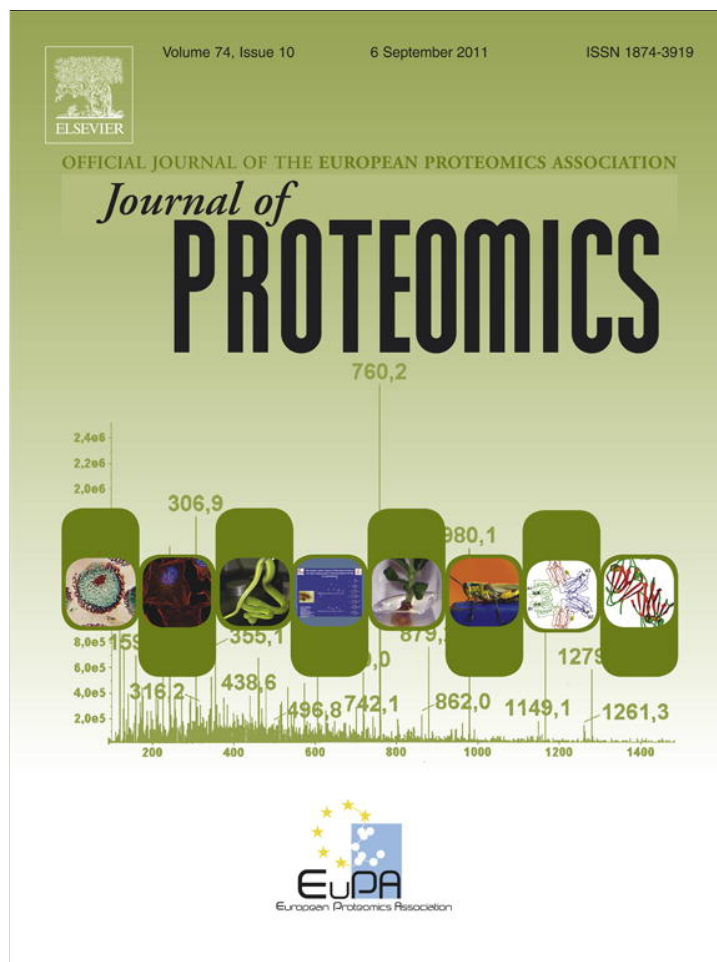


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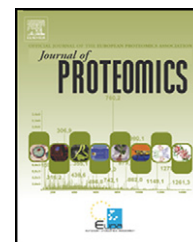
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Response to biotic and oxidative stress in *Arabidopsis thaliana*: Analysis of variably phosphorylated proteins

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ABSTRACT

Protein phosphorylation plays a pivotal role in the regulation of many cellular events; increasing evidences indicate that this post-translational modification is involved in plant response to various abiotic and biotic stresses. Since phosphorylated proteins may be present at low abundance, enrichment methods are generally required for their analysis. We here describe the quantitative changes of phosphoproteins present in *Arabidopsis thaliana* leaves after challenging with elicitors or treatments mimicking biotic stresses, which stimulate basal resistance responses, or oxidative stress. Phosphoproteins from elicited and control plants were enriched by means of metal oxide affinity chromatography and resolved by 2D electrophoresis. A comparison of the resulting proteomic maps highlighted phosphoproteins showing quantitative variations induced by elicitor treatment; these components were identified by MALDI-TOF peptide mass fingerprinting and/or nanoLC-ESI-LIT-MS/MS experiments. In total, 97 differential spots, representing 75 unique candidate phosphoproteins, were characterized. They are representative of different protein functional groups, such as energy and carbon metabolism, response to oxidative and abiotic stresses, defense, protein synthesis, RNA processing and cell signaling. Ascertained protein phosphorylation found a positive confirmation in available *Arabidopsis* phosphoproteome database. The role of each identified phosphoprotein is here discussed in relation to plant defense mechanisms. Our results suggest a partial overlapping of the responses to different treatments, as well as a communication with key cellular functions by imposed stresses.

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1. Introduction

Plants are in intimate contact with the environment; they are constantly being challenged by unfavourable environmental conditions (excess/lack of water, light, nutrients or tempera-

ture extremes) and other organisms eventually causing physical damages (insects or herbivores) or disease development (pathogenic bacteria and fungi). In order to protect themselves from biotic stresses, plants have evolved different lines of defence mechanisms, which are often partly

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overlapped [1]. Usually, recognition of elicitor molecules from pathogens, or Pathogen-Associated Molecular Patterns (PAMPs), triggers a plant basal defence that is non-specific and confers a broad-spectrum resistance [2,3]. Pathogens must circumvent or suppress this first-line surveillance mechanism in order to develop disease. On the other hand, some plant varieties also express a mechanism known as gene-for-gene resistance [4], which relies on the recognition of specific avirulence gene products from the pathogen by complementary R receptors. This recognition elicits a set of biochemical reactions, including generation of H₂O₂ and reactive oxygen species (ROS), lipid peroxidation, ion fluxes, protein phosphorylation and phytoalexin synthesis, which are generally referred to as the Hypersensitive Response (HR) [5]. This response eventually leads to programmed death of infected cells, thereby restricting the favourable environment to pathogens and arresting infection progression. Local response can also generate signals systemically triggering enhanced resistance to secondary infections (SAR) or directed to plant organs not yet colonised by the pathogen [6]. Although different pathways exist for sensing and responding to different environmental cues, the occurrence of cross-tolerance [7], a phenomenon where the exposure to a particular stress also increases the resistance to other ones, suggests that underlying cellular responses may share common mechanisms, at least in part. ROS production and consequent alteration of the plant cell redox balance, for instance, is associated with diverse biotic and abiotic stimuli, such as wounding, fungi challenge or excess-light damage. Response to pathogens underlies a complex reprogramming of plant metabolism, which represents an unnecessary use of cell resources in the absence of pathogen challenge. Plants, although intrinsically inferior to constitutive defences, have evolved inducible mechanisms. In this context, protein phosphorylation is a crucial event for regulation of many processes essential for plant biochemistry and physiology. Increasing evidence indicates that this post-translational modification plays a pivotal role also at different stages of plant response to pathogens, such as signal transduction events, control of the cellular redox status, adjustments to metabolism and accumulation of defense molecules [8]. However, systematic knowledge about *in vivo* plant protein phosphorylation in response to pathogen stimuli is lacking. Recently, functional genomic studies (including proteomics) have greatly contributed to the molecular dissection of plant pathogen interactions, allowing to identify a number of defense-related candidate proteins and helping to clarify specific gene expression patterns [9]. In this respect, the study of sub-proteomes has been greatly recommended to improve technical sensitivity/resolution and reduce the overall system complexity.

In this work, we report on the main quantitative changes of phosphorylated proteins within *Arabidopsis thaliana* leaves as result of its challenge with various elicitors or treatment mimicking different biotic stresses, which stimulate basal resistance responses, or oxidative stress. In particular, plant leaves were treated with i) chitosan, a cell wall component of many fungi able to induce oxidative burst and defense proteins synthesis [10]; ii) benzothiadiazole, a compound known to activate a number of SAR-associated genes and leading to enhanced plant protection against various pathogens [11]; iii) mechanical injury/wounding, mimicking physical

damage by insects/herbivores and known to induce the transcription of wound-responsive genes [12]; iv) methyl viologen (MV), a powerful inducer of oxidative stress, known to be a component of the defense responses [13]. Thus, phosphorylated proteins were enriched by Metal Oxide Affinity Chromatography (MOAC), resolved by 2-DE and resulting proteomic patterns were compared. Differently modified components were identified by various MS approaches; their function is discussed according to previous data on defense responses.

2. Materials and methods

2.1. Plant growth and stress treatments

Arabidopsis thaliana ecotype Columbia (Col-0) was grown in a growth chamber at 22 °C, under a 16 h light/8 h dark cycle. Three-week old plants were subjected to treatment with elicitors as follows: i) chitosan: a solution containing 300 µg/mL chitosan from crab shell (Sigma), which was previously hydrolyzed according to Hadwiger and Beckman [14]; ii) benzothiadiazole: a solution containing 0.4 g/L Bion 50 WG (Syngenta); iii) methyl viologen: a solution containing 50 µM methyl viologen (Sigma). In all cases, elicitor solutions also contained 0.1% w/v Tween 20 and were sprayed on leaves to ensure its complete wetting (about 2 mL for each plant). Aqueous 0.1% w/v Tween 20 spraying was used for control plants. Wounding of rosette leaves was done by piercing small holes through all of the leaf blades to be harvested. Leaves were harvested after a treatment for 8 h.

2.2. Hydrogen peroxide assay

To measure H₂O₂ produced in challenged leaves, the method by Bellincampi et al. [15] was used. Five explant rectangles (2×4 mm for each sample) with the midrib medially placed, were excised from control or treated leaves and incubated in deionised water under vacuum for 2 min and then at ambient pressure, for 30 min. Samples were centrifuged at 10,000×g, for 5 min, and then the supernatant was used for H₂O₂ determination. Supernatant (500 µL) was added to 500 µL of the assay reagent (500 mM ammonium ferrous sulphate, 50 mM sulphuric acid, 200 µM xylenol orange, 200 mM sorbitol); the absorbance of the Fe³⁺-xylenol complex at 560 nm was then determined after incubation for 45 min.

2.3. Protein extraction

Protein mining was performed according to the phenol extraction method [16], with minor modifications. Briefly, three-weeks old rosette *Arabidopsis* leaves were finely powdered in liquid N₂ using a mortar and dried under vacuum. One g of dried leaves was suspended in 20 mL of ice-cold extraction buffer (700 mM sucrose, 500 mM Tris-HCl pH 7.5, 50 mM EDTA, 100 mM KCl, 2% w/v β-mercaptoethanol, 1 mM PMSF, 1% w/v PVP, 0.25% w/v CHAPS, 40 mM NaF, 1 µM okadaic acid). After addition of an equal volume of phenol saturated-500 mM Tris-HCl, pH 7.5, the mixture was stirred for 5 min in Waring blender and then centrifuged at 10,000×g for 10 min, at 4 °C. The upper phenol phase was removed and extracted once with the extraction buffer. Proteins were precipitated from the phenol phase by

addition of 5 vol of saturated ammonium acetate in methanol, overnight at -20°C . Precipitated proteins were centrifuged at $10,000\times g$, for 30 min. Proteins were stored at -80°C , until used. Three biological replicates for each treatment were analyzed, which were then subjected to an independent phenol extraction and subsequent proteomic analysis.

2.4. Metal oxide affinity chromatography

MOAC was performed according to Rohrig et al. [17]. Eighty mg of phenol-precipitated proteins were suspended in 30 mL of incubation buffer (30 mM MES pH 6.1, 0.1 M sodium glutamate, 0.1 M potassium aspartate, 0.25% w/v CHAPS, 8 M urea), sonicated and incubated overnight, under magnetic stirring, at 4°C . After incubation, the suspension was sonicated again and then centrifuged at $15,000\times g$, for 15 min. The supernatant was collected and added to 2 g of $\text{Al}(\text{OH})_3$ previously washed twice with incubation buffer. After incubation on a rotating mixer for 2 h, at 8°C , the matrix was recovered by centrifugation at $6000\times g$ for 10 min and washed four times with 15 mL of 30 mM MES pH 6.1, 0.15 M sodium glutamate, 0.15 M potassium aspartate, 0.25% w/v CHAPS, 8 M urea. Bound proteins were eluted with 20 mL of 200 mM potassium pyrophosphate, 8 M urea, pH 9, for 60 min, at room temperature. After centrifugation at $6,000\times g$ for 10 min, the supernatant was collected and 0.01 vol of 2% w/v sodium deoxycholate and 0.1 vol of 100% w/v TCA were added. Precipitated proteins were recovered in a single tube by stepwise centrifugation at $14,000\times g$, for 10 min. Final pellet was washed with 25% w/v TCA, suspended in Tris-HCl buffer pH 7.5 containing 80% acetone and finally washed with ice-cold acetone. The pellet was dried under reduced pressure and stored at -80°C until used. Protein samples before and after MOAC were analyzed by SDS-PAGE; gels were stained with Pro-Q Diamond (Invitrogen) to reveal phosphorylated proteins and SyproRuby (Invitrogen) to detect total proteins, according to manufacturer's instructions. Fluorescent dyes were visualised using a VersaDoc 4000 scanner (Bio-Rad).

2.5. 2-D electrophoresis and gel image acquisition

Protein pellets were dissolved in IEF buffer (9 M urea, 4% w/v CHAPS, 0.5% v/v Triton X-100, 20 mM DTT, 1% w/v Bio-Rad carrier ampholytes pH 3–10). Protein concentration was estimated by using the Bradford assay, modified according to Ramagli and Rodriguez [18]. IPG strips (17 cm pH 4–7, Bio-Rad ReadyStrip, Bio-Rad) were rehydrated overnight with 300 μL of IEF buffer containing 300 μg of total proteins. Proteins were focused using a Protean IEF Cell (Bio-Rad) at 12°C , by applying the following voltages: 250 V (90 min), 500 V (90 min), 1000 V (180 min) and 8000 V for a total of 52 KVh [16]. After focusing, the proteins were reduced by incubating the IPG strips with 1% w/v DTT in 10 mL of equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30% w/v glycerol, 2% w/v SDS and a dash of bromophenol blue) for 15 min, and alkylated with 2.5% w/v iodoacetamide in 10 mL of equilibration buffer for 15 min. Electrophoresis in the second dimension was carried out on 12% polyacrylamide gels ($180\times 240\times 1$ mm) with a Protean apparatus (Bio-Rad), using electrophoresis buffer (25 mM Tris-HCl pH 8.3, 1.92 M glycine and 1% w/v SDS), with 120 V applied for 12 h, until the dye front reached the bottom of the gel. 2-DE

gels were stained with colloidal Coomassie G250 and/or Pro-Q Diamond to detect total and phosphorylated proteins, respectively; resulting images were acquired by using GS-800 and/or VersaDoc 4000 imaging systems (Bio-Rad). For quantitative analysis, each sample was analyzed in triplicate.

2.6. Gel image analysis

Digitized images of Coomassie-stained gels were analyzed by using the PDQuest (ver 7.4) 2-D analysis software (Bio-Rad), which allowed spot detection, landmarks identification, aligning/matching of spots within gels, quantification of matched spots and their analysis, according to manufacturer's instructions. Manual inspection of the spots was performed to verify the accuracy of automatic gel matching; any errors in the automatic procedure were manually corrected prior to the final data analysis. The spot volume was used as the analysis parameter for quantifying protein expression. The protein spot volume was normalized to the spot volume of the entire gel (i.e., of all the protein spots). Fold changes in protein spot levels were calculated between spot volumes in the treated group relative to that in the control gels. Statistically significant changes in protein amount were determined by using two sequential data analysis criteria. First, a protein spot had to be present in all gels for each sample to be included in the analysis. Next, statistically significant changes in amount were determined by using the distribution of fold-change values in the data. Spots were determined to be statistically significant if the difference between the average intensity of a specific protein spot in the control and treated plants (three technical replicates of three biological samples) was greater than one standard deviation of the spot intensities for both groups. An absolute two-fold change in normalized spot densities was then considered indicative of a differentially modified protein; values >2 or <0.5 were associated with increased or decreased phosphoprotein amounts after treatment, respectively.

2.7. Protein digestion and MS analysis

Spots from 2-DE were manually excised from gels, minced and washed with water. Proteins were *in-gel* reduced, S-alkylated and digested with trypsin, as previously reported [19]. Protein digests were subjected to a desalting/concentration step on ZipTipC18 pipette tips (Millipore Corp., Bedford, MA, USA) before MALDI-TOF-MS and/or nanoLC-ESI-LIT-MS/MS analysis.

During MALDI-TOF peptide mass fingerprinting (PMF) experiments, peptide mixtures were loaded on the instrument target together with CHCA as matrix, using the dried droplet technique. Samples were analysed with a Voyager-DE PRO mass spectrometer (Applied Biosystems, USA). Peptide mass spectra were acquired in reflectron mode; internal mass calibration was performed with peptides derived from trypsin autolysis. Data were elaborated using the DataExplorer 5.1 software (Applied Biosystems). PSD fragment ion spectral analysis of the most abundant mass signal within each MALDI-TOF-MS spectrum was performed as previously reported [20].

Peptide mixtures were eventually analyzed by nanoLC-ESI-LIT-MS/MS using a LTQ XL mass spectrometer (ThermoFinnigan, USA) equipped with Proxeon nanospray source connected to an Easy-nanoLC (Proxeon, Denmark) [21]. Peptide mixtures were

separated on an Easy C₁₈ column (10×0.075 mm, 3 μm) (Proxeon) using a gradient of acetonitrile containing 0.1% formic acid in aqueous 0.1% formic acid; acetonitrile ramped from 5% to 35% over 15 min and from 35% to 95% over 2 min, at a flow rate of 300 nL/min. Spectra were acquired in the range *m/z* 400–2000. Acquisition was controlled by a data-dependent product ion scanning procedure over the three most abundant ions, enabling dynamic exclusion (repeat count 2 and exclusion duration 1 min). The mass isolation window and collision energy were set to *m/z* 3 and 35%, respectively.

2.8. Protein identification

MASCOT software package version 2.2.06 (Matrix Science, UK) [22] was used to identify spots unambiguously from an updated plant non-redundant sequence database (NCBI nr 2009/05/03). MALDI-TOF PMF data were searched using a mass tolerance value of 40–80 ppm, trypsin as proteolytic enzyme, a missed cleavages maximum value of 2 and Cys carbamidomethylation and Met oxidation as fixed and variable modification, respectively. NanoLC-ESI-LIT-MS/MS data were searched by using a mass tolerance value of 2 Da for precursor ion and 0.8 Da for MS/MS fragments, trypsin as proteolytic enzyme, a missed cleavages maximum value of 2 and Cys carbamidomethylation and Met oxidation as fixed and variable modification, respectively. MALDI-TOF PMF candidates with a cumulative MASCOT score >83, which were also confirmed by PSD data, or nanoLC-ESI-LIT-MS/MS candidates with more than 2 assigned peptides with an individual MASCOT score >25, both corresponding to *p* < 0.05 for a significant identification, were further evaluated by the comparison with their calculated mass and pI values, using the experimental values obtained from 2-DE.

3. Results and discussion

3.1. Induction of H₂O₂ production

Since accumulated evidence indicates that hydrogen peroxide is a key signalling molecule involved in plant response to both biotic and abiotic stresses [23], the time-course of H₂O₂ production in challenged *Arabidopsis* leaves was followed to test the effectiveness of administration of elicitors or wounding treatment. Control plants were analyzed for comparison. Results demonstrated that benzothiadiazole, chitosan, methyl viologen or wounding stimuli were able to trigger H₂O₂ production in leaves, even though with partly different time courses (Fig. 1). However, H₂O₂ levels after 8 h were comparable among all treatments; then this time was chosen for comparative proteomic analysis.

3.2. MOAC purification of phosphorylated proteins

Arabidopsis leaf tissues from plants treated with chitosan, benzothiadiazole, wounding, methyl viologen or control were used for the purification of phosphorylated proteins. After 8 h-treatments, total proteins were extracted and phosphoproteins enriched by using a MOAC procedure [17]. Input loads and eluted fractions were analysed by SDS-PAGE; gels were stained with the aspecific fluorescent dye SyproRuby

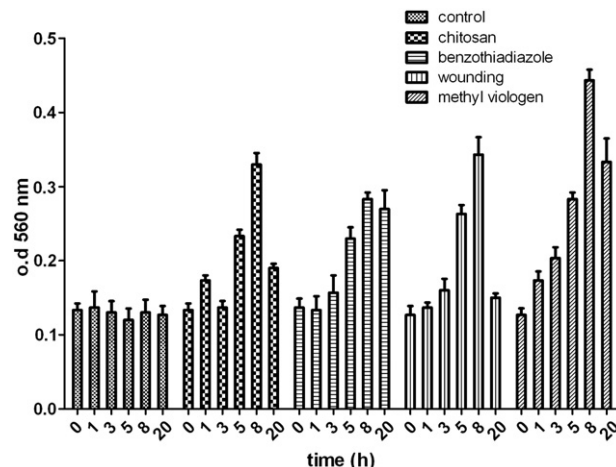


Fig. 1 – Time-course of H₂O₂ production in *A. thaliana* leaves after challenge with elicitors of plant basal defence (chitosan and benzothiadiazole), an inducer of oxidative stress (methyl viologen) or mechanical injury (wounding). At the indicated times after elicitor treatments or wounding, explants from leaves were taken and processed as reported in the experimental section. The release of H₂O₂ in the incubation medium was then determined by measuring the absorbance value of the corresponding Fe³⁺-xylenol complex at 560 nm. Reported values are the mean of three independent experiments from different samples with standard errors.

(Fig. 2A) or the phosphoproteins-specific fluorescent reagent Pro-Q Diamond (Fig. 2B). Results demonstrated that MOAC efficiently enriched phosphoproteins from total leaf extracts.

3.3. Proteomic analysis of elicitor-treated leaves identifies differentially phosphorylated proteins

A proteomic approach was then used to identify candidate phosphoproteins whose abundance changed upon stimulus exerted by various elicitors of plant basal defence. MOAC-enriched proteins from control and challenged leaves were then resolved by 2-D electrophoresis within the pH range 4–7 and mass range 10–200 kDa. Representative gel images obtained after staining with colloidal Coomassie G250 and Pro-Q Diamond (Fig. 3A and B) confirmed optimal enrichment of phosphoproteins within samples.

To ascertain quantitative changes in relative spot densities for elicitor-treated leaves compared to control, colloidal Coomassie-stained gels were subjected to comparative software-assisted image analysis (Fig. 3C–D)(Supporting Information Table S1). Average proteomic maps showed 285 (untreated), 250 (chitosan), 270 (benzothiadiazole), 262 (wounding) and 300 (methyl viologen) spots, with a degree of similarity for the different treatments (compared to the control) of 81, 86, 89, and 77% respectively. Statistical evaluation (*p* < 0.05) of relative spot densities allowed to detect spots differentially modified in challenged leaves with at least a two-fold increase or decrease, when compared to control leaves. In total, 97 differential spots were detected between treated and control plants, among which 29, 27, 24 and 30 after chitosan, benzothiadiazole, wounding and methyl viologen treatment, respectively.

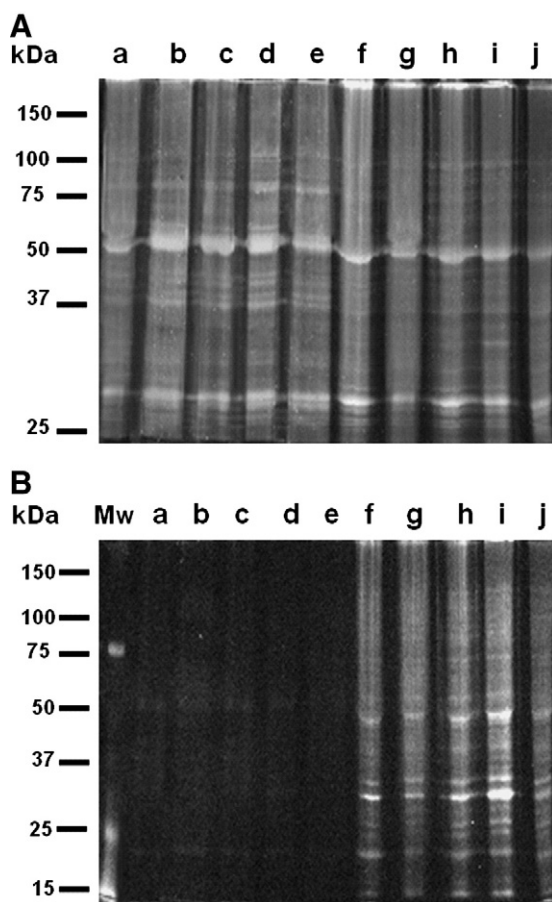


Fig. 2 – SDS-PAGE of total proteins or MOAC-purified phosphoproteins from *A. thaliana* leaves after challenge with elicitors of plant basal defence (chitosan and benzothiadiazole), an inducer of oxidative stress (methyl viologen) or mechanical injury (wounding). Panel A. SyproRuby staining. Lanes a to e: total proteins (5 µg) from control, wounding, chitosan, benzothiadiazole and methyl viologen-treated plants, respectively; lanes f to j: phosphoproteins (5 µg) eluted from MOAC corresponding to control, wounding, chitosan, benzothiadiazole and methyl viologen-treated plants, respectively. Panel B. Pro-Q Diamond staining specific for phosphoproteins. Lanes a to j as in Panel A. Mw, molecular mass markers).

These spots were excised from gels, proteolyzed, subjected to MS analysis and database searching for protein assignment. Nineteen identification derived from MALDI-TOF PMF data, 77 from nanoLC-ESI-LIT-MS/MS data and 5 from gel matching. Identified protein species, together with their quantitative variations as result of the different treatments, are reported in Table 1. All proteins, except three, have been previously described in recent large-scale phosphoproteome studies as being phosphorylated [8,24–31]. Information on protein phosphorylation was deduced by searching *Arabidopsis* phosphoproteome RIPP-DB, PhosPhAt and P3DB database, available on the WEB at the following addresses: <https://phosphoproteome.psc.database.riken.jp>, <http://phosphat.mpimp-golm.mpg.de/phosphat.html> and <http://digbio.missouri.edu/p3db/download>, respectively. Most of the spots contained a single protein component, which

will be discussed below in a dedicated section for each treatment. Those spots containing multiple species (6 in number), for which it was not possible to determine that one responsible for quantitative changes in differential experiments, will be not described. No identification was obtained for spots 30, 62, 64 and 67. In general, a classification according to functional data showed that a large portion of the proteins differentially modified in challenged leaves belong to broad classes involved in: i) energy production and carbon metabolism; ii) response to environmental and biotic stresses; iii) RNA transport and processing; iv) protein synthesis; v) cellular signaling.

3.3.1. Chitosan treatment

Chitosan administration to leaves determined a reduced amount of phosphoproteins involved in energy/carbon metabolism. In fact, ribulose biphosphate carboxylase/oxygenase (RuBisCO) activase (spots 1–3) appeared strongly down-regulated following treatment. This protein is known to be phosphorylated on Ser/Thr/Tyr residues and modified by 5'-phospho-DNA [32]; the occurrence of multiple protein spots was associated with its modification degree. Its molecular target, namely ribulose carboxylase/oxygenase large subunit (spot 12), was also down-regulated. This phosphorylated protein has been already reported to be enriched by MOAC procedures [17]. Other enzymes involved in carbon metabolism, namely cysteine synthase (spot 16), mitochondrial NAD-dependent malate dehydrogenase (spot 15), a probable chloroplastic isoform of fructose biphosphate aldolase (At2g21330) (spot 10) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) subunit A (spots 13 and 14), also resulted down-regulated. The role of their phosphorylated isoforms is not known at present, although in some cases it may be related to a functional regulation of enzyme activity or turn-over. In *Arabidopsis*, for example, it has been shown that GAPDH proteolysis is modulated by binding to regulatory 14-3-3 proteins, through protein phosphorylation [33]. In plants, a cytosolic non-phosphorylating GAPDH is occurring in addition to glycolytic GAPDH; this phosphorylated enzyme is involved in the generation of NADPH and is modulated by oxidative stress [34]. Proteins belonging to the photosynthetic apparatus, namely two components of the photosystem II oxygen-evolving complex (spots 4 and 5), a component of the cytochrome b_6 -f complex (spot 9) and an extrinsic protein associated to the PS II complex (spot 7), also showed lower levels of their phosphorylated species.

In contrast, phosphoproteins involved in stress and defense responses, RNA transport/processing and protein synthesis generally showed increased levels after chitosan administration. In particular, two phospho-isoforms of the heat shock cognate 70 kDa protein 3 (HSC70-3) (spots 23 and 24) and a phosphorylated heat shock protein 70-like component (spot 27) resulted strongly up-regulated. The heat shock protein 70 family (HSP70) contains both heat-inducible and constitutively expressed members, named heat shock cognate proteins (HSC70), which are involved in protein targeting and degradation [35]. *Arabidopsis* genome encodes for five different cytosolic HSC70s, among which three are expressed constitutively (HSC70-1, 2 and 3); interestingly, expression of all genes is increased after environmental stresses [35]. Recently, a specific interaction of HSC70-3 with turnip mosaic virus RNA-dependent RNA polymerase has

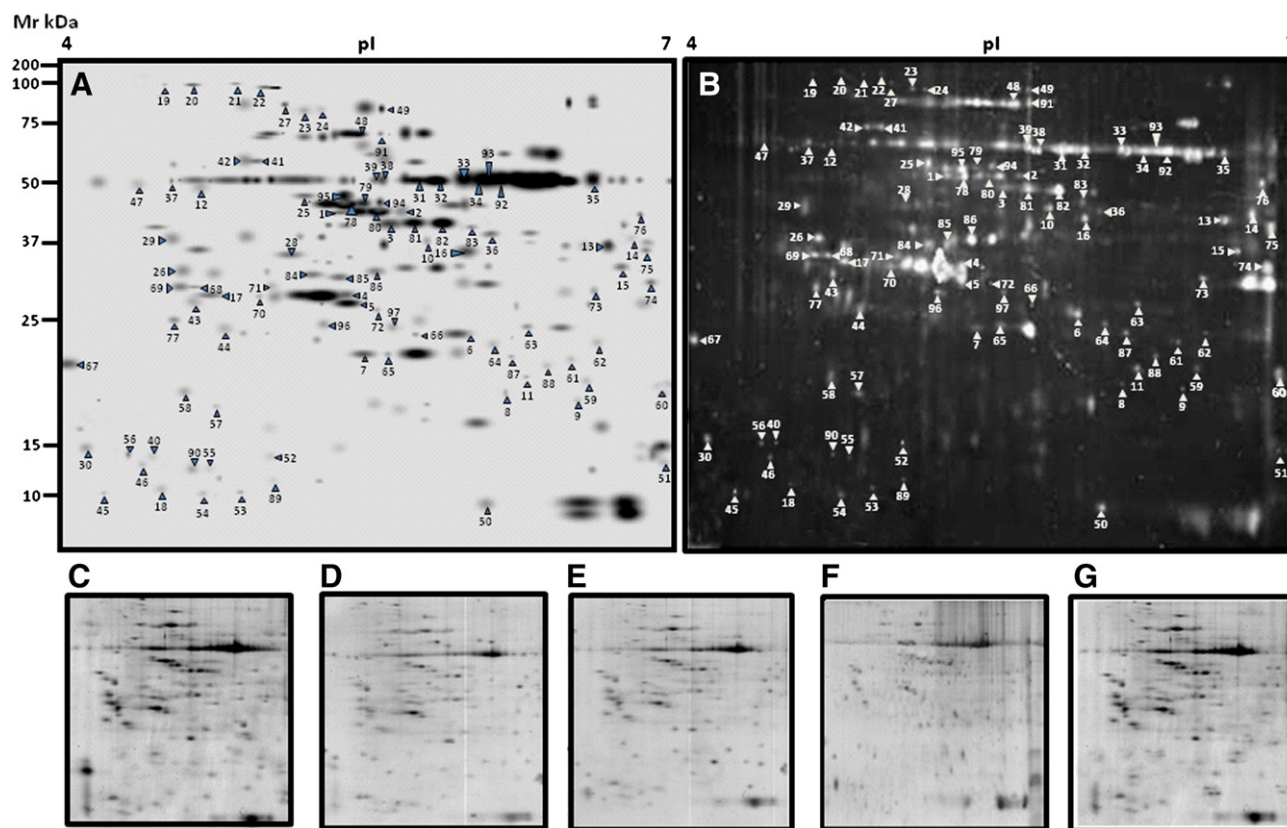


Fig. 3 – 2D proteomic maps of phosphoproteins purified by MOAC from *A. thaliana* leaves after challenge with elicitors of plant basal defence (chitosan and benzothiadiazole), an inducer of oxidative stress (methyl viologen) or mechanical injury (wounding). Panels A and B. Master gel stained with colloidal Coomassie G250 or Pro-Q Diamond to detect total and phosphorylated proteins, respectively; numbers in both gels indicate proteins showing quantitative differences, which were further identified by MS approaches (Table 1). Panels C to G; representative Coomassie G250-stained gels of phosphoproteins from control and chitosan, benzothiadiazole, wounding and methyl viologen treatments. Proteins purified by MOAC were separated in first dimension by using 18 cm IPG strips (pH 4–7 linear); second dimension was performed on a vertical slab gel (12% T), as reported in the experimental section.

been reported [36], suggesting a role of this heat shock cognate protein in pathogenesis.

Two phosphoproteins whose function has been related to RNA processing/transport, namely RNA-binding protein cp33 (spot 26) and Ran-binding protein 1 (spot 28), showed higher modification levels in chitosan-treated samples. The first protein belongs to a family of chloroplast ribonucleoproteins involved in RNA metabolism, which are now emerging as multifunctional regulatory proteins [37]; a member of this class is involved in antioxidative defense in *Arabidopsis* [38]. The second one is a small GTP-binding protein essential for the export of mRNA/ribonucleoprotein complexes from nucleus [39]. Nuclear RNA export is an emerging research topic in abiotic stress response of plants; it is worth noting that specific mRNA/microRNA export under stress conditions is mediated by karyopherins through a Ran-GTPase-dependent pathway [40]. On the other hand, the progesterone-binding protein homolog Atmp2 (spot 17) showed moderately-increased levels upon treatment. This cytochrome b_5 heme- and steroid-binding domains-containing phosphoprotein, present in thylakoid membrane, has been reported being involved in oxidative defense and stress response [41]. Phosphorylated proteasome

RPT5a subunit (spot 25) showed a 5-fold increase; this protein belongs to the AAA-ATPases associated with the proteasome regulatory particle [42]. Recently, Hatsugai and coworkers have demonstrated the involvement of proteasome-dependent tonoplast and plasma membrane fusion in a novel plant defense process triggering bacteria-induced programmed cell death, which leads to a vacuolar release of antimicrobial and death-inducing compounds [43]. Phosphorylated patellin-1 (spot 22) also showed a 7-fold up-regulation. This protein belongs to a small family of Sec14 and GOLD domains-containing lipid-binding proteins probably involved in membrane-trafficking events [44]; phosphoproteomic studies on membrane fractions of salt-stressed *Arabidopsis* demonstrated that patellin-2 phosphorylation levels raise upon stress [28]. Phosphorylated fibrillin-3 (spot 29) also showed increased levels. Fibrillins are thylakoid-associated proteins involved in protection from photo-oxidative damage, which are also known to accumulate in response to different abiotic/biotic stress conditions [45]. Remarkably, a differential phosphoproteomic study on *Arabidopsis* after *Pseudomonas syringae* challenge led to the identification of phosphorylated fibrillin as a component of plant basal defense response [46]. Finally, three spots

related to unknown (spot 19) or putative (spots 20 and 21) proteins showed augmented levels. For the first one (Atg322240), transcriptomic data available from the plant-microbe interaction database (<http://www.phi-base.org>) indicate its strong induction upon inoculation with bacterial pathogens or elicitors.

3.3.2. Benzothiadiazole treatment

Benzothiadiazole treatment determined a variable amount of phosphoproteins involved in energy production/carbon metabolism and up-regulation of components related to stress/defense response. In the first case, multiple phosphorylated isoforms of ribulose 1,5-bisphosphate carboxylase/oxygenase (spots 31, 33, 34, 35, 39 and 47) resulted up-regulated. A similar trend was also observed for mitochondrial NADH ubiquinone dehydrogenase subunit 5 (spot 45), which was the one showing the largest increase following treatment. Conversely, some ATP synthases showed increased (ATPase β subunit—spot 37) or decreased (ATPase CF1 β subunit—spot 32) levels after elicitor treatment.

As far as protein involved in stress response and defense, three heat shock phosphoproteins resulted up-regulated, namely the 70 kDa heat shock cognate protein 3 (HSC70) (spot 24), Hsp 70-like protein (spot 27) and luminal-binding protein (spot 48). For the first two proteins, a similar trend was also observed following chitosan treatment. Other phosphoproteins involved in stress and defense response were highly up-regulated, namely fibrillin-3 (spot 29), the progesterone-binding protein homolog Atmp2 (spot 17) and protein disulfide-isomerase 1 (spot 41). Remarkably, the first two phosphorylated proteins were also identified as strongly induced after chitosan challenge, while the latter was up-regulated in *Arabidopsis* leaves following wounding (see below).

Other phosphoproteins that do not classify in the above-mentioned categories were late embryogenesis abundant protein (spot 42) and cell division control protein homolog A (spot 49); they showed augmented amounts after treatment. Moreover, spots 20 and 21 were associated with an *Arabidopsis* protein of unknown function, which also resulted up-regulated by chitosan treatment. Also in this case, its abundance was highly increased by elicitor treatment.

3.3.3. Wounding

Leave wounding determined an increased amount of phosphoproteins involved in energy production/carbon metabolism and down-regulation of phosphorylated components related to RNA metabolism. In the first case, multiple phosphorylated isoforms of RuBisCO activase (spots 2, 3, 78, 79, 80, 81 and 82) resulted up-regulated. A similar condition occurred for phosphorylated cytosolic triosephosphate isomerase (spot 97), glutamine synthetase (spots 94 and 95) and two forms of putative enolase (At2g36530) (spots 92 and 93). Mutations at LOS2 locus, which encodes for a bifunctional enolase, have been reported to impair freezing tolerance in *Arabidopsis* [47]. In contrast, phosphorylated cytochrome b_6-f complex Fe-S subunit (spot 88) and glycine cleavage system H protein 1 (spot 90), involved in photorespiration, showed a reduced modification.

Among the class of stress response and defense proteins, increased levels were observed for phosphorylated disulfide-isomerase A6 (spot 83), which is an highly conserved protein in eukaryotic cells; it is located in endoplasmic reticulum

(ER), where preserves compartment homeostasis through a mechanism referred to as unfolded protein response [48], and is implicated in seed development and pathogen response [49]. Conversely, phosphorylated luminal-binding protein 1 and 2 (spot 91) levels appeared decreased. These proteins function as ER-located chaperones. *Arabidopsis* genome contains three luminal-binding protein genes, among which one lacks the ER retention motif and has been detected in the cell wall, where it probably plays a different function [50].

Some proteins whose function is related to RNA binding/transport/processing were identified, namely phosphorylated Ran-binding protein 1 (spot 28) and putative chloroplast RNA-binding protein (spots 84 and 85); their abundance diminished in wounded leaves. Levels of the first protein have been also reported to increase in chitosan-challenged leaves. Other proteins that do not classify in the categories mentioned above are a 16.5 kDa thylakoidal protein with unknown function (spot 89), putative uncharacterized protein At1g55480 (spot 86), nascent polypeptide-associated complex subunit α -like protein 2 (spot 77) and BTF3 transcription factor (spot 87). Whereas levels of the two former proteins were decreased, those of the remaining ones were increased upon wounding. Nascent polypeptide-associated complex subunit α -like protein has been shown to be a phosphorylated salt-stress responsive protein in rice roots [51].

3.3.4. Methyl viologen treatment

Methyl viologen administration to leaves determined an increased amount of phosphoproteins involved in: i) carbon metabolism, such as ribulose bisphosphate carboxylase small chain 1B and 1A (spot 50) and triosephosphate isomerase (spot 66); ii) energy production, such as mitochondrial NADH ubiquinone dehydrogenase subunit 5 (spot 57), mitochondrial glycine cleavage system H protein 1 (spot 55), mitochondrial malate dehydrogenase (MDH) (spot 15); iii) some thylakoidal enzymes, such as cytochrome b_6-f complex Fe-S subunit (spot 59), oxygen-evolving enhancer proteins 2-1 (spot 65) and 1-2 (spot 72) [29] and H^+ -transporting ATP synthase chain 9 (spot 53). MDH (At1g53240) plays a key role in the tricarboxylic-acid pathway, glycolysis, gluconeogenesis and lipid metabolism. It has been shown that MDH levels are augmented in salt-stressed *Arabidopsis* roots [52] and following oxidative stress induced by jasmonic acid [53]. On the other hand, experiments on plants deficient in photorespiratory enzymes, such as those belonging to the glycine cleavage system, have demonstrated that these proteins are associated with protection from oxidative damage and resistance to diseases [54]. Photorespiration has a significant impact upon glutathione levels, which in turn are necessary to fuel the ascorbate-glutathione cycle for ROS detoxification. Proteomic studies on *Arabidopsis* response to bacterial challenge classified H^+ -ATPase (At4g32260) as a PAMP-responsive protein associated to basal defense [46].

Another group of up-regulated phosphoproteins after MV treatment was represented by enzymes involved in protection from abiotic/oxidative stress, namely cytosolic ascorbate peroxidase 1 (APX1) (spot 63), thioredoxin M-type 4 (spot 52) and peptidyl-prolyl *cis-trans* isomerase (spot 73). APXs are main enzymes responsible for H_2O_2 removal in plant cytosol, mitochondria and chloroplasts [55]. Previous studies showed that APX1 is a central component of the reactive gene network in *Arabidopsis* [56]. In oxygenic photosynthetic organisms,

Table 1 – Phosphoproteins with changed modification levels in *Arabidopsis thaliana* leaves after challenge with elicitors of plant basal defence (chitosan and benzothiadiazole), mechanical injury (wounding) or an inducer of oxidative stress (methyl viologen). Elicitor, spot number, protein name, gene number according to GI and AT database, organism, identification method, sequence coverage, peptide number, Mascot score, theoretical and experimental pI and Mr values, and fold change for elicited vs control plants are listed. PMF, TMS and GM indicate MALDI-TOF peptide mass fingerprinting, nanoLC-ESI-LIT-MS/MS and gel matching, respectively. Asterisk indicates proteins already characterized as being phosphorylated [8,23–30].

Spot	Protein name	Accession GI code	Accession AT code	Organism	ID method	Coverage/Peptide	Mascot score	Theor. pI/Mr (kDa)	Exp. pI/Mr (kDa)	Relative fold change (treatment/control)
<i>Chitosan</i>										
1	Ribulose biphosphate carboxylase/oxygenase activase *	166834	2G39730.1	<i>A. thaliana</i>	TMS	41 (19)	1154	5.87/52	5.34/43	0.1
2	Ribulose biphosphate carboxylase/oxygenase activase *	166834	2G39730.1	<i>A. thaliana</i>	TMS	33 (13)	773	5.87/52	5.44/44	0.1
3	Ribulose biphosphate carboxylase/oxygenase activase *	166834	2G39730.1	<i>A. thaliana</i>	TMS	31 (12)	733	5.87/52	5.59/42	0.1
4	33 kDa oxygen-evolving protein *	22571	5G66570.1	<i>A. thaliana</i>	TMS	40 (9)	655	5.68/35	5.38/29	0.4
5	Protein 1 photosystem II oxygen/evolving complex *	4835233	3G50820.1	<i>A. thaliana</i>	TMS	49 (14)	1154	5.92/35	5.40/28	0.5
6	Cytosolic triose phosphate isomerase *	414550	3G55440.1	<i>A. thaliana</i>	TMS	44 (10)	601	5.24/27	5.93/24	0.3
	Multicatalytic endopeptidase *	2511574	1G16470.2	<i>A. thaliana</i>	TMS	47 (8)	586	5.53/26		
	Triosephosphate isomerase *	4803926	2G21170.1	<i>A. thaliana</i>	TMS	34 (8)	530	7.67/33		
	Carbonic anhydrase *	14343	3G01500.2	<i>A. thaliana</i>	TMS	16 (5)	260	5.46/36		
	Chlorophyll a/b-binding protein-like *	4538963	4G10340.1	<i>A. thaliana</i>	TMS	11 (2)	199	6.00/30		
	L-ascorbate peroxidase *	145323784	1G07890.6	<i>A. thaliana</i>	TMS	14 (3)	149	5.85/27		
	Expressed protein *	3335374	2G38270.1	<i>A. thaliana</i>	TMS	13 (2)	143	7.72/32		
7	Photosystem II chain P-1, polyU binding *	186478207	1G06680.2	<i>A. thaliana</i>	TMS	32 (4)	222	7.71/24	5.46/21	0.1
8	Cyclophilin (fragment) *	405131	3G62030.1	<i>A. thaliana</i>	TMS	17 (6)	238	8.83/28	6.00/18	0.1
9	Component of cytochrome B6-F complex *	4262156	4G03280.1	<i>A. thaliana</i>	TMS	24 (4)	388	8.80/24	6.50/17	0.1
10	A2g21330/F3K23.9 *	15146282	2G21330.1	<i>A. thaliana</i>	TMS	35 (14)	906	6.18/43	5.76/38	0.2
11	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (fragment) *	7525041	CG00490.1	<i>A. thaliana</i>	TMS	23 (11)	535	5.88/53	6.17/18	0.3
12	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit *	7525041	CG00490.1	<i>A. thaliana</i>	PMF	13 (6)	91	5.88/53	4.94/49	0.4
13	Glyceraldehyde 3-phosphate dehydrogenase A subunit *	166702	3G26650.1	<i>A. thaliana</i>	TMS	14 (5)	347	7.00/38	6.58/37	0.1
14	Glyceraldehyde 3-phosphate dehydrogenase A subunit *	166702	3G26650.1	<i>A. thaliana</i>	TMS	24 (4)	240	7.00/38	6.65/37	0.1
15	Mitochondrial NAD-dependent malate dehydrogenase *	3929649	1G53240.1	<i>A. thaliana</i>	TMS	42 (9)	741	8.54/36	6.75/35	0.1
16	Cysteine synthase *	572517	2G43750.1	<i>A. thaliana</i>	TMS	25 (7)	554	8.14/42	5.94/36	0.1
17	Progesterone-binding protein homolog Atmp2 *	7576227	3G48890.1	<i>A. thaliana</i>	TMS	10 (2)	105	4.56/25	4.62/30	2.9
18	H+-transporting ATP synthase chain9 - like protein *	2864617	4G32260.1	<i>A. thaliana</i>	TMS	33 (7)	455	5.80/24	4.64/11	4.6
19	Unknown protein *	240254562	2G32240.1	<i>A. thaliana</i>	TMS	38 (36)	2325	4.74/109	4.61/98	16.5
20	Putative protein *	4972052	4G29060.1	<i>A. thaliana</i>	TMS	2 (2)	123	4.95/104	4.82/98	10.9
21	Putative protein *	4972052	4G29060.1	<i>A. thaliana</i>	TMS	10 (8)	408	4.95/104	5.03/98	9.1
22	Patellin-1 *	78099065	1G72150.1	<i>A. thaliana</i>	TMS	10 (13)	786	4.82/64	5.06/94	6.3
23	Heat shock cognate 70 kDa protein 3 *	18206367	3G09440.1	<i>A. thaliana</i>	TMS	35 (20)	980	4.97/71	5.16/77	11.0
24	Heat shock cognate 70 kDa protein 3 *	18206367	3G09440.1	<i>A. thaliana</i>	TMS	48 (28)	1486	4.97/71	5.25/77	6.7
25	26S protease regulatory subunit 6A homolog A *	75337114	3G05530.1	<i>A. thaliana</i>	TMS	62 (26)	1232	4.91/47	5.16/47	5.1
26	RNA-binding protein cp33 *	681910	3G52380.1	<i>A. thaliana</i>	TMS	17 (5)	254	4.62/36	4.50/35	7.0
27	Hsp 70-like protein *	5051770	4G24280.1	<i>A. thaliana</i>	TMS	29 (20)	1178	5.07/76	5.05/83	4.5
28	Ran-binding protein 1 homolog a *	75264036	1G07140.1	<i>A. thaliana</i>	TMS	50 (13)	798	4.91/26	5.07/37	4.1
29	Probable plastid-lipid-associated protein 3, chloroplastic, fibrillin 3 *	62900644	2G35490.1	<i>A. thaliana</i>	TMS	29 (11)	628	4.18/34	4.47/37	5.2

(continued on next page)

Table 1 (continued)

Spot	Protein name	Accession GI code	Accession AT code	Organism	ID method	Coverage/ Peptide	Mascot score	Theor. pI/ Mr (kDa)	Exp. pI/Mr (kDa)	Relative fold change (treatment/control)
Benzothiadiazole										
17	Progesterone-binding protein homolog Atmp2 *	7576227	3G48890.1	<i>A. thaliana</i>	TMS	10 (2)	105	4.42/24	4.62/30	7.7
18	H+-transporting ATP synthase chain9 - like protein *	2864617	4G32260.1	<i>A. thaliana</i>	PMF	33 (7)	455	5.80/24	4.64/11	2.7
20	Putative protein *	4972052	4G29060.1	<i>A. thaliana</i>	GM			4.95/104	4.82/98	4.7
21	Putative protein *	4972052	4G29060.1	<i>A. thaliana</i>	GM			4.95/104	5.03/98	3.1
24	Heat shock cognate 70 kDa protein 3 *	18206367	3G09440.1	<i>A. thaliana</i>	GM			4.97/71	5.25/77	6.1
27	Hsp 70-like protein *	5051770	4G24280.1	<i>A. thaliana</i>	GM			5.07/76	5.05/83	2.2
29	Probable plastid-lipid-associated protein 3, chloroplastic, fibrillin 3 *	62900644	2G35490.1	<i>A. thaliana</i>	GM			4.18/34	4.47/37	11.0
31	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit *	7525041	CG00490.1	<i>A. thaliana</i>	PMF	20 (9)	415	5.88/53	5.67/51	2.1
32	ATP synthase CF1 beta subunit *	7525040	CG00480.1	<i>A. thaliana</i>	PMF	34 (12)	91	5.38/54	5.72/51	2.4
33	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit *	7525041	CG00490.1	<i>A. thaliana</i>	PMF	16 (7)	91	5.88/53	5.96/52	2.1
34	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit *	7525041	CG00490.1	<i>A. thaliana</i>	PMF	26 (12)	161	5.88/53	6.08/52	2.2
35	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit *	7525041	CG00490.1	<i>A. thaliana</i>	PMF	23 (9)	1441	5.88/53	6.61/52	2.3
36	Fructose-bisphosphate aldolase *	18420348	4G38970.1	<i>A. thaliana</i>	TMS	36 (12)	825	6.79/43	6.00/38	0.5
	Annexin *	1429207	1G35720.1	<i>A. thaliana</i>	TMS	19 (4)	250	5.19/36		
	Glyceraldehyde 3-phosphate dehydrogenase A subunit *	166702	3G26650.1	<i>A. thaliana</i>	TMS	16 (5)	224	7.00/38		
	ATP synthase gamma-subunit *	166632	4G04640.1	<i>A. thaliana</i>	TMS	12 (3)	204	8.14/41		
	DegP protease precursor *	11994498	3G27925.1	<i>A. thaliana</i>	TMS	11 (4)	204	6.00/47		
	RNA-binding protein *	7406447	5G04430.1	<i>A. thaliana</i>	TMS	9 (3)	162	5.70/34		
37	ATPase beta subunit *	5881701	CG00480.1	<i>A. thaliana</i>	TMS	11 (3)	269	5.38/54	4.70/50	0.4
38	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit *	7525041	CG00490.1	<i>A. thaliana</i>	PMF	11 (6)	91	5.88/53	5.52/51	0.2
39	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit *	7525041	CG00490.1	<i>A. thaliana</i>	PMF	26 (12)	161	5.88/53	5.41/51	4.5
40	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit *	7525041	CG00490.1	<i>A. thaliana</i>	TMS	18 (9)	500	5.88/53	4.54/14	0.3
	H+-transporting ATP synthase chain9 - like protein *	2864617	4G32260.1	<i>A. thaliana</i>	TMS	32 (7)	453	5.80/23		
	Predicted protein of unknown function *	2262151	4G02530.1	<i>A. thaliana</i>	TMS	19 (5)	278	9.44/31		
41	Probable protein disulfide-isomerase 1 *	11134225	1G21750.1	<i>A. thaliana</i>	TMS	12 (6)	306	4.81/56	4.92/57	3.1
42	Late embryogenesis abundant protein	170010		<i>G. max</i>	TMS	12 (5)	214	6.33/51	4.87/57	2.2
43	Oxygen-evolving enhancer protein 2-1, chloroplastic *	18206371	1G06680.1	<i>A. thaliana</i>	TMS	26 (6)	238	6.90/28	4.62/27	5.5
	Nascent polypeptide-associated complex subunit alpha-like protein 3 *	71151986	5G13850.1	<i>A. thaliana</i>	TMS	15 (3)	155	4.41/22		
44	RNA-binding protein-like *	9759027	5G0250.1	<i>A. thaliana</i>	TMS	11 (3)	178	4.90/32	4.77/24	16.4
	Nascent polypeptide-associated complex subunit alpha-like protein 2 *	71151985	3G49470.1	<i>A. thaliana</i>	TMS	27 (3)	152	4.37/24		
45	Elongation factor 1-beta 2 *	75313298	5G19510.1	<i>A. thaliana</i>	TMS	18 (3)	144	4.42/24		
	Probable NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 5, mitochondrial *	25090853	5G52840.1	<i>A. thaliana</i>	TMS	13 (2)	97	4.73/19	4.15/10	19.3
46	Beta-glucosidase (fragment)	25989474		<i>O. europaea</i>	TMS	16 (6)	287	6.30/63	4.58/13	8.3

47	Ribulose biphosphate carboxylase large chain *	3914541	CG00490.1	A. thaliana	TMS	27 (15)	608	5.88/53	4.33/50	4.1
48	Luminal-binding protein 2 *	12643245	5G42020.1	A. thaliana	TMS	24 (14)	668	5.11/73	5.44/70	7.8
49	Cell division control protein 48 homolog A *	1705677	3G09840.1	A. thaliana	TMS	8 (5)	188	5.13/90	5.55/82	3.9
Wounding										
2	Ribulose biphosphate carboxylase/oxygenase activase *	18405145	2G39730.1	A. thaliana	TMS	34 (12)	790	5.09/46	5.46/44	2.5
3	Ribulose biphosphate carboxylase/oxygenase activase, chloroplastic *	30687995	2G39730.2	A. thaliana	PMF	30 (16)	169	5.42/43	5.59/42	2.3
28	Ran-binding protein 1 homolog a *	75264036	1G07140.1	A. thaliana	TMS	53 (12)	778	4.91/26	5.07/36	0.3
77	Nascent polypeptide-associated complex subunit alpha-like protein 2 *	71151985	3G49470.1	A. thaliana	TMS	31 (5)	348	4.37/24	4.60/25	2.5
78	Ribulose biphosphate carboxylase/oxygenase activase, chloroplastic *	18405145	2G39730.1	A. thaliana	PMF	38 (20)	185	5.09/46	5.26/45	2.2
79	Ribulose biphosphate carboxylase/oxygenase activase, chloroplastic *	18405145	2G39730.1	A. thaliana	PMF	39 (20)	196	5.09/46	5.30/45	2.5
80	Ribulose biphosphate carboxylase/oxygenase activase, chloroplastic *	18405145	2G39730.1	A. thaliana	PMF	15 (8)	98	5.09/46	5.36/44	2.3
81	Ribulose biphosphate carboxylase/oxygenase activase, chloroplastic *	30687995	2G39730.2	A. thaliana	PMF	37 (15)	186	5.42/43	5.63/43	2.6
82	Ribulose biphosphate carboxylase/oxygenase activase, chloroplastic *	30687995	2G39730.2	A. thaliana	PMF	32 (17)	188	5.42/43	5.77/43	2.1
83	Probable protein disulfide-isomerase A6 *	2529680	2G47470.1	A. thaliana	TMS	34 (10)	475	5.65/37	5.84/38	5.6
84	Putative chloroplast RNA-binding Protein *	15226983	2G35410.1	A. thaliana	PMF	36 (10)	141	5.55/34	5.16/34	0.3
85	Putative chloroplast RNA-binding protein *	15226983	2G35410.1	A. thaliana	PMF	26 (8)	112	5.55/34	5.24/33	0.3
86	Putative uncharacterized protein At1g55480 *	18405391	1G55480.1	A. thaliana	PMF	50 (17)	215	8.19/37	5.36/35	0.2
87	Putative transcription factor BTF3 (RNA polymerase B transcription factor 3) *	12324314	1G73230.1	A. thaliana	TMS	29 (5)	340	5.91/48	6.13/22	7.6
88	Cytochrome b6-f complex iron-sulfur subunit, chloroplastic *	68566234	4G03280.1	A. thaliana	TMS	24 (5)	386	6.07/19	6.33/20	0.1
89	Thylakoid luminal 16.5 kDa protein, chloroplastic *	84028274	4G02590.1	A. thaliana	TMS	43 (10)	575	4.90/16	5.27/13	0.2
90	Glycine cleavage system H protein 1, mitochondrial *	121075	2G35370.1	A. thaliana	TMS	21 (2)	177	4.73/14	4.73/13	0.3
91	Luminal-binding protein 1 *	1695717	5G28540.1	A. thaliana	PMF	26 (17)	149	5.05/71	5.36/70	0.3
92	Luminal-binding protein 2 *	1695719	5G42020.1	A. thaliana	PMF	26 (16)	132	5.08/71	5.87/52	3.1
93	At2g36530/F1011.16 *	15809970	2G36530.1	A. thaliana	TMS	36 (12)	780	5.74/48	5.75/52	2.9
93	At2g36530/F1011.16 *	15809970	2G36530.1	A. thaliana	TMS	22 (6)	340	5.74/48	5.75/52	2.9
94	Gln synthetase *	228453	5G35630.3	A. thaliana	TMS	43 (14)	714	6.43/47	5.46/45	2.2
95	Gln synthetase *	228453	5G35630.3	A. thaliana	TMS	10 (3)	130	6.43/47	5.29/46	2.1
96	Ribulose biphosphate carboxylase/oxygenase activase (fragment) *	166834	2G39730.1	A. thaliana	TMS	11 (4)	233	5.87/52	5.19/25	2.0
97	Cytosolic triose phosphate isomerase *	414550	3G55440.1	A. thaliana	TMS	23 (4)	263	5.24/25	5.61/25	2.6

(continued on next page)

Table 1 (continued)

Spot	Protein name	Accession GI code	Accession AT code	Organism	ID method	Coverage/Peptide	Mascot score	Theor. pI/Mr (kDa)	Exp. pI/Mr (kDa)	Relative fold change (treatment/control)
Methyl viologen										
15	Malate dehydrogenase 1, mitochondrial *	11133715	1G53240.1	<i>A. thaliana</i>	TMS	26 (9)	643	6.00/33	6.75/34	5.2
17	Progesterone-binding protein homolog Atmp2 *	7576227	3G48890.1	<i>A. thaliana</i>	TMS	31 (5)	365	4.42/24	4.62/29	0.1
50	Ribulose biphosphate carboxylase small chain 1A, chloroplastic *	27735223	1G67090.1	<i>A. thaliana</i>	TMS	41 (12)	624	5.69/15	6.03/9	3.3
	Ribulose biphosphate carboxylase small chain 1B, chloroplastic *	132090	5G38430.1	<i>A. thaliana</i>	TMS	40 (10)	551	5.70/15		
51	60S ribosomal protein L22-2 *	17865568	3G05560.3	<i>A. thaliana</i>	TMS	50 (12)	626	9.57/14	6.92/13	2.9
52	Thioredoxin M-type 4, chloroplastic *	27735268	3G15360.1	<i>A. thaliana</i>	TMS	16 (3)	140	5.47/12	5.20/13	5.1
53	H+-transporting ATP synthase chain 9-like protein *	2864617	4G32260.1	<i>A. thaliana</i>	TMS	28 (6)	393	5.80/24	5.08/10	3.9
54	60S acidic ribosomal protein P2-2 *	12229934	2G27720.1	<i>A. thaliana</i>	TMS	57 (5)	496	4.67/11	4.80/10	11.1
55	Glycine cleavage system H protein 1, mitochondrial *	121075	2G35370.1	<i>A. thaliana</i>	TMS	15 (2)	125	4.73/14	4.81/13	4.4
56	Pollen allergen Lol p VA	4416516		<i>L. perenne</i>	TMS	10 (2)	134	5.39/27	4.40/14	2.7
57	Probable NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 5, mitochondrial *	25090853	5G52840.1	<i>A. thaliana</i>	TMS	49 (8)	291	4.61/18	4.88/18	3.1
58	Translationally-controlled tumor protein homolog *	19883937	3G16640.1	<i>A. thaliana</i>	TMS	27 (4)	226	4.52/19	4.74/19	2.1
59	Cytochrome b6-f complex iron-sulfur subunit, chloroplastic *	68566234	4G03280.1	<i>A. thaliana</i>	TMS	24 (4)	323	6.07/19	6.55/20	5.6
60	60S ribosomal protein L31-3 *	19884146	5G56710.1	<i>A. thaliana</i>	TMS	39 (3)	169	9.95/14	6.87/20	7.5
61	BTF3b-like factor *	5912424	1G17880.1	<i>A. thaliana</i>	TMS	29 (4)	268	6.62/18	6.46/22	4.1
63	L-ascorbate peroxidase 1, cytosolic *	728873	1G07890.1	<i>A. thaliana</i>	TMS	60 (11)	729	5.72/27	6.14/24	2.9
65	Oxygen-evolving enhancer protein 2-1, chloroplastic *	18206371	1G06680.1	<i>A. thaliana</i>	TMS	16 (3)	171	5.72/20	5.58/23	3.4
66	Triosephosphate isomerase, cytosolic *	13432260	3G55440.1	<i>A. thaliana</i>	TMS	52 (8)	392	5.39/27	5.59/27	7.4
	Triosephosphate isomerase, chloroplastic *	13431953	2G21170.1	<i>A. thaliana</i>	TMS	22 (4)	275	5.38/27		
68	Elongation factor 1-beta 2 *	75313298	5G19510.1	<i>A. thaliana</i>	TMS	29 (5)	370	4.42/24	4.55/31	2.6
	Nascent polypeptide-associated complex subunit alpha-like protein 2 *	71151985	3G49470.1	<i>A. thaliana</i>	TMS	31 (5)	305	4.37/24		
69	T14P8.5 *	3193303	4G02450.2	<i>A. thaliana</i>	TMS	14 (3)	158	4.42/28		
	Nascent polypeptide-associated complex subunit alpha-like protein 2 *	71151985	3G49470.1	<i>A. thaliana</i>	TMS	29 (5)	301	4.37/24	4.50/31	2.2
70	14-3-3-like protein GF14 chi *	152112421	4G09000.2	<i>A. thaliana</i>	TMS	10 (2)	107	4.68/30	4.95/29	2.7
71	Ran-binding protein 1 homolog b *	76789667	2G30060.1	<i>A. thaliana</i>	TMS	18 (3)	209	4.83/24	5.02/31	3.7
72	Oxygen-evolving enhancer protein 1-2, chloroplastic *	11134146	3G50820.1	<i>A. thaliana</i>	TMS	42 (11)	709	5.05/27	5.50/27	2.4
73	Peptidyl prolyl cis-trans isomerase *	155334581	5G35100.2	<i>A. thaliana</i>	TMS	8 (2)	143	6.12/27	6.46/31	4.2
74	30S ribosomal protein S5, chloroplastic *	75101015	2G33800.1	<i>A. thaliana</i>	TMS	23 (7)	342	4.68/30	8.99/32	0.1

thioredoxins are small ubiquitous redox proteins encoded by large gene families [57], which are involved in regulation of Calvin cycle enzymes or function as hydrogen donors for antioxidant enzymes [58]. Peptidyl-prolyl *cis-trans* isomerase belongs to the large family of cyclophilins, accounting for 29 genes in *Arabidopsis*. Although their primary function is to assist protein folding [59], they have been recently reported being induced in response to various abiotic and biotic stresses [60]. *Arabidopsis* cyclophilin ROC1 has been also demonstrated to induce the self-cleavage of the product of the avirulence gene AvrRpt2 from *P. syringae*, thus contributing to plant innate immunity [61]. Another phosphoprotein with a function related to protection from stress is Atpm2 (spot 17), whose abundance was decreased after MV treatment, whereas was strongly increased following chitosan and benzothiadiazole treatment.

Methyl viologen administration also determined an increased amount of phosphoproteins involved in protein synthesis. In particular, augmented levels were observed for phosphorylated Ran-binding protein 1 (spot 71), 60S ribosomal protein L31-3 (RPL31C) (spot 60), 60S ribosomal protein L22-2 (RPL22B) (spot 51) and 60S acidic ribosomal protein P2-2 (RPP2B) (spot 54). The first one has been already identified as up-regulated following chitosan treatment and down-regulated in wounded samples. On the other hand, RPL22B and RPP2B have also been reported to play functions related to plant defense, being RPL22B identified as an early-changed component upon elicitor treatments in *Arabidopsis* membrane phosphoproteome [30], whereas RPP2B acts as *Arabidopsis* determinant of *Peronospora parasitica* specific recognition through a gene-for-gene mechanism [62]. Other up-regulated proteins were eukaryotic translation initiation factor 2 subunit β (spot 75), nascent polypeptide-associated complex subunit α -like protein 1 (spot 77) and 2 (spot 69), and BTF3b-like factor (spot 61). As far as the latter proteins, they were found with increased phosphorylation levels also in wounded leaves. Conversely, 30S ribosomal protein S5 (spot 74) was the only protein whose abundance decreased after MV treatment; it was previously identified as down-regulated nuclear protein present in cold-stressed *Arabidopsis* plants [63].

Other two up-regulated phosphoproteins whose function has been related to cell signaling were 14-3-3 protein GF14 χ isoform (spot 70) and calreticulin (spot 76). Highly conserved 14-3-3 proteins are regulatory proteins [64] that bind to a number of phosphorylated target proteins, thereby modifying their activity, localization or interaction [65]. In plants, 14-3-3s are involved in the regulation of fundamental processes [66], together with the response to various stresses. In fact, it has been shown that transcripts of specific 14-3-3 isoforms are induced upon abiotic/biotic stresses [67,68] or pathogen challenge [68,69]. Furthermore, it has been shown that GF14 λ interacts with APX3 [70] and its over-expression in *Arabidopsis* increases drought stress tolerance [71]. Different 14-3-3 interacting proteins involved in response to stress or defense have been identified, which confer resistance to virus and fungal pathogens [72–74]. Recently, GF14 λ has been identified as a responsive phosphoprotein in a study on *Arabidopsis* proteome alterations upon *P. syringae* challenge [46]. On the other hand, calreticulin (CRT) is a ER-resident Ca^{2+} -binding protein that plays an important role in Ca^{2+} signaling and protein folding. Plant CRTs have various properties that differ from their animal counterparts [75]. Endogenous basal expression levels

of both CRT mRNAs and proteins are up-regulated in response to a wide range of stimuli, such as cold, salts and exogenous phytohormones. Rice phosphorylated CRT was shown to be involved in signaling pathways leading to cold stress response [76,77]. CRT was also shown to be induced in response to pathogen attacks [78] and to be involved in tobacco plant defence against TMV infections [79], indicating its potential role in the activation of inducible resistance mechanisms. Other two up-regulated phosphoproteins that do not classify in the above-mentioned categories are pollen allergen Lol pVA (spot 56) and translationally controlled tumor protein homolog (spot 58). A proteomic study on *Arabidopsis* proteome modifications upon bacterial challenge identified the latter as involved in basal defense [46].

4. Conclusions

Reversible protein phosphorylation is a pivotal regulatory mechanism in eukaryotic cells and increasing evidence indicates that it plays a fundamental role also in plant response to biotic and abiotic stresses [8,31]. In this study, we have described the quantitative changes of phosphoproteins present in *A. thaliana* leaves after challenge with elicitors of plant basal defense (chitosan and benzothiadiazole), an inducer of oxidative stress (methyl viologen) as well as mechanical injury (wounding). This analysis relied on selective extraction of phosphoproteins by means of MOAC and their subsequent analysis by a combined 2D electrophoresis/MS approach, which revealed specific changes in modification patterns upon elicitation by different stressors. This investigation allowed to identify 75 differentially modified candidates, which were almost all described previously as being phosphorylated [24–31]. Several of the identified phosphoproteins were shared by different stresses, thereby suggesting a partial overlapping of the responsive pathways (Fig. 4). These findings are in good agreement with other emerging evidences suggesting that mechanisms by which plants respond to various environmental and biotic stresses are not independent, but rather are involved in networks of at least partially superimposed biochemical events [1,7,80,81].

Identified phosphoproteins grouped in various functional classes, with most represented candidates involved in energetic/carbon metabolism and stress/defense response. In chitosan-treated samples, for example, a reduced phosphorylation of enzymes implicated in carbon metabolism, photosynthesis and photorespiration, i.e. GP3DH, MDH, RuBisCO activase and cytochrome b_6-f complex Fe-S subunit, was observed. It is noteworthy that Jones and coworkers already reported that *Arabidopsis* key metabolic enzymes, such as GP3DH and MDH, result PAMP-responsive and down-regulated following plant interaction with *P. syringae* [46]. This finding is in fairly good accordance with the observed down-regulation of primary carbon metabolism transcripts during basal defense [82]. In fact, it has been proposed that chloroplasts may be key players in plant defense and a loss of photosystem functionality can lead to ROS overproduction and to a decreased sugar availability to pathogens [83]. These findings may suggest that the observed quantitative changes in these phosphoproteins should reflect transcriptional phenomena more than being related to an augmented phosphorylation of a specific class of

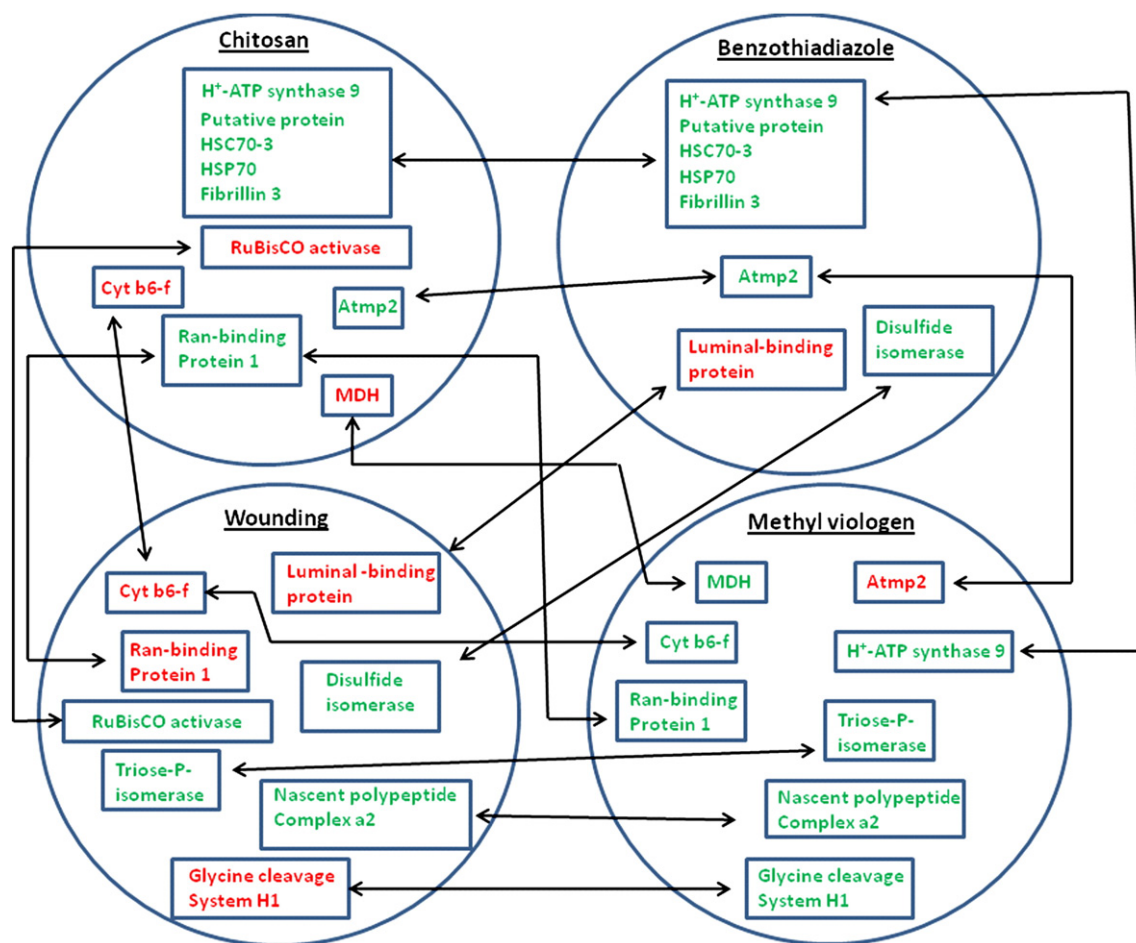


Fig. 4 – Common phosphoproteins changing their quantitative levels after treatment with elicitors of plant basal defence (chitosan and benzothiadiazole), mechanical injury (wounding) and an inducer of oxidative stress (methyl viologen). In green and red are reported phosphoproteins that increased and decreased their levels, respectively.

enzymes. Some of the metabolic enzymes having a reduced modification after chitosan treatment also showed a variable phosphorylation following other stresses, i.e. wounding (RuBisCO activase, cytochrome b_6 -f complex Fe-S subunit) or methyl viologen (MDH, cytochrome b_6 -f complex Fe-S subunit) (Fig. 4). In general, a comparison of the changes associated with the different stressors suggests that down-regulation of enzymes involved in carbon metabolism can be considered as specific for basal defense response to pathogens.

As far as proteins involved in protection from stress or defense responses, a number were found to be regulated by more than one stress. Phosphorylated HSC70s and HSP70s, for example, were identified both in chitosan- and benzothiadiazole-treated samples as strongly up-regulated species, whereas their levels apparently were not influenced by wounding or MV (Fig. 4). In a recent paper, it has been shown that *Arabidopsis* HSC70 chaperones interact with Sgt1, a conserved protein in eukaryotes, which participates into diverse signaling processes mediated by protein-protein interactions [84]. Thus, HSC70 binding to Sgt1 should act as a molecular switch for the response to different stresses, since it has been demonstrated that mutations in Sgt1 and HSC70 genes, altering proteins association, disabled basal

and R-mediated resistance and increased heat shock tolerance [84]. In this context, HSC70-3 phosphorylation may regulate this interaction. Previous studies demonstrated that HSC70-2 and 4 levels increased upon bacterial challenge [84], whereas we observed that chitosan determined up-regulation of the HSC70-3 isoform. Thus, our data seem to confirm that HSC70 chaperones may function as cross points between different stresses and suggest that the specificity of response towards various pathogens should be mediated by diverse HSC70 isoforms. A similarity in the modification profiles of chitosan and benzothiadiazole-treated plants was also highlighted by the common increased phosphorylation observed for fibrillin 3, a putative protein, Atp2 as well as ATPase chain 9 (Fig. 4). Depending on treatment, quantitative differences between elicitors were measured. Changes in the latter two proteins were also observed following MV treatment (Fig. 4). Still regarding proteins related to stress/defense response, a similar trend was observed after MV challenge and wounding for phosphorylated chaperone luminal-binding protein [50] and stress-responsive nascent polypeptide-associated complex subunit α -like protein [51] (Fig. 4), thus suggesting a regulation of common pathways in response to these stresses.

Among the various proteins involved in RNA metabolism identified in this study, phosphorylated Ran-binding protein 1 increased following chitosan and methyl viologen treatment, whereas decreased after wounding (Fig. 4). This protein is a component of the Ran-GTPase-mediated mRNA/miRNA export from nucleus. Emerging evidences support the occurrence in plants of post-transcriptional gene regulation by miRNA and siRNA in response to pathogen and oxidative stresses [85]. Thus, phosphorylated Ran-binding protein 1 should act in these processes.

Peculiar phosphoproteins responsible for response pathways to specific stresses were also identified in this work. This was evident for MV-challenged plants, where up-regulated proteins directly involved in ROS detoxification were identified, such as APX1 and thioredoxin M-type 4. Conversely, protein disulfide-isomerase A6 was identified as strongly modified only in samples from wounded leaves. Interestingly, members of this protein family are involved in ER stress or unfolded protein response, which have also been implicated in pathogen response. Its identification may suggest the implication of related mechanisms also in the response to wounding.

In conclusion, this study has identified a number of candidate phosphoproteins regulated in *planta* by different elicitors or treatments mimicking pathogen attack or oxidative stress. Based on accumulated evidences regarding their function, some phosphorylated proteins are clearly involved in stress response mechanisms, whereas others seem to participate in pathways for which their relationship with defense responses is still relatively novel and/or largely incomplete. Data presented here also suggest a partial overlapping of the different response pathways and the occurrence of cross talk points. Future experiments focused on *gel-free* approaches for quantitative phosphoproteome analysis, extensive characterization of the modified amino acids in the phosphorylated proteins here reported, their functional analysis by site-directed mutagenesis or their use in reverse genetic approaches will be fundamental to validate the role of each identified protein/suggested pathway in plant basal defense responses.

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