Changes in leaf proteome profile of *Arabidopsis thaliana* in response to salicylic acid

RIDDHI DATTA, RAGINI SINHA and SHARMILA CHATTOPADHYAY*

Plant Biology Laboratory, Drug Development/Diagnostics & Biotechnology Division, CSIR-Indian Institute of Chemical Biology, Kolkata 700 032, India

**Corresponding author (Fax, 91-33-2473 5197; Email, sharmila@iicb.res.in)*

Salicylic acid (SA) has been implicated in determining the outcome of interactions between many plants and their pathogens. Global changes in response to this phytohormone have been observed at the transcript level, but little is known of how it induces changes in protein abundance. To this end we have investigated the effect of 1 mM SA on soluble proteins of *Arabidopsis thaliana* leaves by proteomic analysis. An initial study at transcript level has been performed on temporal landscape, which revealed that induction of most of the SA-responsive genes occurs within 3 to 6 h post treatment (HPT) and the expression peaked within 24 HPT. Two-dimensional gel electrophoresis (2-DE) coupled with MALDI-TOF MS/MS analysis has been used to identify differentially expressed proteins and 63 spots have been identified successfully. This comparative proteomic profiling of SA treated leaves versus control leaves demonstrated the changes of many defence related proteins like pathogenesis related protein 10a (PR10a), disease-resistance-like protein, putative late blight-resistance protein, WRKY4, MYB4, etc. along with gross increase in the rate of energy production, while other general metabolism rate is slightly toned down, presumably signifying a transition from 'normal mode' to 'defence mode'.

[Datta R, Sinha R and Chattopadhyay S 2013 Changes in leaf proteome profile of *Arabidopsis thaliana* in response to salicylic acid. *J. Biosci.* **38** 317–328] **DOI** 10.1007/s12038-013-9308-9

1. Introduction

Plants live in complex environments in which they intimately interact with a broad range of invasive pathogens as well as abiotic stresses. This evolutionary arms race between the plant and its attackers has led to highly sophisticated multilayered defence systems in plants (Van den Burg *et al*. 2008; Pieterse *et al*. 2009). This includes preexisting defence mechanisms both in the form of mechanical and chemical barriers as well as defence systems that are activated only after the plant has been challenged by pathogen (Slusarenko *et al.* 2000). Pathogen attack induces expression of a series of defence-related genes, for example, PR genes, some of which encode antimicrobial substances (van Loon *et al*. 2006). It is also an established fact that the plant hormones like SA, jasmonic acid (JA) and ethylene (ET) plays a key role in plant defence response by controlling expression of defence-related genes, which is again somewhat specific for the nature of the pathogen (Glazebrook 2005; Wasternack 2007). JA, along with ET, plays an important role in defence response induced by necrotrophic pathogens; herbivory and insect attack are taken care of mainly by JA, whereas biotrophic pathogen infection turns on defence response that proceeds largely via SA signalling pathway (Glazebrook 2005). While SA is widely recognized as a key player in plant immunity (Moreau *et al*. 2012), genetic studies reveal an increasingly complex network of proteins required for SA-mediated defence signalling (Vlot *et al.* 2009). There are increasing evidences indicating that the elaborate network and cross-talk of signalling molecules can also affect each other and this interplay helps the plant to fine tune its response

Keywords. *Arabidopsis thaliana*; differential proteomics; salicylic acid

Supplementary materials pertaining to this article are available on the *Journal of Biosciences* Website at *http://www.ias.ac.in/jbiosci/jun2013/supp/datta.pdf*

Published online: 25 April 2013

to possible threats (Thomma *et al*. 1998; Bostock 2005; Grant and Jones 2009; Pieterse *et al*. 2009; Ghanta *et al.* 2011; Sharma *et al.* 2013). Mostly, JA and ET work synergistically to combat infection (Penninckx *et al*. 1998), while SA and JA are antagonistic with SA suppressing JA-induced gene expression (Koornneef *et al.* 2008). However, in certain cases SA-JA antagonism is replaced by a concerted activation of both the signalling pathways. For example, application of low concentrations of SA and JA leads to synergistic activation of *PDF1.2*, a JA-responsive gene, and *PR 1*, an SA-responsive gene (Mur *et al*. 2006). The role of SA in plant defence is well studied in *A. thaliana* at the gene expression level (Blanco *et al*. 2009). A loss-of-function mutation of *NOREXPRESSOR OF PR GENES 1* (*NPR1*) gene, the key regulators of plant defence as well as SA signalling, leads to attenuation of SA-mediated signalling, resulting in elevated susceptibility of the plant to biotrophic pathogen infection (Cao *et al*. 1994; Delaney *et al.* 1995). On the other hand, overexpression of *NPR1* gene results in increased resistance to pathogens (Cao *et al.* 1998; Chern *et al.* 2001; Makandar *et al.* 2010). NPR1 plays a dual role: on the one hand, it interacts with a family of bZIP transcription factors and induces expression of SA-responsive genes; on the other hand, it suppresses expression of JA-responsive genes (Spoel *et al*. 2003; Dong 2004). Again, NPR1, being the key player of plant defence, is also involved in JA-mediated but SA-independent induced systemic resistance (ISR) (Pieterse *et al.* 1998). Although NPR1 is central to SA-dependent signal transduction, a branch of SA signalling also proceeds through NPR1-independent pathway.

The dynamic changes induced by SA at the protein level are yet to be studied in details. In this respect, several techniques are available to analyse differential protein expressions. Many techniques like protein microarray or gel free techniques like ICAT and iTRAQ have come up. In spite of all these new techniques, 2-DE remains the method of choice for comparative proteomics (Wu *et al.* 2006). In the present study, 2-DE has been employed to elucidate changes in protein expression that can be induced by SA-mediated defence response in plants.

2. Materials and methods

2.1 *Plant material, growth condition and chemical treatment*

Wild-type *A. thaliana* plants of *Columbia* ecotype (Col-0; Nottingham *Arabidopsis* Stock Centre, N1093) were grown in Murashige and Skoog (MS) media and maintained in growth chamber at 22°C under 16 h light/8 h dark cycles. For treatment, 4-week-old plants were induced by dipping the leaves in a 0.015% (v/v) Silwet L-77 solution containing 1 mM SA and maintained under above mentioned condition. Control plants were similarly treated with 0.015% (v/v) Silwet L-77 solution. Estimation of total chlorophyll was done from control and SA treated leaves 24 HPT according to Lichtenthaler (Lichtenthaler 1987). All experiments were performed in replicates of three.

2.2 *RNA extraction and semi-qRT PCR*

Total RNA was extracted from leaf samples, collected after 0, 3, 6, 12 and 24 h of treatment, using Trizol method. cDNA was synthesized using the Revert Aid H Minus cDNA Synthesis kit (Fermentas, USA). Semi-qRT PCR was performed for selected marker genes presented in table 1. The constitutively expressed actin gene was used as internal loading control.

2.3 *Protein extraction and 2-DE analyses*

Total protein was isolated using phenol extraction method after 24 h of treatment. Briefly, about 1.5 g of leaf tissue was ground in liquid nitrogen and suspended in extraction buffer (700 mM Sucrose, 500 mM Tris–HCl, pH7.5, 50 mM EDTA, 100 mM KCl, 2% (w/v) β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride) and protein extraction was done following standard protocol. The resulting protein was resuspended in IEF buffer consisting of 7 M urea, 2 M thiourea, 4% 3-[(3-cholamido propyl)-dimethylammonio]-1-propane sulfonate (CHAPS), 20 mM DTT and 1% (w/v) Bio-Lyte (3/10) ampholyte (BioRad Laboratories, Hercules, CA, USA) as standardized before (Isaacson *et al*. 2006; Sinha and Chattopadhyay 2011). The protein concentration was determined by Bradford's method (Bradford 1976). 100 μg of protein was used to passively re-hydrate immobilized pH gradient strip (7 cm; pH4–7; BioRad Laboratories, Hercules, CA, USA) for 12 h. IEF was performed as follows: 250 V for 30 min, 4000 V for 2 h, 4000 V for 10000 V-h, 500 V for 1 h on BioRad PROTEAN IEF Cell system (BioRad Laboratories, Hercules, CA, USA). Focused strips were then equilibrated in equilibration buffers I & II (BioRad Laboratories, Hercules, CA, USA) for 15 min each. For running gels in the second dimension, 12% SDS polyacrylamide gels were used and stained with colloidal Coomasie Brilliant Blue (CBB) G-250 (Neuhoff *et al*. 1988).

2.4 *Image analysis*

The gel images were acquired using Versa-Doc Image system (BioRad Laboratories, Hercules, CA, USA) and the images were analysed with PD Quest software version 8.0.1 (BioRad Laboratories, Hercules, CA, USA). Spot detection was carried on by matching the gels automatically which was followed by manual verification. Spot densities

Table 1. Selected marker genes along with primer pairs used

Genes	Forward primer (5′-3′)	Reverse primer (5′-3′)
NPR1-dependent SA-inducible genes		
PR1	ATGAATTTTACTGGCTATTCTCGATT	ATGTACGTGTGTATGCATGATCAC
NPR1	ATGGACACCACCATTGATGG	TTTAACAAGCTCTTCCGGCAA
NPR1-independent SA-inducible genes		
SDRLP	ATGGAGAATAATCCAAGAAGCTC	TTTATTAGACAACAAAGCCTCCAT
bZIP transcription factor		
TGA1	ATGTTCGATCAAGAAGCTTCAAC	AAAGCTTCTAATCTATCCATAGCA

were normalised against whole gel densities. Only the spots detected in at least two replicate gels were annotated; the percentage volume of each spot was averaged for the different (three biological replicates of control and treated samples) gels and statistical analysis was performed to find out significant protein fold changes between control and treated samples.

2.5 *Protein identification using MALDI TOF-TOF MS/MS*

Selected protein spots were manually excised from 2-DE gels and subjected to in-gel digestion with trypsin following the manufacturer's instructions (In-gel trypsin digestion kit, Pierce, USA). Digested proteins were then desalted with Zip-Tip l-C18 (ZipTip, Millipore, USA) and analysed using a 4800 MALDI TOF-TOF MS/MS analyser (Applied Biosystems, Foster City, CA, USA). Before each analysis, the instrument was calibrated with the Applied Biosystems 4700 Proteomics Analyzer Calibration Mixture. Data interpretation was done using the GPS Explorer Software (Applied Biosystems, Foster City, CA, USA), and an automated database search was carried out using the MASCOT program (Matrix Science Ltd., London, UK).

MS/MS data was used for protein identification by searching in a non-redundant protein sequence database (NCBI nr—20070216; 4626804 sequences, 1596079197 residues) using a MOWSE algorithm as implemented in the MASCOT search engine version 3.5 (Matrix science: http://www.matrixscience.com). The following parameters were used for database searches: taxonomy, viridiplantae (green plants; 186963 sequences); cleavage specificity, trypsin with one missed cleavages allowed; mass tolerance of 100 ppm for precursor ions and a tolerance of 0.2 da for the fragment ions; allowed modifications, carbamidomethyl (fixed), oxidation of Met (variable), cleavage by trypsin, cuts C-term side of KR unless next residue is P. According to MASCOT probability analysis, only significant hits ($P<0.05$) were considered.

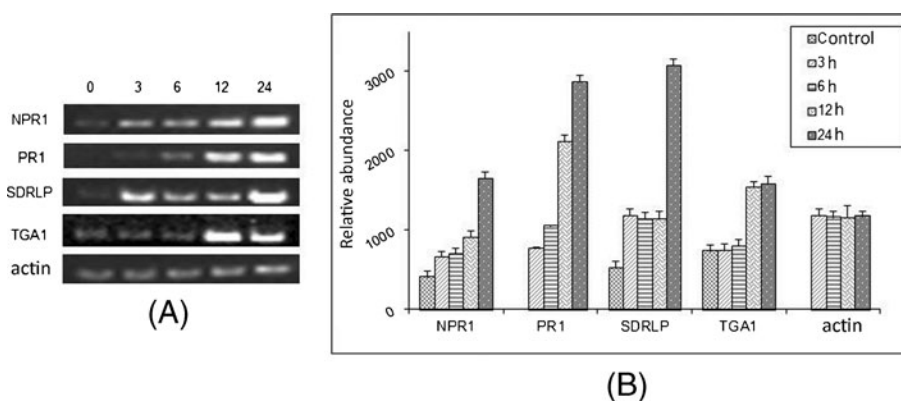

Figure 1. Effect of SA on expression of selected marker genes. Transcript analysis of SA-responsive (NPR1, PR-1, SDRLP) and bZIP transcription factor (TGA1) genes after exogenous application of SA in *A. thaliana* wild-type (Col-0) plants has been done. Four-week-old plants were induced by dipping the leaves in a 0.015% (v/v) silwet L-77 solution containing 1 mM SA. Leaves were harvested for RNA extraction at different time points (0, 3, 6, 12, 24 HPT). Equal loading of RNA samples was checked using the constitutively expressed Actin gene. Experiments have been repeated thrice. (**A**) Representative gel image. (**B**) Relative abundance of gene expression have been obtained from Quantity One software and plotted.

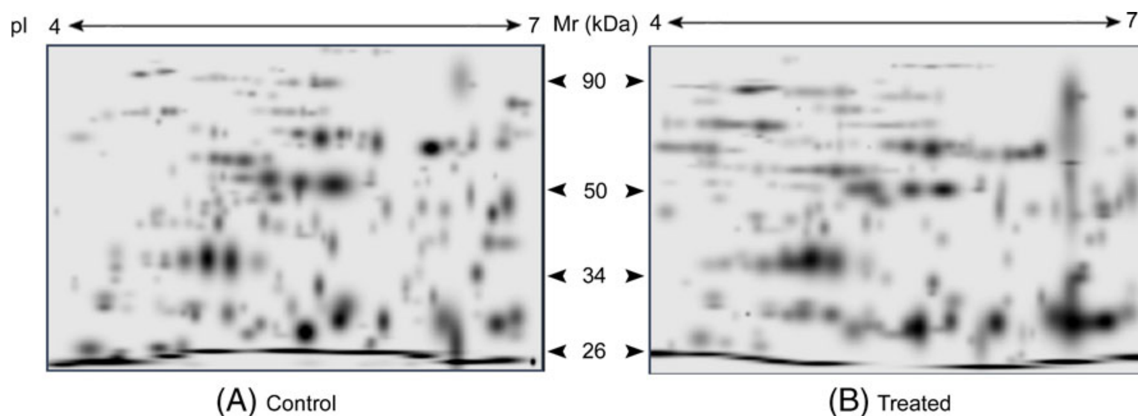

Figure 2. Representative 2-DE gel images of control (**A**) and SA treated (**B**) *A. thaliana* leaves. Results were obtained by separating 100 µg of protein samples in a 7 cm pH4–7 strip followed by SDS-PAGE. The gels were stained by colloidal Coomassie brilliant blue G-250. Mr, molecular marker.

2.6 *Statistical analysis*

Data collected from three biological experiments were compared for control and treated leaves and differences in spot abundance statistically evaluated using the *t*-test function implemented in the PD Quest software. Spots showing differences between control and treated leaves with a *P*-value of <0.05 were chosen for further analysis. Means and standard deviations were calculated from three independent sets of biological replicates.

3. Results and discussion

3.1 *Expression profile of SA-inducible genes on temporal landscape*

To investigate the effect of SA induction at preliminary transcript level, the expression of several known SA-dependent genes have been analyzed 3, 6, 12 and 24 HPT and compared with that of 0 min control, i.e. basal expression level. It has been found that *NPR1* expression is induced within 3 HPT and its expression reaches a maximum within 24 HPT (figure 1). *PR1*, marker gene of SA-dependent pathway, primarily acts in a NPR1-dependent fashion. Although the basal level expression of *PR1* has been found to be very low its expression induced within 3 HPT and the expression peaked at 24 HPT. Expression of the NPR1-independent SA-responsive gene *SDRLP* (for short-chain dehydrogenase/reductase family protein) also exhibited a similar expression pattern (Blanco *et al*. 2009). *TGA1*, a member of bZIP family transcription factors, interacts with NPR1 after being induced by SA and induces transcription of downstream SA-responsive genes. It has been found that the expression of *TGA1* significantly increased after 6 HPT. Taken together, the result indicates that expression of most SA responsive genes peak within 24 HPT and hence further proteomic analysis has been done 24 HPT.

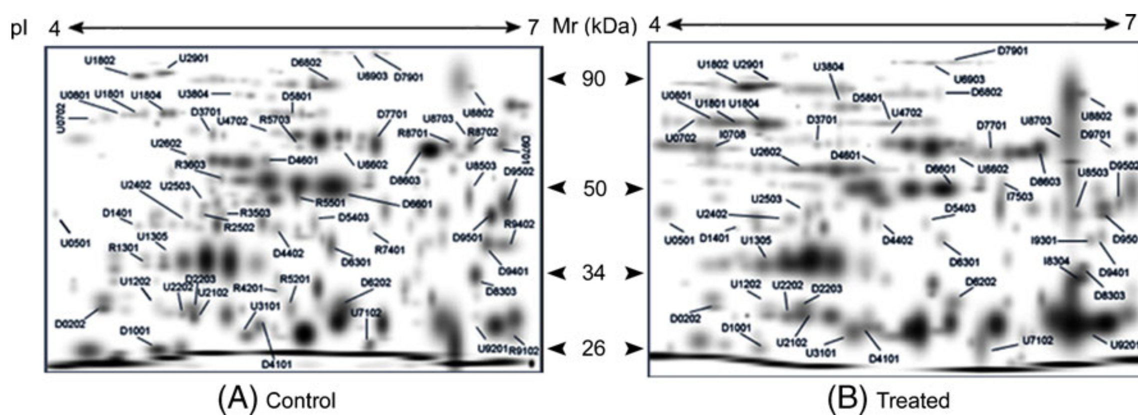

Figure 3. Annotated gel images of control (**A**) and infected (**B**) gels. Significantly altered and identified spots are marked with corresponding spot number. The name of the protein is shown in table 2.

Table 2. Differentially expressed proteins identified by MALDI TOF-TOF MS/MS in *A. thaliana* leaves in response to SA treatment

Functional classes	SSP No.	Accession No.	SC%	Protein	Gene name	Fold change			Species	Score	Theoritical mass (kD)/pI
						Replicate 1	Replicate 2	Replicate 3			
Stress and defence response	U8802	gi|18496467	64	glyceraldehyde 3-phosphate dehydrogenase	gapdh	2.83	3.31	2.22	*Mitthyridium sp. De Sloover 39.082*	61	19.774/9.32
	U6602	gi|11493822	16	transcription factor WRKY4	wrky4	3.37	2.91	2.13	*Petroselinum crispum*	26	37.710/8.78
	RT9102	gi|7649155	37	glutathione S-transferase	gst	0	0	0.16	*Euphorbia esula*	26	23.835/6.71
	U1202	gi|16356667	36	PR10a	pr10	2.22	7.33	2.89	*Nicotiana tabacum*	22	16.945/4.74
	I8304	gi|882248	29	carbonic anhydrase 1	ca1	47.57	nd	23.21	*A. thaliana*	29	24.260/8.53
	D3701	gi|1063400	33	glycolate oxidase	glcD	0.42	0.38	0.49	*Lycopersicon esculentum*	30	31.335/9.32
	D1001	gi|14485509	45	thioredoxin h	trxh	0.33	0.39	0.19	*[Pisum sativum*	24	13.162/4.83
	U2901	gi|2746727	30	ascorbate peroxidase	apx	3.15	16.56	4.38	*Brassica juncea*	24	27.796/5.63
	U4702	gi|4406539	25	ascorbate peroxidase	apx	2.92	nd	3.7	*Glycine max*	24	27.279/5.64
	I708	gi|28628953	19	MYB4	myb4	42.36	7.35	32.11	*Dendrobium sp. XMW-2002–4*	27	119.471/9.36
	D6301	gi|11385505	25	glutathione S-transferase GST 27	gst	0.26	0	0.19	*Zea mays*	25	25.277/5.39
	RT5501	gi|30171231	30	NBS-LRR type R protein	nbs-lrr r	0	0	0.46	*Triticum aestivum/ Thinopyrum intermedium alien addition line*	25	19.801/8.63
	D4402	gi|56784038	23	putative aldose reductase	aldr1	0.22	0.16	0.43	*Oryza sativa (japonica* cultivar-group)	24	33.855/5.85
	RT8702	gi|17065444	13	disease resistance protein, putative	-	0	0.4	0	*thaliana*	23	69.246/6.12
	U6903	gi|48057598	12	putative late blight resistance protein	-	2.74	2.12	3.25	*Solanum demissum*	25	87.960/6.43
	U8703	gi|17065444	21	"disease resistance protein, putative	-	7.84	3.31	2.29	*A. thaliana*	20	113.283/6.31
	U2503	gi|48762871	63	disease resistance-like protein	-	2.32	3.41	2.79	*B. rapa* subsp. *Chinensis*	27	9.945/6.55
	D2203	gi|15219124	23	disease resistance protein (TIR class), putative	-	0.4	0.34	0.22	*A. thaliana*	19	230.199/6.43

Table 2 (continued)

Functional classes	SSP No.	Accession No.	SC%	Protein	Gene name	Fold change Replicate 1	Replicate 2	Replicate 3	Species	Score	Theoritical mass (kD)/ pI
	U9201	gi|38045831	60	resistance protein candidate	-	15.57	3.47	8.66	*Vitis riparia*	32	9.671/8.87
	U501	gi|38045674	30	resistance protein candidate	-	7.99	3.65	5.12	*V. amurensis*	25	19.948/8.07
Signalling, gene and protein regulation	D7701	gi|21388092	10	maturase	mat	0.46	0	0.28	*Canscora diffusa*	22	10.639/9.78
	RT4201	gi|25052685	10	heat stress transcription factor HSFA9	hsfa9	0	0	0.26	*Helianthus annuus*	36	44.001/5.44
	U7102	gi|55419652	24	SBP transcription factor	spl4	4.51	2.39	2.11	*Gossypium hirsutum*	36	19.212/9.58
Protein synthesis	D6202	gi|7576645	25	ribosomal protein system 4	Rps4	0.36	0	0.27	*Leskeodon cubensis*	30	22.074/10.23
	RT5201	gi|42556394	30	small ribosomal protein 4	rps4	0	nd	0	*Balantiopsis cancellata*	35	22.044/10.48
	D4101	gi|2598657	30	elongation factor 1-alpha (EF1-a)	Ef1	0.29	0.46	0.39	*Vicia faba*	37	49.555/9.15
Carbon metabolism	D8603	gi|18158190	33	ribulose-1,5-bisphosphate carboxylase large subunit	rbcl	0.4	0.6	0.27	*Valeriana dioica*	90	66.11/4.99
	D9502	gi|18073654	30	phosphoenolpyruvate carboxylase	pepc	0.18	0.45	0.31	*Sticherus bifidus*	32	40.32/8.74
	D5403	gi|3093462	100	ADP-glucose pyrophosphorylase large subunit	apl	0.37	0	0.26	*O. sativa*	35	4.166/4.17
	RT1301	gi|50978423	40	ribulose 1,5 bisphosphate carboxylase/oxygenase, small subunit	rbcs	0	0	0.14	*Limonium gibertii*	28	17.946/6.07
	U1802	gi|50948109	29	putative immunophilin / FKBP-type peptidyl-prolyl cis-trans isomerase	FKBP12	3.06	2.96	nd	*O. sativa* (*japonica* cultivar)	42	27.614/8.58
	D9501	gi|20563374	31	granule-bound starch synthase	gbss	0.16	0.24	0.13	*Lindleya mespiloides*	42	35.408/5.77
Energy production	U3804	gi|33328384	12	ATP synthase beta subunit	atpF	4.93	2.3	2.11	*Drypetes brownii*	23	52.652/5.28
	U2602	gi|4586602	33	pyruvate kinase	pk	2.88	3.56	2.31	*Cicer arietinum*	29	15.538/5.20
	U2102	gi|4586602	47	pyruvate kinase	pk	2.48	2.99	3.05	*C. arietinum*	31	15.538/5.20
	RT9402	gi|20800444	36	pyruvate decarboxylase protein	pdc	0	nd	0	*Fragaria x ananassa*	24	10.114/5.11
	RT5703	gi|65519	29	cytochrome c - ginkgo (tentative sequence)	cytC	0	0.47	0	*Ginkgo*	23	12.468/9.76

Table 2 (continued)

Functional classes	SSP No.	Accession No.	SC%	Protein	Gene name	Fold change Replicate 1	Replicate 2	Replicate 3	Species	Score	Theoritical mass (kD)/ pI
	D5801	gi|60498740	33	Cytochrome c biogenesis C	cytC	0.36	0.11	0	*O. sativa* (*japonica* cultivar-group)	24	27.314/8.48
	RT2502	gi|52421699	63	NADH dehydrogenase subunit 1	nad1	0	0.42	0.17	*Salix reticulata*	23	4.191/8.23
	U2202	gi|9652180	18	NADH-ubiquinone oxidoreductase subunit PSST	nad	2.38	2.94	2.19	*Lupinus luteus*	22	23.782/9.51
Hypothetical and unknown proteins	D4601	gi|21537186	18	unknown	-	0.36	0.44	0.35	*A. thaliana*	32	51.275/6.49
	D9701	gi|50944617	52	hypothetical protein	-	0.18	0	0.21	*O. sativa* (*japonica* cultivar-group)	18	6.359/7.88
	RT8701	gi|50915422	23	hypothetical protein	-	0	0.19	0	*O. sativa* (*japonica* cultivar-group)	26	10.274/9.21
	U8503	gi|51091293	42	hypothetical protein	-	2.67	2.19	3.36	*O. sativa* (*japonica* cultivar-group)	24	14.273/10.54
	U1305	gi|6006848	21	"unknown protein, 5' partial	-	3.37	nd	3.33	*A. thaliana*	32	57.150/9.19
	U3101	gi|52076780	38	hypothetical protein	-	2.61	2.95	4.66	*O. sativa (japonica* cultivar-group)	33	15.636/10.53
	D1401	gi|9759174	66	unnamed protein product	-	0.38	0.51	0.32	*A. thaliana*	35	16.765/4.78
	D7901	gi|21592433	22	unknown	-	0.26	nd	0.11	*A. thaliana*	26	36.700/9.47
Others	D0202	gi|4406372	25	thiosulfate sulfurtransferase	tst	0.47	0.52	0.21	*Datisca glomerata*	27	41.528/6.51
	U2402	gi|51091339	20	putative chaperonin 21 precursor	Cpn21	2.02	3.1	2.36	*O. sativa (japonica* cultivar-group)	31	25.479/5.97
	D9401	gi|21592909	30	lysine decarboxylase-like protein	ldc	0.42	0.29	0.23	*A. thaliana*	24	25.459/5.89
	U1801	gi|1568478	60	cdc2-related protein kinase	crk	2.71	3.11	2.26	*Beta vulgaris* subsp. *vulgaris*	37	13.773/8.69
	D8303	gi|7529243	15	putative protein	-	0.36	0.14	0.21	*A. thaliana*	29	40.596/4.79
	RT3503	gi|8885534	30	GTP-binding protein-like	-	0	0.47	0.11	*A. thaliana*	28	23.183/5.73
	RT7401	gi|7573380	28	putative protein	-	0	0	0.39	*A. thaliana*	27	34.237/9.112
	D6802	gi|34914966	59	OSJNBa0091E23.25	-	0.21	0.43	nd	*O. sativa (japonica* cultivar-group)	26	70.15/10.68
	U1804	gi|50935143	26	myosin-like protein	-	3.42	2.32	2.91	*O. sativa (japonica* cultivar-group)	36	44.605/8.97
	D6601	gi|57869096	45	pollen-specific kinase partner protein-like protein	-	0.43	nd	0.27	*Lycopersicon esculentum*	36	13.349/5.0
	U0801	gi|21928055	34	AT4g03150/F4C21_7	-	21.41	3.21	16.23	*A. thaliana*	32	20.886/5.41

Table 2 (continued)

Functional classes	SSP No.	Accession No.	SC%	Protein	Gene name	Fold change			Species	Score	Theoritical mass (kD)/pI
						Replicate 1	Replicate 2	Replicate 3			
	U0702	gi|21928055	34	AT4g03150/F4C21_7	-	3.53	2.13	9.32	*A. thaliana*	32	20.886/5.41
	I9301	gi|21928055	34	AT4g03150/F4C21_7	-	14.67	nd	6.45	*A. thaliana*	32	20.886/5.41
	I7503	gi|8439885	29	Contains similarity to a mitochondrial ribosomal protein from gb|Z30582.	-	20.02	6.77	13.12	*Saccharomyces cerevisiae*	41	21.582/9.79
	RT3603	gi|7269236	31	putative protein	-	0	0	0.36	*A. thaliana*	23	13.538/5.76

SSP No.: Spot number generated by PD Quest software; SC%: Sequence coverage; Fold change: Fold change with respect to control calculated from PD Quest Software (spots detected only in control gel (restricted to control) have been assigned a value of 0 by the software); Score: Score obtained from MALDI TOF-TOF MS/MS; nd: Not detected.

In column 2: U,Up-regulated (spot intensity in treated is higher than control by 2-fold or more); D, down-regulated (spot intensity in treated is lower than control by 2-fold or more); RT, restricted to control (spots detected solely in control gel); I, induced (spots newly detected in treated gel only).

3.2 *Proteomic analysis of differentially expressed proteins*

A comparative analysis of the proteome has been performed to investigate the profiles of SA-responsive, differentially expressed proteins. Figure 2 shows the representative gel images of control and SA-treated leaf samples respectively. Analysis of 2-DE pattern has revealed defence-related changes in several protein spots which appeared in three independent biological experiments. The number of resolved spots is approximately 209 in control gels and 204 in SA-treated gels. 139 spots have matched successfully, and 63 identified spots have been found to be significantly altered in intensity between the treatments while several spots have appeared in treated sample and several others have been detected solely in control sample. The overall mean coefficient of variation for intensity of the spots matched has been determined to be 46.50%. Spots showing statistically significant increases or decreases in response to treatment, as shown in figure 3, have been excised from gels of control and SA-treated samples. Sixty-three of these spots have been identification through MALDI TOF MS/MS.

Table 2 shows the list of the identified proteins by MS/MS; the fold change represents the ratio of change of spot intensity in comparison to the control. The distribution of proteins according to their functional category in control and SA-treated leaves is shown in figure 4. Appearance of several spots more than once probably represents different post translational modifications of proteins.

About 39% of identified proteins are up-regulated (i.e. spot intensity in treated is higher than control by 2-fold or more) among which majority are related to stress and defence (41%). Also 50% of the induced proteins (6%), i.e. proteins which were newly detected in treated sample, are related to this class. About 36% proteins are down-regulated (i.e. spot intensity in treated is lower than control by 2-fold or more) and 19% are restricted to control, i.e. they are detected in control sample only. These proteins placed mostly into stress and defence, signalling, gene and protein regulation, nucleic acid binding and carbon metabolism categories.

3.2.1 *Stress and defence:* Induction of defence response is accompanied by a redox imbalance and subsequent changes in the expression of redox-related proteins, which on the one hand turns on downstream signalling pathway and tries to restrict further advancement of the attacker on the other hand (Vranová *et al.* 2002; Foyer and Noctor 2005). A similar pattern of change in the expression levels of several redox proteins like ascorbate peroxidase and thioredoxin h has been induced by exogenous SA treatment in the present study. This observation can be corroborated with the previous report that SA treatment induces change in expression of redox-related proteins in cucumber cotyledons (Hao *et al.* 2012). Such a change may be because exogenously

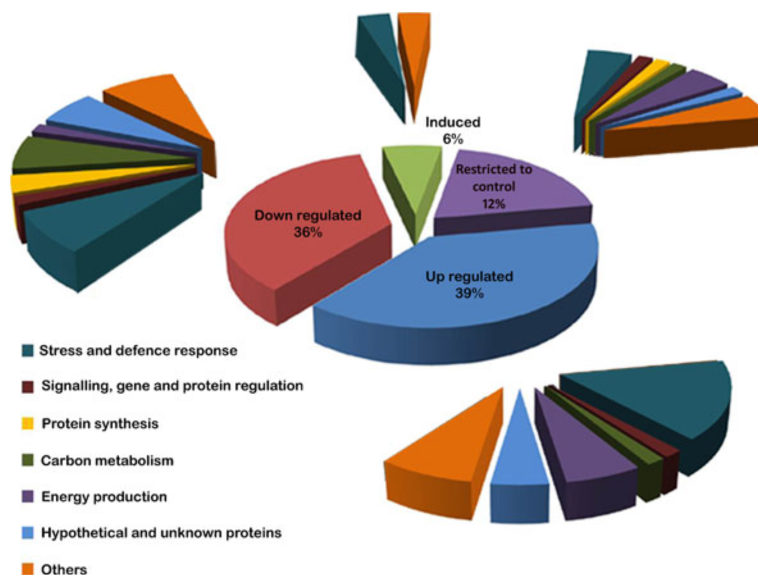

Figure 4. Functional classification of identified proteins from *A. thaliana*. Pie chart of proteins identified as up-regulated, down-regulated, induced and restricted to control in *A. thaliana* in response to SA treatment.

applied SA induces oxidative stress to the plant, which can also be one of the reasons for slight yellowing of the plant (supplementary figure 1) (Rajjou *et al*. 2006), and a drop in the total chlorophyll content noted in the present study complements this observation (supplementary figure 2). Figure 5 shows the identified differentially expressed stress and defence related proteins. The synthesis and accumulation of PR proteins and resistance proteins are ubiquitous plant response to pathogen infection (van Loon and van Strien 1999). In our study various proteins like PR10a, disease-resistance-like protein, putative late blight-resistance protein, etc., have been found to be up-regulated, which is supported by previous reports (Poupard *et al.* 2003, Tarchevsky *et al.* 2010). Also, changes in expression of receptor proteins like NBS-LRR-type R protein and disease-resistance protein (TIR class) have been observed, while up-regulation of defence-related transcription factor WRKY4 and induction of MYB4 indicates that there has been a transition to defence mode which requires activation of selected downstream genes, thus preparing the plant for upcoming challenges. A recent report reveals that proteomic analysis of SA-treated rice leaves unveiled 36 differentially expressed proteins implicated in various functions, including defence, antioxidative enzymes and signal transduction (Li *et al.* 2012), which also complements our present observation.

3.2.2 *Signalling, gene and protein regulation:* Changes in expression of several proteins like SBP transcription factor, heat stress transcription factor HSFA9, etc., indicate that transition to defence mode requires several changes at gene and protein regulation levels.

3.2.3 *Carbon metabolism:* A series of proteins related to photosynthesis has been altered, suggesting dynamic influence of SA on photosynthesis. Down-regulation of proteins like RubisCO large subunit, phosphoenolpyruvate carboxylase, ADP-glucose pyrophosphorylase large subunit and detection of RubisCO small subunit to control sample only indicates that there has been a reduction in photosynthetic rate after treatment, which is also at par with the observed drop in chlorophyll content of SA-treated plant. Such a role of SA has also been observed in barley seedlings where treatment with SA reduced the rate of photosynthesis as well as RubisCO activity but increased the CO_2 compensation point and stomatal resistance (Pancheva *et al.* 1996). Like many other stress factors, SA effects thylakoid membranes and light-induced activities associated with them, which finally leads to reduction of carbon fixation (Popova *et al*. 1997). Another probable explanation is due to the influence of SA on protein synthesis. It has been shown that in presence of 1 mM SA RubisCO synthesis is reduced to 50% (Pancheva and Popova 1997). Presumably the host plant curtails some of its energy expenditure from normal metabolism which is utilized by for defence.

3.2.4 *Energy metabolism:* It has been observed that there has been a general induction of proteins related to respiration and energy metabolism like ATP synthase beta subunit, pyruvate kinase, cytochrome c, NADH dehydrogenase subunit 1, etc., which is also supported by literature (der Straeten *et al.* 1995) It is a well-known

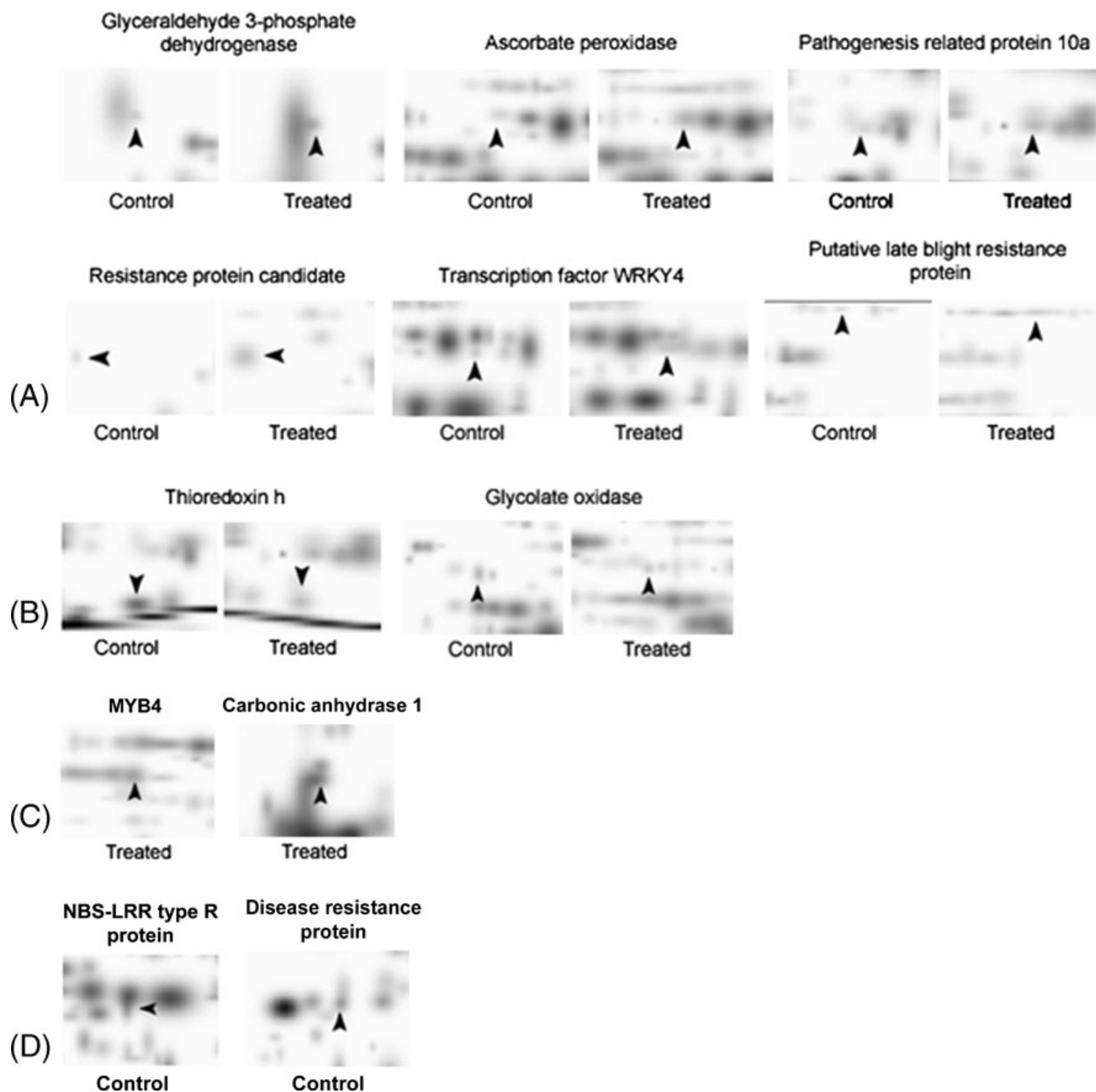

Figure 5. Representative proteome profile of *A. thaliana* in response to SA treatment. Close- up of the area with spots of Stress and defence-related proteins that were up-regulated (**A**), down-regulated (**B**), induced (**C**) and restricted to control (**D**) in *A. thaliana* in response to SA treatment.

fact that induction of defence is cost-intensive and the host plant accelerates its energy production which will eventually be utilized for generating defence-related metabolites.

3.2.5 *Protein synthesis:* Down-regulation of proteins involved in translation, like small ribosomal protein 4 and elongation factor 1-alpha, has been observed, which indicates a possible influence of SA on protein synthesis. Indeed, a decrease in the content of total soluble protein has been observed in our study, as noted earlier (Pancheva and Popova 1997).

Environmental stress presents a major challenge in our quest for sustainable food production as it reduces the potential yields as high as 70% in crop plants (Agarwal *et al.* 2006). The present study provides a better understanding of physiological changes induced by SA-dependent signalling including a redox imbalance and concomitant changes in expression of redox-related proteins, transcriptional activation of defence-related genes and a gross increase in the rate of energy production to meet the increasing energy expense for defence. Together, it can be said that proteins related to stress and defence, carbon metabolism and energy synthesis have been significantly affected in the plant in response to exogenous SA treatment, which together dictate transition to a 'defence mode'.

Acknowledgements

We acknowledge Department of Science and Technology (DST), and Council of Scientific and Industrial Research (CSIR), India, for necessary funding. Research activities have been supported by fellowships to RS and RD from CSIR, and ICMR, India, respectively. Central Proteomic facility of CSIR-IICB is acknowledged herewith.

References

Agarwal PK, Agarwal P, Reddy MK and Sopory SK 2006 Role of DREB transcription factors in abiotic and biotic stress tolerance in plants. *Plant Cell Rep.* **25** 1263–1274

Blanco F, Salinas P, Cecchini N, Jordana X, Van Hummelen P, Alvarez M and Holuigue L 2009 Early genomic responses to salicylic acid in *Arabidopsis*. *Plant Mol. Biol.* **70** 79–102

Bostock RM 2005 Signal crosstalk and induced resistance: straddling the line between cost and benefit. *Annu. Rev. Phytopathol.* **43** 545–580

Bradford MM 1976 A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem*. **72** 248–254

Cao H, Bowling SA, Gordon AS, and Dong X 1994 Characterization of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired resistance. *Plant Cell* **6** 1583–1592

Cao H, Li X, and Dong X 1998 Generation of broad-spectrum disease resistance by overexpression of an essential regulatory gene in systemic acquired resistance. *Proc. Natl. Acad. Sci. USA* **95** 6531–6536

Chern M, Fitzgerald HA, Yadav RC, Canlas PE, Dong X and Ronald PC 2001 Evidence for a disease-resistance pathway in rice similar to the NPR1-mediated signaling pathway in *Arabidopsis*. *Plant J.* **27**(2) 101–113

Delaney TP, Friedrich I and Ryals JA 1995 *Arabidopsis* signal transduction mutant defective in chemically and biologically induced disease resistance. *Proc. Natl. Acad. Sci. USA* **92** 6602–6606

Dong X 2004 NPR1, all things considered. *Curr. Opin. Plant Biol.* **7** 547–552

Foyer CH and Noctor G 2005 Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. *Plant Cell* **17** 1866–1875

Ghanta S, Bhattacharyya D, Sinha R, Banerjee A and Chattopadhyay S 2011 *Nicotiana tabacum* overexpressing γ-ECS exhibits biotic stress tolerance likely through NPR1-dependent salicylic acid-mediated pathway. *Planta* **233** 895–910

Glazebrook J 2005 Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol.* **43** 205–227

Grant M and Jones J 2009 Hormone (dis)harmony moulds plant health and disease. *Science* **324** 750–752

Hao JH, Dong CJ, Zhang ZG, Wang XL and Shang QM 2012 Insights into salicylic acid responses in cucumber (*Cucumis sativus* L.) cotyledons based on a comparative proteomic analysis. *Plant Sci.* **187** 69–82

Isaacson T, Damasceno CMB, Saravanan RS, He Y, Catalá C, Saladié M and Rose JKC 2006 Sample extraction techniques for enhanced proteomic analysis of plant tissues. *Nat. Protoc.* **1** 769–774

Koornneef A, Leon-Reyes A, Ritsema T, Verhage A,Otter FCD, Van Loon LC and Pieterse CMJ 2008 Kinetics of salicylate-mediated suppression of jasmonate signalling reveal a role for redox modulation. *Plant Physiol***.** **147** 1358–1368

Li Y, Zhang Z, Nie Y, Zhang L and Wang Z 2012 Proteomic analysis of salicylic acid-induced resistance to *Magnaporthe oryzae* in susceptible and resistant rice. *Proteomics* **12** 2340–2354

Lichtenthaler HK 1987 Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. *Methods Enzymol.* **148** 350–382

Makandar R, Nalam V, Chaturvedi R, Jeannotte R, Sparks AA and Shah J 2010 Involvement of salicylate and jasmonate signaling pathways in *Arabidopsis* interaction with *Fusarium graminearum. MPMI* **23** 861–870

Moreau M, Tian M, Klessig DF 2012 Salicylic acid binds NPR3 and NPR4 to regulate NPR1-dependent defense responses. *Cell Res.* **22** 1631–1633

Mur LAJ, Kenton P, Atzorn R, Miersch O and Wasternack C 2006 The outcomes of concentration-specific interactions between salicylate and jasmonate signalling include synergy, antagonism, and oxidative stress leading to cell death. *Plant Physiol*. **140** 249–262

Neuhoff V, Arold N, Taube D and Ehrhardt W 1988 Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis* **9** 255–262

Pancheva TV, Popova LP and Uzunova AN 1996 Effects of salicylic acid on growth and photosynthesis in barley plants. *J. Plant Physiol*. **149** 57–63

Pancheva TV and Popova LP 1997 Effect of salicylic acid on the synthesis of ribulose-1, 5-bisphosphate carboxylase/oxygenase in barley leaves*. J. Plant Physiol*. **152** 381–386

Penninckx IAMA, Thomma BPHJ, Buchala A, Métraux JP and Broekaert WF 1998 Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in *Arabidopsis*. *Plant Cell* **10** 2103–2113

Pieterse CMJ, Leon-Reyes A, Van der Ent S and Van Wees SCM 2009 Networking by small-molecule hormones in plant immunity. *Nat. Chem. Biol.* **5** 308–316

Pieterse CMJ, van Wees SCM, van Pelt JA, Knoester M, Laan R, Gerrits H, Weisbeek PJ, and van Loon LC 1998 A novel signalling pathway controlling induced systemic resistance in *Arabidopsis*. *Plant Cell* **10** 1571–1580

Popova L, Pancheva T and Uzunova A 1997 Salicylic acid: properties, biosynthesis and physiological role. *Bulg. J. Plant physiol*. **23** 85–93

Poupard P, Parisi L, Campion C, Ziadi S, Simoneau P 2003 A wound- and ethephon-inducible PR-10 gene subclass from apple is differentially expressed during infection with a compatible and an incompatible race of *Venturia inaequalis*. *Physiol. Mol. Plant Pathol*. **62** 3–12

Rajjou L, Belghazi M, Huguet R, Robin C, Moreau A, Job C, and Job D 2006 Proteomic investigation of the effect of salicylic acid on *Arabidopsis* seed germination and establishment of early defense mechanisms. *Plant Physiol*. **141** 910–923

Sharma R, Vleesschauwer DD, Manoj K. Sharma MK and Ronald PC 2013 Recent advances in dissecting stress regulatory crosstalk in rice. *Mol. Plant* doi:10.1093/mp/sss147

Sinha R and Chattopadhyay S 2011 Changes in the leaf proteome profile of *Mentha arvensis* in response to *Alternaria alternata* infection. *J. Proteomics* **74** 327–336

Slusarenko AJ, Fraser RSS and Van Loon LC 2000 *Mechanisms of resistance to plant diseases* (Dordrecht: Kluwer Academic Publishers) p 620

Spoel SH, Koornneef A, Claessens SMC, Korzelius JP, Van Pelt JA, Mueller MJ, Buchala AJ, Métraux JP, Brown R, Kazan K, Van Loon LC, Dong X and Pieterse CMJ 2003 NPR1 modulates cross-talk between salicylate- and jasmonate dependent defense pathways through a novel function in the cytosol. *Plant Cell* **15** 760–770

Tarchevsky IA, Yakovleva VG, and Egorova AM 2010 Proteomic analysis of salicylate induced proteins of pea (*Pisum sativum* L.) leaves. *Biochemistry* **75** 590–597

Thomma B, Eggermont K, Penninckx I, Mauch-Mani B, Vogelsang R, Cammue B and Broekaert W 1998 Separate jasmonate dependent and salicylate-dependent defense-response pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogens. *Proc. Natl. Acad. Sci. USA* **95** 15107–15111

van den Burg HA, Tsitsigiannis DI, Rowland O, Lo J, Rallapalli G, MacLean D, Takken FLW and Jones JDG 2008 The f-box protein acre189/acif1 regulates cell death and defense responses activated during pathogen recognition in tobacco and tomato. *Plant Cell* **20** 697–719

Van der Straeten, Chaerle DL, Sharkov G, Lambers H and van Montagu M 1995 Salicylic acid enhances the activity of the alternative pathway of respiration in tobacco leaves and induces thermogenicity. *Planta* **196** 412–419

van Loon LC, Rep M and Pieterse CMJ 2006 Significance of inducible defense-related proteins in infected plants. *Annu. Rev. Phytopathol.* **44** 135–162

van Loon LC and van Strien EA 1999 The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. *Physiol. Mol. Plant Pathol.* **55** 85–97

Vlot AC, Dempsey DA and Klessig DF 2009 Salicylic acid, a multifaceted hormone to combat disease. *Annu. Rev. Phytopathol.* **47** 177–206

Vranová E, Inzé D and Breusegem FV 2002 Signal transduction during oxidative stress. *J. Exp. Bot*. **53** 1227–1236

Wasternack C 2007 Jasmonates: an update on biosynthesis, signal transduction and action in plant stress response, growth and development. *Ann. Bot.* **100** 681–697

Wu WW, Wang G, Baek SJ and Shen RF 2006 Comparative study of three proteomic quantitative methods, DIGE, cICAT, and iTRAQ, using 2D Gel- or LC-MALDI TOF/TOF. *J. Proteome Res.* **5** 651–658

MS received 27 November 2012; accepted 04 February 2013

Corresponding editor: Utpal Nath