuest software. Proteins with a spot number on the gels have more than three times higher or lower levels in wild type than those in *cdr2* mutants. **C**, Quantitative analysis of spot 8 during lesion formation. Spot 8 was encircled on each gel. These spots were quantified using the PDQuest software. The highest value was set equal to 100.

A**Class I** **B****Class II** **C****Class III**

Fig. 3. Classification of the identified proteins based upon expression pattern. The identified proteins were categorized into three classes; **A**, Class I or **B**, Class II were increased or reduced, respectively, along with lesion formation, and **C**, Class III was increased in the stage of NL (no lesions) and gradually decreased at later stages. 1, wild type; 2, *cdr2*-NL; 3, *cdr2*-FL (few lesions); 4, *cdr2*-ML (many lesions). The highest value was set equal to 100.

lates ROS production and spreading of cell death in the *eto1* and *eto3* mutants that overproduce ethylene in *Arabidopsis* (Wang et al. 2002).

Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) is a glycolytic enzyme. However, this protein also possesses a number of secondary functions including a cellular protein kinase, an mRNA binding protein for translation regulation, and a DNA repair enzyme with uracil DNA glycosylase activity (Meyer-Siegler et al. 1991). Recently, GAPDH has been shown to function as a positive mediator for apoptotic cell death in animals (Dastoor and Dreyer 2001). As shown in Figure 3 the level of GAPDH protein (spot 11) increased during the lesion formation of *cdr2*, suggesting that GAPDH may stimulate cell death in rice.

In this work, we identified three enzymes, putative transketolase, putative cytosolic 6-phosphogluconate dehydrogenase, and sedoheptulose-1,7-bisphosphatase precursor, involved in pentose phosphate pathway. Among them, 6-phosphogluconate dehydrogenase provides NADPH, which is required for the activity of plasma membrane NADPH oxidase to produce ROS

during a defense response (de Pinto et al. 2002). The protein level of 6-phosphogluconate dehydrogenase (spot 10) was up-regulated at the ML stage. Because the nature of *cdr2* mutation is assumed to be associated with the regulation of NADPH oxidase activity (Takahashi et al. 1999), it is possible that the up-regulation of 6-phosphogluconate dehydrogenase contributes to the activation of NADPH oxidase in lesion formation.

Aldehyde dehydrogenase (ALDH; spot 21) was highly accumulated in the ML stage. ALDH catalyzes the oxidation of various toxic aldehydes to carboxylic acids (Liu and Schnable 2002; Nakazono et al. 2000; Sunkar et al. 2003). A well-studied ALDH is betaine aldehyde dehydrogenase, which is induced in response to osmotic stress (Chen and Murata 2002). Over-expression of *Arabidopsis* ALDH results in the reduction of oxidative stress by scavenging toxic aldehydes (Sunkar et al. 2003). Thus, the up-regulation of ALDH in *cdr2* is likely one of the responses against oxidative stress, which occurs in lesion formation.

Ribulose-1,5-bisphosphate carboxylase (Rubisco; spot 37) was down-regulated during lesion formation. The spot was de-

Table 1. List of identification proteins

Protein type	Spot no.	Homologous protein	Source	Accession	Class	Mascot score	No. of peptide	Coverage (%)
Defense related	1	Probenazole-induced protein (PBZ1)	<i>Oryza sativa</i>	gi 7442204	I	914	7	38
	2	Caffeic acid 3-O-methyltransferase	<i>Oryza sativa</i>	AK061859	I	185	4	12
	4	Probable superoxide dismutase (EC 1.15.1.1) (Mn) 2 precursor Mn-SOD	<i>Oryza sativa</i>	gi 7433347	II	268	4	28
	5	Glutathione S-transferase ? (GST)	<i>Oryza sativa</i>	gi 3746581	I	243	4	22
Chaperone	6	Putative heat shock protein 70 (HSP70)	<i>Oryza sativa</i>	AK060410	I	399	8	16
	7	Mitochondrial chaperonin-60	<i>Oryza sativa</i>	gi 22758324	I	304	7	15
	8	DnaK-type molecular chaperone BiP	<i>Oryza sativa</i>	gi 7441868	I	535	8	16
Metabolism related	3	S-adenosylmethionine synthetase 2	<i>Oryza sativa</i>	gi 3024122	I	396	7	22
	9	Putative transketolase	<i>Oryza sativa</i>	gi 11875148	III	626	14	18
	10	Putative cytosolic 6-phosphogluconate dehydrogenase	<i>Oryza sativa</i>	gi 7363280	I	162	3	8
	11	Putative glyceraldehyde-3-phosphate dehydrogenase	<i>Oryza sativa</i>	gi 23306126	I	467	7	20
	12	Phosphoglycerate kinase	<i>Oryza sativa</i>	gi 50905557	II	226	4	18
	13	Phosphoglucomutase	<i>Oryza sativa</i>	gi 13324798	I	675	6	12
	14	Glutamine synthetase shoot isozyme	<i>Oryza sativa</i>	gi 121349	I	494	4	9
	15	NADP dependent malic enzyme	<i>Oryza sativa</i>	gi 12082337	I	236	5	12
	16	Cytoplasmic malate dehydrogenase	<i>Oryza sativa</i>	AC037425	I	642	9	14
	17	Putative oxalyl-CoA decarboxylase	<i>Oryza sativa</i>	AP003054	II	320	8	19
	18	Putative glycine dehydrogenase	<i>Oryza sativa</i>	gi 13366201	III	280	6	24
	19	Putative methionine synthase	<i>Oryza sativa</i>	AK067726	III	146	2	2
	20	Putative thiamine biosynthesis protein	<i>Oryza sativa</i>	dbj BAC45141.1	II	175	4	19
	21	Aldehyde dehydrogenase	<i>Oryza sativa</i>	gi 8163730	I	630	9	19
	22	Ferritin	<i>Oryza sativa</i>	gi 14091661	I	380	6	23
	23	Methylenetetrahydrofolate reductase (MTHFR)	<i>Oryza sativa</i>	AK062082	I	174	5	12
	24	Putative isoflavone reductase homolog IRL	<i>Oryza sativa</i>	gi 10945249	I	238	6	22
	25	Putative fructokinase ?	<i>Oryza sativa</i>	gi 16566704	II	327	6	22
	26	Vacuolar ATP synthase catalytic subunit A (V-ATPase A subunit)	<i>Oryza sativa</i>	gi 1352830	II	262	5	11
	27	Putative H ⁺ transporting ATP synthase	<i>Oryza sativa</i>	gi 13366196	I	571	5	8
	28	ATP synthase beta subunit	<i>Oryza sativa</i>	gi 21684925	I	367	7	16
	29	Sedoheptulose-1,7-bisphosphatase precursor	<i>Oryza sativa</i>	gi 27804768	II	411	6	20
	30	ATP synthase CF1 alpha chain	<i>Oryza sativa</i>	NC_001320	III	322	12	26
	31	Rubisco subunit binding-protein alpha subunit, chloroplast precursor	<i>Oryza sativa</i>	AK061410	I	394	7	14
	33	Ferredoxin-NADP reductase	<i>Oryza sativa</i>	gi 729478	I	237	9	19
	34	Photosystem II stability/assembly factor HCF136	<i>Arabidopsis thaliana</i>	NM_122218	I	212	5	11
	37	Ribulose 1,5-bisphosphate carboxylase/oxygenase large chain (fragment)	<i>Oryza sativa</i>	gi 11466795	II	78	2	6
Proteasome and protease	32	Putative ftsH chloroplast protease	<i>Oryza sativa</i>	AK061771	III	439	7	16
	35	ATP-dependent Clp protease	<i>Oryza sativa</i>	gi 32489942	I	366	7	8
	36	Proteasome subunit alpha type2	<i>Oryza sativa</i>	gi 12229921	II	320	6	25

tected at a lower molecular mass position (16 kDa) compared with the expected size (53 kDa) of Rubisco. This may be possibly explained by the observation that oxidative stress induces fragmentation of the Rubisco protein in vitro (Ishida et al. 1997, 1999).

Proteasome and protease.

We identified 20S proteasome subunit, alpha type 2 (spot 36), which was down-regulated in the initial stage of lesion formation. The 20S proteasome is one of the components of 26S proteasome involved in the recognition and degradation of ubiquitinated protein (Grune 2000). The ubiquitin/26S proteasome pathway has important roles in plant growth, development, and defense through protein degradation (Sullivan et al. 2003). Recently, it has been reported that the inhibition of proteasome activities results in the activation of programmed cell death in plants (Moonil et al. 2003), which is coincident with the down-regulation of a 20S proteasome subunit during the lesion formation of *cdr2*.

FtsH protease (spot 32) and Clp protease (spot 35) are known to be chloroplastic proteases involved in the removal of oxidatively damaged proteins and settlement of quantity of certain chloroplast proteins in response to a large variety of environmental conditions (Adam and Clarke 2002). The level of tobacco chloroplastic FtsH protease is reduced in *Tobacco mosaic virus*-infected leaves before the HR lesion appears, which results in the acceleration of cell death (Seo et al. 2000). The phenomenon is consistent with the fact that rice FtsH protease (spot 32) decreased during the lesion formation of *cdr2*. Clp protease (spot 35), an ATP-dependent serine protease, was up-regulated in the *cdr2* mutant. Clp protease has been reported to be increased at both the mRNA and protein levels by high light, cold, and ozone (Agrawal et al. 2002; Zheng et al. 2002). Therefore, clp protease is likely a positive factor to stress responses in plants.

In the present work, we have found a total of 37 proteins that were differentially expressed between the wild type and *cdr2*. All of them were identified by mass spectrometric analysis. Thus, this type of proteome analysis is shown to be useful for the identification of proteins that have important functions. Among 37 proteins that we have identified, four are likely to be defense-related. Interestingly, many metabolic enzymes were changed during lesion formation. These results suggested that the programmed cell death occurring in the *cdr2* mutant is accompanied with active metabolic changes. The comprehensive proteome analysis of defense-related mutants may provide new insights to understand signaling networks involved in programmed cell death and defense signaling in plants.

MATERIALS AND METHODS

Plant material.

The rice cultivar Kinmaze (wild type) and the *cdr2* mutant were used in this work. The plants were grown in a greenhouse, and their leaves were harvested at three different stages according to the levels of lesion formation.

Protein extraction.

Frozen rice leaves were ground in liquid nitrogen. The resultant powder was suspended in an extraction buffer (7 M urea/2 M thiourea; 4% (wt/vol) 3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulfonate (CHAPS); 20 mM dithiothreitol (DTT); 2% (vol/vol) immobilized pH gradient (IPG) buffer, pH 3 to 10. Crude homogenates were centrifuged at 4°C (9,000 × g for 30 min). The supernatants were precipitated by 10% TCA for 1 h at -30°C, followed by centrifugation at 13,000 × g for 30 min. The pellets were washed twice

with cold-acetone and were allowed to air dry. The dried samples were resuspended with extraction buffer and were kept frozen at -80°C. Protein contents were determined by the Bradford (1976) method using bovine serum albumin as a standard.

2-DE and image analysis.

For analytical separations, precast 13-cm immobilized pH gradient strips (pH 4 to 7, immobilized DryStrips; Amersham Biosciences, Piscataway, NJ, U.S.A.) were rehydrated overnight with 1.2 mg of sample proteins and 250 µl of a rehydration buffer (7 M urea/2 M thiourea; 4% (wt/vol), CHAPS; 20 mM DTT; 2% (vol/vol) IPG buffer, pH 4 to 7; bromophenol blue). Isoelectric focusing was performed for 36 kVh at 20°C with a Multiphor II unit (Amersham Biosciences). The strips were placed in a reduction buffer (1% DTT, 6 M urea, 2% SDS, 30% glycerol, 50 mM Tris-HCl, pH 8.8) for 20 min with gentle shaking. The strips were then transferred to an alkylation buffer (2.5% iodoacetamide, 6 M urea, 2% SDS, 30% glycerol, 50 mM Tris-HCl, pH 8.8) and were shaken for 20 min before being placed on a 12% polyacrylamide gel slab (160 × 160 × 1.0 mm). Separation in the second dimension was carried out using a Tris-glycine buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) at a current setting of 10 mA/gel.

The 2-DE gels were stained with Coomassie brilliant blue R-350 (Amersham Biosciences). 2-DE images were acquired by scanning with a scanner (GS-800 calibrated imaging densitometer; Bio-Rad, Hercules, CA, U.S.A.). Noise reduction, background subtraction, spot detection, quantification, gel-to-gel matching, and differential protein display analysis were carried out using the PDQuest software (Bio-Rad). Intensities of individual spots were normalized based upon the total density of all spots detected in each gel and compared between gels. Selected spots were excised and stored at 4°C in deionized water.

Protein identification.

Gel pieces excised from the CBB-stained gels were destained with 30% acetonitrile (Wako, Osaka, Japan), were washed with 100% of acetonitrile, and were dried in a vacuum concentrator. The dried gel pieces were absorbed with 2 µl of trypsin/50 mM ammonium bicarbonate (0.5 µg/µl) (Promega, Madison, WI, U.S.A.) (Shevchenko et al. 1996). After the digestion reaction at 37°C, the digested peptides were extracted with 5% formic acid and 50% acetonitrile. Trypsin-digested peptides were loaded on the column (PEPMAPC18, 5 µm, 75 µm internal diameter, 15 cm; Dionex, Sunnyvale, CA, U.S.A.) using the CapLC system (Waters, Wythenshawe, U.K.). Buffers were 0.1% HCOOH in water (A) and 0.1% HCOOH in acetonitrile (B). A linear gradient from 5 to 45% B for 25 min was applied, and peptides eluted from the column were introduced directly into a Q-tof mass spectrometer (Waters) with a flow rate of 100 nL/min. Ionization was performed with a PicoTip nanospray source (New Objective; Cambridge, MA, U.S.A.). Masses of precursor ions were scanned from m/z 400 to 1,500; scan time was 1.0 s. The collision voltage was 20 to 40 V, depending on the precursor mass. MS/MS spectrums were subjected to the Mascot server against a protein database from the NCBI. Mascot search parameters included digestion by trypsin with allowance of one missed cleavage, peptide tolerance ±0.2 Da, and MS/MS tolerance ±0.2 Da.

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