Identification of differentially expressed proteins in wheat after benzothiadiazole treatment

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Abstract

The systemic acquired resistance (SAR) mechanism is stimulated by biological and chemical agents in response to pathogen infection as a part of the innate immunity response system of plants. The externally applied synthetic chemical, benzo-(1,2,3)-thiadiazole-7-carbothioic acid-S-methyl ester (BTH), is also known to induce a SAR response in plants. Studies identifying genes induced or suppressed by BTH are limited. Only few genes responding to BTH are determined. The focus of this preliminary study is to identify the gene products affected by BTH in wheat. A 2D-polyacrylamide gel electrophoresis (PAGE) analysis was carried out with BTH treated *Triticum aestivum* cv. Gerek-79 and mock treated samples. Following 2D-PAGE image analysis, the selected differentially expressed protein spots were identified by nanoLC-ESI-MS/MS. Among the 26 protein spots distinguished, five were found to be increased upon BTH treatment, another set of seven spots were absent in the control sample.Thus, they were apparent only in the gel of the BTH treated sample (+BTH), whereas five protein spots disappeared in the gel of the BTH treated plants (-BTH). Up-regulation of some proteins such as OEE2 (oxygen evolving enhancer protein) and COR (cold-responsive) LEA (late embryogenesis abundant)/RAB (responsive to abscisic acid, ABA) and down-regulation of some proteins such as RuBisCo LSU (large subunit), fructose 1, 6-biphosphate aldolase (AldP), methyl binding domain protein 6 (MBD6), and 3-isopropylmalate dehydrogenase are shown for the first time in BTH treatment of wheat.

Key words: Benzo-(1,2,3)-thiadiazole-7-carbothioic acid-S-methyl ester (BTH), 2D-PAGE, nanoLC-ESI-MS/MS analysis, proteomics, wheat.

Introduction

Rusts, mildews, and septoria belong to the most damaging fungal diseases in wheat. Plants protect themselves by activating various defense mechanisms.The two of the intertwined and preconditioned responses are induced systemic resistance (ISR) and systemic acquired resistance (SAR), differing in the induction pathways depending on the type of elicitor. SAR can be activated by biotic or abiotic elicitors and dependent on both resulted in the accumulation of salicylic acid (SA) and PR proteins. On the other hand, ISR functions independently of SA, but utilises jasmonic acid (JA) and ethylene (ET) by biotic elicitors such as plant growth promoting rhizobacteria (reviewed by Vallad & Goodman 2004). SAR leads to pathogen resistance in the entire plant after biological or chemical treatment (Oostendorp et al. 2001). The most important and the most commercially popular chemicals for induction and enhancement of the defense mechanisms of plants against a wide variety of pathogens are benzo-(1,2,3)-thiadiazole-7-carbothioic acid-S-methyl ester (BTH), 2,6-dichloroisonicotinic acid (INA), and SA. INA and BTH are structurally similar to SA (Görlach et al. 1996). They are also the most thoroughly investigated chemical inducers of SAR (Friedrich et al. 1996, Görlach et al. 1996). In contrast to fungicides that directly affect the pathogen metabolism, BTH induces resistance responses against wheat and other grass pathogens such as powdery mildew (*Blumeria graminis*), leaf rust (*Puccinia triticina*), and Septoria leaf spot (Görlach et al. 1996) by activating SAR in the host plant. Thus, analysis of the effects of BTH on the molecular level could be informative for the elucidation of the BTH induced defense mechanism. Most of the molecular studies on determining differentially expressed genes focus on describing differences of RNA levels. However, recent advances in mass spectroscopic analysis on proteins and the accumulation of genome or transcriptome sequence data allow the identification of proteins directly. Thus, the objective of our study was to investigate changes of the proteome of wheat leaves upon treatment with BTH. The results show that BTH affects different cellular functions including defense response.

Materials and methods

Plant material and treatment of seedlings

Wheat seedlings of the cultivar Gerek-79 were used in the experiments. The plants were grown in growth chambers in soil and maintained with a 16 h photoperiod. Ten days after sowing 25 seedlings were treated with 250 ml of 0.3 mM BTH in water (+BTH) and 25 control seedlings were treated

with water. Both groups of plants were placed into different boxes. Leaves were harvested 7 days after treatment and stored at −80°C.

Protein extractions

The TCA (trichloro acetic acid)-acetone protein precipitation method was considered to be the best in our hands after testing various soluble protein extraction methods. The total protein was extracted according to Damerval et al. (1986). Plant leaves (1 g) were ground to a fine powder in liquid nitrogen. Ten ml of TCA (10%) in cold acetone containing 0.07% DTT was added and protein precipitation was performed at −20°C overnight. The precipitate was collected by centrifugation at 35 000 × g for 20 min at 4°C. The supernatant was gently decanted and the pellet was washed six times with cold acetone containing 0.07% DTT and dried under vacuum. The resulting powder (1 g) was dissolved in 4 ml solubilisation buffer (7 M urea, 2 M thiourea, 1.25% SDS, 0.5% DTT, 5 mM potassium carbonate, 6% triton X-100, 1% ampholyte pH 3-10 (BioRad Laboratories, Hercules CA, USA)). The sample was then centrifuged at 14 000 × g for 10 min at 4°C. Undissolved material appeared as precipitate and was discarded. The final protein content was determined using the Bradford dye binding assay (Sigma). The protein sample was aliquoted and stored at −80°C.

Two dimensional gel electrophoresis (IPG-IEF) and image analysis

The protein sample was diluted with rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 65 mM DTT and 1% ampholyte pH 3-10) to a final concentration of 1 mg ml^{-1} IEF strips (17 cm, BioRad Laboratories, Hercules CA, USA) were rehydrated on a BioRad rehydration/focusing tray under a layer of mineral oil at 20°C for 12 h and focused for a total of 75 kVh on BioRad Protean-IEF. After isoelectric focusing, strips were removed and equilibrated as recommended by the manufacturer. The equilibrated strips were then placed onto the second dimension 12% SDS-PAGE. The SDS-PAGE was conducted in a standard Tris-Glycine-SDS buffer in Protean II xi Cell (BioRad Laboratories, Hercules CA, USA) at a constant current setting of 5 mA per gel for 1 h, then at 25 mA per gel until the bromophenol blue dye front disappeared at the bottom. The stepwise colloidal Coomassie Brilliant Blue (CBB) G250 stained gels (Neuhoff et al. 1985) were analysed using PdQuest version 8.0.1. Two biological replicates and three 2D-gel technical replicates were studied. Experimental pI and molecular weight values were determined by comparing the co-migrated 2D-SDS-PAGE standards (Bio-Rad) in 2D-PAGEs electrophoresed in the same running conditions. Differences between density of protein spots in mock and BTH treated samples were tested by Student's T-test with 95% confidence using SPSS (statistical packages for social sciences, version 10.0). The spots having 1.5 fold up- or down-regulation with 95% confidence level were selected for further analysis.

NanoLC-ESI-MS/MS analysis

The spots were identified by Proteome Factory (Proteome Factory AG, Berlin, Germany; http://www.proteomefactory.com) on a system consisting of an Agilent 1100 nanoLC system (Agilent, Germany), PicoTip emitter (New Objective, USA) and an Esquire 3000 plus ion-trap MS (Bruker, Bremen, Germany). The proteins were identified using MS/MS ion search of Mascot search engine (Matrix Science, London) and either UniProtKB database or nr protein database (National Center for Biotechnology Information, Bethesda, MD, USA) against to either *Triticum aestivum* or whole plant database (within which blast is performed (Altschul et al. 1990)). Search parameters which were used for MS/MS ion search of Mascot search engine are as in the followings: Variable modifications: oxidation (M), mass values: monoisotopic, protein mass: unrestricted, peptide mass tolerance: ± 0.1%, fragment mass tolerance: ± 0.5 Da, max missed cleavages: 1, instrument type: ESI-QUAD-TOF, number of queries: 300. Subcellular localisation predictions were obtained from PSORT web server (http://wolfpsort.seq. cbrc.jp) (Horton et al. 2007).

Results

Triticum aestivum cv. Gerek-79 was selected since it is a commonly planted cultivar in the country. Although, it does only possess moderately disease resistance genes, it can withstand drought. Therefore, it is still a preferred cultivar by farmers. We studied the samples 7 days after the BTH treatment based on the report of Vechet et al. (2005), where it was indicated that a period of seven day after BTH treatment was effective against pathogen infection (powdery mildew) in wheat. Although, 2D-gels using IEF strips with pH range of 4−7 were also investigated, since more variations were observed beyond the pH range of 4−7, we decided to focus our analysis on the gels using the pH range of 3−10 of IEF strips. In the gel profiles presented in Fig. 1 (A: −BTH (control) and B: +BTH) and Fig. 2, the most obvious differential spots are indicated. The ones further investigated for the peptide fragment identification are presented in Table 1.

Among all the differentially expressed proteins identified in this study, 12 spots were detected as enzymes involved in oxidative phosphorylation and glycolysis, the spots 5 (putative thioredoxin peroxidase) and 14 (putative dehydroascorbate reductase) are involved in protection of the cells against oxidative stress (Zhang et al. 1997, Cervilla et al. 2007). The spot 15 (ferredoxin-NADP(H) oxidoreductase) was identified as a protein involved in photosynthesis related reactions, including the reduction of NADP$^+$, cyclic photophosphorylation, and the light regulation (Hosler & Yocum 1987). Spot 16 was identified as osr40c1 protein which is a member of a novel abscisic acid (ABA)-responsive protein family. The spots 20, 29, 41, and 51 are glycolysis and Calvin Cycle enzymes; others, 10, 52, and 53 are H(+) transporting ATP synthase and ATP synthase CF1 alpha chain fragments, respectively. The hits possessing a higher Mascot Score are
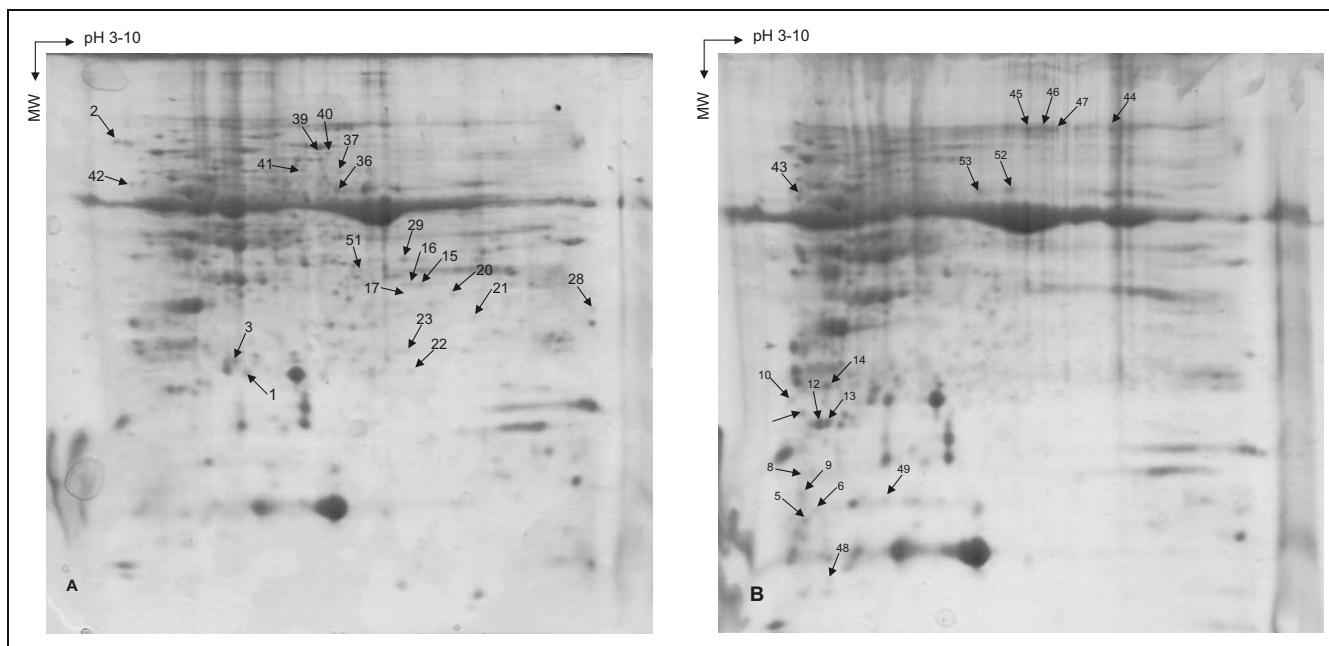
Fig. 1:   2D-PAGE patterns of control and BTH treated plants of total soluble proteins from (A) wheat leaves mock treated with water (17 days post treatment) and (B) wheat leaves treated with 0.3 mM BTH (17 days post treatment). Both gels show patterns using pH 3–10 in the first dimension and 12% SDS-PAGE in the second, stained with colloidal Coomassie Brilliant Blue G250. Spots indicated with arrows were excised for MS.

considered to be the likely identity of the spots. The spots 11, 37, and 43 did not show any significant hit. Subcellular localisations of the identified proteins were predicted according to Horton et al. (2007). Table 1 summarises the information obtained on the differentially expressed spots as the best identity hits, peptide matched, sequence coverage, Mascot score, ID and taxonomy, fold of induction or suppression, the theoretical versus experimental molecular weight and pI values, and the subcellular localisations.

Discussion

Proteins in energy production and consumption

Many spots identified in this study are involved in oxidative phosphorylation and/or electron transfer chain (ETC) leading to ATP synthesis. Previous findings of the increased expression level of oxygen evolving enhancer protein 2 (OEE2), spot 1, in methyl jasmonate treated cocoa leaves (Verica et al. 2004) and rice also under salt stress (Abbasi & Komatsu 2004) support the established consensus of BTH stimulating the induced resistance responses (Walters & Heil 2007). It was also demonstrated that OEE2 interacts with WAK1 (wall associated kinase 1) acting as a substrate for WAK1, which is known to be up-regulated together with the pathogenesis related protein-1 (PR-1), an induced marker protein upon avirulent pathogen infection. The phosphorylation of OEE2, *via* a glycine-rich secreted protein, AtGRP-3 which is mediated by WAK1, was enhanced in Arabidopsis infected with avirulent *P. syringae* (Yang et al. 2003). These findings suggest that OEE2 plays a significant role in defense signaling.

H+-ATPase (in our case, identified as a putative H+-transporting ATPase; spot 10) was identified as a chloroplast thylakoid membrane protein and was found to be induced as a response to pathogen infection (Jones et al. 2006). The lowered expression of Ferredoxin-NADP reductase (FNR) (spot 15 and spot 17) was previously shown in *P. syringae* treated *A. thaliana* leaves (Jones et al. 2006). BTH by reducing the FNR expression in turn may mimic pathogen induced plant response.

Spot 41, identified as succinate dehydrogenase (SDH1-1) enzyme, was decreased after BTH treatment. This enzyme, as part of the electron transport chain complex II, plays an important role in both the TCA cycle and the respiratory chain. It was shown that SDH inhibitors are increasing the resistance against abiotic stress in plants (www.faqs.org/patents/app/20110196000).

Spot 51, which is absent in BTH treated plant, was identified as 3-isopropylmalate dehydrogenase. It is involved in the biosynthesis of branched-chain amino acids including leucine (Nozawa et al. 2005). Jiang et al. (2007) showed that the abundance of this protein was decreased in salt stressed *A. thaliana*. The transcription level of 3-isopropyl malate dehydrogenase was shown to be increased in potato infected with *Phytophthora infestans* when BTH treated sample was used as control (Beyer et al. 2001).

It has been shown that SA very rapidly induces the inhibition of ATP synthesis in tobacco cells *via* rapid inhibition of mitochondrial electron transport and oxidative phosphorylation (Xie & Chen 1999). Our observation of the reduced expression level of ATP synthase CF1 alpha chain (spots 52 and 53) upon BTH treatment may suggest lowering of ATP synthesis. The proteins of the spots, 52 and 53 have the

Table 1: Proteins identified as up- and down-regulated upon BTH treatment using nanoLC-ESI MS/MS analysis.

Spots	Putative identity	PM	QM	MOSP	FDR (%)	SC	Mascot-Score	ID	Taxonomy	MW (kDa) Exp/Theor	pI Exp/Theor	Fold change[a]	Presence & absence	Subcellular localisation[b]
1	Oxygen-evolving enhancer protein 2 (OEE2)	6	8	2	0.00	35	299	Q00434	T. aestivum	31/27.253	5.7/8.84	2.34x↑		Chloroplast
2	Methly binding domain 6 (MBD 6)	1	1	–	0.00	9	119	A4UQN7	T. aestivum	90/20.684	5.3/4.88		–BTH	Nucleus
3	Putative chaperonin 21 precursor	2	2	–	0.00	4	66	BAD35228	O. sativa	30/26.303	5.5/7.71	1.79x↓		Chloroplast
5	Putative thioredoxin peroxidase	3	7	2	0.00	21	207	BAD16416	O. sativa	21/23.165	4.7/5.15		+BTH	Chloroplast
7	Cold-responsive LEA/RAB-related protein	3	3	–	0.00	32	209	Q9M4T9	T. aestivum	16/17.150	4.7/4.84		+BTH	Nucleus
8	Gibberellic acid responsive protein (ES2A clone)	2	2	–	0.00	16	88	CAA55976	H. vulgare	16/18.372	4.9/4.84		+BTH	Nucleus
9	Group 3 late embryogenesis related protein	2	2	–	0.00	19	140	A7VL25	T. aestivum	21/18.325	4.7/5.00		+BTH	Nucleus
10	Predicted protein	1	5	1	12.56	9	198	F2DQX8	H. vulgare	25/26.640	4.5/4.98		+BTH	Chloroplast
	Putative H(+)-transporting ATP synthase	–	–	–	–	7	46	BAD15636	O. sativa	25/26.202				–
11	No significant hit	–	–	–	–	–	–	–	–	23/–	4.8/–		+BTH	–
12	2-cys peroxiredoxin BAS1, chloroplast precursor	9	61	5	0.00	58	522	P80602	T. aestivum	22/23.284	5.1/5.48	1.89x↑		Cytosol
13	2-cys peroxiredoxin BAS1, chloroplast precursor	6	42	3	0.00	69	626				5.4/5.48	1.91x↑		Cytosol
14	Putative dehydroascorbate reductase	2	3	1	50.00	11	50	ABB47766	O. sativa	23/23.546	5.4/5.44		+BTH	Chloroplast
15	Ferredoxin-NADP(H) oxidoreductase	6	16	5	11.11	27	307	Q8RVZ9	T. aestivum	39/38.792	6.4/8.29	2.21x↓		Chloroplast
16	osr40c1(member of ABA responsive family or Euonymus Lectin domain containing proteins (ELUs))	4	5	1	0.00	3	65	CAA64683	O. sativa	39/38.799	6.5/6.30	1.48x↓		Cytosol
17	Ferredoxin-NADP(H) oxidoreductase	1	1	–	20.00	1	303	Q8RVZ9	T. aestivum	38/38.782	6.4/8.29	1.31x↓		Chloroplast
20	RuBisCo large subunit	4	5	1	0.00	13	129	P11383	T. aestivum	38/52.851	6.5/6.22	1.73x↓		Cytosol
21	ATP Synthase subunit E	5	6	1	0.00	17	210	Q2XP43	T. aestivum	38/26.245	7.3/6.57	2.05x↓		Cytosol
28	Voltage dependent anion channel (VDAC)	3	3	–	0.00	19	137	Q41590	T. aestivum	38/29.298	9.3/9.33		–BTH	Cytosol
29	Fructose-bisphosphate aldolase	3	8	2	16.67	5	93	ACO44684	T. aestivum	40/38.300	6.4/6.48		–BTH	Cytosol
37	No significant hit	–	–	–	–	–	–	–	–	71/–	6.2/–		–BTH	–
41	Succinate dehydrogenase (SDH A); ATP binding	3	3	–	0.00	8	103	F2EL27	T. aestivum	71/67.967	6.0/5.86		–BTH	Mitochondria
43	No significant hit	–	–	–	–	–	–	–	–	65/–	4.8/–		–BTH	–
48	Hypothetical prot: Thylakoid lumenal 15 kDa prot	2	3	1	0.00	14	124	BAF03916	O. sativa	18/20.562	5.2/6.71	2.94x↓		Chloroplast
49	Cytochrome b6-f complex iron-sulfur subunit or Rieske iron-sulfur protein	3	7	1	0.00	25	165	AAM88439	T. aestivum	23/23.711	5.6/8.47	2.34x↑		Chloroplast
51	3-isopropylmalate dehydrogenase	5	8	2	0.00	22	228	NP_001149947	Z. mays	39/39.883	6.4/6.33		–BTH	Mitochondria
52/53	ATP synthase CF1 alpha chain	12	28	7	0.00	53	542	P12112	T. aestivum	65/55.261	6.1/6.11	1.79x↓		Chloroplast

Spot numbers are the same as in the 2D-PAGE in Fig. 1. Proteins were identified by MS/MS analysis and MASCOT search of MS/MS spectra with BLAST. All identifications met statistical confidence criteria according to MASCOT (Perkins et al. 1999) and BLAST (Altschul et al. 1990) scoring schemes. PM: Peptides matched, QM: Queries matched, MOSP: Multiple observations of same peptides, SC: Sequence coverage, FDR: False discovery rate (Peptide matches above homology or identity threshold (Peptide matches above identity threshold values are 0% for all the spots). x↑ fold up-regulated with BTH treatment, x↓ folddown-regulated with BTH treatment, +BTH: only detectable/observed in BTH treated sample, absent in control sample, –BTH: only detectable/ observed in control sample, absent in BTH treated sample, a: $p < 0.05$, b: Subcellular localisation is predicted by wolf-PSORT
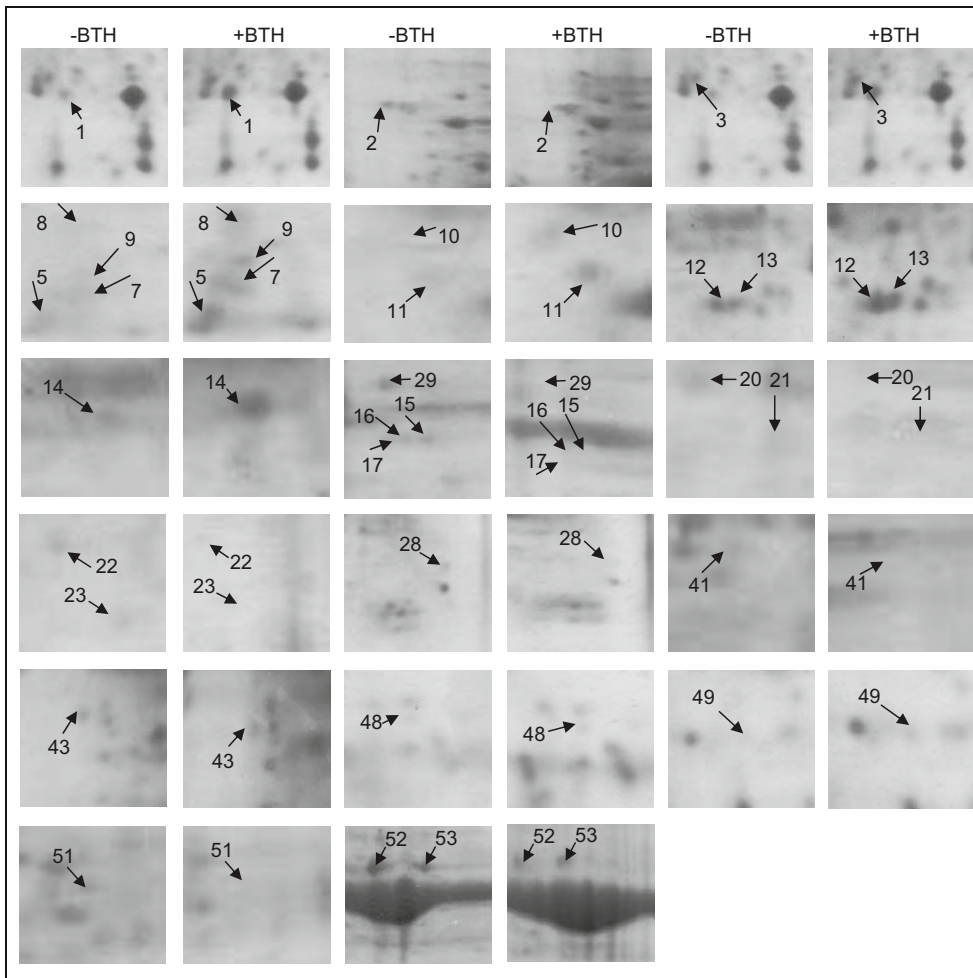
Fig. 2:   Comparisons of spots on 2D-PAGE: control (-BTH) and after BTH treatment (+BTH).

same molecular weights, and also reveal the same identity with very similar pI values. The expression levels of ATP synthases and voltage dependent anion channel (VDAC) (spot 28) were also found as down-regulated when *Arabidopsis thaliana* was treated with *P. syringae* (Jones et al. 2006). In that study, another protein that we have detected as vacuolar proton ATPase subunit E (Spot 21), responsible for generating energy for the transport of ions and metabolites and having an important role as a stress response enzyme, was also shown to be decreased (Jones et al. 2006).

Another set of enzymes identified with reduced expression levels were the RuBisCo (spot 20) and AldP (spot 29), both are part of the Calvin Cycle. There are substantial reports of these enzymes being regulated under stress conditions or by treatments of defense compounds. In previous studies, the expression of RuBisCo LSU was shown to decrease upon JA treatment in barley, in turn reducing carbon fixation (Maslenkova et al. 1992, Rakwal & Komatsu 2000). Down-regulation of RuBisCo LSU was also observed in ozone stressed *Oryza sativa*, determined *via* proteomics study (Agrawal et al. 2002). The transcript level of RuBisCo LSU also decreased in *Nicotiana attenuate* when attacked by several insects and when treated with several elicitors such as SA, JA, and BTH, as shown by microarray analysis (Heidel & Baldwin 2004). Our observation of the reduction of RuBisCo LSU (spot 20) upon BTH treatment may suggest

that BTH is stimulating similar responses as JA treatment. Interestingly, spot 3 with decreased expression level, identified as mitochondrial chaperonin 21 precursor protein assisting the folding of assembly proteins, as subunits of cytochrome b2, oligomeric yeast mitochondrial F1-ATPase, and the mitochondrial Rieske Fe/S protein (Cheng et al. 1989), also known to involve in assembling RuBisCo LSU (Sieger et al. 2005). The down-regulation of molecular chaperones was also shown in fungal elicitor-treated Arabidopsis cell cultures (Chivasa et al. 2006). In previous studies, it was shown that AldP decreased in ozone stressed *Oryza sativa* (Agrawal et al. 2002). AldP (in our study, spot 29) increased in the first 48 h with 50 mM NaCl treatment and then decreased (Abbasi & Komatsu 2004). Also, this enzyme has two isozymes in *Nicotiana* known as AldP1 and AldP2, under salt stress mRNA accumulation of AldP1 is decreased, whereas mRNA AldP2 accumulation is increased (Yamada et al. 2000).

Stress responsive proteins

In addition to the proteins and enzymes involved in energy consumption, we have also identified enzymes that are particularly responding to stresses. The spots 5, 12, and 13, all were shown up-regulated after BTH treatment, identified as

peroxiredoxin family proteins which are localised in chloroplasts (Baier & Dietz 1997, Horling et al. 2003). Peroxiredoxins are known to reduce H_2O_2, alkyl hydroperoxides, and hydroxyl radicals. Peroxiredoxins were also shown to have antioxidant activity in protection of lipids, enzymes, and DNA against radical attacks (Lim et al. 1993, Netto et al. 1996, Baier & Dietz 1997) and preventing damage to the chloroplast due to oxidative stress (Dietz et al. 2002). In rice against *X. oryzae* pv. *oryzae* (Mahmood et al. 2006) peroxiredoxin was induced by means of regulating the oxidative stress. Spot 5 was identified as thioredoxin peroxidase. This enzyme was shown to prevent H_2O_2 accumulation in cells, and thus it protects cells from apoptosis in response to reactive oxygen species (ROS) (Zhang et al. 1997). Spots 12 and 13 are both identified as 2-cysperoxiredoxin, chloroplast (BAS1) precursor (thiol-specific antioxidant protein) which are shown to be stress-induced (Balmer et al. 2004). A possible role for 2-cys peroxiredoxin in defense signaling lies in their ability to reduce reactive nitrogen peroxides, such as peroxynitrite (Bryk et al. 2000), and were generated during incompatible interactions.

Spot 14, which was up-regulated after BTH treatment was identified as dehydroascorbate reductase (DHAR), a stress responsive protein.DHAR is one of the key enzymes responsible for ascorbate regeneration. The enzyme is known to be increased under stress conditions such as wounding and salt stress in tomato (Grantz et al. 1995, Mittova et al. 2003), and upon methyl jasmonate treatment in broccoli (Nishikawa et al. 2003), in Arabidopsis (Sekimoto et al. 2005), and ozone stress in Arabidopsis (Yoshida et al. 2006). It acts as a diffusible signal that leads to SAR (Noctor & Foyer 1998). Another stress responding protein shows similarity to late embryogenesis related proteins. These are the cold-responsive LEA/RAB related COR protein (spot 7) and the group 3 late embryogenesis related protein (spot 9). Previously, it has been shown that SA treatment decreased the effects of chilling injury in maize (Janda et al. 1999, Szalai et al. 2000), in wheat leaves (Tasgin et al. 2003), and sweet peppers (Fung et al. 2004). The cold-responsive LEA/RAB-related COR protein was shown to be up-regulated responding to ABA and GA3 in common wheat (Tsuda et al. 2000). It was also found that LEA proteins were able to suppress protein aggregation and inactivation under water-stress conditions (Goyal et al. 2005). Spot 7 also shows similarity to the ES2A clone, a gibberellic acid (GA3)-inducible protein, which has a region showing homology to late embryogenesis abundant proteins (LEA) (Speulman & Salamini 1995).

Spot 16 showed hit to Osr40c1 protein, which was identified by Moons et al. (1997) as a 40 kDa histidine-rich ABA-responsive salt stress induced protein in rice roots. As other Osr40 proteins were found later, recently Osr40 s were classified as a multigene family of Euonymus lectin (EUL) domain containing proteins (EULs). EUL domains are present in many stress response proteins suggesting a role in stress signalling (Fouquaert et al. 2009). In this study, it was found to be down-regulated upon BTH treatment.

Spot 2 was identified as a methyl binding domain 6 protein (MBD6). In Arabidopsis, it specifically binds to methylated CpG sites and considered that MBDs infer CpG methyla-

tion-induced formation of repressive chromatin (Luo et al. 2012). The involvement of MBDs in epigenetic regulation of transcription of genes was predicted through the interactions with histone acetylation and deacetylation mechanisms. Interestingly, in Yasuda et al. (2008), it has been shown that AtMBD6 level is increased in absisic acid (ABA) treatment or ABA induced stress condition, such as salt stress. It is also known that ABA behaves antagonistically to SAR in plants (Yasuda et al. 2008). Thus, BTH may be induce SAR by reducing the level of MBD with which the altered DNA methylation resulted in the regulation of transcription. This may also suggest, not only the observed change in DNA methylation caused by several abiotic stress factors is also mimicked by BTH treatment, but also by doing so, it is aiding to retain long lasting stress memory of plants.

In a proteomics study by Li et al. (2012) analysing SA treated susceptible and resistant cultivars of rice seedlings to *Magnaporthe oryzae*, it has been noticed that few of the differentially expressed proteins are common as in our study, which are up-regulated (chaperonin 21 precursor, RuBisCo LSU, ATP synthase CF1 alpha subunit), except 2-cysperoxiredoxin BAS1, which is down-regulated. Interestingly, all of these proteins are found to be regulated in an opposite manner in our study. We found that the protein spot identified as RuBisCo LSU, spot 20 in our study, has experimental MW value of 38 kDa. It is known that the excess radical oxygen species can cause fragmentation of LSU (Cohen et al. 2005). In a study it was shown that light and hydroxyl-radical exposed chloroplast extracts lead the purification of 37 kDa fragment of RuBisCo LSU in green algae (Ishida et al. 1999). Our finding of reduced fragmented RuBisCo is in accordance with other studies such as Pancheya et al. (1996) and Bilgin et al. (2010). However, the fragment length of the increased RuBisCo LSU in Li et al. (2012) was not indicated. In another study, the decline in ATP synthase, fructose bisphosphate aldolase were also observed in BTH treated plants in addition to the reduced levels of triose phosphate isomerase and glutamine synthetase and were suggested that BTH causes significant changes in primary metabolism explaining for the observed decrease of vegetative growth of foliage and rhizome result of BTH treatment (Hukkanen et al. 2008). Our data present consistency with the well-known detected expression levels of proteins or activities in SAR mechanism, such as up-regulation of peroxidases, and down regulation of photosynthesis. Thus, the discrepancies are most likely resulting from the use of different plant species and duration of potentiation period and concentration of BTH treatment which differ in priming of SAR or the inducers. This should raise even more awareness on the complexity of the plant immunity and should lead to more system biology studies.

In conclusion, the down-regulated proteins identified here are mostly involved in energy metabolism, thus BTH may sustain the plant to down-regulate its metabolic activities to guard itself for potential stress factors mimicking similar plant responses observed when they are attacked by pathogens or under abiotic stress. On the other hand, most of the identified proteins, which are found as up-regulated are the

stress responding proteins by which plants are further prepared against pathogen attacks.

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