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Research article

Comparative phosphoproteome analysis upon ethylene and abscisic acid treatment in *Glycine max* leaves

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ABSTRACT

Abscisic acid (ABA) and ethylene play key roles in growth and development of plants. Several attempts have been made to investigate the ABA and ethylene-induced signaling in plants, however, the involvement of phosphorylation and dephosphorylation in fine-tuning of the induced response has not been investigated much. Here, a phosphoproteomic analysis was carried out to identify the phosphoproteins in response to ABA, ethylene (ET) and combined ABA + ET treatments in soybean leaves. Phosphoproteome analysis led to the identification of 802 phosphopeptides, representing 422 unique protein groups. A comparative analysis led to the identification of 40 phosphosites that significantly changed in response to given hormone treatments. Functional annotation of the identified phosphoproteins showed that these were majorly involved in nucleic acid binding, signaling, transport and stress response. Localization prediction showed that 67% of the identified phosphoproteins were nuclear, indicating their potential involvement in gene regulation. Taken together, these results provide an overview of the ABA, ET and combined ABA + ET signaling in soybean leaves at phosphoproteome level.

1. Introduction

Abscisic acid (ABA) and ethylene (ET) are involved in the regulation of diverse biological pathways including normal growth, development and stress response (Bari and Jones, 2009; Müller and Munné-Bosch, 2015; Salazar et al., 2015; Sharp, 2002). Growing body of evidence suggests a potential cross-talk between ABA and ET signaling during several biological processes including seed dormancy, germination and fruit development (Arc et al., 2013; Weng et al., 2015; Zhang et al., 2009). In most of the cases, if not all, ABA and ET work in an antagonistic manner (Arc et al., 2013). It was reported that exogenous application of ET precursor counteracts the inhibitory effects of ABA on endosperm cap weakening and endosperm rupture in *Arabidopsis* and *Lepidium sativum* (Arc et al., 2013; Linkies et al., 2009). ABA limits the ET production by inhibiting the ACC oxidase (ACO) activity and reduces ACO transcription, a major enzyme involved in ET biosynthesis (Arc et al., 2013). Moreover, recent reports have shown that the exogenous application of ethephon (ET precursor) leads to the accumulation of dietary isoflavones in soybean leaves and the effect was partially inhibited by the application of ABA (Gupta et al., 2018a; Yuk et al., 2016). Because of the antagonistic effects of these two hormones, our previous study investigated the effect of ABA, ET and combined ABA + ET on soybean leaves using a high-throughput proteomics approach. This study highlighted the involvement of MAP kinase in the regulation of these hormone signaling. In particular, phosphorylation of MAPK3, MAPK4, and MAPK6 was observed in response to ET treatment while ABA treatment led to the dephosphorylation of these MAP kinases. These results indicate a potential involvement of phosphorylation and dephosphorylation in the regulation of these hormone induced signaling in soybean leaves.

Phosphorylation is a well-known post–translational modification that regulates the activity of proteins involved in the myriad of biological pathways (Kline-Jonakin et al., 2011). Phosphorylation and dephosphorylation of proteins by kinases and phosphatases respectively, act as a biological switch that regulates the functional properties of proteins (Kline-Jonakin et al., 2011). Attempts have been made

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previously to identify the phosphoproteins in response to ABA or ET treatments (Bhaskara et al., 2017; Li et al., 2009; Minkoff et al., 2015; Qing et al., 2016; Qiu et al., 2017; Umezawa et al., 2013; Wang et al., 2013). A recent study where phosphoproteomic changes in grapevine leaves were analyzed after ABA treatment identified 33 differential proteins using a label-free quantitative proteomic approach (Rattanakan et al., 2016). Of these differential proteins, abundance of 20 and 13 phosphoproteins was increased and decreased respectively, upon ABA treatment. Proteins related to the serine family amino acid metabolic process showed decreased abundance while protein folding related proteins were upregulated in response to ABA treatment (Rattanakan et al., 2016). Similarly, several other studies led to the identification of hundreds of phosphopeptides in response to ABA or ET treatments, however, none of these studies focused on investigating the combined effect of ABA and ET. Moreover, there is no report of either ABA or ET phosphoproteome analysis in soybean.

Therefore, here we carried out a phosphoproteome analysis to analyze the changes in soybean (*Glycine max*) leaf upon ET, ABA, and combined ABA + ET treatments. Results presented here provide new insights into the molecular action of ET and ABA signaling and their consequences on the metabolic pathways in soybean leaves.

2. Materials and methods

2.1. Plant growth and hormone treatments

Soybean seeds (*Glycine max* cv. Daewon) were planted in the soil and allowed to grow in a growth chamber at 25 °C (16/8 h day/light cycle, 70% relative humidity) for one month. Ethephon (5 mM, a natural precursor of ET) and ABA (500 μM) were prepared in the deionized water and 50 mL of solution was evenly sprayed, using a foliar spray, over six pots containing five soybean plants per pot. An equal volume of deionized water was sprayed as a control. Whole trays were shifted to transparent acrylic chambers and sealed to prevent the evaporation of ET produced. Leaves were harvested after 3 h for phosphoproteome analysis.

2.2. Protein extraction and phosphopeptide enrichment

Control and hormone-treated leaves (1 g) were homogenized in 5 mL RIPA buffer containing phosSTOP phosphatase inhibitor cocktail (Roche, Basel, Switzerland) and protease inhibitor cocktail (Thermo Fisher Scientific, USA) and subjected to methanol-chloroform precipitation. The pellets so obtained were solubilized in 1 × SDS-loading buffer or 6 M urea for SDS-PAGE or in-solution trypsin digestion respectively. Protein concentration in each fraction was determined by 2D-Quant Kit (GE Healthcare, Uppsala, Sweden) and 30 μg protein from each fraction was loaded on 12% SDS-PAGE. Gels were either stained with the PRO-Q diamond stain (Invitrogen, OR, USA) as per the manufacturer's instructions or electroblotted on PVDF membranes and probed with antiphospho-serine (Q5) or antiphospho-threonine (Q7) antibodies (Qiagen SJ, USA). For shotgun proteome analysis, 1 mg protein from each sample was used for in-solution trypsin digestion as described previously (Gupta et al., 2016). In brief, proteins were first reduced using 1 mM dithiothreitol (DTT) and then alkylated with 5.5 mM iodoacetamide for 45 min. Urea concentration was reduced to 0.6 M by the addition of deionized water and digested overnight with Trypsin Gold (Promega, Madison, USA). Protein digest was acidified to a final concentration of 0.1% TFA to stop the trypsin digestion. Peptides were centrifuged at 2000 g for 10 min to remove the undigested proteins and desalted using C18 columns (Empore, MN, USA) prior to phosphopeptide enrichment. TiO_2 based phosphopeptide enrichment kit (Pierce Biotechnology) was used for enrichment of phosphopeptides following manufacturer's protocol.

2.3. Phosphopeptide identification

Enriched phosphopeptides were desalted using zip-tips, dissolved in solvent-A (water/ACN, 98:2 v/v; 0.1% formic acid), and separated by reversed-phase chromatography using a UHPLC Dionex UltiMate® 3000 (Thermo Fisher Scientific, USA) instrument (Gupta et al., 2016). For trapping the sample, the UHPLC was equipped with Acclaim PepMap 100 trap column (100 μm × 2 cm, nanoViper C18, 5 μm, 100 Å) and subsequently washed with 98% solvent A for 6 min at a flow rate of 6 μL/min. The sample was continuously separated on an Acclaim PepMap 100 capillary column (75 μm × 15 cm, nanoViper C18, 3 μm, 100 Å) at a flow rate of 400 nL/min. The LC analytical gradient was run at 2%–35% solvent B over 90 min, then 35%–95% over 10 min, followed by 90% solvent B for 5 min, and finally 5% solvent B for 15 min. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was coupled with an electrospray ionization source to the quadrupole-based mass spectrometer QExactive™ Orbitrap High-Resolution Mass Spectrometer (Thermo Fisher Scientific, USA). Resulting peptides were electro-sprayed through a coated silica emitted tip (PicoTip emitter, New Objective, USA) at an ion spray voltage of 2000 eV. The MS spectra were acquired at a resolution of 70,000 (200 m/z) in a mass range of 350–1800 m/z. A maximum injection time was set to 100 ms for ion accumulation. Eluted samples were used for MS/MS events (resolution of 17,500), measured in a data-dependent mode for the 10 most abundant peaks (Top10 method), in the high mass accuracy Orbitrap after ion activation/dissociation with Higher Energy C-trap Dissociation (HCD) at 27 collision energy in a 100–1650 m/z mass range (Min et al., 2017).

2.4. Data processing using MaxQuant software

The obtained raw data were analyzed by MaxQuant software (Cox and Mann, 2008) v.1.5.0.0 using Andromeda as a search engine (Cox et al., 2011). The acquired MS/MS spectra were searched against the soybean protein database (Gmax_275_Wm82. a2. v1, 88647 entries), obtained from Phytozome and quantification of peptides and proteins was performed by MaxQuant with an FDR < 0.01 for proteins, peptides, and modifications. Search parameters included trypsin as a cleavage enzyme, cysteine carbamidomethylation as a fixed modification and oxidation of methionine, acetylation (protein N-term), and phosphorylation of Ser, Thr, Tyr residue (phosphoSTY) as variable modifications. A minimum peptide length of six amino acids was specified and “match between runs” (MBR) was enabled with a matching time window of 1 min. Obtained data were analyzed using Perseus software and phosphopeptides that were reproducibly identified in at least two out of three replicates of at least one sample with score > 40 and delta score > 7 were considered as valid identification and used for the further analysis.

2.5. Functional annotation of the identified proteins

Significantly enriched phosphorylation motifs were extracted from phosphopeptides with confidently identified phosphorylation residues (class I) using the Motif-X algorithm (http://motif-x.med.harvard.edu/). The phosphopeptides were centered at the phosphorylated amino acid residues and aligned, and six positions upstream and downstream of the phosphorylation site were included. A data set containing the Arabidopsis homologs of the identified phosphoproteins were retrieved from phytozome and used for protein − protein interaction (PPI) analysis by the Search Tool for Retrieval of Interacting Genes/Proteins (STRING) database (http://string-db.org/) and arranged using Cytoscape. Functional analysis of the identified proteins was carried out using DAVID functional annotation tool (https://david.ncifcrf.gov/tools.jsp) with integrated PANTHER Gene Ontology (GO) and KEGG pathways.