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Analysis of abscisic acid responsive proteins in *Brassica napus* guard cells by multiplexed isobaric tagging

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ABSTRACT

Guard cells, which form stomata on the leaf epidermis, play important roles in plant gas exchange and defense against pathogens. Abscisic acid (ABA) is a phytohormone that can be induced by drought and leads to stomatal closure. Guard cells have been a premier model system for studying ABA signal transduction. Despite significant progress on the identification of molecular components in the ABA signaling pathway, our knowledge of the protein components is very limited. Here, we employ a recently developed multiplexed isobaric tagging technology to identify ABA-responsive proteins in *Brassica napus* guard cells. A total of 431 unique proteins were identified with relative quantitative information in control and ABA-treated samples. Proteins involved in stress and defense constituted a major group among the 66 proteins with increased abundance. Thirty-eight proteins were decreased in abundance and fell into several functional groups including metabolism and protein synthesis. Many of the proteins have not been reported as being ABA responsive or involved in stomatal movement. A large percentage of the protein-coding genes contained ABA-responsive elements. This study not only established a comprehensive inventory of ABA-responsive proteins, but also identified new proteins for further investigation of their functions in guard cell ABA signaling.

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1. Introduction

Plants are sessile organisms and have evolved a suite of molecular mechanisms to transduce external signals and adapt to environmental conditions. The environmental signals include water availability, light, CO_2 and pathogen attack. Stomata play an essential role in plant interactions with the environment through adjustment of their apertures in response to these signals. The opening and closing movement is controlled by the surrounding pair of guard cells, which can change shape due to solute influx and efflux [1]. The ability of guard cells to change shape efficiently is a prerequisite for their functions. Consistent with this function, guard cells usually exhibit high activities of energy metabolism and solute transport [2]. Recent proteomic studies of guard cells revealed the dominant groups of guard cell proteins involved in energy (respiration),

Abbreviations: ABA, abscisic acid; ABRE, ABA-responsive element; ARG1, altered responsive to gravity 1; DPI, diphenyleneiodonium; GST, glutathione S-transferase; ROS, reactive oxygen species; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; TRX, thioredoxin.

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signaling, transport and cell structure [3,4]. It is clear that guard cells possess the machinery to transduce environmental signals and regulate stomatal movement.

In addition to its function in seed dormancy, growth regulation, leaf senescence and plant stress responses, ABA plays a key role in regulating stomatal movement [5]. Classic genetic screens, reverse genetics and cell biological analyses have revealed many molecular components involved in guard cell ABA signaling [1,6–8]. This information has been synthesized into a network model of ABA-induced stomatal closure [8]. Briefly, ABA is perceived by ABA receptors [9–11] and induces stomatal closure via messengers that include cytosolic Ca^{2+} (Ca_i) and pH increases. Ca_i increase is due to Ca^{2+} influx from outside and its release from internal stores. Ca^{2+} influx is mediated by Ca^{2+}-permeable channels and prompted by reactive oxygen species (ROS). ABA-induced ROS production relies on NADPH oxidases downstream of the ABA-activated protein kinase, open stomata 1 (OST1). ROS production promotes the synthesis of nitric oxide (NO), which in turn elicits Ca^{2+} release from internal stores. Downstream components responding to cytosolic Ca^{2+} increase include vacuolar K^+-permeable channels, plasma membrane K^+-influx channels and anion efflux channels. An increase in cytosolic pH promotes the opening of anion and K^+ efflux channels in the plasma membrane [8,12]. Guard cell volume reduction and stomatal closure occur upon water efflux induced by K^+ and anion efflux, sucrose removal, and conversion of malate to osmotically inactive starch. Phosphatidic acid and ROS negatively regulate a protein phosphatase 2C, which regulates anion efflux and feedback regulates ROS production [8]. In this assembled network, which closely represents current knowledge of guard cell ABA signaling, there are only about 30 proteins known to be involved. The regulatory mechanisms of many proteins and the involvement of other proteins remain to be determined.

To date, the only available large-scale transcriptomic analysis of ABA-responsive genes in guard cells was carried out in *Arabidopsis* using a microarray that covered approximately one-third of the *Arabidopsis* genome [13]. ABA was found to regulate 133 genes specifically in guard cells, including genes encoding protein phosphatase 2C, a 14-3-3 protein, oxidase and reductase family members, ribosomal proteins and several heat shock proteins [13]. *Brassica napus*, the most important oilseed crop, is genetically closely related to *Arabidopsis*. The two species share extensive co-linearity and 87% sequence identity in their protein-coding regions [14]. The rich source of genomic sequences available for both organisms, as well as the success in isolating high quality guard cell protoplasts [3] dramatically improve our ability to apply functional proteomics tools. Currently no large-scale proteomic analysis of guard cell ABA-responsive proteins has been reported. In this study, we employed a recently developed 8-plex isobaric tag for relative and absolute quantitation (iTRAQ) method and a 2D LC-MS/MS approach to analyze ABA-responsive proteins in *B. napus* guard cells. The 8-plex iTRAQ isobaric tagging employs the same chemistry to label peptides via free amine groups [3] to enable eight different samples to be relatively quantified in a single LC-MS experiment. In the 8-plex reagent, reporter ion masses are 113.1–119.1, and 121.1. The mass at 120.1 is omitted, to avoid contamination from phenylalanine immonium ion (*m*/*z* 120.08) [15]. The advantages of this technology are that it allows eight samples to be analyzed simultaneously and it is unbiased toward membrane proteins and very basic or acidic proteins. A major disadvantage is that not all the identified proteins have confident quantitative data. In this study, the iTRAQ LC-MS analysis established a comprehensive inventory of ABA-responsive proteins and identified new proteins that are potentially involved in ABA signaling. In addition, the results complemented the guard cell microarray data and highlighted a redox regulatory mechanism in guard cell ABA signaling.

2. Material and methods

2.1. *Plant growth, guard cell preparation and ABA treatment*

B. napus var Global seeds were obtained from Svalöv Weibull AB (Svalöv, Sweden). Seeds were germinated in Metro-Mix 500 potting mixture (The Scotts Co., USA) and plants were grown in a growth chamber under a photosynthetic flux of 160 μmol photons m^{-2} s^{-1} with a photoperiod of 8 h at 22 °C light and 20 °C dark. Fully expanded young leaves from two month old plants (Supplemental Fig. 1) were used for preparation of guard cell protoplasts as previously described [3,16]. For ABA treatment, ABA was added to a final concentration of 100 μM in the second enzyme digestion mixture, and the solutions used in the following steps. The treatment time was 3 h.

2.2. *Stomatal bioassays*

Aperture and H_2O_2 measurements were carried out as previously described [17,18] with slight modifications. A couple of leaves from a plant were blended and the epidermal strips were washed with cold tap water [16]. The freshly prepared epidermal strips were incubated in degassed medium (50 μM $CaCl_2$, 10 mM KCl and 10 mM MES-KOH, pH 6.2) for 3 h under light to promote stomata opening. After checking the stomatal aperture, the following chemicals were added: diphenyleneiodonium (DPI) 20 μM, catalase 200 U/ml, and ascorbic acid 10 mM, respectively. Tissues were incubated for 20 min before addition of ABA. Images of stomata were captured using a Zeiss Axiostar Plus microscope (Carl Zeiss Inc., USA). At least 60 stomata were analyzed in each experiment and three replicate experiments were conducted.

2.3. *Protein digestion, 8-plex iTRAQ labeling, and strong cation exchange fractionation*

Three independent guard cell preparations were pooled to yield one biological replicate, which contained 80 μg total protein as measured by a CB-X protein assay kit (Genotech, USA). Four control replicates and four ABA-treated replicates were used for overnight acetone precipitation. After protein precipitation, the pellet was dissolved in 1% SDS, 100 mM triethylammonium bicarbonate, pH 8.5. The samples were reduced, alkylated, trypsin-digested and labeled using the 8-plex iTRAQ reagent kit according to manufacturer's instructions (Applied Biosystems Inc., USA). The control samples were labeled with iTRAQ tags 113, 114, 115 and 116 and ABA-treated samples were labeled with tags 117, 118, 119 and 121. After labeling, the samples were combined and lyophilized. The peptide mixture was dissolved

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