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Comparative proteomic analysis of two rice cultivars (*Oryza sativa* L.) contrasting in Brown Planthopper (BPH) stress resistance

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Abstract

The brown planthopper (BPH) is a pest which destroys rice paddy fields. To determine the molecular mechanism of the resistance of rice plants to BPH, the proteomic profiles of two contrasting rice cultivars, TN1 (susceptible) and PTB33 (resistant), were compared. This information was then used to investigate protein expression during BPH feeding. Rice seedlings were inoculated with BPH nymphs to screen for resistance behavior among five selected cultivars. Following inoculation, small BPHs were introduced to the rice seedlings at a ratio of 8:1 (BPH: rice seedling) and the hopperburn symptom on each cultivar was measured over the fourteen day period of observation. The samples demonstrating the highest resistance to BPH (PTB33) and susceptibility (TN1) were then chosen for protein extraction and comparison. An observed total of nineteen protein spots demonstrated significant differences between the two cultivars, with a two-dimensional electrophoresis (2-DE) and mass spectrometry (LC-MS/MS) approach adopted to identify the results of the experiment. The protein spots were then categorized into different groups according to the presence of a carbohydrate metabolism, antioxidants, protein synthesis, ATP synthesis, photosynthesis and stress response proteins. 2-Cys peroxiredoxin BAS1 (2-CysPrx-BAS1), putative inorganic pyrophosphatase (IPPase) and eukaryotic elongation factor 1 (EF-1) were reported to be 2.09, 2.25 and 2.22-fold up-regulated in resistant cultivar, respectively. It has also been shown that fructokinase (FK) and phosphoglyceratemutase (PGmutase) were only found using CBB staining in resistant cultivar, but not in susceptible cultivar. The protein up-regulation observed in the resistant cultivar might promote glycolysis and lignin biosynthesis via the phenylpropanoid pathway, which offers such plants protection against BPH infestation.

Keywords: Brown Planthopper; Protein Maker; Resistant cultivar; Rice.
Abbreviations: ACN_acetronitrile; BPH_brown Planthopper; BSA_bovine serum albumin; CBB_coomassie brilliant blue; CHAPs_ 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate; CNT1_chainat1; DTT_dithiothreitol; EDTA_Na_2- sodium 2,2'-1,2-ethanediylbis[(carboxymethyl) imino]di-acetate hydrate; ESI-TRAP_electrospray ion trap; Hepes_KOH, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid-potassium hydroxide; IPG_immobilized pH gradient; LC/MS_MS- liquid chromatography-tandem mass spectrometry; MS_mass spectrometry; $MgCl_2$_magnesium dichloride; PMSF_phenylmethylsulfonyl fluoride; PSL2_phitsanulok2; PVP_ polyvinylpyrrolidone; SDS_sodium dodecyl sulfate ; SP90_suphanburi 90; TCA_trichloro acetic acid; TFA_tri fluoro acetic acid; TN1_thai chung native 1; Tris_tris(hydroxymethyl)aminomethane.

Introduction

The brown planthopper (BPH), *Nilaparvata lugens* (Stål) is the most serious rice crop insect pest in South East Asia, which includes Indonesia, Philippines, Vietnam and Thailand. It attacks leaves and sucks phloem sap, and rice infested by BPH becomes yellow, dry and even dies due to "hopper burn" symptoms (Duan et al., 2008, 2014; Wang et al., 2010; Lin et al., 2014a). In the period, 2010-2013, BPH infestation in Thailand was estimated to have affected at least 5 million Rai (approximately 2 million acres), with a loss of 60% of rice production and income of 26,000 million baht per year. Insecticides, including organochlorines, organophosphates, cabamates and pyrethroids, are mainly used for continuous control of BPH in rice paddy fields. However, they cause a reduction of natural enemies, BPH insecticide resistance (Duan et al., 2008, 2014) and ecological problems (Wang et al., 2010). In addition, pesticide foliar sprays cause increased BPH infestation, also called pesticide-induced susceptibility (Cheng et al., 2012). Rice cultivar resistance to BPH has been realized as one of the most economically effective and environmentally friendly strategies for controlling the BPH (Jairin et al., 2009). Developing rice containing resistance genes is an efficient strategy for controlling BPH (He et al., 2013). Previous reports have identified genes related to BPH resistance in rice such as salicylic acid (SA) synthesis-related genes, phenylalanine ammonia-lyase (*PAL*), *NPR1*, *EDS1* and *PAD4*

genes (Duan et al., 2014), pathogenesis defense gene, antioxidant genes (Hao et al., 2011), and *BPH* resistance genes (He et al., 2013). Moreover, the activation speeds and expression levels of the defense gene vary in different plant-pathogen insect interactions (Hao et al., 2011). Previous research reported that plants defend themselves against a pathogen or an insect by activation of a defense response pathway (Lee et al., 2007). The defense mechanism of rice cultivar resistance to BPH has many hypotheses: (1) BPH infestation will activate the primary and secondary metabolite production such as shikimate mediated biosynthesis of phenylpropanoids, polyphenols, oxalic acid, phenols, apigenin-C, glycoside and volatile compound affect rice resistance to BPH (Schwachtje and Baldwin, 2008; Cheng et al., 2013). Volatile compounds control rice against BPH by attracting the natural enemies of BPHs such as parasitoid and BPH predators (Lou et al., 2005; Cheng et al., 2013) after BPH feeding. (2) Moreover, higher expression of protease inhibitors such as the trypsin gene in resistant cultivar will be inhibited the BPH infestation (Yang et al., 2005). (3) Protein plugging and callose decomposition is one of a plant mechanism for sealing the phloem sieve pore during pathogen attack in resistant cultivars. Callose deposition in sieve plates will be occluded, injuring the sieve tube in the rice plant carrying a BPH gene, preventing attack on the sap by BPH. Meanwhile, β-1,3 glucanase will be promoted the callose hydrolysis in the susceptible cultivar, as a result of continuous BPH feeding (Hao et al., 2008; Luna et al., 2011; Cheng et al., 2013). However, this hypothesis contrasts with previous report that proteins related to the callose metabolism remain unchanged in the BPH infestation of resistant lines (Wei et al., 2009). Therefore, the mechanism of plant resistance to pests is poorly understood and the rice plant has developed many defense mechanisms against BPH and other invaders (Cheng et al., 2013). This study, the proteomic technique is used for determining the mechanism involved in the BPH resistance characteristic of rice. Differential expression of proteins may identify those responsible for protection from the rice pathogen. Proteomic is a tool for identifying proteins which are responsible for both the structure and the functions of all living organisms. It has also enabled the identification of biomarkers and allowed the discovery of novel target genes. Recently, the proteomic approach successfully analyzed glyceraldehyde-3-phosphate dehydrogenase B (OsGAPDHB), a unique protein in aroma rice, which may serve as one of the proteins that contribute to the aroma phenotype (Lin et al., 2014b). This tool also allows the categorization of protein groups such as metabolism, stress response, growth and differentiation and signal transduction during stress conditions (Hwang et al., 2011). Most rice proteomic studies have been used to identify and characterize differentially expressed proteins under stress conditions in order to find the protein marker responsive to various types of biotic and abiotic stress. Rice proteins respond to abiotic stress such as salt (Sarhadi et al., 2012; Salekdeh et al., 2002), cold (Lee et al., 2009), drought (Salekdeh et al., 2002; Faghani et al., 2015), anoxia (Sadiq et al., 2011), ozone (Sawada et al., 2012), space flight (Wang et al., 2008; Ma et al., 2007) and also various high temperatures (Han et al., 2009) were widely reported. In addition, hormone and chemical stress such as probenazole (Lin et al., 2008), cadmium (Xue et al., 2014; Lee et al., 2010; Ge et al., 2009), mercury (Chen et al., 2012), copper (Ahsan et al., 2007), jasmonic acid (Rakwal et al., 1999) and abscisic acid (He and Li, 2008; Li et al., 2010a; Zhang et al., 2012) have also successfully employed the proteomic tool to identify differentially expressed proteins. Moreover, proteins associated with rice pathogen interaction have been also identified. For example, proteins being induced by blast fungus *Magnaporthe oryzae* (Koga et al., 2012; Kim et al., 2003; Konishi et al., 2001), rice xylem sap against Xanthomonas PV oryzae (González et al.,2012; Xu et al., 2013) and bacterial blight pathogen (Mahmood et al., 2006) were extensively reported. However, a few proteomic researches have been useful in investigating proteins responsive to BPH, which is a serious crop pest (Wei et al., 2009; Sangha et al., 2013). For example, proteins involved in response to BPH feeding were reported to include jasmonic acid synthesis, oxidative stress response proteins, β-glucanases, kinases, clathrin, glycine cleavage system protein, photosynthesis s and aquaporins, while proteins related to the callose metabolism remained unchanged (Wei et al., 2009). Furthermore, differential changes in twelve proteins, including S-like RNase, glyoxalase I, EF-Tu1 and salt stress root proteins were found in a BPH mutant resistant line. These proteins may be involved in an enhanced level of resistance against BPH in resistant line (Sangha et al., 2013). In the present study, proteins associated with rice plant interaction with BPH were identified, using a proteomic approach. These results may provide new information about the interaction between rice and BPH in order to better understand rice in terms of the BPH resistance mechanism and may lead to the development of new rice varieties, offering an effective means for the long-lasting control of BPH.

Results and Discussion

Feeding behaviors of BPH nymphs on rice plants with different cultivars

After rice plants were exposed to BPH for 14 days, the plant survival rates were monitored by using the IRRI scale, as shown in Table 1. Among the selected five cultivars, PTB33 expressed the highest resistance to BPH infestation (Supplementary fig.1). In previous studies, PTB33 is reported to have three genes (*BPH* 2, *BPH* 3 and *BPH* 9) associated with BPH resistance (Sai et al., 2013), whereas TN1 was completely destroyed by BPH, indicating that it is a highly susceptible cultivar. Other cultivars such as PSL2 showed moderate resistance but SP90 and CNT1 were moderately susceptible to BPH. PTB33 and TN1 were selected for further study and proteome analysis.

Proteomic analysis of proteins in rice seedling

To determine a protein's underlying difference between resistant and susceptible cultivars, all proteins were extracted from a 2-week rice seedling of both resistant and susceptible cultivars and then subjected to two-dimensional electrophoresis (2-DE). Highly reproducible protein spots across an 18 cm strip of PI 3-11 NL and molecular weight range 245-11 kDa were detected on CBB staining, shown in Fig.1. Nineteen different protein spots were observed between resistant and susceptible cultivars. These differentially regulated protein spots were successfully identified using LC-MS/MS analysis, followed by a Mascot database search of *Oryza sativa* in the NCBI database (Table 1). Among the 19 proteins, only two proteins were detected in the resistant cultivar (spots 18, 19), which were identified as proteins related to carbohydrate metabolism. Four proteins (spots 1, 2, 3 and 4) were up-regulated in the resistant cultivar involved in the stress response, antioxidant and protein synthesis. Thirteen spots (spots 5-17) showed high expression

Table 1. Survival rate of rice seedling after BPH feeding under cage condition.

Cultivars	BPH inoculation (days)													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
CNT1	HR[1]	HR	HR	HR	HR	HR	MR[3]	MS[4]	MS	MS	S[5]	HS[6]	HS	HS
SP 90	HR	HR	HR	HR	HR	R[2]	MR	MS	MS	MS	S	HS	HS	HS
PTB33	HR	HR	HR	HR	HR	HR	HR	HR	R	R	R	R	R	R
PSL2	HR	HR	HR	HR	HR	HR	R	R	R	R	MR	MR	MS	MS
TN1	HR	HR	HR	HR	HR	R	R	MR	MS	MS	S	HS	HS	HS

1= Highly resistance:HS, 2= Resistance:R, 3= Moderately resistance; MR, 4= Moderately susceptible; MS, 5= Susceptible; S, 6= Highly susceptible;HS

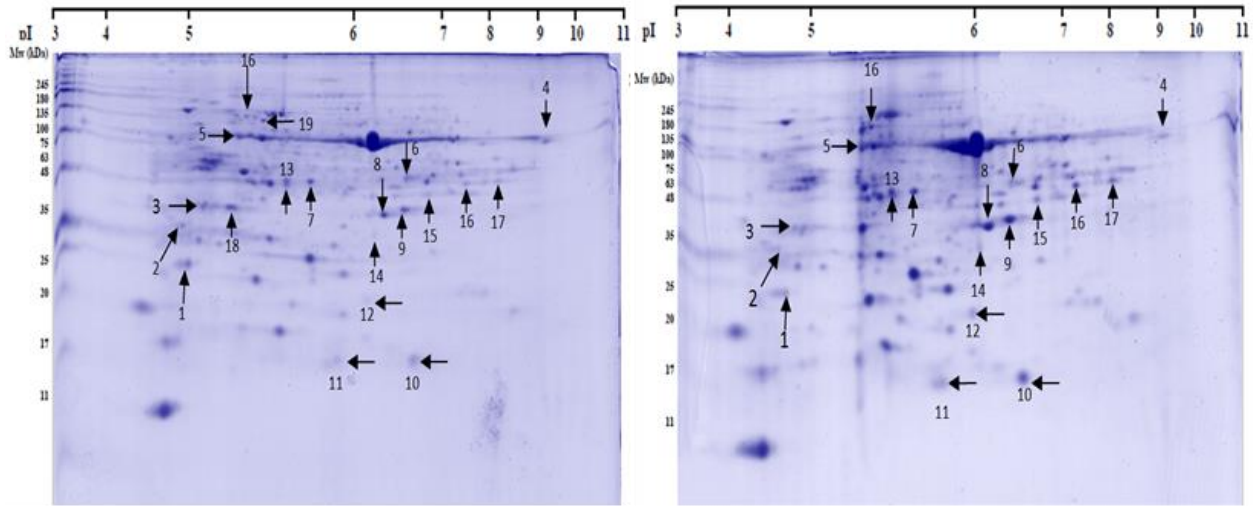

Fig 1. 2-D gel electrophoresis of (A) rice cultivar resistance to BPH and (B) susceptible cultivar were separated on 18 cm of the immobilized dry strip pH 3-11 NL and 12.5% SDS-PAGE in the second dimension.

in the susceptible cultivar. Most of them (spots 9-17) were photosynthesis-related proteins such as ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco) and their fragments (Fig. 1;supplementary fig.2). These 19 proteins were sorted and categorized according to their putative functions such as antioxidant proteins (spots 1, 2), response stress protein (spot 3), protein synthesis (spots 4, 8), ATP biosynthesis protein (spot 5), carbohydrate metabolism (spots 6, 13, 18 and 19) and photosynthesis proteins (spots 9-17) and other stress response proteins that may be related to resistance to pathogen attack at the seedling stage. However, proteins were categorized into the following three groups: (1) Proteins found only in the resistant cultivar; (2) high expression proteins in the susceptible cultivar; and (3) high expression proteins in the resistant cultivar, and all three will now be discussed.

Proteins found only in the resistant cultivar

Fructokinase (FK) and phosphoglyceratemutase (PGmutase) were only detectable in the PTB33 cultivar. Both proteins found in the fructose metabolism and the glycolysis pathway may be responsible for protecting rice plants from the effects of BPH attack. The main function of FK (spot 18) is in the fructose metabolism. There are two isoforms (FK1 and FK2) which have different enzymatic properties. The enzyme catalyzes the transfer of the phosphate group from ATP to produce fructose-1-phosphate (F-1-P). F-1-P is converted to fructose-6-phosphate (F-6-P) and consequently, to glucose 6-phosphate (G-6-P) by phosphoglucoisomerase (PGI) in cytoplasm. Also, PGmutase, (spot 19), catalyzes 3-phosphoglycerate (3PG) to 2-phosphoglycerate (2PG) in the glycolysis pathway. Up-regulation of both proteins in the resistant cultivar may activate the integrated metabolisms (glycolysis, shikimate and phenylpropanoid pathways) to promote resistant protein production (Fig. 2) (Mutuku and Nose, 2012). Brogue et al., 1991 suggested that a plant attempts to integrate a set of metabolic alterations to adapt to stress during stress response. For example, the glycolytic pathway is activated followed by the activation of the phenylpropanoid pathway of the rice infected with *Rhizoctonia solani* to produce lignin (Mutuku and Nose, 2010). A high amount of lignin accumulation in the plant cell wall may be responsible for improving rice resistance from pathogens. These results have combined with the previous investigations in identifying the differences between the resistant and susceptible cultivar in terms of the amount of lignin which higher levels detected in the resistant line (Mutuku and Nose, 2012). In addition, the expression of other glycolysis enzymes, including phosphofructokinase (PFK), triosephosphate isomerase (TPI), phosphoglycerate kinase (PGK), enolase and pyruvate kinase (PK) were also found in leaf sheaths infected with *R.solani* (Mutuku and Nose, 2010, 2012). Also, previous studies showed an increased expression of FK in rice cultivars that respond to salt stress (Sarhadi et al., 2012), pollen grain development (Kerim et al., 2003), high CO_2 conditions (Karni and Aloni, 2002), and Al^{3+} stress (Wang et al., 2014). Previous reports indicated that stress has a direct effect on the glycolysis pathway, leading to an enhanced generation of lignin in the phenylpropanoid pathway (Mutuku and Nose, 2010, 2012).
Thus, regulation of the glycolysis pathway may lead to the activation of glycolysis associated with a resistant response. Up-regulation of several enzymes in the glycolysis pathway is one of the strategies to increase pathogen resistance (Bolton, 2009).

Table 2. List of differentially expressed proteins of resistant and susceptible cultivars under BPH infestation condition.

Spot ID	Protein name	Theoretical Mr/pI	Score	Peptide Match	Accession number	Expression ratio	Biological functions
Proteins up-regulated in the resistant cultivar							
1	2-Cysperoxiredoxin BAS1	28079/5.67	146	12	NP_001047050	2.09	Antioxidant protein
2	Os02g0328300	30791/5.44	59	3%	NP_001046714	2.55	Electron transfer
3	Putative inorganic pyrophosphatase	31762 /5.80	88	14%	BAD16934	2.25	Energy production
4	Eukaryotic elongation factor 1	49590/9.09	111	6%	BAA23658	2.22	Protein synthesis
Proteins up-regulated in the susceptible cultivar							
5	Putative ATP synthase beta subunit	45937 /5.33	191	21	BAD82521	1.12	ATP synthesis
6	Glyceraldehyde-3-phosphate dehydrogenase	36641/6.61	59	7	AAA82047	1.52	Carbohydrate Metabolism
7	Putative chaperonin 21 precursor	26360/7.71	141	9	BAD35228	1.08	Protein folding
8	Proteasome subunit alpha type 6	32472/7.05	90	6	ABF94306	1.32	Programmed cell death
Proteins up-regulated in the susceptible cultivar							
9	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	53421/6.22	378	13	CAA28475	1.88	Photosynthesis
10	Chain S, Structure Of Rice Rubisco	15222/5.89	193	37	3AXM_S	1.20	Photosynthesis
11	Chain S, Structure Of Rice Rubisco	15269/5.89	81	8	3AXK_S	1.56	Photosynthesis
12	Ribulose-1,5-bisphosphate carboxylase/oxygenase	53428/6.22	87	4	NP_039391	1.20	Photosynthesis
13	Ribulose-1,5-bisphosphate carboxylase/oxygenase	53428/6.22	87	4	NP_039391	2.50	Photosynthesis
14	Ribulose-1,5-bisphosphatecarboxylase/oxygenase large subunit	53418/6.22	291	11	NP_039391	1.12	Photosynthesis
15	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	45615/8.43	126	4	AAB02583	1.81	Photosynthesis
Proteins up-regulated in the susceptible cultivar							
16	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	45615 /8.43	120	7	AAB02583	2.56	Photosynthesis
17	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	45615/8.43	108	4	AAB02583	2.50	Photosynthesis
Proteins expressed only in the resistant cultivar							
18	Fructokinase	35893/5.02	87	7	NP_001060837	-	Carbohydrate Metabolism
19	2,3-bisphosphoglycerate-independent phosphoglyceratemutase	60980/5.42	136	3	NP_001044625	-	Carbohydrate Metabolism

High expression proteins in the resistant cultivar

2-Cysperoxiredoxin BAS1 (2-CysPrx-BAS1), Os02g0328300, Putative inorganic pyrophosphatase (IPPase), and Eukaryotic elongation factor 1 (EF-1) were found 2.09, 2.55, 2.25, and 2.22 -fold up-regulated in resistant cultivar, respectively. In 2-Cys Prx-BAS1 (spot1), thiol-specific antioxidant enzyme (Dietz et al., 2006), the structure consists of two conserved cysteine residues associated with defense protein and expressed in the leaf during the rice seedling stage (Lim et al., 1993; Shao et al., 2008). This protein belongs to a family of peroxidases which are localized in the soluble chloroplast fraction (Baier and Dietz, 1997), plastid and mitochondria (Dietz et al., 2006). They also function to reduce alkyl hydroperoxide, scavenge reactive oxygen species (ROS) (Fan et al., 2011) and hydrogen peroxide (H_2O_2) (Dietz et al., 2006). Previous findings reported that, 2-CysPrx-BAS1 participates in cell multiplication, polarization, adjusting the concentration of H_2O_2 in cells and also protects against DNA damage (Dietz et al., 2006). Increased expression of 2-Cys Prx-BAS1 in a resistant cultivar might contribute to H_2O_2 detoxification, following plant cell injuries. Previous studies support the idea that antioxidant proteins are often up-regulated after elicitor inoculation and insect feeding in rice to prevent oxidative injuries in the cell (Mahmood et al., 2006; Liao et al., 2009; Wei et al., 2009). Moreover, Hao et al., 2011 reported that the antioxidant enzyme also appeared to eliminate ROS more efficiently in rice resistant to rice stripe disease, as a result, oxidative damage is less than in susceptible variety. The 2-CysPrx-BAS1 protein may be helpful in protecting against oxidative damage, caused by BPH in PTB33 cultivars, in the current study. EF-1 (spot 4) was slightly up-regulated in the rice resistant cultivar. EF-1 is responsible for protein synthesis as a function of the transportation of aminoacyl-tRNA to ribosome. It has been reported that the elongation factor-thermo unstable protein (EF-Tu), EF-1 related-protein family, was slightly up-regulated in rice leaf tissue and involved in protein synthesis (Lee et al., 2010). They have been found to relate to heat tolerance in plants by acting as a molecular chaperone and protecting heat-labile proteins from inactivation by thermal aggregation (Ristic et al., 2007; Lee et al., 2010). In addition, EF-Tu was up-regulated in rice roots and Aizaizhan roots responding to 0.1 mM Cd and 1 mM Cd stress, respectively (Ge et al., 2009). It is indicated that the expression of EF-Tu has an important role in plant adaptation to environmental stress (Singh et al., 2004). The higher expression of EF-1 in this study might promote the production of resistant proteins and repair damaged proteins in resistant cultivars**.** IPPase (spot 3), is an enzyme that catalyzes pyrophosphate to phosphate. It helps to decrease cell membrane damage and protect membrane integrity under cold stress. A previous study found that the *OVP1* gene encoding rice vascuolar-H^+-translocating IPPase was being induced by anoxia and chilling stress (Carystinos et al., 1995). Meanwhile, Zhang et al., 2011a reported that *OVP1*-transgenic rice has a higher survival rate than the wild type, under cold stress. Furthermore, overexpression of the *OVP1* gene in Arabidopsis, tomato, bentgrass and alfafa resulted in enhanced tolerance to salt and drought stress (Bao et al., 2009; Li et al., 2010b). The present study found that IPPase increased in resistant rice, and this may be because BPH disruption to the cell membrane leads to solute leakage, similar to rice under cold stress. Therefore, IPPase might be one of the proteins that is responsible for repairing cell injury after BPH feeding in a resistant cultivar.

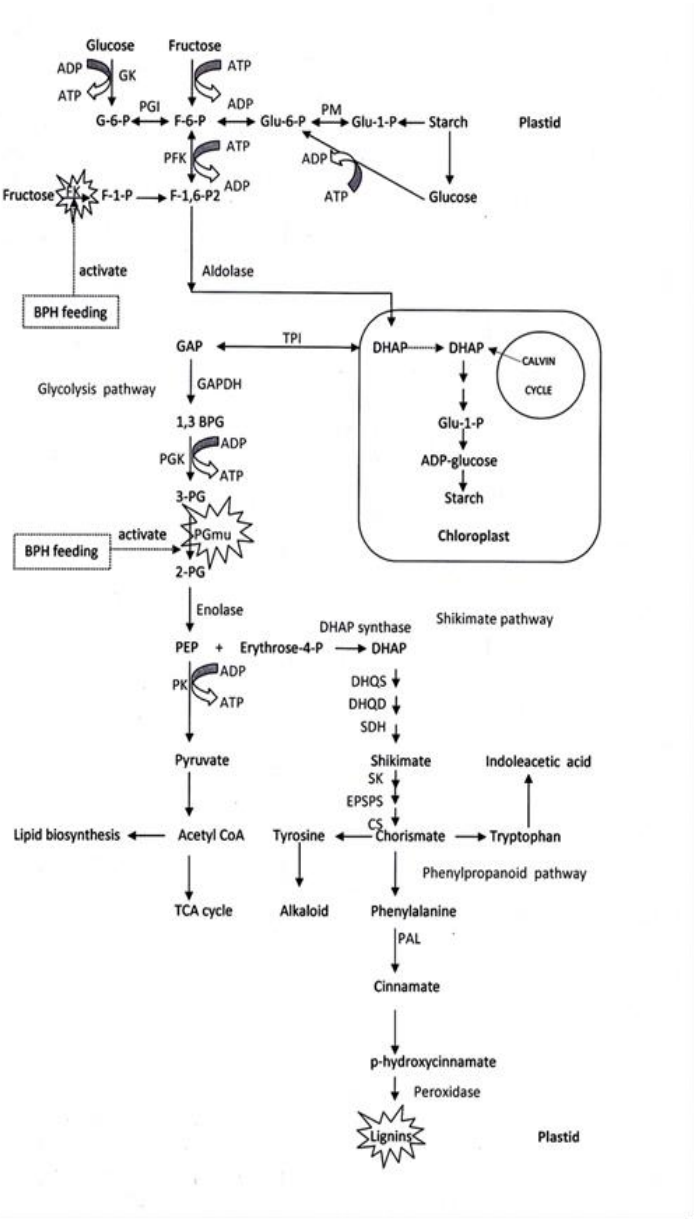

Fig 2. The activation of fructokinase (FK, EC 2.7.1.4) and phosphoglyceratemutase (PGmutase, EC 2.7.5.3) in fructose metabolism and glycolysis pathway connect to the tricarboxylic acid (TCA), Shikimate and phenylpropanoid pathway. The lignin is the end product of integrated pathway in current studies. The other related enzyme in glycolysis pathway were shown following: 6-Phosphofructokinase (PFK, EC 2.7.1.11), Phosphoglucomutase (PM, EC 2.7.5.1), Phosphoglucose isomerase (PGI, EC 5.3.1.9), Aldolase (EC 4.1.2.13), Triosephosphate isomerase, (TPI, EC 5.3.1.1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12), phosphoglycerate kinase (PGK, EC 2.7.2.3), Enolase (EC 4.2.1.11), Pyruvate kinase (PK, EC 2.7.1.40), In shikimate pathway; Dehydroquinate synthase (DHQS, EC 4.2.3.4), Dehydroquinase (DHQD, *EC* 4.2.1.10), Shikimate dehydrogenase (SDH, *EC* 1.1.1.282), Shikimate kinase (SK, *EC* 2.7.1.71), 5-Enolpyruvylshikimate 3-Phosphate synthase (EPSPS, *EC* 2.5.1.19), Chorismate synthase (CS, EC 4.2.3.5). Finally, enzyme in Phenylpropanoid pathway; Phenylalanine ammonia lyase (PAL, EC EC 4.3.1.24). and peroxidae. Adapted from Mukutu and Nose (2012).

High expression proteins in the susceptible cultivar

Five identified proteins were found at 1.12, 1.52, 1.08, 1.32 and > 1.12 –fold up-regulated in susceptible cultivar, including putative ATP synthase beta subunit (spot 5), glyceraldehyde-3-phosphate dehydrogenase (spot 6), putative chaperonin 21 precursor (spot 7), proteasome subunit alpha type 6 (spot 8)and photosynthesis related proteins (spot 9-17), respectively (Table 2). ATPase-β (spot 5) is the main enzyme for ATP biosynthesis in the presence of a proton gradient, across the thylakoid membranes (del Riego et al., 2006; Li et al., 2011; Chen et al., 2011). It is located in the inner membrane of mitochondria and thylakoid membranes of chloroplasts. The catalytic reaction of ATPase-β involves the production of ATP from ADP in the presence of a proton gradient across the membrane via β-subunits as catalytic sites. ATPase-β was found in rice responsive to heat (Chen et al., 2011), drought (Ji et al., 2012), water deficit (Ngamhui et al., 2012), cold (Cui et al., 2005) and rice leaf senescence (Liu et al., 2008). Senescence is the last stage of plant development, tissues, organs or the entire plant goes through a series of programmed cell death (PCD) processes. In the susceptible cultivar from this research, there was destruction from BPH, resulting in the senescence phenomenon rather than resistant cultivars. Thus, ATPase-β showed higher expression in the susceptible cultivar, which might be because they are acting as energy producers in all kinds of activities in cells, especially, senescence processes (Liu et al., 2008). However, Ngamhui et al., 2012 reported that two isoforms of ATPase-β and ATPase-α subunits were up-regulated under water deficit, indicating that linear electron flow was coupled to the generation of electrochemical gradients across the thylakoid membranes, leading to an increased abundance of both subunits. In addition, the susceptible cultivar becomes dryer than the resistance in this study; one possible explanation of this is that dehydrated water subsequently occurred, resulting in a high abundance of ATPase-β expression. We also identified proteasome subunit alpha type 6 (PSMA 6), (spot 8), in the susceptible cultivar. The proteasome is a multicatalytic proteinase complex that is involved in ATP/ubiquitin-dependent proteolytic pathways (Lee et al., 2007). The function of proteasome involves ubiquitin mediated proteolysis in phytochrome action, pathogen infection, stress responses and floral senescence (Bahrami and Gray, 1999). The up-regulation of PSMA 6 may promote proteolysis that is activated by ROS, associated with oxidative stress (Palma et al., 2002). This might be due to the low abundance of antioxidant proteins in the susceptible cultivar, as a result of the higher ROS generation associated with higher proteasome expression in this study.

Glyceraldehydes-dehydrogenase-3-phosphate (GAPDH) (spot 6) is an enzyme that catalyzes glyceraldehydes-3-phosphate (GAP-3-P) to D-glycerate-1,3-biphosphate (GAP-1,3 bi-P) in the glycolysis pathway to produce energy for the cell. GAPDH was found in the shoot, root and leaf of the rice seedling, but rarely in the stem. It has been shown to control the transcription and post translational level in various organelle of the plant cell. Previous reports showed GAPDH in rice plant up-regulation response to stress such as ROS (Su et al., 2014), abiotic stresses (PEG 6000 salt, heat, abscisic acid and methyl viologen treatments) (Zhang et al., 2011b) and also contributed aroma phenotype to the rice cultivar (Lin et al., 2014b). Recently, mRNA expression of a cytoplasm GAPC2 gene, in response to blast infection in rice leaves, was reported, suggesting that the expression of this gene may be relative to the disease resistance in the resistant cultivar (Su et al., 2014). In contrast, this research found that the increase of GAPDH in the susceptible cultivar may have the following two hypotheses: firstly, higher ROS production and accumulation in the susceptible cultivar than in the resistant cultivar, results in the activation of GAPDH protein. The second hypothesis is that there is a high amount of substrate (GAP-3-P) of this enzyme in susceptible cultivars. GAP-3-P accumulation consequently results from the high amount of Rubisco in the susceptible cultivar (Table 1). Rubisco catalyzes the fixation of one molecule of CO_2 to the pentose-bisphosphate sugar ribulose-1,5-bisphosphate (RuBP), yielding two molecules of the three-carbon phosphoglyceric acid (PGA) (Cleland et al.,1998). PGA is then reduced to glyceraldehyde-3- phosphate by ATP and NADPH produced in the light reactions (Ji et al., 2012). Therefore, the high expression of GAPDH might depend on the Rubisco abundance in the susceptible cultivar.

The Putative Chaperonin 21 (spot 7) precursor is a chloroplast co-chaperonin protein, composed of two chaperonin 10-like domains and involved in heat shock stress (Sano et al., 2013). They can bind to the RuBisCO large subunit of RuBisCO subunits holoenzyme assembly (Han et al., 2009). However, the main functions of the putative chaperonin 21 precursor are responsible for protein folding and assembly (Ma et al., 2012). Previous reports found that the putative chaperonin 21 precursor of rice leaf up-regulated under high-temperature (45°C) (Sano et al., 2013) and salt stress (Ma et al., 2012). In this study, up-regulation of the putative chaperonin 21 precursor in susceptible cultivars implied that protein folding and assembly in this cultivar was enhanced. Furthermore, the current study also found high abundance of photosynthesis-related protein in the susceptible cultivar such as Rubisco (spots 9,14,15,16,17), Chain-S-structure of rice Rubisco (spots10,11), Ribulose-1,5-bisphosphate carboxylase/oxygenase (spots 12,13), Rubisco and RuBisCO activase small isoform precursor (spots 10,11), respectively. Rubisco plays an important role in photosynthesis, since it catalyzes the carboxylation or oxygenation of riburose 1,5 biphosphate (RuBP) (To et al., 1999; Wang and Portis, 2006). The activity of Rubisco directly affects the rate of CO_2 fixation of plants (Wang et al., 2008b; Law and Crafts-Brandner, 2001). Therefore, Rubisco abundance in the susceptible cultivar might increase the photosynthesis rate. Furthermore, Rubisco-related proteins were migrated in multiple spots on 2-DE gel with different MW and PI, suggesting that the degradation of the large subunit of Rubisco was caused by higher oxidative stress and H_2O_2 accumulation (Desimone et al., 1996) in susceptible rather than resistant cultivars. However, Rubisco is particularly critical for proteomic analysis in green leaf tissue (Zhang et al., 2013). Zhang et al., 2013 developed and evaluated polyethyleneimine (PEI) assisted Rubisco cleanup (PARC) column as a new method for the removal and fractionation of Rubisco from a green leaf. The results reported in this work seem to support the view that the Rubisco existing in a green rice leaf does not involve in plant defense against a BPH herbivorous insect.

Materials and Methods

Plant materials and growth conditions

Five cultivars of rice were obtained from Phitsanulok Rice Research Center, Thailand. TN1 and PTB33 cultivars were used as susceptible and resistant reference cultivars, respectively. Rice seeds were soaked and grown in a seed tray containing soil from rice paddy fields, for 2 weeks. Ten

seedlings per row were separately grown for each cultivar, in a cage (Fan et al., 2011 and some modification).

Screening for BPH resistance capacity

The BPH were collected from rice paddy fields, Thailand. The insects were grown on TN1 cultivar in a cage until they laid their eggs, allowing the eggs to grow to the first instars nymph stage. First instars nymphs of BPH were inoculated onto the 2-week rice seedlings at a ratio of 8-10 insects per one rice seedling (Jairin et al., 2009 with some modification). Hopper burn symptom was monitored, based on the scoring system of the International Rice Research Institute (IRRI, 1996) until the susceptible cultivar showed wilting. Each seedling was scored as 0=no visible damage, 1=less than 50% of total rice showed yellow color of the first leaf, 2=more than 50% of total rice showed yellow color of the first leaf, 3=more than 50% of total rice showed quarter yellow color of the first and second leaves, 4=more than 50% of total rice showed half yellow color of the first and second leaves, 7=55-75% rice death, 8=80-95% rice death and 9=complete death or wilting. Interpretation of results was based on the standard evaluation system, with the rating scores of 0, 1-2, 3, 4-6, 7 and 8-9 being designated as HR=Highly Resistant, R=Resistant, MR=Moderately Resistant, MS=Moderately Susceptible, S=Susceptible and HS=Highly Susceptible, respectively.

Protein extraction

Each rice seedling (0.2g) was ground in liquid nitrogen and suspended in 1 mL of extraction buffer containing 50 mM Hepes-KOH pH 7.4, 10 mM $MgCl_2$, 1 mM EDTA-Na_2, 10% (w/v) glycerol and 5%(w/v) insoluble PVP, 5 mM DTT, 5 mM PMSF and protease inhibitor mix (GE healthcare BIO-science) was added. The crude extract was centrifuged at 10,000×g for 30 min at 4^{o}C, twice. The pellet was discarded and the supernatant was collected for protein precipitation. Briefly, 200 µl of the supernatant was mixed with 12% (v/v) TCA-24 mM DTT in acetone and allowed to precipitate overnight at -20^{o}C. The precipitated proteins were collected by centrifugation at 10,000×g for 20 min at 4^{o}C. The pellet was collected and washed with 1 mL of ice-cold acetone containing 20 mM DTT, three times, then centrifuged at 10,000×g for 15 min at 4^{o}C. The protein pellet was solubilized in lysis buffer (7M urea, 2M thiourea, 4%(v/v) CHAPs, 2%(v/v) IPG buffer, 65 mM DTT). Protein concentrations were determined by Bradford assay (Bio-Rad) with BSA as the standard. Each cultivar study was extracted in triplicate for proteome analysis (Sadiq et al., 2011; Hwang et al., 2011; Fan et al., 2011; Lin et al., 2008 with some modification).

Two-dimensional electrophoresis

2DE was performed according to the GE healthcare BIO-science manual and some modification. Briefly, proteins (450 µg) were mixed with rehydration buffer containing 7M urea, 2M thiourea, 2%(v/v) CHAPs, 2%(v/v) IPG buffer, 0.002% (v/v) bromophenol blue and placed into an IPG focusing tray. Isoelectric focusing (IEF) was carried out on the immobilized dry strip (18 cm, pH3-11NL) (GE healthcare BIO-science). The IEF conditions were set as follows: 500 V for 1 h, followed by 1000 V for 8 h, 8000 V for 3 h, 8000 V for 1.30 h and finally, 10000 V for 3 h. After IEF, the IPG strips were separated immediately in the second dimension of SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Prior to SDS-PAGE, the focused strips were equilibrated with gentle shaking at room temperature in 10 mL equilibration buffer solution (6M urea, 75mM Tris-HCl pH 8.8, 29.3% (v/v) glycerol, 2% (v/v) SDS, 0.002% (v/v) bromophenol blue) containing 10 mg/ml of DTT for 15 min, followed by equilibration in buffer containing 25 mg/ml of iodoacetamide for 15 min, respectively. The equilibrated strips were separated in the second dimension by 12.5% SDS-PAGE in a vertical slab. The protein spots were visualized by staining with colloidal Coomassie Blue G-250.

Gel image and data analysis

The Coomassie-stained gels were scanned using a scanner with a resolution of 300 dpi. The data and comparative analysis was performed using Image master 2D-Platinum version 5.0 software (GE Healthcare BIO-Science). The spot detection parameters were set as follows: 2 of smooth, 5 of min area, 1.00 of saliency and artifact spots were also removed. Gel matching was carried out using 15 landmark spots. The reproducibility and variation of data were considered from the scattering plot between %volume of spots in reference gel (y value) and % vol of spots in interested gel (x value).

Ingel digestion

Selected protein spots were manually excised from the CBB-stained gel by cutting into small pieces. Small gel pieces were de-stained with 10 mM ammonium bicarbonate in 50% (v/v) ACN for 1 h. The gel pieces were washed with ultrapure water for 2 min and followed by dehydration with 100% acetonitrile for 5 min or until gel piece turned opaque. The protein in the gel piece was reduced with 10 mM DTT in 10 mM ammonium bicarbonate for 1 h at room temperature. Cabamidomethyl reagent containing 100 mM Iodoacetamide in 10 mM ammonium bicarbonate was added for 1 h at room temperature, in the dark. The gel pieces were dehydrated twice for 5 min with 100% ACN before digestion with 10 ng trpsin (10-ng/mL trypsin in 50% ACN/10 mM ammonium bicarbonate) and immersed in 20 µl of 30% ACN overnight at 37^{o}C. The solution of extracted peptides was pipetted into a microcentrifuge tube and residual digested proteins were extracted by adding 30 µl of 0.1% TFA in 50% ACN (v/v) by shaking three times for 10 min. Supernatant was pooled with the first extract and dried at 40^{o}C overnight. The peptide sample was dissolved in 0.1% (v/v) formic acid for LC/MS-MS analysis (Ngamhui et al., 2012 with some modification).

Protein identification and database search

Peptide samples were injected into Ultimate 3000 LC System (Dionex, Sunnyvale, CA) coupled to an ESI-Ion Trap MS, HCT Ultra PTM Discovery System (Bruker, Rheinstetten, Germany) with electrospray at a flow rate of 300 nl/min to a nanocolumn (Acclaim PepMap 100 C18, 3 mm,100A˚, 75 mm id;150 mm). The solvent gradient utilized in this analysis was (0.1% formic acid in water) to 80% of solvent B (0.1% formic acid in 80% ACN) with the gradient running within 40 min. Mass Spectrometry (MS) raw data was obtained from LC-MS/MS analysis and analyzed by Mascot software (Matrix Science). Mascot MS/MS ion analyzed by searching the NCBI database and *O.sativa* was chosen for the taxonomic category. Other parameters were set according to a maximum of 1 missed cleavage per peptide, a fixed

modification of carbamidomethyl (C), variable modifications of oxidation (M), the experimental mass values are monoisotopic, Peptide charge of 1+ 2+ 3+, error for MS/MS fragment ion mass values (MS/MS total) of 0.6 Da, error on experimental peptide mass values of 1.2 Da and an instrument was set at ESI-TRAP. In addition, other parameters were set according to their default value in the software. The matches with high score and significant levels were reported as protein names and functions (Ngamhui et al., 2012 with some modification).

Conclusion

In this study, we successfully identified proteins in rice cultivar that might be related to BPH resistance. 2-DE coupled MS were applied to identify differentially expressed proteins in the rice cultivars resistant to BPH, compared with susceptible cultivars. Lignin production by activated glycolysis connected to a phenylpropanoid pathway may be responsible for rice resistance to the BPH mechanism. These findings suggested that the identified proteins are providing important information for plant breeders to develop rice resistant to BPH.

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