Journal of Plant Physiology 173 (2015) 33–40

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Molecular Biology

Cantharidin, a protein phosphatase inhibitor, strongly upregulates detoxification enzymes in the *Arabidopsis* proteome

Joanna Bajsa[1], Zhiqiang Pan, Stephen O. Duke*

USDA, ARS, Natural Products Utilization Research Unit, Cochran Research Center, University, MS 38677, USA

ARTICLE INFO

Article history:
Received 14 May 2014
Received in revised form 19 August 2014
Accepted 7 September 2014
Available online 16 September 2014

Keywords:
Cantharidin
Glutathione-*S*-transferase
Protein phosphatase
Proteome
Serine/threonine protein phosphatase

ABSTRACT

Cantharidin, a potent inhibitor of plant serine/threonine protein phosphatases (PPPs), is highly phytotoxic and dramatically affects the transcriptome in *Arabidopsis*. To investigate the effect of cantharidin on the *Arabidopsis* proteome, a combination of two-dimensional difference gel electrophoresis (2-D DIGE) and matrix-assisted laser desorption ionization time-of-flight (MALDI/TOF) mass spectrometry was employed for protein profiling. Multivariate statistical analysis identified 75 significant differential spots corresponding to 59 distinct cantharidin-responsive proteins, which were representative of different biological processes, cellular components, and molecular functions categories. The majority of identified proteins localized in the chloroplast had a significantly decreased presence, especially proteins involved in photosynthesis. Detoxification enzymes, especially glutathione-*S*-transferases (GSTs), were the most upregulated group (ca. 1.5- to 3.3-fold). Given that the primary role of GSTs is involved in the process of detoxification of both xenobiotic and endobiotic compounds, the induction of GSTs suggests that cantharidin promoted inhibition of PPPs may lead to defense-like responses through regulation of GST enzymes as well as other metabolic pathways.

Published by Elsevier GmbH.

Introduction

Posttranslational modification (PTM) is one method by which cells can control properties of proteins. Modifications of specific amino acids by covalent binding or removal of functional groups such as phosphate, acylate, N-glycans, methyl, nitrate oxide and ubiquitin in plants are common PTMs (Ying et al., 1999; Wang et al., 2006; Sugiyama et al., 2008; Burén et al., 2011; Wu et al., 2011; Furlan et al., 2012). These changes can alter protein conformations and may modify as well as enable interactions with other proteins. PTMs of enzymes may also change their activity. With phosphorylation, the most common PTM, kinases and phosphatases coordinate the phosphorylation status of targeted proteins. In eukaryotes, mostly Ser, Thr and Tyr residues undergo phosphorylation (Hunter, 2012). Sugiyama et al. (2008) analyzed the *Arabidopsis* phosphoproteome and discovered 1346 proteins with unique phosphorylation sites representing an average content of phosphoserine (pSer), phosphotreonine (pThr) and phosphotyrosine (pTyr) of 85.0, 10.7 and 4.3%, respectively. Changes in the phosphorylation state of protein may lead to conformational alterations which can trigger protein–protein interactions or activate/deactivate an enzyme. Various types of proteins contain domains that enable interaction with pSer/pThr motifs. The most well known domains are 14-3-3, WW, forkhead-associated and WD40 (Yaffe and Elia, 2001).

Serine/threonine protein phosphatases (PPPs) are indispensable in cell signal transduction. *In planta*, PPPs are proposed to be involved in various cellular processes such as cell cycle regulation, hormone signaling and transport, flowering, photoregulation, cellular proliferation, plant defenses, cell differentiation, and apoptosis (Pernas et al., 2007; Iki et al., 2012; Ballesteros et al., 2013; Dai et al., 2013). Endothall and cantharidin are specific inhibitors

Abbreviations: ACC, 1-aminocyclopropane carboxylate; ACO, 1-aminocyclopropane carboxylate oxidase; ACS, 1-aminocyclopropane-1-carbonylate synthase; AER, alkenal reductase; APX, ascorbate peroxidase; ATPA, ATP synthase subunit alpha; CDPK, calcium-dependent protein kinase; DIGE, 2-D difference gel electrophoresis; FBA2, fructose-bisphosphate aldolase 2; FNR, ferredoxin-NADP(+)-oxidoreductase; GST, glutathione-*S*-transferase; JA, jasmonic acid; LOX, lipoxygenase; MALDI/TOF, matrix-assisted laser desorption ionization time-of-flight; MAPK, mitogen activated protein kinase; PAR, photosynthetically active radiation; PGK, phosphoglycerate kinase; PPP, serine/threonine protein phosphatase; PRK, phosphoribulokinase; PSBP-1, photosytem II subunit P-1; PTM, posttranslational modification; RCA, rubisco activase; SAM, S-adenosylmethionine, TGGthioglucoside glucohydrolase; TPX, thioredoxin-dependent peroxidase.

* Corresponding author at: USDA, ARS, Rm 1012, Cochran Center, School of Pharmacy, Univ. Mississippi, University, MS 38677, USA. Tel.: +1 662 915 1036.
E-mail address: Stephen.Duke@ars.usda.gov (S.O. Duke).
[1] Present address: Life Sciences Bldg., Dept. Biological Sciences, Louisiana State University, Baton Rouge, LA 70803, USA.

http://dx.doi.org/10.1016/j.jplph.2014.09.002
0176-1617/Published by Elsevier GmbH.

of PPP (Bajsa et al., 2011a,b, 2012) that have been used to probe the role of PPP in specific processes such as pathogen defenses (MacKintosh et al., 1994; Rakwal et al., 2001), responses to cold treatment (Manzanero et al., 2002), and regulation of anthocyanin synthesis (Vitrac et al., 2000). PPPs are strongly conserved, having highly homologous amino acid sequences across various species and even across kingdoms. Cantharidin interacts with the binding site of PPPs (Bertini et al., 2009). In the *Arabidopsis* genome, 19 PPPs belonging to five different classes (PP1, PP2A, PP4, PP5, and PP6) have been identified that represent potential cantharidin targets. However, until the publication of recent papers by Bajsa et al. (2011a,b), there were no studies demonstrating the full capacity of a PPP inhibitor to affect the entire transcriptome.

Both cantharidin and its herbicidal structural analog endothall are very phytotoxic to *Arabidopsis* due to their effects on PPPs (Bajsa et al., 2011a, 2012). When plants were sprayed with 200 μM (IC_{30} for chlorophyll accumulation) cantharidin, transcription of 1509 genes were significantly affected within 2 h, and within 24 h transcription of ca. 10% (2577 genes) of the genome was significantly up- or down-regulated. All 19 *Arabidopsis* PPPs were inhibited by cantharidin, indicating that a number of signal transduction pathways were also affected. However, how these transcriptome effects influence the proteome has yet to be reported. In this article, data on this topic are provided to complement the papers of Bajsa et al. (2011a,b) on transcription effects. The same plants from which RNA was sampled for effects of cantharidin on the transcriptome (Bajsa et al., 2011a,b) were also sampled for proteins, and the proteome profile was determined by two-dimensional protein electrophoresis and mass spectrometry (MS) techniques.

Materials and methods

Plant growth and cantharidin treatment

Procedures were identical to those described by Bajsa et al. (2011a). Briefly, wild type *Arabidopsis thaliana* (L.) Heynh. Columbia ecotype was grown on MS medium, 200 seedlings per 10 cm × 8 cm glass container (PYREX no. 3250 storage dishes with Pyrex lid, Corning Glass Works, Corning, NY, USA) at 24 °C and under constant light intensity of 100 μmol m^{-2} s^{-1} photosynthetically active radiation. Twelve-day-old seedlings were sprayed with the IC_{30} of cantharidin (200 μM). Seedling shoots were harvested at three time points after spraying: 2, 10 and 24 h. Each experiment (time point) had three independent replicates of both treated and untreated (control) samples.

Protein isolation and 2-D DIGE protein profiling

Whole *Arabidopsis* seedlings were ground to a powder in liquid nitrogen, and 1 g of the powder was mixed with 20 mL of precipitation solution containing 10% (w/v) TCA and 0.07% (w/v) 2-mercaptoethanol in acetone. Aliquots (1.8 mL) of the suspension were chilled in liquid nitrogen for 15 s and then incubated at −20 °C for 1 h. The suspension was mixed after 5, 10, and 15 min. Precipitated material was collected by centrifugation (25,000 × *g*, 4 °C, 15 min). The pellet was washed twice with acetone containing 0.07% (w/v) 2-mercaptoethanol. The precipitate was dried in a vacuum centrifuge. Protein extraction was performed according to Schlesier and Mock (2006). The 2-D difference gel electrophoresis (DIGE) protein profiling was done by Applied Biomics, Inc. (Hayward, CA). Protein extracts from untreated and treated samples were labeled with different CyDye DIGE fluors (Cy5 untreated (red color) and Cy3 treated samples (green color). Pairs of untreated and treated samples were separated in the first dimension strips 4–7 pH and then run on the 10.5% SDS gels. Gels were scanned on a Typhoon image scanner and analyzed with ImageQuant software.

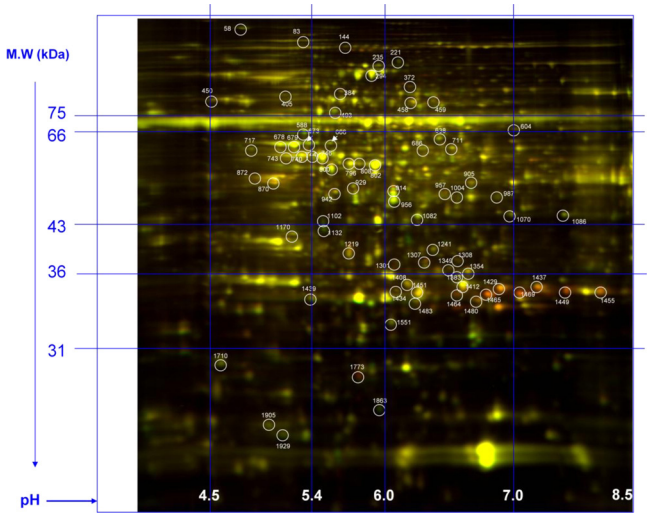

Fig. 1. 2-D gel profiling using a 10.5% SDS gel: *Arabidopsis* seedlings proteome 10 h after cantharidin treatment. Circles mark spots with $p < 0.05$ levels of significant changes in protein expression that were picked for mass spectrometry analysis. Samples treated (labeled with Cy5, red) and untreated (labeled with Cy3, green) were run on the same gel.

Protein spots were digested with trypsin, and then samples were spotted on a MALDI plate and analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI/TOF) mass spectrometer (MS). GPS Explorer software was used to search the MASCOT database to analyze MS and MS/MS spectra. The spots with significant statistical changes ($p < 0.05$) were determined by one way or two way ANOVA methods. The ANOVA values depended on treatment, time point, or treatment plus time point.

Immunoblotting

Forty micrograms of total protein of each sample was loaded on a 10% SDS-PAGE precast gel (BioRad). After electrophoresis proteins were transferred onto a Hybond-P PVDF membrane (Amersham). The membrane was incubated with polyclonal anti-PP2A (Millipore). An enhanced chemiluminescence reagent (Pierce ECL Western Blotting Substrate from Thermo Scientific) was used for signal detection. The experiment was replicated three times.

Results

Effects of cantharidin on the proteome of Arabidopsis

In total, there were 75 protein spots (Fig. 1, Table 1) for which there were statistically significant changes in abundance between treated and control (untreated) plants. Their peptides were identified with the MASCOT database and subjected to further analysis. They corresponded with 59 different gene products, as annotated in TAIR, the *Arabidopsis* database. Eight of the proteins were found to be in two or more posttranslationally modified forms. Two types of rubisco activase (RCA) isoforms were detected: the long RCA (46 kDa; detected 4 spots) and short RCA (42 kDa; detected 3 spots) (Fig. 2). All together, RCA produced seven spots, the highest number of PTM forms of a single protein in the experiment (Fig. 2). Glutathione-*S*-transferase 6 (GSTF6) and phosphoglycerate kinase 1 (PGK1) each had three spots, while glutathione-*S*-transferase 2 (GSTF2), ascorbate peroxidase 1 (APX1), ferredoxin-NADP(+)-oxidoreductase 2 (FNR2), 1-aminocyclopropane carboxylate (ACC) oxidase 2 (ACO2) and thioglucoside glucohydrolase 1 (TGG1) had two each (Table 1).