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Methyl Salicylate Esterases in Plant Immunity

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ABSTRACT

Salicylic acid (SA) is an important signal in various plant processes. It is well known and widely studied for its role in plant disease resistance. Several proteins, which physically interact with SA has been identified and characterized for their possible role in disease resistance signaling. These plant proteins bind to SA with varying affinity and they differ considerably in their structure and activity. The protein, which binds to SA with highest affinity amongst all the characterized SA-binding proteins, is SABP2. It is a 29-kDa protein and has esterase like enzymatic activity. It is able to use plant synthesized methyl salicylate as a substrate and convert it into SA, which triggers disease resistance in plants. Silencing of SABP2 makes plants more susceptible to pathogens and their capacity to induce SAR is severely compromised. The esterase activity of SABP2 is required to process the phloem mobile signal, MeSA in distal uninoculated tissues to induce resistance. The binding of SA to SABP2 is important for activation of SAR in distal tissues.

Keywords: disease resistance, systemic acquired resistance, SABP2, SA-methyl transferase, SA-binding protein
Abbreviations: **MeSA**, methyl salicylate; **PR-1**, pathogenesis-related protein-1; **RNAi**, ribonucleic acid interference; **SA**, salicylic acid; **SABP2**, SA-binding protein 2; **SAR**, systemic acquired resistance; **TMV**, *Tobacco mosaic virus*

CONTENTS

INTRODUCTION

Salicylic acid (SA) has been known to humans for a long time as a plant compound, which could cure or relieve symptoms associated with many diseases. It was encouraged by Hippocrates to be used as a child birth pain reliever as early as fourth century B.C. (Vlot *et al.* 2009). Extracts of willow bark, which contain 'Salicylate' was extensively used to relieve the pain. Possible role of salicylic acid in plants was first demonstrated by the induction of resistance against tobacco mosaic virus by pretreating tobacco plants with aspirin (White 1979). Many plants synthesize and accumulate high levels of SA upon pathogen infection. How this newly synthesized SA activates downstream signaling pathway leading to activation and establishment of robust resistance in plants has been subject of interest for last several decades? Which components of signaling pathways are influenced by the increased levels of SA? Besides analysis of Arabidopsis mutants defective in SA signaling pathways, another bold approach was to identify and characterize cellular proteins, which physically interact with SA. In this approach radioactive labeled SA was used as ligand to detect, identify and purify proteins, which interacted with it.

SALICYLIC ACID-BINDING PROTEIN: CATALASE

Following this biochemical approach several plants proteins, which physically interacted with SA were identified. ^{14}C-labeled SA was used to detect and identify these proteins. First such protein to be identified from extracts of tobacco leaves was salicylic acid binding protein (SABP) (Chen and Klessig 1991; Chen *et al.* 1993a). Further analysis and characterization of SABP showed that it is a catalase like protein, which could degrade H_2O_2 into H_2O and O_2 (Chen 1993; Conrath *et al.* 1995). Biochemical characterization of tobacco catalase showed that binding of SA lead to the inhibition of its H_2O_2 degrading activity. Other biologically active (ability to induce resistance response in plants) analogs of SA also specifically inhibited H_2O_2 degrading activity of catalase. These observations lead Chen *et al.* to propose that inhibition of H_2O_2 degrading activity of catalase resulted in accumulation of H_2O_2 triggering a resistance response in plants known as SA mediated resistance response (Chen *et al.* 1993b). Durner and Klessig (1996) showed that mammalian catalases are also inhibited by SA and so are other scavenging enzymes e.g. ascorbate peroxidase in the cell (Durner and Klessig 1995, 1996). Inhibition of these enzymes by SA generates SA free radicals,

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which may induce lipid peroxidation leading to activation of PR proteins and defense response (Anderson and Klessig 1998). This hypothesis was quickly challenged by the observations that concentrations of SA (10^{-3} M) necessary to inhibit H_2O_2 degrading activity is never achieved in the cell (Ruffer *et al.* 1995). Levels of SA in systemic plants tissue are probably too low (0.5-9.0 μM) to bind and cause inhibition of catalase and ascorbate peroxidase (Malamy *et al.* 1990; Enyedi *et al.* 1992; Vernooij *et al.* 1994). Other studies also did not support SA mediated inhibition of ascorbate peroxidase and catalase (Miyake 1996; Tenhaken and Rubel 1997). Several other studies have also reported SA mediated inhibition of catalase activity but its role in activation of disease resistance is unclear (Sánchez-Casas and Klessig 1994; Chen *et al.* 1997). More precise studies are needed to understand the role of SA binding to catalase and its effect on disease resistance or other plant processes.

SA-BINDING PROTEIN 3 (SABP3): A CHLOROPLASTIC CARBONIC ANYHYDRASE

To detect binding proteins with much higher affinity for SA compared to catalase (SABP), ^{3}[H]-SA, which has higher specific activity was used. Using ^{3}[H]-SA as ligand, another SA-binding protein (SABP3) was identified and characterized from tobacco leaves. SABP3 is a chloroplast localized carbonic anyhydrase (Slaymaker *et al.* 2002). SABP3 showed higher binding affinity to SA (K_d of 3.7 μM) compared to SABP (K_d of 14 μM) (Chen and Klessig 1991; Du and Klessig 1997; Slaymaker *et al.* 2002). Although biochemical analysis did not show any effect of SA binding on the carbonic anhydrase activity of SABP3 but its silencing resulted in suppression of *pto*:*avrPto* mediated hypersensitive response in disease resistance in *Nicotiana benthamiana* and enhanced susceptibility to *Phytophthora infestans* (Slaymaker *et al.* 2002; Restrepo *et al.* 2005). Although binding of SA did not affect the enzymatic activity of SABP3 but its modification through S-nitrosylation in Arabidopsis resulted in its reduced SA binding and enzymatic activity (Wang *et al.* 2009). This may suggest that S-nitrosylation and SA-binding may help modulate carbonic anhydrase activity of SABP3.

SA-BINDING PROTEIN 2 (SABP2): A METHYL SALICYLATE ESTERASE

Of all the SABP's studied to date, SABP2 showed the highest affinity (K_d of 90 nM) for SA (Du and Klessig 1997). Affinity of SABP2 to SA is 150 times higher compared to SABP (catalase). SABP2 is present in excruciatingly low abundance (0.01 pmol/mg) in tobacco leaves compared to SABP (5 pmol/mg soluble protein) and SABP3 (0.4 pmol/mg). Low abundance of SABP2 made its purification much harder compared other SA-binding proteins. Indeed, it took several years to standardize a working purification scheme to purify small amounts of SABP2 enough to determine its amino acid sequence (Kumar and Klessig 2003). Based on size exclusion chromatography, SABP2 appeared to be a monomeric protein of 25-30 kDa as reported by Du *et al*. (Du and Klessig 1997). A combination of degenerate primers were synthesized based on the peptide sequences obtained and RT-PCR was used to amplify a partial sequence of putative SABP2. Full- length cDNA was obtained following 5′-RACE.

For biochemical characterization of putative SABP2, its full length coding sequence was cloned into *E.coli* expression vector and protein was expressed and purified. Recombinant purified SABP2 bound specifically to SA and its active analogs and not to its inactive analogs. DNA analysis showed that SABP2 belongs to α/β hydrolase superfamily of proteins. This family of proteins exhibit a range of enzymatic activities, which include carboxylic acid ester hydrolases e.g. cholineterases; lipid hydrolases e.g. lipases; thioester, hydrolases e.g. thioesterases; peptide hydrolases e.g. serine carboxipeptidases; hydroxylnitrile lyases etc. Recombinant SABP2 showed low level activity with several artificial substrates, which included paranitrophenol acetate, paranitrophenol butyrate, paranitrophenol myristate, paranitrophenol palmitate and 4-methylumbelliferone butyrate (Kumar and Klessig 2003). Several of the compounds, which are naturally produced by plants, were also tested as potential substrates of SABP2. Of the three potential substrates e.g. methyl salicylate (MeSA), methyl jasmonate (MeJA) and methyl indole acetic acid (MeIAA) tested; SABP2 showed highest activity with MeSA as a substrate (Forouhar *et al.* 2005). MeSA, commonly known as oil of wintergreen is naturally synthesized by plants and its level increases upon pathogen infection (Shulaev *et al.* 1997).

To understand the effect of SA binding to SABP2, its 3-D structure alone and in complex form with SA was determined at 2.1 Å resolution (Forouhar *et al.* 2005). As expected based on its similarity to other known proteins, SABP2 belonged to α/β hydrolase superfamily of proteins. The structure of SABP2 consisted of six-stranded parallel β-sheet flanked on both sides by six α-helices. It has catalytic triad consisting of active site Ser-81, His-238 and Asp-210. Another surprising observation was existence of SABP2 as a dimer. Size exclusion chromatography of purified recombinant SABP2 suggested it to be a dimer while native SABP2 purified from tobacco leaves was reported to be a monomer (Du and Klessig 1997; Forouhar *et al.* 2005). Most surprising observation was the binding of SA in the active site of SABP2. SABP2 was completely shielded from the solvent. SABP2 bound SA was located in the hydrophobic environment surrounded by side chains of Asn-123, Trp-131, Phe-136, Met-149 and Leu-181. This observation explained why SABP2 bound to SA with such a high affinity (K_d of 90 nM). Binding of SA to SABP2 did not result in a major structural change. This observation was also supported by NMR analysis, which showed only minor chemical shifts for few residues in presence of SA (Forouhar *et al.* 2005). MeSA could also readily fit into the active site of SABP2 while MeJA and MeIAA were not easy to fit. Tight binding of SA in the active site of SABP2 suggested that it could inhibit enzymatic activity of SABP2. *In vitro* studies using recombinant SABP2 clearly suggested that its enzymatic activity is inhibited by SA (Forouhar *et al.* 2005). Synthetic SA analog, acibenzolar-S-methyl (ASM) binds to SABP2 with higher affinity compared to SA. SABP2 catalyzes the conversion of ASM to its acid form acibenzolar to induce resistance in plants (Tripathi *et al.* 2010).

Role of SABP2 in plant disease resistance

To determine if SABP2 has any role in disease resistance signaling, tobacco plants silenced in SABP2 expression were used. RNAi was used to stably silence the expression of SABP2 in tobacco plants (Smith *et al.* 2000). Transgenic SABP2 silenced tobacco plants upon challenge with *Tobacco mosaic virus* (TMV) resulted in compromise in their ability to mount robust systemic acquired resistance compared to the control plants containing only empty silencing vector (Kumar and Klessig 2003). Expression of pathogenesis-related protein-1 (PR-1) was also compromised in SABP2-silenced plants. These silenced plants allowed enhanced replication/movement of TMV but did not allow systemic movement to distal tissues. Results from silencing experiments made it clear that SA synthesized by SABP2 in tobacco plants is essential for a robust SAR response. A synthetic version of SABP2 was used to trans-complement the loss of SAR inducing activity of SABP2 silenced (Kumar *et al.* 2006). This synthetic version of SABP2 escaped silencing in SABP2 silenced plants due to sufficiently different DNA sequence compared to wild type tobacco SABP2 sequence while coding for the same amino acid sequence (Kumar *et al.* 2006). Arabidopsis contains a gene family of 18 members sharing 32-57% identity with tobacco SABP2 (Vlot *et al.* 2008). Five of these show preference for MeSA as a substrate and their activity is inhibited by SA binding. Three of these family members (At2g23620,

At2g23560 and At4g37150) when expressed in SABP2 silenced tobacco plants could complement for its SAR deficiency. These results suggest that several Arabidopsis genes (At2g23620, At2g23560 and At4g37150) are orthologs of tobacco SABP2 and are redundant for hydrolysis of MeSA and SAR. This, in part explains why SABP2 like proteins were never identified in several exhaustive genetic screens for *Arabidopsis* mutants with enhanced disease susceptibility phenotype (Glazebrook *et al.* 1996; Volko *et al.* 1998; Ton *et al.* 2002).

MeSA is a phloem mobile signal for SAR

Since SABP2 catalyzes conversion of MeSA to SA and MeSA is a volatile compound, question arises if MeSA instead of SA is the signal for SAR in distal uninfected tissue. The earlier observation that SA accumulates in phloem tissues in plants exhibiting SAR led to the believe that SA is the mobile signal for SAR (Yalpani *et al.* 1991). However, later studies using grafting experiments with *NahG* transgenic plants did not support this hypothesis because NahG rootstock containing chimera plants lacking SA were still able to generate signal for SAR (Vernooij *et al.* 1994). MeSA has been shown to be an airborne signal involved in inducing disease resistance in neighboring plants (Shulaev *et al.* 1997). MeSA does not induce SAR by itself and works by being converted to salicylic acid. It was observed that levels of MeSA increases dramatically in TMV infected tissue and also in uninfected distal tissues (Seskar *et al.* 1998). This increase in MeSA levels paralleled the increase in SA levels in plants infected with TMV. Chimera plants were made by grafting SABP2-silenced plants, and control tobacco plants expressing wild type levels of SABP2. Grafted plants were assayed for SAR response and plants lacking SABP2 in distal tissues (scion) failed to convert MeSA to SA and was defective in inducing resistance to pathogen and expression of PR-1 was also compromised (Park *et al.* 2007). Chimera plants lacking SABP2 in rootstock and expressing wild type levels of SABP2 in scion developed normal SAR. These observations suggested that while SABP2 is not required for generating systemic signal for SAR but it is required for processing the SAR signal in distal tissues (Park *et al.* 2007; Kumar and Klessig 2008). Mutant plants with uncontrolled esterase activity in primary tissue were compromised in SAR response. These plants did not have increased levels of MeSA in primary inoculated tissue, phloem exudates and upper distal tissues. Transgenic tobacco plants silenced in SA-methyl transferase expression failed to develop SAR (Park *et al.* 2007). SA-methyl transferase is required to convert SA into MeSA. All these results indicated that MeSA is phloem mobile signal for SAR and SABP2 is required to convert this signal into SA leading to resistance response (Park *et al.* 2007). A synthetic analog of SA, 2,2,2,2'-tetrafluoroacetophenone (tetraFA), binds SABP2 with high affinity as SA and inhibits its esterase activity but it could not induce resistance response as SA. Treatment with tetraFA effectively blocked SAR in tobacco and *Arabidopsis* plants showing the importance of MeSA and SABP2 in SAR (Park *et al.* 2009).

Several studies using *Arabidopsis* mutants have suggested a lipid-derived signal for SAR. SAR was compromised in *Arabidopsis* mutant *dir1-1* defective in an apoplastic lipid transfer protein (Maldonado *et al.* 2002). Recent studies have implicated plant volatile compound JA in SAR (Truman *et al.* 2007). Several recent studies however rule out the possibility of JA as an SAR signal (Cui *et al.* 2005; Mishina and Zeier 2007; Chaturvedi *et al.* 2008; Attaran *et al.* 2009). Another study completely ruled out MeSA and JA as a phloem mobile signal in Arabidopsis (Attaran *et al.* 2009). A new study suggests role of length exposure to light as a deciding factor if plant uses MeJA or MeSA mediated pathway for SAR (Liu *et al.* 2011).

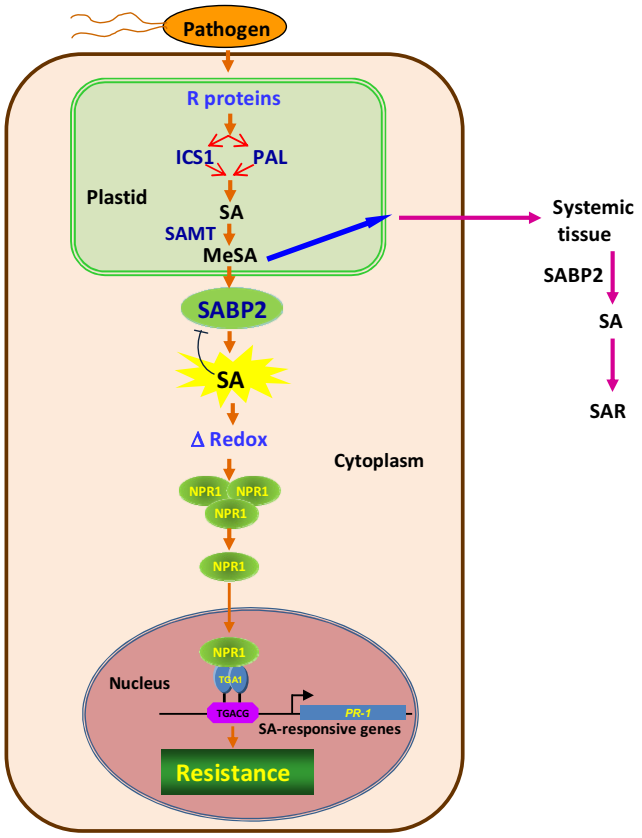

Fig. 1 A working model for SA-dependent resistance pathway. Interaction between pathogen derived *avr* (avirulence) molecule and plant derived 'R' (resistance) protein results in synthesis of SA via ICS1/PAL pathways. SA is then converted into MeSA by an SAMT (SA-methyl transferase). SABP2 catalyzes conversion of MeSA to SA, which triggers downstream signaling leading to expression of SA responsive genes. MeSA being lipid mobile, travels to uninfected (systemic) parts of the plant where it is converted to SA by SABP2, which triggers expression of resistance proteins leading to SAR. ICS1: isochorismate synthase 1; PAL: phenyl ammonia lyase; NPR1: non expressor of pathogenesis related protein 1.

Effect of pathogen infection on SABP2 expression

Tobacco SABP2 expression was induced upon infection by TMV (Kumar and Klessig, 2003). This expression was dependent on SA (Kumar and Klessig unpublished results). Three of the Arabidopsis orthologs of SABP2, *AtMES*1 (At2g23620), *AtMES*7 (At2g23560) and *AtMES*9 (At4g37150) were also induced upon infection by avirulent *Pseudomonas syringae*. Treatments with SA did not induce the expression of tobacco SABP2 but MeJA treatment induced its expression (Kumar and Klessig, unpublished results). Induction of SABP2 expression by MeJA suggests a possible crosstalk between SA and JA pathway. Further studies may be required to explore this role of SABP2. Recently, SABP2 homlogs of poplar plants, *Pt*SABP2-1 and *Pt*SABP2-2 were shown to be differentially expressed. *Pt*SABP2-1 was highly expressed in leaves while *Pt*SABP2-2 was in roots (Zhao *et al.* 2009). *Pt*SABP2-2 was significantly upregulated by wounding and MeJA treatments. In a global gene expression analysis of tomato genes, a SABP2 homolog was highly expressed in NaCl treated plants indicating a possible role in abiotic stress (Sun *et al.* 2010).

Is SABP2 expression needed for basal defense?

Role of SA is well established in basal defense in plants. SA-mediated basal defense is specifically targeted by plants pathogens to gain entry into the cell. Analysis of SABP2 silenced plants for expression of defense genes revealed that these transgenic plants were compromised in expres-

sion of PR-1 (D Kumar unpublished results). This may indicate role of SABP2 in synthesis of basal levels of SA in cell. This SABP2 synthesized SA may help in maintaining basal defense levels in tobacco plants.

CONCLUSIONS

Discovery of tobacco SABP2 has helped to identify the long sought phloem mobile signal in plant immunity. Plants appear to communicate by synthesizing MeSA as a signal and require SABP2 to decipher this plant immunity signal. High affinity binding of SA to SABP2 is critical in enhancing the levels of MeSA in local and distal tissues. In distal tissues MeSA is converted back to SA, which results in the downstream signaling leading to activation of resistance (**Fig. 1**). SABP2 proteins are present in many of the plant species. Its role in tobacco, Arabidopsis and potato is now well studied (Kumar and Klessig 2003; Vlot *et al.* 2008; Manosalva *et al.* 2010). SA synthesized due to activity of SABP2 appears to be critical for activation of defense responses because plants silenced in SABP2 expression are compromised in local and systemic resistance (Kumar and Klessig 2003). Further studies may be needed to determine if SABP2 is also required for other SA mediated plant processes.

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