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Proteomics of *Arabidopsis* redox proteins in response to methyl jasmonate

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ABSTRACT

Protein redox regulation is increasingly recognized as an important switch of protein activity in yeast, bacteria, mammals and plants. In this study, we identified proteins with potential thiol switches involved in jasmonate signaling, which is essential for plant defense. Methyl jasmonate (MeJA) treatment led to enhanced production of hydrogen peroxide in *Arabidopsis* leaves and roots, indicating *in vivo* oxidative stress. With monobromobimane (mBBr) labeling to capture oxidized sulfhydryl groups and 2D gel separation, a total of 35 protein spots that displayed significant redox and/or total protein expression changes were isolated. Using LC–MS/MS, the proteins in 33 spots were identified in both control and MeJA-treated samples. By comparative analysis of mBBr and SyproRuby gel images, we were able to determine many proteins that were redox responsive and proteins that displayed abundance changes in response to MeJA. Interestingly, stress and defense proteins constitute a large group that responded to MeJA. In addition, many cysteine residues involved in the disulfide dynamics were mapped based on tandem MS data. Identification of redox proteins and their cysteine residues involved in the redox regulation allows for a deeper understanding of the jasmonate signaling networks.

1. Introduction

Plants produce jasmonic acid and methyl jasmonate (MeJA) in response to many abiotic and biotic stresses, particularly pathogen and insect herbivores [1,2]. Jasmonates are plant hormones biosynthesized from linolenic acid through the octadecanoid pathway [3]. They function as signaling molecules to activate genes involved in plant defense responses [4,5]. Over the past decades, intensive research has been focused on the jasmonate signaling pathway in *Arabidopsis* and tomato [6–8]. The perception of stress signal, the induction and regulation of jasmonate biosynthesis, and the genes differentially expressed by jasmonates have been well-studied [3,6,7,9–11]. However, the molecular details of downstream regulatory proteins and pathways remain to be discovered. It was suggested in 1994 that jasmonates could induce oxidative stress in parsley suspension cells [12]. Later, jasmonate induced hydrogen peroxide (H_2O_2) accumulation was observed in the cell wall of tomato plants [13]. H_2O_2, the most stable form of reactive oxygen species (ROS), is well known to function as a signaling molecule to activate cellular antioxidant mechanisms, and can be used as an indicator of cellular oxidative stress [14].

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For instance, the production of H_2O_2 in the cell wall was shown to induce the activity of polygalacturonases [15]. The result indicates that H_2O_2 can function as a secondary messenger and play a role in MeJA mediated metabolic processes [16].

Cellular protein redox cycling is regulated by several well-known systems such as the NADPH/ferredoxin–thioredoxin and glutathione–glutaredoxin systems [17,18]. Whereas the thioredoxin and glutaredoxin systems seem to function mainly in the reduction of disulfide bonds, other thiol/disulfide containing proteins (*e.g.*, oxidoreductases) and ROS act to oxidize thiol groups. Although it is known that H_2O_2 can regulate the activities of certain plant proteins, little is known about the molecular mechanisms underlying the regulation. It was suggested that H_2O_2 mediated redox state change of protein thiols may play a role in oxidative stress signaling [19]. Thiol based redox proteome is relatively complex. Under oxidative stress, cysteine free thiols can be reversibly oxidized to form disulfides, sulfenic acids, S-nitrosylated and S-glutathiolated adducts or irreversibly oxidized to form sulfinic acids and sulfonic acids [17,20].

To date, many redox proteomics studies have been focused on the identification of direct protein targets of thioredoxin and glutaredoxin function [18,21,22]. Major approaches include affinity purification with mutant thioredoxin affinity column, 2D gel separation of proteins with thiol groups fluorescently labeled with a fluorescent dye monobromobimane (mBBr), and diagonal gel electrophoresis [22]. Mass spectrometry (MS) is used for protein identification in all the approaches. Progress has been made in identifying disulfide proteins [23] and thioredoxin regulated proteins [21]. However, the cysteines involved were largely unknown and whether the redox regulation is a direct effect of sensing cellular redox state was not clear. Characterization of redox proteins in plant jasmonate signal transduction has not been reported. Here we present the identification of changes in protein redox regulation in response to oxidative stress induced by MeJA in *Arabidopsis* shoots and roots using a 2DE-based proteomics approach [24]. The fluorescent mBBr was used to label the thiol groups of proteins obtained after alkylation of free thiol groups and reduction of reversible oxidized thiol groups. The labeled proteins were separated on 2D gels, followed by visualization of mBBr-labeled proteins. Total proteins were stained using SyproRuby to determine protein expression changes and to compare with the mBBr signal indicative of oxidation of protein thiol groups. A comparative proteomic map of potential redox proteins regulated by MeJA was established and the cysteine residues involved in the oxidative regulation were localized.

2. Experimental procedures

2.1. Plant growth and treatment

Seeds from *Arabidopsis thaliana* ecotype Col-0 were obtained from the *Arabidopsis* Biological Resource Center (Stock number: CS3879). The seeds were sterilized in 50% bleach for 10 min, and washed four times with sterilized water. They were then germinated on a half strength Murashige-Skoog agar medium containing 1% sucrose, and transferred to a growth chamber under a photosynthetic flux of 140 μmol photons m^{-2} s^{-1} with a photoperiod of 16 h at 24 °C and 20 °C at night for nine days. MeJA was applied evenly to the agar medium at a final concentration of 500 μM. After 24 h of incubation, the seedlings were dissected into shoots and roots, weighed and immediately frozen in liquid nitrogen. For control samples, all the steps were the same except that MeJA was replaced with 0.004% (v/v) ethanol in water. For all the experiments, at least three independent replicates were conducted.

2.2. Hydrogen peroxide detection

Hydrogen peroxide was visualized by staining with 3,3-diaminobenzidine (DAB). Once DAB encounters H_2O_2, it undergoes oxidative polymerization to produce a dark-brown precipitate [25]. After MeJA treatment, some seedlings were collected and immediately incubated with 1 mg/ml DAB for 2 h in the dark. Then shoots were separated from the roots. The leaf stain was fixed in 95% ethanol for 30 min, and the root stain was fixed by rinsing with distilled water. The leaves and roots were imaged using a Leica DMRE microscope coupled to a digital camera and a computer with Leica Qwin Imaging software (Leica Ltd., USA).

2.3. Protein extraction and mBBr labeling of protein thiols

Shoot and root samples were ground in liquid nitrogen to fine powders. One milliliter 10% trichloroacetic acid in acetone was added, followed by incubation on ice for 30 min. The samples were centrifuged at 14,000 rpm for 10 min, the supernatant was removed and 1 ml cold acetone was added to the pellet. After centrifugation, the pellet was washed twice in 80% acetone, and resuspended in a 2D gel sample solubilization solution containing 5 M urea, 2 M thiourea, 2% CHAPS, 2% SB 3–10, 40 mM Tris, 0.2% Bio-Lyte 3/10 ampholyte (Bio-Rad, CA, USA). Protein amounts were quantified using a CB-X Protein Assay kit (GenoTech, MO, USA).

Two hundred microliters of alkylation buffer (100 mM Tris–HCl pH 7.5, 200 mM iodoacetamide) were added to 200 μL (about 250 μg) protein sample. Alkylation was performed at 75 °C for 5 min, then at 37 °C for 1 h in the dark. Proteins were precipitated by adding 1 ml cold 80% acetone at −20 °C for 1 h. The tubes were centrifuged at 14,000 rpm at 4 °C for 10 min. After removing the supernatant, the pellet was dried briefly, and resuspended in 200 μL of reduction buffer (100 mM Tris–HCl pH 7.5, 10 mM DTT). Reduction was performed at room temperature for 1 h. Labeling of the proteins was performed by adding 20 μl of mBBr solution (1 μg/100 μL) to each sample and the samples were incubated at room temperature for 30 min in the dark. The labeling reaction was terminated by adding 10 μl 10% SDS. Proteins were then precipitated by incubating with 1 ml cold acetone at −20 °C overnight. After centrifugation, the protein pellet was resuspended in a destreak rehydration buffer (GE Healthcare, NJ, USA).

2.4. Two dimensional gel electrophoresis

For shoot samples, 1 mg protein was loaded onto a 24 cm Bio-Rad ReadyStrip (pH range 3–10 NL). The focusing conditions were: 200 V for 30 min, then ramping to 500 V for 30 min, and