High-affinity salicylic acid-binding protein 2 is required for plant innate immunity and has salicylic acid-stimulated lipase activity

Dhirendra Kumar and Daniel F. Klessig*

Boyce Thompson Institute for Plant Research, Tower Road, Ithaca, NY 14853

Salicylic acid (SA) is a critical hormone for signaling innate immunity in plants. Here we present the purification and characterization of SA-binding protein 2 (SABP2), a tobacco protein that is present in low abundance and specifically binds SA with high affinity. Sequence analysis predicted that SABP2 is a lipase belonging to the α/β fold hydrolase super family. Confirming this prediction, recombinant SABP2 exhibited lipase activity against several synthetic substrates. Moreover, this lipase activity was stimulated by SA binding and may generate a lipid-derived signal. Silencing of *SABP2* expression suppressed local resistance to tobacco mosaic virus, induction of pathogenesis-related 1 (*PR-1*) gene expression by SA, and development of systemic acquired resistance. Together, these results suggest that SABP2 is an SA receptor that is required for the plant immune response. We further propose that SABP2 belongs to a large class of ligand-stimulated hydrolases involved in stress hormone-mediated signal transduction.

Mounting evidence suggests that the innate immune system of plants shares many parallels with that of vertebrates and insects (1–3). In plants, the ability to recognize a pathogen and activate an effective defense is often governed by an interaction (direct or indirect) between the products of a plant resistance gene and a pathogen avirulence gene (4, 5). After this gene-for-gene interaction, the inoculated leaves may exhibit ion fluxes, the production of reactive oxygen species, salicylic acid (SA), nitric oxide, and increased expression of defense-associated genes, including those encoding pathogenesis-related (*PR*) proteins (1, 6, 7). In addition, the cells surrounding the site(s) of pathogen entry usually undergo apoptotic-like cell death, thereby forming the necrotic lesions characteristic of the hypersensitive response (8, 9). Generally, the pathogen is restricted to these lesions. Subsequent to the local responses, plants frequently develop a broad-based long-lasting resistance to secondary pathogen infection known as systemic acquired resistance (SAR). Concurrent with SAR development, the systemic leaves accumulate SA and *PR* gene transcripts. The correlation between *PR* gene expression and systemic resistance makes these genes excellent markers for SAR.

Many studies have demonstrated that SA is a critical signal for the activation of both local and systemic resistance responses. For example, tobacco and *Arabidopsis* plants that are SA-deficient or are unable to accumulate SA after infection fail to develop SAR, do not express *PR* genes in the uninoculated leaves, and display enhanced susceptibility to pathogen infection (7, 10–12). Recent evidence suggests that SA also regulates cell death, possibly via a positive feedback loop that involves reactive oxygen species (13–15). Additionally, SA cross regulates the ethylene and jasmonic acid-dependent defense pathways (12, 16).

To investigate how SA exerts its effects, several putative effector proteins have been identified, including catalase (17, 18), ascorbate peroxidase (19), and carbonic anhydrase (20). All three bind SA with low to moderate affinity (K_d of 14–3.7 μM) and appear to have antioxidant activity. In comparison, SA-binding protein 2 (SABP2) was identified as a very low-abundance (10 fmol/mg) soluble protein of $\approx$25 kDa that exhibits high affinity for SA (K_d = 90 nM) (21). This binding was reversible and specific to SA and its SAR-inducing analogs. Here we report the purification of SABP2 and cloning of its encoding gene. Furthermore, we demonstrate that SABP2 has SA-stimulated lipase activity and that SABP2 is required for full local and systemic resistance to pathogen infection.

Materials and Methods

Plant Material, Pathogen Infection, and Chemical Treatments. All plants were grown and inoculated with tobacco mosaic virus (TMV), as described (22). Induction of *PR-1* expression was performed by infiltrating leaves with 0.25 mM SA (pH 7.0).

Purification of Tobacco SABP2. Fully expanded leaves from 7- to 8-wk-old tobacco plants were powderized in liquid N_2 and homogenized in 3 vol (wt/vol) of buffer A [20 mM sodium citrate/5 mM $MgSO_4$/1 mM EDTA, pH 6.3/14 mM 2-mercaptoethanol/0.1 mM phenylmethylsulfonylflouride (PMSF)/1 mM benzamidine-HCl] with 1.5% (wt/wt) polyvinylpolypyrrolidone. The homogenate was filtered and then centrifuged at 11,000 $\times$ *g* for 30 min, and the supernatant was fractionated by ammonium sulfate [$(NH_4)_2SO_4$] precipitation, as described (21). The 50–75% fraction was applied to a Sephadex G-100 column [Amersham Pharmacia (AP)] in buffer A. Fractions with high [^{3}H]SA-binding activity were concentrated by precipitation with 75% $(NH_4)_2SO_4$. Further chromatographic steps were carried out by using a FPLC system (AP). The concentrated protein from Sephadex G-100 step was resuspended and desalted by using buffer B (10 mM bicine, pH 8.5/14 mM 2-mercaptoethanol/0.1 mM PMSF/1 mM benzamidine-HCl) and applied to a Q Sepharose column (AP) preequilibrated with buffer B. The bound proteins were eluted with a linear gradient of 15–180 mM $(NH_4)_2SO_4$ in buffer B. To the fractions with high [^{3}H]SA-binding activity, $(NH_4)_2SO_4$ was added to a concentration of 1 M and applied to a Butyl Sepharose column (AP) preequilibrated with 1.2 M $(NH_4)_2SO_4$ in buffer B. The bound proteins were eluted with a linear gradient of decreasing $(NH_4)_2SO_4$ (1–0 M). The fractions with high binding activity were again concentrated, desalted, and applied to a Mono Q column (HR 5/5, AP). The bound proteins were eluted with a linear gradient of 15–180 mM $(NH_4)_2SO_4$ in buffer B. Fractions with high binding activity were concentrated, desalted, and loaded onto a Superdex 75 column (HR 10/30, AP), preequilibrated with buffer C (buffer B plus 150 mM [$NH_4]_2SO_4$). The proteins were eluted with buffer C. Fractions containing high binding activity were further

PLANT BIOLOGY

Table 1. Purification of SABP2 from tobacco leaves

Fraction	Total protein, mg	Total activity, dpm	Specific activity, dpm·mg^{-1}	Purification, fold	Recovery, %
Crude extract	31,626.250	7,509,343	237	1.0	100
50–75% $(NH_4)_2SO_4$	4,723.252	1,892,720	400	1.7	25
Sephadex G-100	2,216.825	1,385,625	625	2.6	18.4
Q Sepharose	56.925	1,023,300	17,976	75.8	13.6
Butyl Sepharose	4.398	123,840	28,158	118.8	1.6
Mono Q	0.368	1,386,700	3,762,930	15,877.3	18.4
Superdex 75	0.013	55,460	4,160,420	17,554.5	0.7
Mono Q	0.008	45,515	5,689,375	24,005.8	0.6

purified on a Mono Q column after concentration and desalting in buffer B.

SA Binding, Protein Estimation, and SDS/PAGE Analysis. SA binding, in the absence or presence of its analogs, was measured as described (20, 21). Protein concentration was determined by Bradford analysis, and SDS/PAGE analysis was performed on 12.5% SDS-polyacrylamide gels by using Laemmli's protocol followed by Coomassie blue R-250 or silver staining to visualize the proteins.

Amino Acid Sequencing. Fractions from the second Mono Q column containing peak [^{3}H]SA-binding activity were separated by SDS/PAGE and stained with 0.1% Coomassie blue R-250. The protein bands that copurified with SA-binding activity were excised and sequenced at the Biotechnology Resource Center, Cornell University, after in-gel digestion with trypsin.

Cloning *SABP2*, Sequence Alignments, and Database Searches. Based on the amino acid sequences of the tryptic peptides, several degenerate oligonucleotides were custom synthesized (Invitrogen). First-strand cDNA was synthesized from DNase-treated total RNA extracted from young leaves by using SuperScript II Reverse Transcriptase (Invitrogen) and the universal oligo-dT (14) with an adapter sequence. A combination of degenerate primer ACWCARTTYTTRCCHTAYGG (W, A or T; R, A or G; Y, C or T; H, A, C, or T), and the universal adapter primer (GACTCGAGTCGACATCGA) was used to PCR amplify the *SABP2* cDNA. The amplified products were cloned into pGEMT (Promega), and several independent clones were sequenced and analyzed. The full-length *SABP2* gene was cloned by performing 5′ RACE by using the SMART RACE cDNA amplification kit (Clontech). Sequence comparisons were carried out with the DNA STAR software (DNAstar, Madison, WI). Multisequence alignments were constructed with the CLUSTALW program of the European Bioinformatics Institute, and database searches were performed with the BLAST program from National Center for Biotechnology Information.

Expression of Recombinant SABP2 (rSABP2) in *Escherichia coli.* Full-length tobacco *SABP2* was PCR amplified to introduce *Bam*H1 enzyme sites at both ends by using the primers F2 (CGCG-GATCCATGAAGGAAGGAAAACACTTTG) and F3 (GCGGGATCCAGATCAGTTGTATTTATGGGC). The amplified product was cloned into the *Bam*H1 site of pET28a (Novagen) and sequenced. rSABP2 was synthesized as a soluble protein in *E. coli* strain BL21 (DE3) and affinity purified by Ni-NTA agarose chromatography (Novagen) as described by the manufacturer. rSABP2 was further purified on a Mono Q column as described earlier.

Lipase Activity Assay. The lipase activity assay was performed as described (23) with modifications to allow SA binding to SABP2. The standard 1-ml assay mixture consisted of 1 mM substrate in 50 mM bicine, pH 8.0, 0.05% Triton X-100. After a 60-min preincubation on ice in the absence or presence of 1 mM SA, the reaction was allowed to proceed at 24°C for 60 min. A 100 mM stock solution of *para*-nitrophenyl myristate (*p*-NPM) or *para*-nitrophenyl palmitate (pNPP) was prepared in acetonitrile. Lipase activity was estimated colorimetrically (Unicom UV1, Spectronic Unicom, Cambridge, U.K.) by measuring the liberation of *para*-nitrophenol from *p*-NPP or *p*-NPM at 410 nm. Measurements from control reactions without SABP2 were subtracted from each reaction. For non SA-binding conditions, Tris·HCl, pH 8.0 (50 mM), was used in place of bicine buffer.

Plasmid Construction and Plant Transformation. The *RNAi-SABP2* construct was made in the pHANNIBAL vector (24) by inserting a 404-bp fragment corresponding to the 5′ portion of *SABP2*. The fragment corresponding to the sense arm of the hairpin loop was generated by using the primers F6 (CCGCTCGAGAT-GAAGGAAGGAAAACACTTG) and F7 (GGGGTACCA-GATCAGTTGTATTTATGGGC) and cloned in the *Xho*I-*Kpn*I site. The fragment for the antisense arm was generated by using the primers F2 and G2 (GCGGGATCCCTGAGTATC-CAACCAATTCTCGG) and was cloned into the *Bam*H1 site. The *Not*I fragment from pHANNIBAL containing *SABP2* was then subcloned into binary vector pART27 and transformed into Agrobacterium strain LBA4404. Plant transformations and regeneration and maintenance of the transgenic lines were carried out as described (25).

RNA Blot Analysis and RT-PCR Analysis. Total RNA was extracted from tobacco leaves as described in ref. 26. Ten micrograms of total RNA per lane was used for RNA blot analyses. Blots were hybridized with desired probes as specified in Figs. 4 and 5 legends. Hybridizations were carried out as described (27) and exposed to a PhosphorImager screen.

First-strand cDNA was synthesized by using 2 μg of total RNA isolated from control and silenced plants as described above. Semiquantitative RT-PCR analysis was performed by using 1 μl of the cDNA in a 20-μl reaction mixture containing primers G6 (TGGCCCAAAGTTCTTGGC) and E6 (AGAGATCAGTT-GTATTTATG), which anneal outside the region used for silencing SABP2 expression. Control reactions to normalize RT-PCR amplifications were run with the primers derived from constitutively expressed translation elongation factor 1α (*EF1α*) (forward, TCACATCAACATTGTGGTCATTGGC; reverse, TTGATCTGGTCAAGAGCCTCAAG). PCR was performed for 30 cycles at 55°C annealing temperature.

Results

Purification of SABP2. The tobacco SA-binding activity was purified ≈24,000-fold from 7.5 kg of leaves after a seven-step protocol (Table 1). The partially purified SA-binding activity was not due to catalase (CAT) or carbonic anhydrase (CA) (SABP3), because CAT was precipitated in the 0–50% $(NH_4)_2SO_4$ frac-
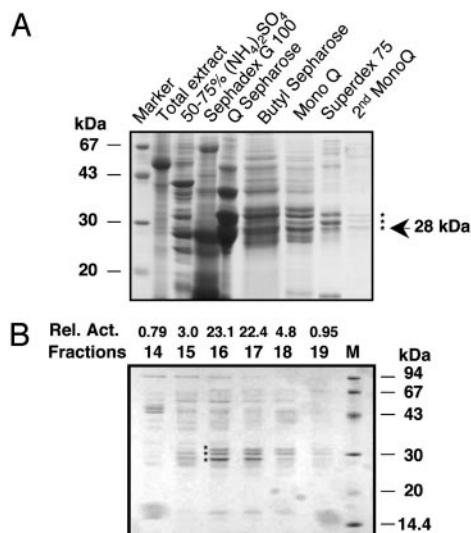
A

Fig. 1. Protein profiles from the purification of tobacco SABP2. (*A*) SDS/PAGE analysis of fractions from each step of the SABP2 purification protocol. Aliquots from pooled fractions containing peak SA-binding activity were analyzed by SDS/PAGE on a 12.5% gel that was subsequently stained with Coomassie blue. After the final Mono Q chromatography step, three proteins (indicated by *) copurified with the SA-binding activity. An arrow marks the putative SABP2 protein, which is 28 kDa. (*B*) Protein profiles and SA-binding activity in fractions from the final Mono Q chromatography step. SA-binding activity (dpm, ×1,000) is shown above each fraction. The three proteins copurifying with the SA-binding activity are marked by *.

Fig. 2. Sequence alignment of the tobacco SABP2 with related proteins. Aligned from top to bottom are the deduced amino acid sequences of the tobacco SABP2 and putative α-hydroxynitrile lyases (hnls) from *Arabidopsis thaliana* (GenBank accession no. AAO22676), *Manihot esculenta* (CAA11219), *Hevea brasiliensis* (P52704), and an esterase from *Oryza sativa*, Pir7b (Q43360). The residues of the catalytic triad are shown in bold and are indicated by arrowheads; the lipase signature sequence of SABP2 is boxed. The sequences corresponding to the five tryptic peptides obtained by microsequencing the 28-kDa protein are overlined. Sequence alignment was performed by using the CLUSTALW program. The symbols used to show the degree of conservation are: *, residues identical in all sequences in the alignment; :, conserved substitutions; ., semiconserved substitutions; and no symbol, nonconserved substitutions.

tions, and CA activity eluted from the Sephadex G-100 column in different fractions from those containing SABP2.

Analysis of fractions from the last two chromatography steps revealed three proteins of 32, 30, and 28 kDa that copurified with the SA-binding activity (Fig. 1*A*). Careful comparison of the prevalence of these proteins in fractions from the final Mono Q column with the level of SA-binding activity in each fraction suggested that the 28-kDa protein was SABP2 (Fig. 1*B*). Consistent with this conclusion, a prior purification scheme identified a 28- but not a 30- or a 32-kDa protein that copurified with SA-binding activity (data not shown). Partial amino acid sequence analysis of tryptic peptides from both 28-kDa proteins indicated that they are the same. Gel filtration chromatography also indicated that the native molecular weight of SABP2 is ≈25–30 kDa (data not shown; ref. 21). Based on this result, SABP2 appears to be a monomeric protein.

Cloning and Characterization of the *SABP2* Gene. To identify the gene encoding this 28-kDa protein, RT-PCR was performed by using a degenerate primer derived from the partial amino acid sequence and a universal adapter primer. The RT-PCR-amplified 639-bp product was found to contain a complete 3′ untranslated region and a polyA tail. Using 5′ RACE, an ≈850-nt product containing the missing 5′ portion of the *SABP2* gene was obtained. Combined sequence analysis of the 5′ RACE product and the original cDNA clone indicated that the *SABP2* mRNA contains a single large ORF encoding a 260-aa protein with a calculated molecular mass of 29.3 kDa (Fig. 2). Sequence analysis indicates that SABP2 is a member of the α/β fold hydrolase super family, whose members contain a catalytic triad consisting of Ser, Asp, and His. SABP2 shares substantial homology to several members of this super family, including plant α-hydroxynitrile lyases and esterases. SABP2 also contains the lipase signature sequence GXSXG (amino acid residues 79–83) found in lipases belonging to this super family.

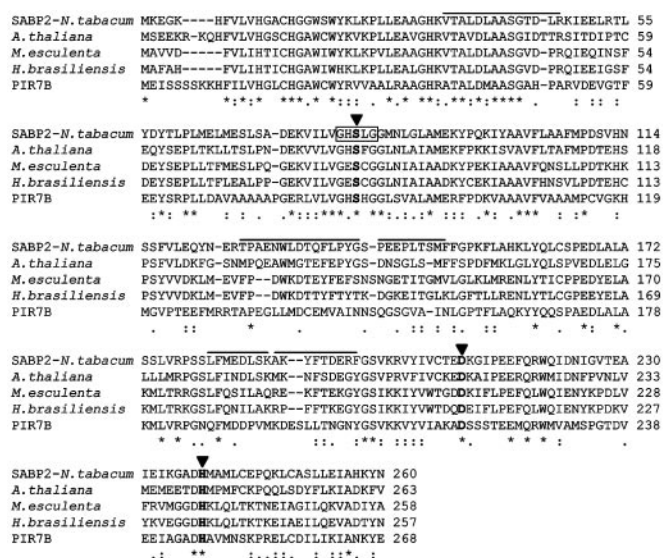To rigorously establish that the 28-kDa protein purified by the

seven-step protocol was SABP2, its encoding cDNA was expressed in *E. coli* as a His-6-tagged fusion protein. The purified rSABP2 exhibited high affinity for [³H]SA (Table 2). Moreover, this binding was effectively competed by a large molar excess of unlabeled SA or its biologically active analogs, which induce *PR* gene expression and resistance. By contrast, biologically inactive SA analogs were poor competitors for SA binding.

SABP2 Exhibits Lipase Activity. Because SABP2 contains the catalytic triad and the lipase signature sequence, the recombinant protein was tested for lipase activity. Highly purified rSABP2 exhibited lipase/esterase activity with 4-methylumbelliferone butyrate in an in-gel assay, and with *para*-nitrophenyl (*p*NP) butyrate in a solution assay (data not shown). rSABP2 also cleaved esters containing long carbon chains such as *p*NP palmitate (C-16) and *p*NP myristate (C-14) (Fig. 3), thereby demonstrating true lipase activity. Addition of SA to the reaction

Table 2. Specificity of SA-binding activity of recombinant SABP2

Assay	Bound [³H]SA, dpm	Biological activity of competitor
rSABP2 + no competitor	140,206	—
rSABP2 + 1 mM unlabeled SA	160	Active
rSABP2 + 1 mM unlabeled 5-CSA	260	Active
rSABP2 + 1 mM unlabeled 2,6-DHBA	924	Active
rSABP2 + 1 mM unlabeled 2,5-DHBA	90,839	Inactive
rSABP2 + 1 mM unlabeled 4-HBA	95,634	Inactive

5-CSA, 5-chlorosalicylic acid; 2,6-DHBA, 2,6-dihydroxybenzoic acid; 2,5-DHBA, 2,5-dihydroxybenzoic acid; 4-HBA, 4-hydroxybenzoic acid.
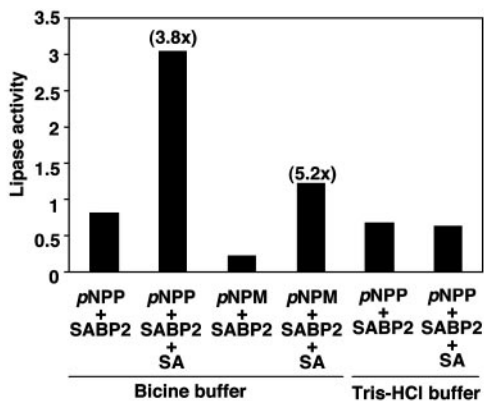
Fig. 3. SA stimulates the lipase activity of recombinant SABP2. Results from lipase assays using pNP palmitate (para-nitrophenyl palmitate) as the substrate in the presence or absence of SA are presented as an average with three separate preparations of rSABP2, whereas those using pNP myristate (para-nitrophenyl myristate) as the substrate were done with one preparation of rSABP2. Lipase activity detected in each sample is presented in relative units, and fold stimulation by SA is shown in parentheses above the bars. One relative unit is the amount of enzyme that releases 0.017 μmol/min p-nitrophenol. Note that when 50 mM bicine is replaced with 50 mM Tris·HCl, pH 8.0, SABP2 does not bind SA.

stimulated lipase activity 3- to 6-fold (Fig. 3). This stimulation required SA binding to rSABP2, because it was abolished in reaction conditions that prevented SA binding. In contrast to rSABP2, the lipase from *Mucor meihei* did not exhibit stimulation by SA, indicating that SA stimulation of lipase activity is not a general phenomenon (data not shown).

SABP2 Expression Is Required for Complete Local and Systemic Resistance to TMV. To assess the role of SABP2 in defense signaling, *SABP2* expression was silenced by using RNA interference (RNAi) (28). RNA blot analysis of 16 independently generated T_1 lines expressing the *RNAi-SABP2* construct revealed that *SABP2* expression was suppressed >75% compared with the empty vector control plants (data not shown). Strikingly, the TMV-induced lesions on these plants were 41% larger than those observed on the empty vector control plants. RNA blot analysis of five independent T_2 lines similarly revealed little *SABP2* transcript accumulation before or after TMV infection, and the lesions were on average 34% larger than those on the control lines (Fig. 4A). Moreover, transcripts for the TMV coat protein (*CP*) accumulated to higher levels in the inoculated leaves of *SABP2*-suppressed lines as compared with control plants.

SA induction of *PR-1* expression, which is associated with local resistance to TMV, was also affected in *SABP2*-silenced plants. Suppression of SA-induced *PR-1* expression was readily detected in the T_1 generation of the five lines of *SABP2*-silenced plants in which little, if any, *SABP2* transcripts could be detected by RT-PCR under the conditions used (Fig. 4B). In the T_2 generation of these five lines, the level of *SABP2* silencing was more variable (Fig. 4C and data not shown). In plants in which *SABP2* transcript was undetectable, SA induction of *PR-1* expression was suppressed (see Fig. 4C, e.g., transgenic 1–2). However, in plants in which silencing was less effective, suppression of *PR-1* induction was poor (e.g., transgenic 1–3 and 1–4). These results suggest that silencing was less effective in the T_2 generation and that suppression of SA-induction of *PR-1* expression depended on the level of *SABP2* silencing.

Whether SAR development also is suppressed in the *SABP2*-silenced lines was then assessed. In control plants, the lesions formed after a secondary infection were ≈50% smaller than those produced after a primary infection (Fig. 5 A and B); this
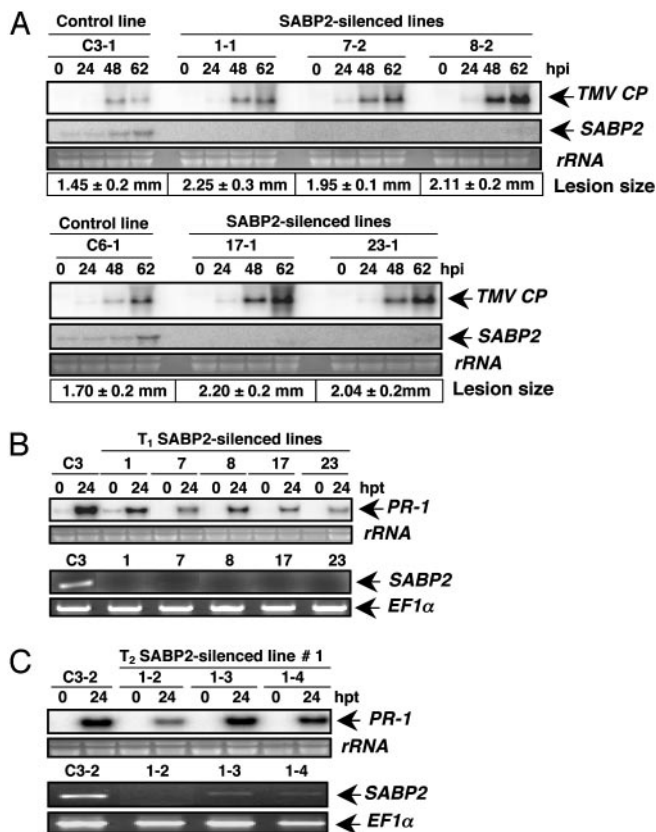

Fig. 4. Silencing of *SABP2* suppresses local resistance to TMV and SA-induced *PR-1* expression. (A) RNA blot analysis of TMV *CP* and *SABP2* transcript accumulation in control plants (transformed with empty vector) and various *SABP2*-silenced lines from the T_2 generation. Total RNA was isolated from TMV-inoculated leaves harvested at the indicated time points. After transfer, the membrane was hybridized with probes for the *SABP2* and TMV *CP*. The size of TMV-induced lesions presented is an average of 50 lesions per line. Lesion diameter is presented ± standard deviation. (B) Comparison of SA-induced *PR-1* expression and *SABP2* silencing in the T_1 generations of control and *SABP2*-silenced lines. Total RNA was isolated from all lines at 0 and 24 h posttreatment (hpt) with 0.25 mM SA. *PR-1* expression was monitored by RNA blot analysis, whereas *SABP2* expression was determined by RT-PCR analysis by using cDNA generated from untreated plants. The level of *EF1α* product was monitored as an internal control to normalize the amount of cDNA template. Please note that *PR-1* induction by SA was tested at least twice in two independent experiments for both the T_1 and T_2 generations to confirm the difference in SA responsiveness. (C) Comparison of SA-induced *PR-1* expression and *SABP2* silencing in three different T_2 generation plants for line 1; see *B* for details.

reduction in secondary lesion size is a common marker for SAR. By contrast, the lesions formed on secondarily inoculated *SABP2*-silenced plants were as large as those formed after a primary infection and 2.5-fold larger than those exhibited by control plants. *SABP2*-silenced plants also exhibited increased viral replication, as indicated by higher levels of TMV movement protein (*MP*) transcript in the systemic leaves of *SABP2*-silenced plants than control plants after secondary inoculation (Fig. 5C). In addition, systemic expression of the *PR-1* gene, another common marker for SAR, was reduced in *SABP2*-silenced plants. Unlike control plants, whose uninoculated leaves accumulated low-to-moderate levels of *PR-1* transcript after a primary infection (Fig. 5C), the systemic leaves of *SABP2*-silenced plants contained little to no *PR-1* mRNA. After a secondary infection with TMV, however, the challenge-inoculated leaves of *SABP2*-silenced plants accumulated more *PR-1* transcripts than those of control plants.
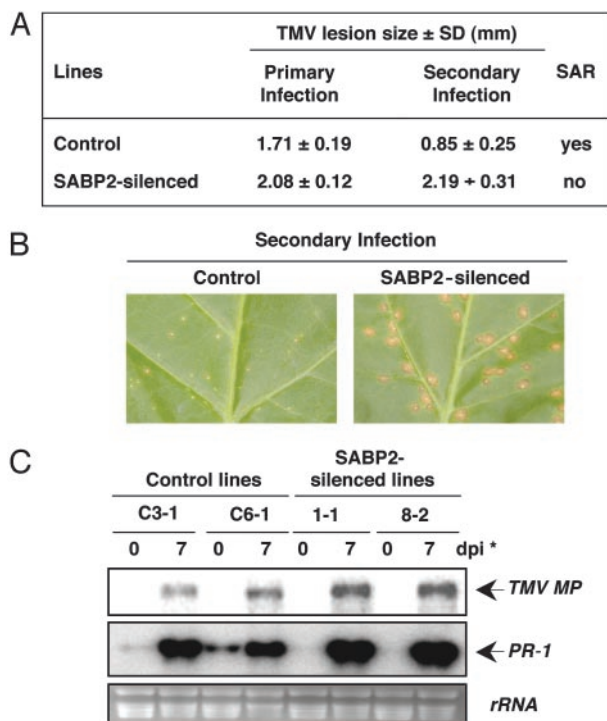
A

| Lines | TMV lesion size ± SD (mm) | | SAR |
	Primary Infection	Secondary Infection	
Control	1.71 ± 0.19	0.85 ± 0.25	yes
SABP2-silenced	2.08 ± 0.12	2.19 + 0.31	no

B Secondary Infection

Control / SABP2-silenced

C Control lines / SABP2-silenced lines

C3-1 C6-1 1-1 8-2

0 7 0 7 0 7 0 7 dpi *

← TMV MP

← PR-1

rRNA

Fig. 5. Silencing *SABP2* expression blocks SAR development. (*A*) The size of primary lesions developed by TMV-inoculated control and *SABP2*-silenced plants (T$_2$ generation) was measured at 7 dpi. Seven days after the primary TMV infection, the upper previously uninoculated leaves received a secondary inoculation with TMV. The diameter of the secondary lesions was then measured 7 days after challenge infection. Each value represents the average size (in mm ± standard deviation) of 50 lesions per line. (*B*) Morphology of TMV-induced lesions in control and *SABP2*-silenced tobacco. The leaves were photographed 7 days after secondary infection with TMV. (*C*) RNA blot analysis of TMV *MP* and *PR-1* transcript accumulation in control and *SABP2*-silenced T$_2$ lines. Total RNA was isolated from systemic leaves before (0 day) or 7 days after a secondary inoculation with TMV (*). After transfer, the membrane was hybridized with probes for the TMV *MP* and *PR-1*.

Taken together, these results suggest that SABP2 plays a role(s) in restricting viral replication/spread in TMV-inoculated leaves, as evidenced by increased lesion size and greater accumulation of transcripts for TMV *CP*. They also indicate that *SABP2* expression is required for systemic *PR-1* expression and the characteristic reduction in lesion size and viral replication associated with SAR.

Discussion

In this study, we describe the purification, cloning, and characterization of tobacco SABP2. SABP2 is a previously undescribed member of the α/β fold hydrolase super family. This super family consists of proteins with diverse enzymatic activities that, despite limited sequence homology, share structural homology (29). The presence of a lipase signature sequence, combined with SABP2's ability to hydrolyze artificial lipase substrates, argues that this protein is a lipase. Intriguingly, SABP2's lipase activity is enhanced by the binding of SA.

Is SABP2 a Resistance-Signaling Receptor for SA? The combined observations that SABP2 binds SA with high affinity, is present in exceedingly low concentrations, and displays SA-stimulated enzymatic activity suggest that it might be a receptor for SA. Further supporting this hypothesis, local and systemic resistance in *SABP2*-silenced plants was disrupted at least as effectively as in SA-deficient tobacco expressing the *nahG* transgene. After a

primary infection with TMV, the lesions formed on T$_1$ and T$_2$ generations of *SABP2*-silenced plants were on average 41% and 34% larger, respectively, than those of control plants, whereas those formed on NahG tobacco were only 23% larger on average (30). In addition, neither *SABP2*-silenced nor *nahG*-expressing plants developed SAR. Further arguing that SABP2 is an SA receptor is the reduced ability of SA to induce *PR-1* expression in plants effectively silenced for *SABP2*. Interestingly, *SABP2* silencing was more variable and generally less effective in T$_2$ vs. T$_1$ plants. Less effective silencing of *SABP2* correlated with poor suppression of *PR-1* induction, suggesting that the residual SABP2 level in these T$_2$ plants is at or above a threshold required for SA induction of *PR-1*, whereas in the T$_1$ plants and a minority of T$_2$ plants (e.g., transgenic 1–2 in Fig. 4*C*), it is below this level. Because local and systemic resistance was impaired in all T$_2$ plants tested, the level of SABP2 required for resistance appears to be higher than that needed for *PR-1* gene activation. However, the efficiency of *SABP2* silencing did appear to influence how severely resistance was impaired, because the primary TMV lesions on T$_2$ plants were not as large as those on T$_1$ plants.

The reduction in local resistance, inability to activate SAR, and loss of SA responsiveness exhibited by *SABP2*-silenced plants is very similar to the phenotype of SA-insensitive SAR-defective *npr1/nim1/sai1 Arabidopsis* mutants (31–34). NPR1 is an important signal transducer that functions downstream of SA in the defense signaling pathway. This protein contains ankyrin repeats and shares limited homology with the IκBα subclass of transcription factors/inhibitors in animals, which regulate immune and inflammatory responses (35, 36). Recent studies have revealed that NPR1 is maintained in the cytoplasm as an oligomer formed through intermolecular disulfide bonds (37). Treatment with SA (or its analog 2,6-dichloro isonicotinic acid) or infection with pathogens alters the cellular reduction potential, thereby promoting monomerization of NPR1; these monomers then are translocated to the nucleus, a prerequisite for *PR-1* gene activation (37, 38). Although these findings provide one mechanism of action for SA, an additional mechanism(s) also must exist to account for the poorer induction of *PR-1* expression and disease resistance in transgenic *npr1-1* mutant *Arabidopsis* that constitutively accumulate monomeric, nuclear-localized NPR1 than in 2,6-dichloro isonicotinic acid-treated WT plants (37). Furthermore, NPR1 was recently shown to regulate SA-mediated suppression of jasmonic acid signaling via a mechanism that does not require nuclear localization (39). Future analyses with an *Arabidopsis* knock-out mutant(s) lacking the SABP2 ortholog(s) are needed to elucidate the respective contributions and locations of NPR1 and SABP2 in the SA signaling pathway(s). Nonetheless, the similarities between *SABP2*-silenced and *npr1/nim1/sai1* plants strongly argue that SABP2 is an important component of this pathway that functions at a point downstream of SA.

If SABP2 has a direct role in SA signaling, the question arises why its orthologs were not identified by genetic screens for *Arabidopsis* mutants displaying enhanced disease susceptibility (7, 11, 12). A likely explanation is that *Arabidopsis* contains an 18-member gene family whose encoded proteins are 46–71% similar to SABP2. Thus, if some of these proteins are functionally redundant, mutation of a single family member would be unlikely to produce a detectable phenotype. Supporting this possibility, 10 of the *Arabidopsis* SABP2-like (*SABP2L*) genes contain the lipase signature sequence and several display specific SA-binding activity (data not shown).

Possible Role(s) for SABP2 During Defense Signaling. The discovery that SABP2 displays SA-stimulated lipase activity and SABP2 is required for local and systemic resistance suggests SABP2's lipase is required to signal resistance. One possible mechanism for resistance-specific SABP2 activation is by means of direct

PLANT BIOLOGY

stimulation of its lipase activity by SA. This might be mediated by SA-facilitated displacement of the lid, a surface loop found on many lipases and other α/β fold hydrolases that covers the active site and regulates substrate selection and binding (29). SABP2 activity also may be increased by enhanced gene expression, because *SABP2* transcript levels increased in TMV-infected tobacco plants (Fig. 4*A*).

The mechanism through which SABP2's lipase activity transduces the defense signal is not known. However, there is growing evidence that lipids play an important role in signaling disease resistance. The EDS1 and PAD4 proteins of *Arabidopsis*, which are putative lipases, are required to transduce the resistance signal after pathogen recognition by a specific class of resistance (*R*) genes. Although these proteins share little homology with SABP2, all three contain the catalytic triad and the lipase signature sequence (40, 41). Additionally, the *ssi2* mutation in *Arabidopsis*, which impairs fatty acid (FA) desaturase activity and thereby alters cellular FA content, confers constitutive activation of several SA-associated defense responses and suppression of certain jasmonic acid-dependent defenses (42, 43). More recently, a defect in a putative apoplastic lipid transfer protein caused by the *dir1-1* mutation was shown to impair systemic, but not local, resistance in pathogen-infected *Arabidopsis* (44). *DIR1* therefore appears to play a role in generating or translocating the SAR signal that moves from inoculated leaves to other parts of the plant. Given that both *dir1-1* mutant and *SABP2*-silenced plants are defective in developing SAR, it is tempting to speculate that SABP2's SA-stimulated lipase activity generates a SAR-inducing lipid (or lipid derivative) that

is translocated by the *DIR1*-encoded lipid transfer protein to the uninoculated parts of the plant.

Because some SABP2L proteins do not bind SA (data not shown), certain family members might bind other ligands, such as stress-associated hormones like jasmonic acid or abscisic acid. Indeed, these proteins may comprise a family of receptors that, on binding their cognate ligand, exhibits enhanced hydrolase (e.g., lipase/esterase) activity. Different members, or sets of members, would likely display distinct substrate specificities, thereby ensuring that the proper response is signaled. Alternatively, sequence similarity between SABP2 and other known proteins, including several plant hydroxynitrile lyases and lecithin (phosphatidylcholine) cholesterol acyl transferase (45) from animals, raises the possibility that some SABP2/SABP2L family members have other enzymatic activities.

In summary, our results suggest that SABP2 is a resistance signaling receptor for SA. The steps activated downstream of this SA effector protein are not yet known. However, the ability of SA to regulate SABP2's lipase activity suggests a mechanism through which lipids/fatty acids are linked to the SA-dependent defense signaling pathway.

1. Dangl, J. L. & Jones, J. D. G. (2001) *Nature* **411,** 826–833.
2. Holt, B. F., III, Hubert, D. A. & Dangl, J. L. (2003) *Curr. Opin. Immunol.* **15,** 20–25.
3. Cohn, J., Sessa, G. & Martin, G. B. (2001) *Curr. Opin. Immunol.* **13,** 55–62.
4. Flor, H. (1971) *Annu. Rev. Phytopathol.* **9,** 275–296.
5. Keen, N. T. (1990) *Annu. Rev. Genet.* **24,** 447–463.
6. Hammond-Kosack, K. E. & Jones, J. D. G. (1996) *Plant Cell* **8,** 1773–1791.
7. Dempsey, D., Shah, J. & Klessig, D. F. (1999) *Crit. Rev. Plant Sci.* **18,** 547–575.
8. Lamb, C. & Dixon, R. A. (1997) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **48,** 251–275.
9. Mittler, R., Shulaev, V., Seskar, M. & Lam, E. (1996) *Plant Cell* **8,** 1991–2001.
10. Dong, X. (2001) *Curr. Opin. Plant Biol.* **4,** 309–314.
11. Glazebrook, J. (2001) *Curr. Opin. Plant Biol.* **4,** 301–308.
12. Kunkel, B. N. & Brooks, D. M. (2002) *Curr. Opin. Plant Biol.* **5,** 325–331.
13. Van Camp, W., Van Montagu, M. & Inzé, D. (1998) *Trends Plant Sci.* **3,** 330–334.
14. Draper, J. (1997) *Trends Plant Sci.* **2,** 162–165.
15. Shirasu, K., Nakajima, H., Rajasekhar, V. K., Dixon, R. A. & Lamb, C. (1997) *Plant Cell* **9,** 261–270.
16. Devadas, S. K., Enyedi, A. & Raina, R. (2002) *Plant J.* **30,** 467–480.
17. Chen, Z., Ricigliano, J. W. & Klessig, D. F. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 9533–9537.
18. Chen, Z., Silva, H. & Klessig, D. F. (1993) *Science* **262,** 1883–1886.
19. Durner, J. & Klessig, D. F. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 11312–11316.
20. Slaymaker, D. H., Navarre, D. A. Clark, D., del Pozo, O., Martin, G. B. & Klessig, D. F. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 11640–11645.
21. Du, H. & Klessig, D. F. (1997) *Plant Physiol.* **113,** 1319–1327.
22. Guo, A., Salih, G. & Klessig, D. F. (2000) *Plant J.* **21,** 409–418.
23. Yang, J., Koga, Y., Nakano, H. & Yamane, T. (2002) *Protein Eng.* **15,** 147–152.
24. Smith, N. A., Singh, S. P., Wang, M. B., Stoutjesdijk, P. A., Green, A. G. & Waterhouse, P. M. (2000) *Nature* **407,** 319–320.
25. Shah, J. & Klessig, D. F. (1996) *Plant J.* **10,** 1089–1101.
26. Kumar, D., Verma, H. N., Tuteja, N. & Tewari, K. K. (1997) *Plant Mol. Biol.* **33,** 745–751.
27. Tang, X., Xie, M., Kim, Y. J., Zhou, J., Klessig, D. F. & Martin, G. B. (1999) *Plant Cell* **11,** 15–29.
28. Wesley, S. V., Helliwell, C. A., Smith, N. A., Wang, M. B., Rouse, D. T., Liu, Q., Gooding, P. S., Singh, S. P., Abbott, D., Stoutjesdijk, P. A., *et al.* (2001) *Plant J.* **27,** 581–590.
29. Nardini, M. & Dijkstra, B. W. (1999) *Curr. Opin. Struct. Biol.* **9,** 732–737.
30. Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessmann, H. & Ryals, J. (1993) *Science* **261,** 754–756.
31. Cao, H., Bowling, S. A., Gordon, A. S. & Dong, X. (1994) *Plant Cell* **6,** 1583–1592.
32. Delaney, T. P., Friedrich, L. & Ryals, J. A. (1995) *Proc. Natl. Acad. Sci. USA* **2,** 6602–6606.
33. Glazebrook, J., Rogers, E. E. & Ausubel, F. M. (1996) *Genetics* **143,** 973–982.
34. Shah, J., Tsui, F. & Klessig, D. F. (1997) *Mol. Plant–Microbe Interact.* **10,** 69–78.
35. Cao, H., Glazebrook, J., Clark, J. D., Volko, S. & Dong, X. (1997) *Cell* **88,** 57–64.
36. Ryals, J., Weymann, K., Lawton, K., Friedrich, L., Ellis, D., Steiner, H.-Y., Johnson, J., Delaney, T. P., Jesse, T., Vos, P. & Uknes, S. (1997) *Plant Cell* **9,** 425–439.
37. Mou, Z., Fan, W. & Dong, X. (2003) *Cell* **113,** 935–944.
38. Kinkema, M., Fan, W. & Dong, X. (2000) *Plant Cell* **12,** 2339–2350.
39. Spoel, S. H., Koornneef, A., Claessens, S. M. C., Korzelius, J. P., Van Pelt, J. A., Mueller, M. J., Buchala, A. J., Métraux, J.-P., Brown, R., Kazan, K., *et al.* (2003) *Plant Cell* **15,** 760–770.
40. Falk, A., Feys, B. J., Frost, L. N., Jones, J. D., Daniels, M. J. & Parker J. E. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 3292–3297.
41. Jirage, D., Tootle, T. L., Reuber, T. L., Frost, L. N., Feys, B. J., Parker, J. E., Ausubel, F. M. & Glazebrook, J. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 13583–13588.
42. Kachroo, P., Shanklin, J., Shah, J., Whittle, E. J. & Klessig, D. F. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 9448–9453.
43. Shah, J., Kachroo, P., Nandi, A. & Klessig, D. F. (2001) *Plant J.* **25,** 563–574.
44. Maldonado, A. M., Doerner, P., Dixon, R. A., Lamb, C. J. & Cameron, R. K. (2002) *Nature* **419,** 399–403.
45. Jonas, A. (2000) *Biochim. Biophys. Acta* **1529,** 245–256.