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Proteomic analysis of rice defense response induced by probenazole

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Abstract

Here, we report the first proteomic analysis of rice defense response induced by probenazole (PBZ), an agricultural chemical that has been widely used to protect rice plants from rice blast and the bacterial blight pathogen. Two-dimensional gel electrophoresis (2-DE) was utilized to identify a total of 40 protein spots including 9 protein spots that are up-regulated by PBZ and 31 abundant protein spots. A total of 11 unique proteins from these 9 spots were identified by LC–MS/MS, and the majority of them were classified and/or possessed orthologs in defense-related functions. Five protein spots with only one protein species identified in each spot appear to be PBZ-regulated proteins. They are a putative glutathione *S*-transferase GSTU17, a putative phenylalanine ammonia-lyase (PAL, XP_466843), a putative caffeic acid 3-*O*-methyltransferase (COMT), a putative NADH-ubiquinone oxidoreductase, and a putative glucose-1-phosphate adenyltransferase. However, the other six protein species identified from the remaining four protein spots could not be conclusively described as PBZ-regulated proteins due to either the co-migration of two protein species in one spot or the presence of one protein species in two spots. Through real-time reverse transcription polymerase chain reaction (RT-PCR), it was determined that PAL (XP_466843) is likely regulated at the protein level, whereas GSTU17 and COMT were regulated at the mRNA level after PBZ application. Interestingly, the mRNA transcripts of two PAL paralogs were found to be up-regulated by PBZ. We propose that PAL, COMT, and GSTU17 are likely to confer PBZ-induced disease resistance via such functions as biosynthesis and transport of flavonoid-type phytoalexin and/or lignin biogenesis.

Keywords: Rice; *Oryza sativa*; Graminaceae; Defense; Disease resistance; Probenazole; Proteomics

1. Introduction

For more than 20 years, probenazole **1**(PBZ, 3-allyloxy-1,2-benzisothiazole-1,1-dioxide), an agricultural chemical, has been widely used to protect rice plants from the rice blast fungus *Magnaporthe grisea* (Watanabe et al., 1977, 1979) and the bacterial blight pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) (Nakashita et al., 2003). It is believed that PBZ (**1**) induces the resistance response to protect plants from pathogen infection by inducing the systemic acquired resistance-like (SAR-like) response (Yoshioka et al., 2001). Interestingly, little is known about how PBZ serves as a plant activator and its mode of action. The accumulating evidence, however, demonstrates that PBZ (**1**) enhances the activities of defense-related enzymes such as peroxidase (Watanabe et al., 1979), lipoxygenase, phenylalanine ammonia-lyase (PAL) and catechol-*O*-methyltransferase (Iwata et al., 1980; Sekizawa et al., 1987). PBZ (**1**) induces the expression of pathogenesis-related gene *PR10 (PBZ1)* (Midoh and Iwata, 1996; Nakashita et al., 2001), the lipoxygenase-encoding *RCI-1* gene (Schaffrath et al., 2000), the mitogen-activated protein kinase gene *OsBIMK1* (Song and Goodman, 2002) and *RPR1,* a gene encoding a nucleotide binding site and leucine-rich repeats sharing structural similarity with known resistance genes (Sakamoto et al., 1999). More recently, cDNA microarray analysis discovered new PBZ-induced genes encoding a receptor kinase-like protein, caffeic acid 3-*O*-methyltransferase (COMT), and β-1,3-glucanase, although only 1265

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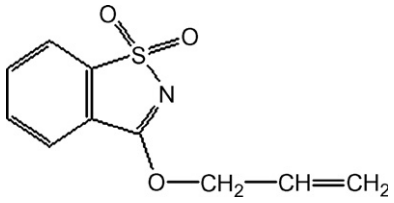
rice cDNA clones were analyzed (Shimono et al., 2003). Based on genetic studies in Arabidopsis and tobacco, PBZ (**1**) acts upstream of the salicylic acid (SA)-mediated signaling pathway (Nakashita et al., 2002; Yoshioka et al., 2001). The molecular mechanisms involved in PBZ-induced SAR-like response in rice, however, remain unclear.



Based on the concepts and evidence that mRNA levels do not always represent the levels of proteins (Gygi et al., 1999; Ideker et al., 2001; Kolkman et al., 2006; Nishizuka et al., 2003), proteomic analysis of protein profiling is equally important to understand how genes/proteins are regulated, since proteins usually carry out the functions. Of late, proteomics approaches have been adapted to address many questions in plant biology. With the availability of rice genome sequences (International Rice Genome Sequencing Project, 2005), the combination of high resolution two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) has been successfully employed to identify proteins expressed differentially in response to many biotic and abiotic stresses in rice (Agrawal and Rakwal, 2006; Komatsu et al., 2003). As a result, several known or putative defense-related proteins were identified as being induced by blast fungus (Kim et al., 2003, 2004; Konishi et al., 2001), sheath blight fungus (Lee et al., 2006), bacterial blight pathogen (Mahmood et al., 2006), ozone (Agrawal et al., 2002b), metal (Hajduch et al., 2001), and jasmonic acid (JA) (Rakwal et al., 1999). To date, however, no proteomic studies have been undertaken to reveal proteins regulated by PBZ.

Here we use proteomic approaches to provide an overview of PBZ-induced defense response in rice. Nine protein spots were up-regulated by PBZ (**1**) and result in 11 unique protein identifications. Further analysis indicated that *GSTU17*, *COMT*, and two *PAL* paralogs were induced at the mRNA level, while another PAL paralog may be regulated at the protein level by PBZ. The putative functions and roles of identified proteins played in the PBZ-induced disease resistance are discussed.

2. Results

2.1. Induction of rice defense response by PBZ

Oryza sativa subsp. *japonica* cv. Tainung 67 (TNG67), a rice cultivar widely grown in Taiwan, is susceptible to rice blast and bacterial blight. To determine whether PBZ (**1**) is capable of inducing a SAR-like response in TNG67, we assayed its effect in resistance to pathogen infections and induction of marker genes. Bacterial blight disease was chosen as the test disease, and the inoculation was performed 7 days after PBZ application. The plants treated with PBZ (**1**) had obviously shorter lesions than untreated plants (Fig. 1a). Real-time RT-PCR showed that the transcripts of two known PBZ-inducible genes, *PBZ1* and *RPR1*, increased significantly after PBZ(**1**) treatment (Fig. 1b), as compared with non-treated plants (C0D and C6D). Thus, PBZ (**1**) effectively induced a SAR-like response in TNG67.

2.2. Identifications of PBZ-regulated and abundant proteins of rice seedlings

The effectiveness of the PBZ-induced disease resistance against *Xoo* allows us to search for PBZ-regulated proteins. We compared the 2-DE proteome profiles from three independent experiments using 2D analysis software ImageMaster 2D and GeneSpring. The differential enrichment value, or fold change, of treatment over controls was obtained for the corresponding spot pairs and used to set the selection criteria for PBZ-regulated protein spots. While no significant PBZ-repressed protein spots were detected, we identified nine protein spots (spots 1–9) that accumulate $\geqslant$2-fold at 1 day (P1D), 3 days (P3D), and/or 6 days (P6D) after PBZ (**1**) treatments in comparison to the spots of non-treated samples (C0D and C6D) (Figs. 2 and 3) in each of three independent experiments. Although there are some variations of fold changes at certain time points among three independent biological repeats (shown as high standard errors), all nine protein spots indicates at least one time points with clear up-regulation ($\geqslant$2-fold with low standard errors).

Analysis of these nine protein spots by LC–MS/MS results in 11 unique protein identifications. All proteins identified were previously uncharacterized but had been assigned putative functions (Table 1), and the majority of them were classified and/or possessed orthologs in defense-related functions (Table 2) according to the classifications previously described (Bevan et al., 1998). Five protein spots (spots 1, 4, 6, 7, 9) with only one protein species identified appear to be PBZ-regulated proteins, while the other six protein species identified from the remaining four protein spots (spots 2, 3, 5, 8) could not be conclusively described as PBZ-regulated proteins (Fig. 3 and Table 1).

Spot 1, a putative glutathione *S*-transferase GSTU17, falls into the functional categories of metabolism and defense (Tables 1 and 2). GSTU17 is a PBZ-inducible protein as it is not detected or is only present at very low levels in the absence of PBZ (Fig. 3). Induction of the GSTU17 protein spot started as early as day 1 (P1D), but reached the highest expression at day 3 (P3D) after PBZ treatment. Spot 6 is a putative phenylalanine ammonia-lyase PAL (XP_466843) and spot 7 is a putative caffeic acid 3-*O*-methyltransferase COMT; both proteins are classified in the

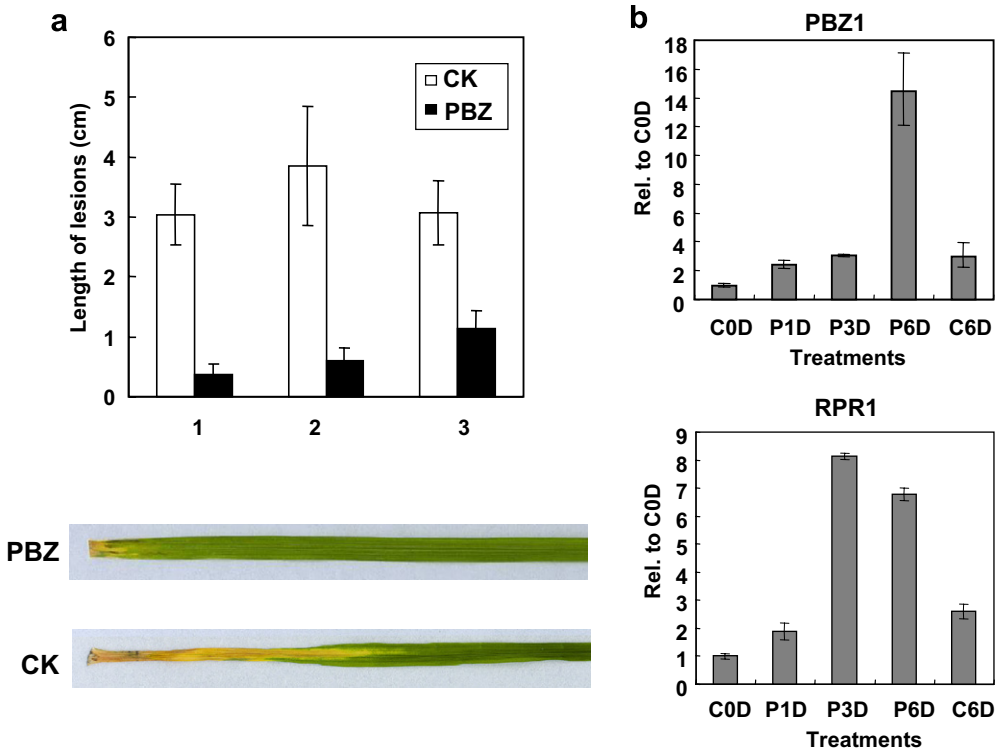


Fig. 1. Induction of rice defense response by PBZ. (a) Rice seedlings were inoculated with the bacterial blight pathogen *Xoo* 7 days after PBZ application. The length of lesions on infected leaves with (PBZ, black bar) or without (CK, white bar) PBZ treatments was measured from three independent experiments. The data are shown in average with standard errors from a minimum of 10 plants in each independent experiment. Photographs indicate representative disease symptoms. (b) The mRNA expression patterns of *PBZ1* and *RPR1* were quantified by real-time RT-PCR. The relative expression ratios of PBZ-treated samples at 1day (P1D), 3 days (P3D), 6 days (P6D) or untreated control for 6 days (C6D) to the sample before PBZ application (C0D) were determined in triplicates and were shown as average values with standard deviations. The expression levels were normalized using *OsS5A* as an internal control.

functional categories of secondary metabolism and defense (Tables 1 and 2). Both protein spots were up-regulated approximately 2- to 4-fold at day 3 (P3D), but were slightly down-regulated at day 6 (P6D) after PBZ (**1**) treatments (Fig. 3). The identification of these two proteins correlates with previous studies showing that PBZ (**1**) induced the enzymatic activity of PAL (Iwata et al., 1980) and that the cDNA of *COMT* was up-regulated by PBZ (**1**) as determined by microarray analysis (Shimono et al., 2003). Spot 4, a putative reductase with homology to NADH-ubiquinone oxidoreductase that catalyzes the redox reaction in the mitochondrion (Kerscher, 2000) and classified in the functional category of energy, is up-regulated by PBZ at least 3- to 4-fold (Fig. 3). Interestingly, spot 9, a putative glucose-1-phosphate (G1P) adenylyltransferase belong to the category of metabolism and involved in starch synthesis whose wheat ortholog is down-regulated by heat (Majoul et al., 2004) , is up-regulated by PBZ about 3-fold as early as day 1 (P1D) (Fig. 3).

Due to the co-migration of two protein species in one spot or the presence of one protein species in two spots, we cannot conclude whether the proteins identified from spots 2, 3, 5, 8 are PBZ-regulated proteins. Spot 5 was identified as a putative leucine aminopeptidase (LAP); this protein is also present in spot 3, co-migrating with a putative ATPase subunit B (AtpB), complicating the expression pattern of the putative LAP. It seems, however, that the putative LAP is likely involved in the PBZ-induced defense response, as LAP was also found to be induced by pathogen infection in tomato (Pautot et al., 1993) and wounding response in potato (Hildmann et al., 1992). Putative coproporphyrinogen III oxidase and a probable fructose-bisphosphate aldolase were found in spot 2, and a protein with homology to glycosyl hydrolase and a putative NADP-dependent malic enzyme were present in spot 9. Although it cannot be determined from the current data which protein is PBZ-regulated in spots 2 and 9, both coproporphyrinogen III oxidase and NADP-dependent malic enzyme have been identified as related to plant defense (Ishikawa et al., 2001; Walter et al., 1988).

Intrigued by the observation that the detected PBZ-regulated protein spots were low abundance proteins (Fig. 2), we also made efforts to identify the abundant protein spots (spots 10–40) present in the 2-DE proteome (Fig. 2, Supplementary Table 1). In contrast to that PBZ-regulated proteins are mostly classified in the defense functional category or possess orthologs that are induced by biotic and abiotic stress (Table 2), these abundant proteins mostly correspond to photosynthetic or chloroplast-related proteins that fall into the functional category of energy (Table 2). The most abundant protein Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) large subunit (spots

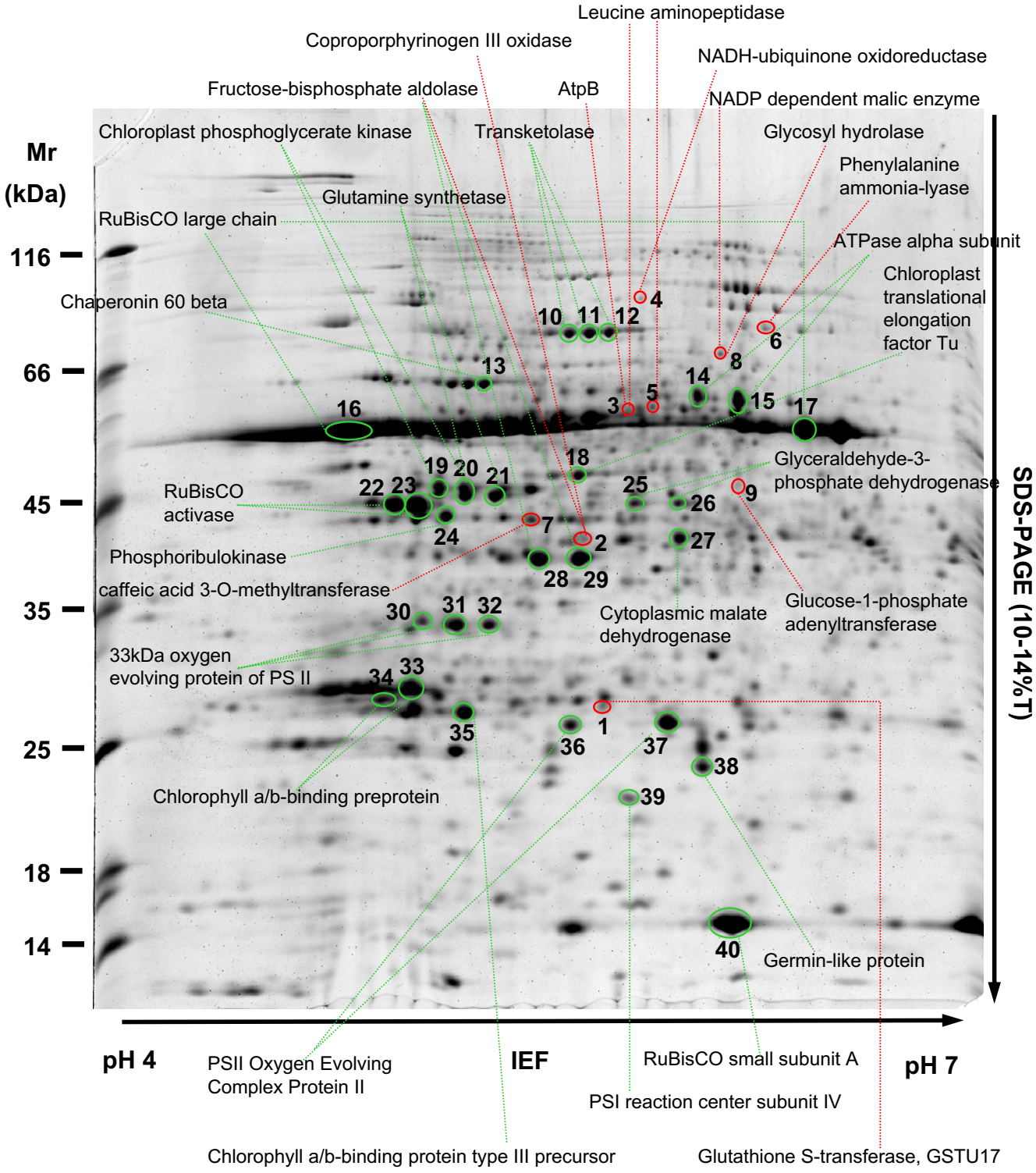


Fig. 2. 2-DE mapping of 9 PBZ-regulated and 31 abundant protein spots. SYPRO Ruby-stained 2-DE of PBZ-treated samples (P6D) is presented and total of 40 protein spots are identified. Spots 1–9 are PBZ-regulated protein spots that are indicated in red. Spots 10–40, the selected abundant proteins, are indicated in green.

16, 17) likely spans over the entire IEF, probably due to its high abundance. In addition, many of these abundant photosynthesis-related proteins such as RuBisCO activase, photosystem II oxygen-evolving complex protein I and II, chlorophyll *a*/*b* binding protein and transketolase were migrated in multiple spots with similar molecular masses but different p*I*s, suggesting the occurrence of posttranslational modifications (Fig. 2).

Taken together, we identified five proteins (GSTU17, PAL, COMT, NADH-ubiquinone oxidoreductase, G-1-

P adenylyltransferase) that are clearly up-regulated by PBZ along with 2-DE mapping of abundant proteins in rice seedlings. Surprisingly, the known PBZ-induced proteins PBZ1 (Mr 16.7 kD, p*I* 4.95) and RPR1 (Mr 103.3 kDa, p*I* 6.60) were not identified as PBZ-regulated proteins by our proteomic analysis, although their transcripts were clearly induced (Fig. 1). The failure to identify PBZ1 and RPR1 as PBZ-regulated proteins by the 2-DE proteome analysis could be due to their low abundance, no significant changes of their protein levels, their co-migration with other proteins in our 2-DE map, or the loss of these proteins during sample preparation or 2-DE process.

2.3. Identification of PBZ-induced PAL paralogs

As PAL belongs to a family of proteins with similar masses and p*I*s (data not shown), we were curious whether the specific PAL protein species XP_466843 were unambiguously identified in spot 6 by LC–MS/MS. Therefore, seven rice PAL paralogs annotated by NCBI were aligned and the peptides identified by MS/MS ions were noted (Fig. 4). PAL XP_466843 contains matched MS/MS ions with both common and specific peptides, while the other PAL paralogs only matched with the common peptides. Therefore, it is clear that spot 6 contains PAL XP_466843 although we cannot exclude the possibility that

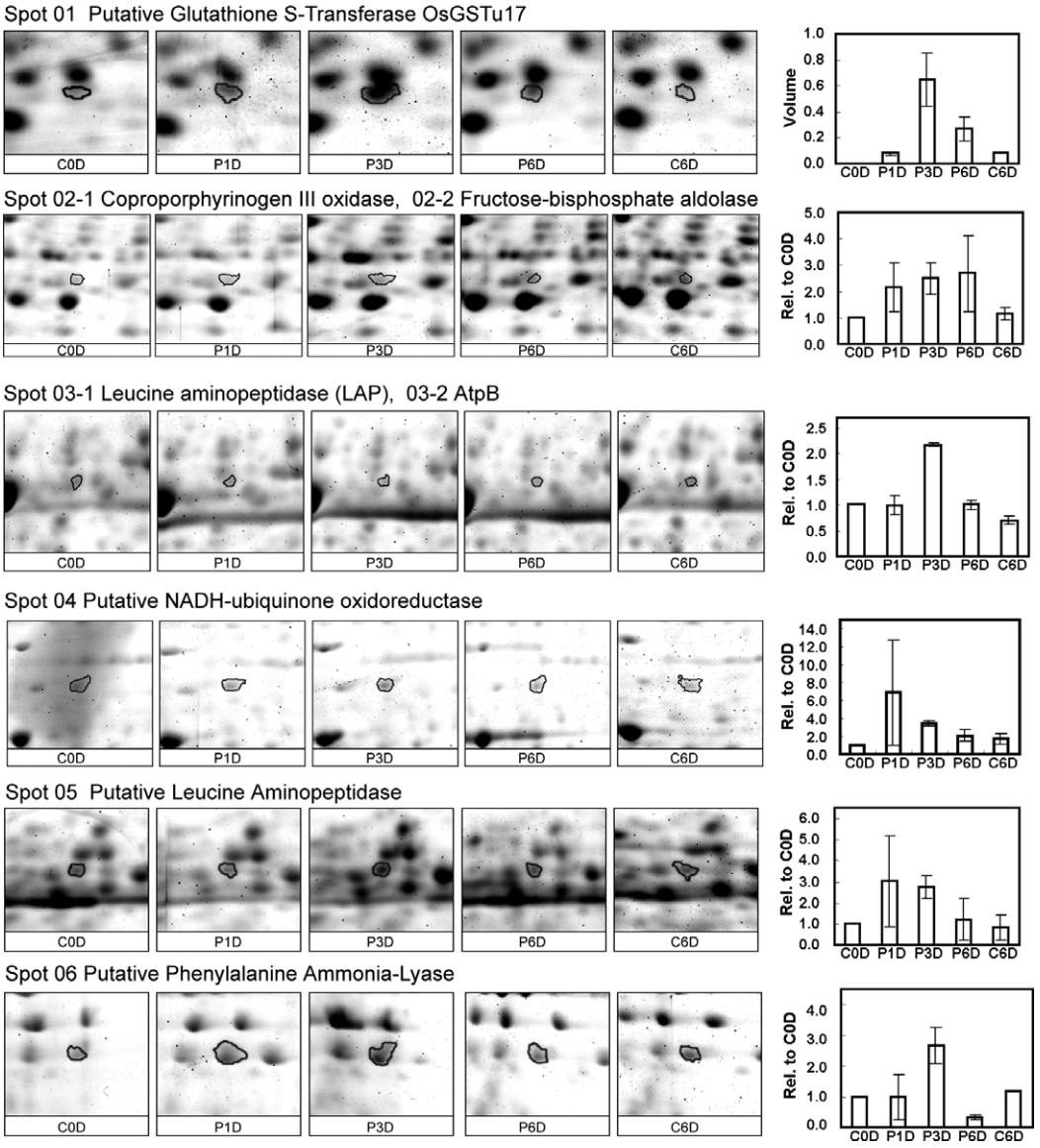


Fig. 3. The protein expression patterns of 9 PBZ-regulated protein spots. The expression level of spot 1 is shown as an absolute value in both untreated controls (C0D and C6D) and PBZ-treated samples (P1D, P3D, P6D). Spots 2–9 are shown as relative expression ratios of PBZ-treated samples at 1day (P1D), 3 days (P3D), 6 days (P6D) or untreated control for 6 days (C6D) to the sample before PBZ application (C0D). The data obtained from three independent experiments are averaged and shown with standard errors with the exception of spot 1 at C6D, spot 6 at C6D, and spot 9 at P3D and C6D which were detected in only one independent experiment. The representative images of the Sypro Ruby-stained gels with selected regions showing the quantified proteins circled are also shown.

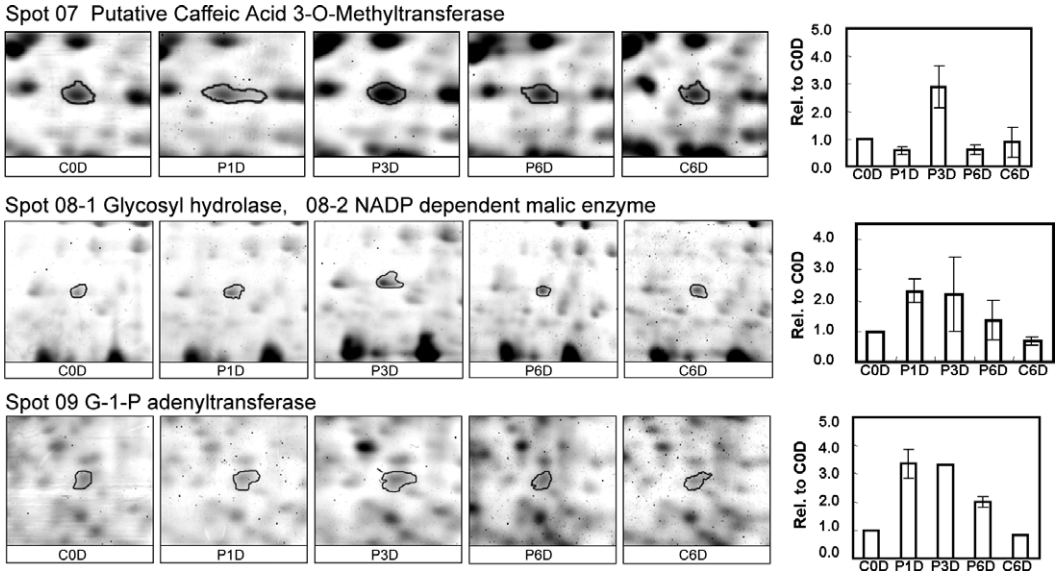


Fig. 3 (*continued*)

other PAL paralogs were also present in lower amounts in the same spot.

Next, we systematically screened all seven rice *PAL* genes for mRNA expression in response to PBZ. Gene-specific primers were designed to detect specific expression of each of the seven *PAL* genes (XM_466843, XM_466845, XM_466846, XM_466849, XM_473192, XM_473196, XM_475254) in response to PBZ by gel-based or real-time RT-PCR. Surprisingly, *PAL* XM_466843 was not induced by PBZ at any time points, although the down regulation at day 6 (P6D) was observed (Fig. 5). Interestingly, *PAL* paralogs XM_466849 and XM_475254 were strongly induced after PBZ treatments (Fig. 5), while no transcripts of the other four *PAL* paralogs were detected (data not shown). The XM_466849 transcript was induced at day 3 (P3D), and increased to 3-fold at day 6 (P6D), while XM_475254 was up-regulated by PBZ with the highest expression levels, approximately 6-fold induction, at day 3 (P3D, Fig. 5). It is clear that XM_466849 and XM_475254 were induced by PBZ (**1**) at the mRNA level; their protein products, however, were not identified (Figs. 2 and 4). It is possible that these two PAL proteins accumulate at lower levels in comparison to highly expressed PAL XP_466843 such that no specific tryptic peptides of XP_466849 and XP_475254 could be identified in spot 6. Alternatively, they may migrate at positions other than spot 6 and were not detected as PBZ-regulated protein spots.

2.4. Real-time RT-PCR of GSTU17 and COMT

The mRNA expression levels of *GSTU17* and *COMT* were also analyzed by real-time RT-PCR. Results showed that *GSTU17* transcript was also clearly induced by PBZ as early as day 1 (P1D), with the highest expression level at day 3 (P3D), consistent with the expression pattern of the protein (Figs. 3 and 5). The expression of the *COMT* transcript also correlates with its protein expression pattern (Figs. 3 and 5). Taken together these data indicated that *GSTU17*, *COMT*, and *PAL* paralogs XP_466849 and XP_475254 were induced at the mRNA level, while PAL XP_466843 may be regulated at the protein level by PBZ.

3. Discussion

Here we report the first proteomic analysis of PBZ-induced rice defense response and identify 9 PBZ-regulated protein spots resulting in the identification of 11 unique proteins. The presence of more than one protein species in one spot or of one protein species migrating at several positions, however, complicated the quantitative analysis. We noticed that some protein spots determined to contain two protein species by LC–MS/MS only matched one significant protein species when analyzed by MALDI-Q-TOF (data not shown). Therefore, caution should be taken in assignment of protein identity and their respective protein expression levels especially when MALDI-TOF MS other than LC–MS/MS is used for protein identifications.

Identifications of 31 abundant protein spots (spots 10–40) of rice seedlings revealed the majority of these proteins correspond to photosynthetic or chloroplast-related proteins (Fig. 2 and Table 2). Many of these proteins migrated at more than one spots with similar molecular masses but different p*I*s, suggesting the occurrence of post-translational modifications (Fig. 2). Protein isoforms migrated as a chain of spots are indeed commonly observed in 2-DE analysis (Jones et al., 2004); yet the identities and/or biological significances of these post-translational modifications are mostly unclear. The occurrence of more than one protein species in one spot and one protein species migrating at several spots has raised a potential problem on quantitative protein profiling. Therefore, we performed a parallel transcript analysis to provide useful information to confirm some proteins that are regulated at either

Table 1
Identifications of 9 PBZ-regulated protein spots

Spot #.	Mr/p*I*(O)[a]	MS/MS		Protein Identity	Accession. # Mr/pI (T)[a]
		Score	PM (%C)[b]		
1	27,320/5.65	95	2 (7%)	OJ1005_D12.39 gene product, putative glutathione *S*-transferase, GSTU17	XP_507428 25170/5.50
2	43,625/5.59	233	5 (13%)	Putative coproporphyrinogen III oxidase	XP_473852 43844/6.23
		78	3 (6%)	Probable fructose-bisphosphate aldolase (EC 4.1.2.13) precursor, chloroplast	Q40677 42122/7.6
3	63,932/5.75	653	13 (23%)	Putative leucine aminopeptidase	XP_468246 61780/8.29
		246	6 (12%)	Putative ATPase subunit B, AtpB	NP_920970 53951/5.38
4	87,341/5.79	211	6 (8%)	Putative NADH-ubiquinone oxidoreductase	XP_469533 81065/5.86
5	64,433/5.83	471	10 (16%)	Putative leucine aminopeptidase	XP_468246 61780/8.29
6	78,306/6.22	822	20 (26%)	Putative phenylalanine ammonia-lyase	XP_466843 75451/6.07
		234	7 (8%)	Phenylalanine ammonia-lyase	XP_473192 75991/6.60
7	46,434/5.40	455	11 (26%)	Putative caffeic acid 3-O-methyltransferase	XP_480185 39724/5.41
8	74,145/6.06	373	8 (8%)	OSJNBb0003B01.22, with homology to Glycosyl hydrolase family 3	CAE03631 80869/6.42
		146	4 (6%)	NADP dependent malic enzyme	BAB20887 65368/5.79
9	50,201/6.13	70	3 (6%)	Putative glucose-1-phosphate adenyltransferase	XP_481806 52917/5.87
		70	3 (6%)	Putative glucose-1-phosphate adenyltransferase	XP_481807 56069/6.58

[a] Mr/pI(O) or Mr/p*I*(T): Observed (O) or theoretical (T) relative molecular mass/isoelectric point.
[b] PM (%C): Number of peptides matched (% of coverage of matched peptides) in MS/MS ion search.

mRNA levels or to provide the possibility of translational or posttranslational regulation. When possible, MS identifications of all protein spots resolved on 2-DE by LC–MS/MS may be necessary to unambiguously determine the quantitative data of identified regulated proteins. In conclusion, one should consider to confirm the results obtained by quantitative proteomic analysis with alternative experiment such as Western blotting or isotopic labeling before performing further biological experiments.

The up-regulation of putative PAL and COMT suggests that PBZ (**1**) may induce either flavonoid-type phytoalexin and/or lignin biosynthesis through co-inducing these two key enzymes in the phenylpropanoid pathway (Dixon, 2001; Vom Endt et al., 2002). PAL, as the first enzyme in the phenylpropanoid pathway, may also contribute to the synthesis of SA derived from phenylalanine via cinnamic and benzoic acid (Metraux, 2002). It is clear that PAL plays a key role in induced disease resistance based on its induced expression by pathogens or elicitors, and on studies using transgenic approaches in tobacco (Howles et al., 1996; Maher et al., 1994; Shadle et al., 2003). In rice, PAL was found to be up-regulated at activity levels or expression levels in response to pathogen or defense signals. The early studies showed that the activity of PAL is markedly increased in PBZ-treated rice plants (Iwata et al., 1980). *PAL* paralog *ZB8* cloned from *O. sativa* subsp. *indica* cv. IR¸ corresponding to XP_466849, was induced by wounding, virus, and fungal elicitors in a promoter-GUS activity assay (Zhu et al., 1995). Recent expression studies also showed that PAL transcripts were up-regulated by the bacterial flagellin (Tanaka et al., 2003) and in *OsWRKY03*-overexpressing transgenic rice (Liu et al., 2005). Until recently, with the exception of *ZB8,* however, it has remained unclear which PAL paralog is responsible for either the induced enzymatic activity or mRNA accumulation. The results of our 2-DE and real-time RT-PCR analysis suggest that PBZ (**1**) may regulate PAL expression at both the mRNA and protein levels. While the mRNA transcripts of PAL XP_466849 and XP_47525 were up-regulated by PBZ (**1**), PAL XP_466843 is likely regulated at the protein level. Whether PAL XP_466843 is induced by PBZ (**1**) at translational or post-translational levels remains unclear. Nevertheless, our results agree with several studies revealing significant number of genes without good correlations between mRNA and protein levels in yeasts or mammals (Gygi

Table 2
Functional categories of the identified proteins

Functional category (# of proteins)	Protein name (spot #)	Known or putative defense-related expression or function	Also in Defense functional category
Energy (16)	Probable fructose-bisphosphate aldolase (2, 28, 29)		
	Putative AtpB (3)		
	Putative NADH-ubiquinone oxidoreductase (4)	Antioxidant defense Babiychuk et al. (1995)	
	NADP dependent malic enzyme (8)	Induced by fungal elicitor Schaaf et al. (1995)	
	Putative chloroplast phosphoglycerate kinase (19, 20)	Up-regulated by H_2O_2 Paron et al. (2004)	
	PSI reaction center subunit IV (39)		
	Putative transketolase (10, 11, 12)		
	Putative ATPase alpha subunit (14, 15)		
	RuBisCO activase (22, 23)		
	Putative glyceraldehyde-3-phosphate dehydrogenase (25, 26)		
	Putative 33 kDa oxygen evolving protein of photosystem II (30, 31, 32)		
	Chlorophyll *a*/*b*-binding preprotein (33, 34)		
	PSII Oxygen Evolving Complex Protein II (36, 37)		
	RuBisCO large chain (16,17)		
	Putative chlorophyll *a*/*b*-binding protein type III precursor (35)		
	RuBisCO small subunit A (40)		
Metabolism (6)	Putative glutathione *S*-transferase, GSTU17 (1)	Protector against cellular oxidative stress Dixon et al. (2002), Edwards et al. (2000), Marrs (1996)	Yes
	Putative Glycosyl hydrolase (8)		
	Putative glucose-1-phosphate adenyltransferase (9)	Heat-decreased protein Majoul et al. (2004)	
	Putative glutamine synthetase (20, 21)		
	Phosphoribulokinase precursor (24)		
	Cytoplasmic malate dehydrogenase (27)		
Secondary metabolism (3)	Putative coproporphyrinogen III oxidase (2)	Lesion formation Ishikawa et al. (2001))	Yes
	Putative phenylalanine ammonia-lyase (6)	Phytoalexin and/or lignin biosynthesis synthesis of SA Dixon, (2001), Metraux (2002), Vom Endt et al. (2002)	Yes
	Putative caffeic acid 3-*O*-methyltransferase (7)	Phytoalexin and/or lignin biosynthesis Dixon (2001), Vom Endt et al. (2002)	Yes
Protein destination and storage (3)	Putative leucine aminopeptidase (3, 5)	Induced by pathogen, wounding, abscisic acid (ABA), or JA Hildmann et al. (1992), Pautot et al. (1993)	
	Putative chaperonin 60 beta precursor (13)		
Protein synthesis (1)	Chloroplast translational elongation factor Tu (18)		
Cell structure (2)	Germin-like protein (38)	Oxalate oxidase activity; resistance to fungal infection and heavy metal stress Dumas et al. (1995), Lane (1991), Patnaik and Khurana (2001)	Yes
	PSI reaction center subunit IV (39)		

et al., 1999; Ideker et al., 2001; Kolkman et al., 2006; Nishizuka et al., 2003). Although it was not demonstrated in rice, PAL was indeed phosphorylated at a threonine residue by a calcium-dependent protein kinase (Allwood et al., 1999, 2002). We, however, failed to identify any phosphopeptides by MS/MS ion searches (data not shown). Future work employing MS-based quantitative proteomics using isotope labeling or immunoblot analysis with antibodies specific to PAL XP_466843, XP_475254, and XP_466849 may provide evidence to identify the PBZ-induced expression or modifications of PAL paralogs.

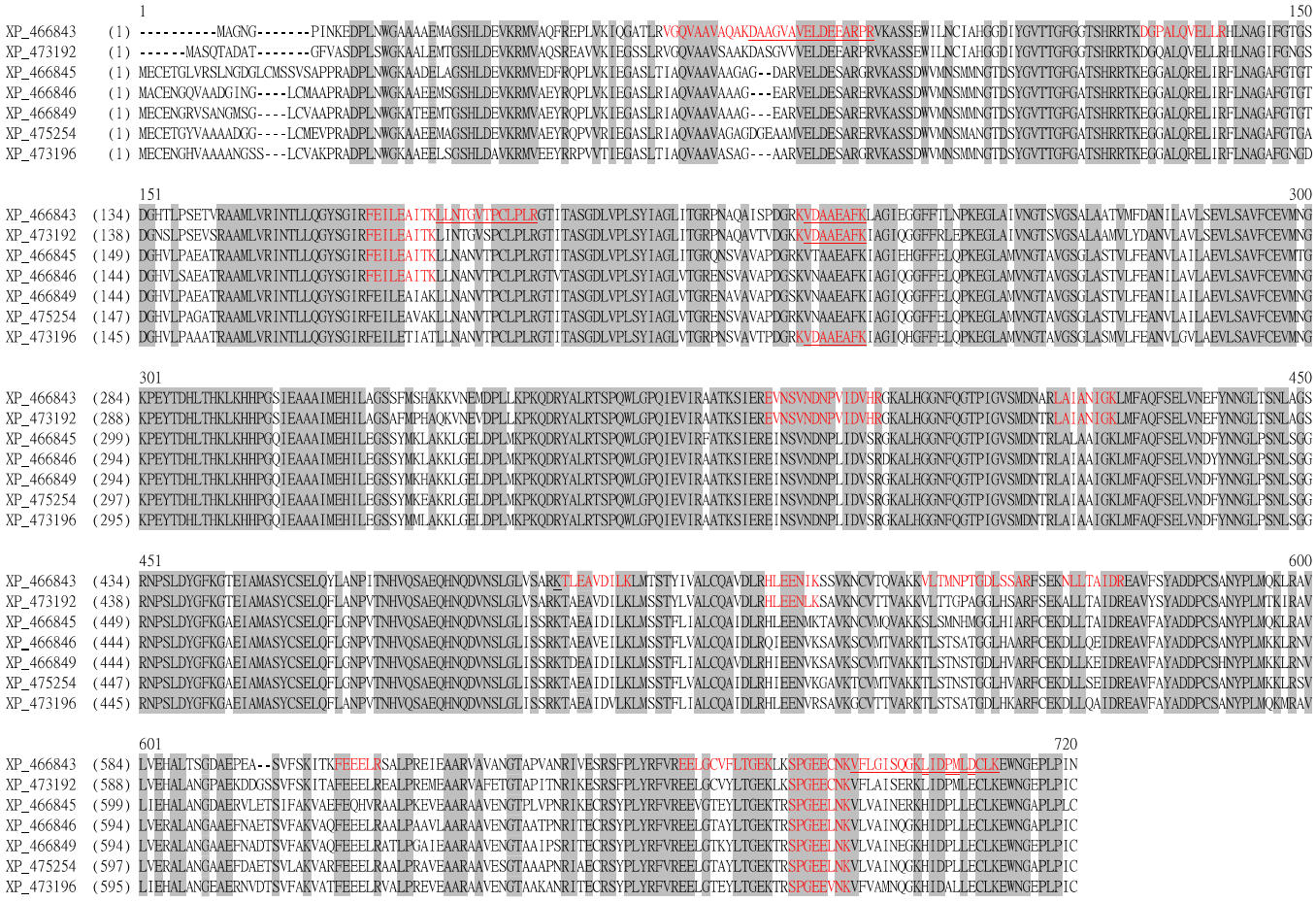


Fig. 4. The MS-identified common and specific peptides of putative PAL paralogs. seven rice PAL paralogs are aligned to present the peptides identified by MS/MS analysis. The identified peptides are shown in red. When two or three identified peptides are consecutive, each identified peptide is underlined. The conserved amino acids are highlighted in gray. The respective NCBI accession # is indicated for each protein sequence.

COMT is another key enzyme in the phenylpropanoid pathway for biosynthesis of lignin in the cell wall, playing a role in response to pathogen attack. *COMT* was previously identified as up-regulated by PBZ in a rice cDNA microarray analysis (Shimono et al., 2003) and its protein product was shown to be induced in rice lesion mimic mutants by 2-DE analysis (Tsunezuka et al., 2005). Interestingly, COMT is highly homologous to a JA-induced NOMT (naringenin 7-*O*-methyltransferase), an enzyme in phenylpropanoid pathway that catalyzes the methylation of naringenin to sakuranetin, a flavonoid-type phytoalexin shown to be one of major phytoalexins in rice (Rakwal et al., 2000). Therefore, the PBZ-induced COMT protein may function in the phenylpropanoid pathway but may involve two possible steps, one leading to the biosynthesis of lignin in the cell wall and the other acting on the synthesis of a flavonoid-type phytoalexin sakuranetin.

The strong induction of GSTU17 suggests its important role in PBZ-induced defense response. Plant GSTs have various functions such as detoxifying xenobiotics or endogenous secondary metabolites via conjugation with the tripeptide glutathione GSH, or in stress metabolism via acting as glutathione peroxidases, antioxidant activity, or signaling (Edwards and Dixon, 2005). In rice, there are at least 59 putative *GST* genes organized into four main phylogenetic classes (tau, phi, zeta, and theta) (Soranzo et al., 2004). In rice, a type II GST *OsGST2* was induced by blast pathogen (Agrawal et al., 2002), two tau class GSTs, *OsGSTU3* and *OsGSTU4*, were heavy metal- and hypoxic stress induced (Moons, 2003), and a putative GST protein was also shown to be related to lesion formation by 2-DE analysis (Tsunezuka et al., 2005). GSTU17, a tau class GST identified in this study, was not previously characterized. In general, the plant specific phi and tau class GSTs have the classic conjugating activities toward a diverse range of xenobiotics including pesticides and endogenous secondary metabolites such as flavonoids (Edwards and Dixon, 2005). Therefore, it is likely that the induction of GSTU17 by PBZ may be responsible to conjugate PBZ or flavonoid that is then transported from the cytosol into the vacuole for sequestration or storage. Indeed, an isoflavonoid phytoalexin (medicarpin) from mung bean has been demonstrated to be the substrate for GST and transported into vacuole (Li et al., 1997). Since many phytoalexins are toxic to the host plant as they are to its pathogen, the phytoalexin or its precursor stored in vacuole may provide a good strategy for defense. Therefore, it is possible that PBZ-induced GSTU17 may conjugate the flavonoid-type

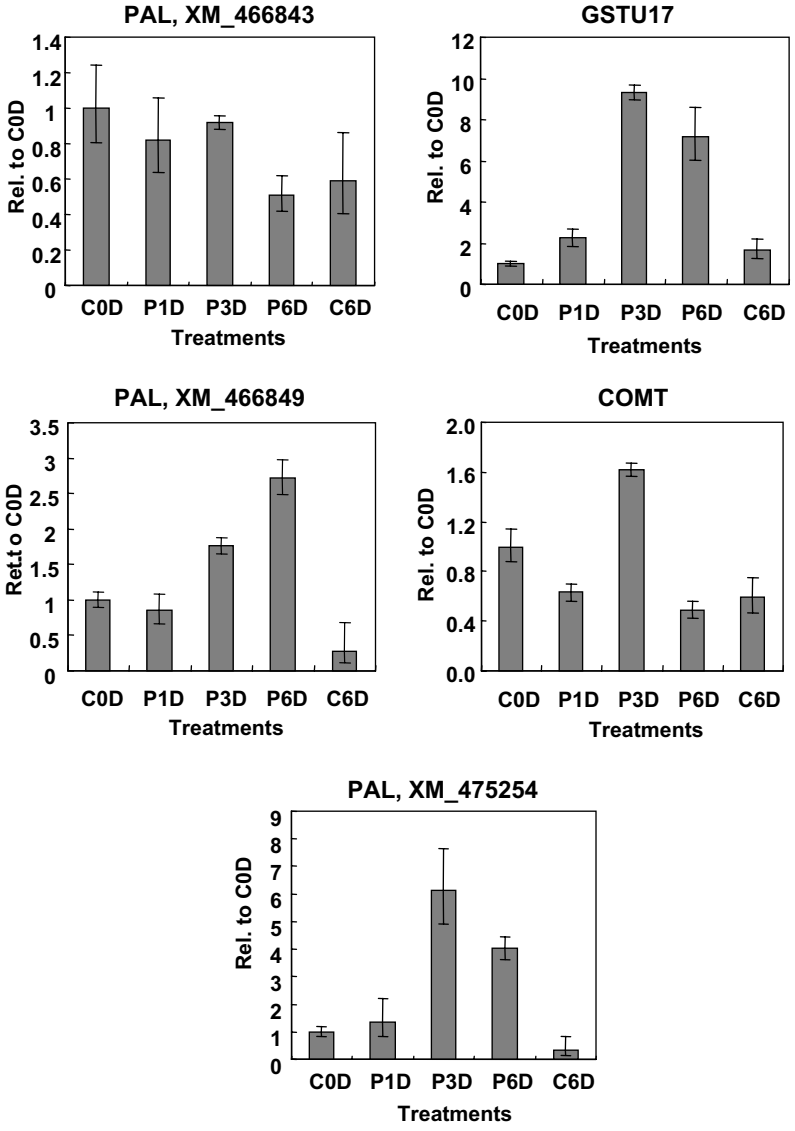


Fig. 5. Real-time RT-PCR analysis of *PAL, COMT,* and *GSTU17*. The mRNA expression patterns of each gene were quantified by RT-PCR. The relative expression ratios of PBZ-treated samples at 1day (P1D), 3 days (P3D), 6 days (P6D) or untreated control for 6 days (C6D) to the sample before PBZ application (C0D) were determined in triplicates and were shown as average values with standard deviations. The expression levels were normalized using *OsS5A* as an internal control.

phytoalexin or precursor that is overproduced by the up-regulation of PAL and COMT, and transport it from the cytosol into the vacuole that will be released to attack the invading pathogens after infections. In the future, it would be interesting to test this hypothesis and shall provide new insights to understand the action mode of PBZ-induced disease resistance.

4. Experimental

4.1. Plant materials, growth conditions, and treatments

O. sativa subsp. *japonica* cv. TNG67 was used for chemical treatment and pathogen inoculation. The seeds were washed with tap water several times and sterilized in 0.5% NaCl for 15 min followed by extensive rinses in sterile H_2O. The sterilized seeds were germinated at 37 °C for 2–3 days and then grown in a plastic box (32 x 24 × 6.5 cm) in a 4:2:2:1 mixture of peat moss, vermiculite, perlite and organic fertilizer ($N:P_2O_5:K_2O = 1:1:1$) in a growth chamber (30 °C, 14 h, light/25 °C, 10 h, dark). PBZ (Yih Fong Chemical Corp., Taichung County, Taiwan) was applied to 2-week-old rice seedlings at leaf stage 4 (3 true leaves) at 0.4 g/m^2 by the soil-drench method (Sakamoto et al., 1999), and seedlings were harvested in liquid N_2 at 1 day (P1D), 3 days (P3D), and 6 days (P6D), respectively. Water-treated plants before PBZ application (C0D) and after 6 days (C6D) were collected as controls. The materials were used immediately or stored at −80 °C prior to protein and RNA isolation.

To evaluate the effect of PBZ in disease resistance, rice seedlings were inoculated with the XM1 isolate of bacterial blight pathogen *Xoo* (gift from Taiwan Agricultural Research Institute, Taichung County, Taiwan) 7 days after PBZ treatment by the scissor-clipping method (Kauffman et al., 1973) in the greenhouse. Briefly, newly matured leaves from PBZ-treated and non-treated plants were chosen for cutting 3 cm from the tip with scissors that had been dipped into the bacterial suspension (OD600 = 1, grown in 523 medium (Kado and Heskett, 1970)). Mock-inoculated plants were treated under the same conditions, except that the bacterial suspension was replaced by H_2O. The length of lesions on infected leaves was measured two weeks after pathogen inoculation.

4.2. Total protein extraction and 2-DE

The methodology for total protein extraction for 2-DE was modified from the literature (Porubleva et al., 2001). Briefly, frozen rice seedlings (0.2 g) were ground with liquid N_2 in ice-cold acetone (1.35 ml containing 10% w/v trichloroacetic acid (TCA) and 30 μl 1 M DTT). After incubation at −20 °C for 45 min, the proteins were precipitated by centrifugation at 19,000*g* for 15 min at 4 °C. The pellet was washed with 1 ml of ice-cold acetone containing 20 μl 1 M DTT, 10 μl 100 mM PMSF, and 4 μl 0.5 M EDTA. The wash step was repeated five times with centrifugation at 19,000*g* for 15 min at 4 °C until no chlorophyll was present in the acetone. The protein pellet was dried in vacuo and then resuspended in lysis buffer (7 M urea, 2 M thiourea, 2% CHAPS, 1% ASB-14, 40 mM Tris base, 2 mM tributyl phosphine [TBP; Bio-Rad, Hercules, CA, USA], and 1 mM PMSF) by sonication. The insoluble debris was removed by ultracentrifugation at 45,000*g* for 15 min. The protein concentration was determined by the Bradford method (Bio-Rad) with bovine serum albumin (BSA) as the standard.

The 2-DE experiment was performed essentially according to the manufacturer's instruction (GE Healthcare Amersham Biosciences) except where indicated. To proceed with isoelectric focussing (IEF), protein (500 μg) was resuspended in lysis buffer containing 0.5% [v/v] IPG buffer pH 4–7, and 0.01% bromophenol blue, and rehydrated with each 18-cm pH 4–7 immobilized pH gradient (IPG) strip (GE Healthcare Amersham Biosciences). IEF was carried out with the Multiphor II system (GE Healthcare Amersham Biosciences) at 20 °C in a stepwise manner: 150 V (0.5 h), 300 V (0.5 h), 600 V (0.5 h), 1000 V (1 h), 2500 V (1 h), 4000 V (1 h), and 6000 V for total of 80,000 V h. After IEF, the IPGs were either separated immediately in either the second dimension or kept at −20 °C until use. Prior to SDS–PAGE, the IPGs were equilibrated with shaking at room temperature in equilibration buffer (6 M urea, 2% [w/v] SDS, 30% [v/v] glycerol, 50 mM Tris–HCl [pH 8.8]) containing 1% (w/v) DTT for 20 min followed by equilibration in buffer containing 3% (w/v) iodoacetamide for another 20 min. The equilibrated IPGs were separated on 10–14% SDS–PAGE using Protean II Multicells (Bio-Rad) at 16 °C. The protein spots were visualized by staining with Sypro Ruby (Molecular Probes).

4.3. Gel image comparison and data analysis

Gel images were digitized and stored as TIF files with a resolution of 300 dpi using a UMAX Power Look scanner and MAGIC SCAN software (UMAX Technologies Inc., Dallas, TX, USA). The quantitative analysis of protein spots among PBZ-treated and non-treated samples was carried out using ImageMaster 2D Elite IV (GE Healthcare Amersham Biosciences). Spot detection and matching was automatic initially, followed by manual detection as necessary. To correct variations due to staining, spot volumes were normalized as a percentage of the total volume of all spots on the gel. Following acquisition of quantitative data, the PBZ-regulated protein spots from three independent experiments were selected using GeneSpring 6.0 software (Silicon Genetics, Red Wood, CA, USA). The protein spots not detected in control samples, but expressed in one of the PBZ-treated samples, were classified as PBZ-induced spots. The PBZ-repressed spots are those only detected in controls. A spot intensity ratio for PBZ-treated samples versus non-treated of $\geqslant 2$ or $\leqslant 0.5$ is defined as PBZ up- or down-regulated, respectively. The observed molecular masses (Mr) and isoelectric points (p*I*s) of the protein spots were also calculated using ImageMaster on the basis of co-electrophoresis of molecular weight protein markers (Fermentas Inc., Hanover, MD, USA) and migration on pH 4–7 IPGs.

4.4. Protein identification and database searches

Trypsin in-gel digestion was performed (Lai et al., 2004) and the digested peptides were analyzed by MALDI-Q-TOF MS or LC–MS/MS following the method described (Lee et al., 2003). For MALDI-Q-TOF MS, the digested peptides were premixed 1:1 with matrix solution (5 mg/ml CHCA in 50% CH_3CN, 0.1% v/v TFA and 2% w/v ammonium citrate), spotted onto the 96 well format MALDI sample stage, and analyzed by MALDI-Q-TOF MS (Ultima, Micromass, Manchester, UK) following the method described (Lee et al., 2003). For 1-D LC-nanoESI-MS/MS (Q-TOF Ultima API MS, Micromass, Milford, MA, USA), injected samples were separated on an analytical C18 capillary column (15 cm 675 mm id, packed with 5 mm Zorbax 300 SB C18 particles; Micro-Tech Scientific, Vista, CA, USA), connected in-line to the mass spectrometer, at 300 nL/min using a 50 min gradient of 5–80% CH_3CN in 0.1% formic acid. On-line nanoESI-MS survey scan and data dependent acquisition of CID MS/MS were fully automated and synchronized with the nanoLC runs under the full software control of MassLynx 4.0. After data acquisition, the individual MS/MS spectra acquired for each of the precursors within a single LC run were

combined, smoothed, deisotoped and centroided using the Micromass ProteinLynx Global Server (PGS) 2.0 data processing software resulting in a single Mascot-searchable peak list (.pkl) file.

The peak list file containing the m/z ratios of precursor ions and MS/MS fragmented ions was used in searches of the web-based search engine Mascot (http://www.matrixscience.com/search_form_select.html) against the most recent database in the National Center for Biotechnology Information (NBCI). A maximum of one missed trypsin cleavage, variable modification including carbamidomethylation and oxidation (M), and a mass accuracy of either 100 ppm for peptide mass fingerprinting (PMF) or 0.25 Da for both precursor and daughter ions in MS/MS ion search were used. When available, the NCBI Reference Sequence (RefSeq) accession number was used to assign the respective protein ID in order to provide a comprehensive, integrated, non-redundant set of sequences. The proteins were unambiguously identified as significant hits ($P < 0.05$) by PMF and/or MS/MS ion search. The obtained score, number of matched peptides, and peptide coverage for each identified protein spot are listed in Table 1 and Supplementary Table 1.

4.5. Real-time RT-PCR

Total RNA was isolated from frozen plant materials by the TRIzol method (Chomczynski and Sacchi, 1987). Reverse transcription reactions were performed with SuperScript™ III RNase H⁻ Reverse Transcriptase (Invitrogen Corp., Carlsbad, CA, USA). All primers were designed with the software primer express 2.0 (Applied Biosystems) to the following stringent criteria: melting temperature 58–60 °C, primer length 20–24 nucleotides, guanine–cytosine (GC) content 45–55%, PCR amplicon length 60–150 bps. The suggested primers were checked by use of BLASTN in a rice NCBI nr database to ensure their specificity to target genes. The sequences of each primer pair follow. *OsS5a* encoding 26S proteasome regulatory subunit S5a (Accession # AB010740) (5′- CTTCCTTgCAACCACCTCTTg -3′; 5′- CCAAATAAAgCgAgACCAgATATCT -3′), *PBZ1* (Accession # AF274850) (5′- TggTCAgTAgAgTgATCAgTTgCAA -3′; 5′- gCCATCACTgAAgATATAATCTAACTAgCT- 3′), *RPR1* (Accession # AB019186) (5′- AACATgTTCCAgAgCTgCATgT -3′; 5′-gCCCATAATCACCAgACAgAAAT - 3′), *GSTU17* (Accession # AF402804) (5'-AgAAgTgCAACgCTgCTCTgT-3'; 5'-gCTCCTCTCCggTCgTCAT-3'), *PAL* (Accession # XM_466843) (5'-AggAgCTCggCTgCgTATT-3'; 5'-ATgCCgAggAACACCTTgTT-3'), *PAL* (Accession # XM_466849) (5'- gAATTCTTCgTTgCATAgCgg-3'; 5'-gCCACTCgTACCAAgTTTTgC -3'), *PAL* (Accession # XM_475254) (5'- TgggTggAAgCgATggTAg-3'; 5'-gCgTgTTCTTggCACgAAg-3'), *COMT* (Accession # XM_480185) (5'-gCTTCAACCgCgTCTTCAA-3'; 5'-TgATgATgACggAgTggTTCTTC-3'). PCR was performed in MicroAmpR optical Tubes using an ABI PRISM® 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and SYBR® Green to monitor dsDNA synthesis. Reactions contained 25 μl SYBR® Master Mix (Applied Biosystems), specific primers, and template cDNA in a final volume of 50 μl. The following standard thermal profiles were used for all reactions: 50 °C for 2 min; 95 °C for 10 min; 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Data were analyzed with SDS 2.0 software (Applied Biosystems). In order to compare data from different PCR runs or cDNA samples, C_T values for all target genes were normalized to the C_T value of *OsS5a*, a constitutively expressed gene with approximately equal PCR efficiency in all samples.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2007.09.005.

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