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SABP2, a methyl salicylate esterase is required for the systemic acquired resistance induced by acibenzolar-*S*-methyl in plants

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ABSTRACT

Tobacco SABP2, a 29 kDa protein catalyzes the conversion of methyl salicylic acid (MeSA) into salicylic acid (SA) to induce SAR. Pretreatment of plants with acibenzolar-*S*-methyl (ASM), a functional analog of salicylic acid induces systemic acquired resistance (SAR). Data presented in this paper suggest that SABP2 catalyzes the conversion of ASM into acibenzolar to induce SAR. Transgenic SABP2-silenced tobacco plants when treated with ASM, fail to express PR-1 proteins and do not induce robust SAR expression. When treated with acibenzolar, full SAR is induced in SABP2-silenced plants. These results show that functional SABP2 is required for ASM-mediated induction of resistance.

1. Introduction

Plants resisting pathogen infection synthesize and accumulate high levels of salicylic acid (SA) [1–3]. Involvement of SA in both local resistance (LR) and systemic acquired resistance (SAR) has been widely studied [4]. Exogenous treatment with SA makes the plants more resistant to many pathogens and leads to expression of the same set of pathogenesis related (PR) genes/proteins as induced by tobacco mosaic virus (TMV) inoculation [5,6]. Expression of PR protein/genes, especially PR-1, with the development of SAR has made them an excellent marker for SAR in plants. Endogenous Levels of methyl salicylic acid (MeSA) increases in plants resisting pathogen infection [7,8]. SA-binding protein 2 (SABP2) catalyzed conversion of MeSA into SA is essential for inducing the SAR in tobacco plants [9,10]. Recently, SABP2 homologs have been identified and studied in several plants including Arabidopsis and poplar plants [11,12]. While Arabidopsis has several SABP2 like proteins (3–5), only two (Pt-SABP2-1 and Pt-SABP2-2) were identified from poplar. Poplar Pt-SABP2-1 is highly expressed in leaves while Pt-SABP2-2 is normally expressed in roots [12]. Arabidopsis homologs of SABP2 (AtMES1, -9) were induced upon infection by avirulent *Pseudomonas syringae* [11].

Besides infection with biotrophic pathogens, SAR could also be induced upon treatment with chemicals such as SA, 2-6-dichloroisonicotinic acid (INA) [6] or acibenzolar-*S*-methyl (ASM)/(BTH: Benzo (1,2,3) thiadiazole-7 carbothionic acid *S*-methyl ester) (CGA 245704) [13]. Due to low phytotoxic effects, ASM is favored as an elicitor of defense against microbial pathogens and is widely used in agriculture [14]. ASM when used as a plant protection agent has very low toxicological risk for the environment compared to commonly used pesticides used to control various pests [15]. Public interest and a growing number of human diseases linked to pesticide use have generated considerable interest in finding alternative means to control plant pests [16–18].

The mechanism by which ASM induces resistance in plants is largely unknown. ASM treatment induces SAR in nahG transgenic plants which fail to accumulate SA, suggesting that accumulation of SA is not required for ASM-mediated induction of SAR [19]. ASM/BTH inhibits the activity of catalase and ascorbate peroxidase. It was hypothesized that inhibition of catalase resulted in buildup of H_2O_2 leading to activation of disease resistance pathway [20]. In 2001, an BTH affinity column was used to purify a BTH-binding protein kinase (BBPK) from tobacco leaves [21]. BBPK was hypothesized to regulate *NPR1* activity through phosphorylation. Recently, ASM has been shown to inhibit mitochondrial NADH:ubiquinone oxidoreductase resulting in increased production of reactive oxygen species (ROS) [22,23]. Increased production of ROS, confers enhanced disease resistance through various mechanisms including lignification, cell wall crosslinking, cell death, and direct killing of the infecting pathogen [24,25].

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Recent discovery of SABP2's role in local and systemic acquired resistance has provided additional information on the SA signaling pathway [9]. SA synthesized by SABP2 may be responsible for increased cytosolic levels of SA which results in a change in the redox potential of the cytoplasm. This change in redox potential leads to the monomerization and migration of NPR1 monomers into the nucleus where it induces expression of SA- responsive genes with the help of TGA transcription factors [26,27]. Besides SA, treatment with INA or ASM results in nuclear migration of NPR1 [26]. In the nucleus, the monomeric NPR1 is further regulated by phosphorylation and proteosome-mediated degradation [28]. ASM-mediated induction of resistance in plants requires a functional NPR1. Because, ASM activates the SAR signal pathway at the site of or downstream from SA accumulation, we hypothesize that SABP2 catalyzes the conversion of acibenzolar-*S*-methyl (CGA 245 704) into acibenzolar (CGA 210 007) which, in turn, activates the disease resistance signaling pathway as activated by SA through the activation of NPR1.

2. Materials and methods

2.1. Plant material and treatment

The control tobacco plant line, C3, containing an empty silencing vector in *Nicotiana tabacum* cv. Xanthi nc (NN) and line 1–2 as the SABP2-silenced plants were used [9] in the experiments. Autoclaved soil (Fafard F15) was used to grow the plants from seeds. All plants were grown in autoclaved soil (Fafard F15) in plant growth chamber (PGW36, Conviron, Canada) maintained at 22 °C with a 16-h day cycle. Six to eight week old plants were used for experiments.

Acibenzolar-*S*-methyl (Benzo (1,2,3) thiadiazole-7 carbothionic acid S-methyl ester- CGA 245704) was kindly supplied by Syngenta Crop Protection, NC. Plants were sprayed either with 0.1 mM ASM (50% active ingredient (a.i.) in wettable powder formulation or with acibenzolar, dissolved in 20 mM sodium phosphate buffer (pH 7.2).

The inoculation of tobacco plants with TMV was carried out as described by [29]. The three upper leaves were evenly dusted with carborundum (Fisher Scientific) and TMV solution (2 μg/ml) in 20 mM sodium phosphate buffer (pH 7.2) was rubbed onto the leaves with cheesecloth. Plants were kept at 22 °C in a light-controlled plant growth room throughout the experiment.

2.2. HPLC analysis of conversion of ASM into acibenzolar by SABP2

HPLC was used to monitor the reaction catalyzed by SABP2 using ASM as a substrate. The method was performed as described by Scarponi et al. [30] with minor modifications. Acetonitrile (HPLC grade) and methanol were obtained from Fisher scientific. For HPLC analysis, a C-18 analytical column (C-18, 250 × 4.6 mm, Microsorb MV 100-5, Varian) was equilibrated with 80% methanol containing 0.3% TFA at a flow rate of 0.7 ml/min. Pure ASM (0.4 mM) or acibenzolar (0.4 mM) were incubated at 25 °C for 40 min in 20 mM sodium phosphate buffer (pH 7.2) before injecting into HPLC. The absorbance of the samples was monitored at 255 nm. ASM was incubated with SABP2 (5 μM) for 10, 20 and 40 min in 20 mM sodium phosphate buffer (pH 7.2). Retention time of the reaction products were determined and compared with retention times of pure ASM and acibenzolar. To further confirm the identity of the products, fractions of the peaks matching the retention times of ASM and acibenzolar were collected and analyzed by NMR. SABP2 used in this analysis was expressed in BL21(DE3) cells as a C-terminal 6xHis tagged protein from pET21 (Novagen). Recombinant SABP2 was affinity purified using Ni-NTA agarose followed by purification using Q-sepharose [10]. Purified SABP2 was analyzed by SDS–PAGE followed by silver staining (data not shown).

2.3. ASM/acibenzolar induced expression of PR-1 in SABP2-silenced plants

SABP2-silenced (1–2) and control (C3) tobacco plants were treated by foliar spray with either 0.1 mM ASM or acibenzolar in 20 mM sodium phosphate buffer (pH 7.2). As a control, 1–2 and C3 plants were treated with only 20 mM sodium phosphate buffer. After 48 h, samples (two leaf discs with cork borer # 7) were collected from the treated and untreated leaves and homogenized in 0.2 ml extraction buffer (50 mM Tris–Cl, pH 8.0, 1 mM EDTA, 100 μM PMSF, 14.4 mM βMe) using Fast Prep 24 (MP Bio). The homogenate was centrifuged at 12 000×*g* for 15 min using a table top centrifuge (Eppendorf). Protein content of the supernatant was determined using Bradford reagent (Bio-Rad, CA) following manufacturer's instructions. Proteins were separated on a 12% SDS–polyacrylamide gel and transferred to a PVDF membrane (Millipore) following manufacturer's instructions. The blot was probed with monoclonal anti-PR-1 antibodies (1:5000) overnight at 4 °C and with HRP conjugated secondary antibody (1:1000) (Sigma) for 30 min at 24 °C and visualized by ECL (GE Healthcare). Prior to incubation with antibodies, the blot was stained with 0.1% Ponceau-S and photographed to verify equal loading of the proteins. Equal loading was visually assessed by the abundance of the large subunit (LSU) of ribulose bisphosphate carboxylase/oxygenase.

2.4. Induction of SAR in ASM/acibenzolar treated SABP2-silenced plants

To induce SAR, three lower leaves of SABP2-silenced (1–2) and control (C3) tobacco plants were spray treated with 0.1 mM ASM or 0.1 mM acibenzolar diluted in 20 mM sodium phosphate buffer (pH 7.2). Plants were maintained at 22 °C with 16 h of light. After seven days, the upper untreated leaves of the tobacco plants were dusted with carborundum (Fisher Scientific) and mechanically inoculated with TMV in 20 mM phosphate buffer, (pH 7.2) using cheese cloth. The sizes (diameters) of the TMV induced lesions (~20) were measured after 5–6 dpi (days post inoculations) using a digital caliper.

3. Results

3.1. SABP2 catalyzes the conversion of acibenzolar-S-methyl into acibenzolar

HPLC was used to monitor the SABP2 catalyzed conversion of ASM to acibenzolar. The retention time for pure ASM and acibenzolar on C-18 column was 8.65 and 5.2 min, respectively (Fig. 1A and E). When ASM was incubated for 10 min at room temperature with purified SABP2 in phosphate buffer, more than 60% of ASM was converted into acibenzolar as evident by the reduction in the ASM peak and simultaneous increase in acibenzolar peak height (Fig. 1B). Incubation of ASM with SABP2 for longer reaction times (20 and 40 min) resulted in >98–99% conversion of ASM into acibenzolar (Fig. 1C and D). Incubation of ASM in phosphate buffer without SABP2 did not result in any detectable conversion of ASM into acibenzolar at room temperature (Fig. 1A). These data show that SABP2 catalyzes the conversion of ASM into acibenzolar.

3.2. Induction of PR-1 upon ASM treatment require SABP2

ASM treatment induces the expression of PR-1 protein [19]. Using transgenic tobacco plants silenced in SABP2 expression, it