

# The tobacco salicylic acid-binding protein 3 (SABP3) is the chloroplast carbonic anhydrase, which exhibits antioxidant activity and plays a role in the hypersensitive defense response

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In plants, salicylic acid (SA) plays an important role in signaling both local and systemic defense responses. Previous efforts to identify SA effector proteins in tobacco have led to the isolation of two soluble cytoplasmic SA-binding proteins (SABPs): catalase, SABP, and an  $\approx 25$ -kDa protein, SABP2. Here we describe the identification of an SA-binding protein, SABP3, in the stroma of tobacco chloroplasts. SABP3 bound SA with an apparent dissociation constant ( $K_d$ ) of 3.7  $\mu$ M and exhibited much greater affinity for biologically active than inactive analogs. Purification and partial sequencing of SABP3 indicated that it is the chloroplast carbonic anhydrase (CA). Confirming this finding, recombinant tobacco chloroplast CA exhibited both CA enzymatic and SA-binding activities. Expression of this protein in yeast also demonstrated that CA/SABP3 has antioxidant activity. A second gene encoding CA was also cloned, and its encoded protein was shown to behave similarly to that purified as SABP3. Finally, silencing of CA gene expression in leaves suppressed the *Pto:avrPto*-mediated hypersensitive response in disease resistance. These results demonstrate that SA may act through multiple effector proteins in plants and shed further light on the function of CA in chloroplasts.

Recognition of avirulent microbial pathogens by plants can trigger both local and systemic plant defense responses. The local responses, which serve direct or indirect roles in containing the invading pathogen, may include ion fluxes, strengthening of cell walls, accumulation of salicylic acid (SA), activation of various defense genes, and induction of host cell death [the hypersensitive response (HR)] (1–3). After these events, the uninoculated portions of the plant frequently exhibit increased defense gene expression, elevated levels of SA, and a long-lasting resistance to a broad range of pathogens known as systemic acquired resistance (SAR).

Many studies have implicated SA as an important signal for activation of both local and systemic defense responses. For example, transgenic plants expressing the SA-degrading enzyme salicylate hydroxylase exhibit delayed HR development and increased viral spread in the inoculated leaf (4), loss of SAR and pathogenesis-related gene expression in the uninoculated leaves, and heightened disease susceptibility (4–6). Exogenously supplied SA also induces various defense-associated responses, including an increase in cytosolic calcium levels in tobacco suspension cells (7), activation of the mitogen-activated protein kinase SIPK in tobacco plants (8), and induction of many defense genes, including those encoding the pathogenesis-related proteins (2, 3). In addition, if SA is provided before or at the time of pathogen infection or elicitor challenge, it can potentiate various defense responses, such as the generation of reactive oxygen species (ROS) (9, 10), activation of hypersensitive cell death (10, 11), and expression of certain defense genes (10, 12, 13). Because SA either fails to induce or only weakly induces these responses on its own, potentiation likely occurs via a different mechanism from that used by SA for direct activation. However, a proposed positive feedback loop, involving SA,  $H_2O_2$ , and cell death, may serve to enhance both the potentiation and direct

activation pathways (10, 14, 15). Beyond its central role in the SA signaling pathway, there is growing evidence that SA crossmodulates the activity of the ethylene and/or jasmonic acid (JA)-dependent defense response pathway(s). In particular, SA has been shown to antagonize JA's synthesis and/or action (16, 17), and SA and the JA derivative methyl JA mutually antagonize each other's ability to induce gene expression in tobacco (18).

To elucidate the mechanisms through which SA induces defense responses, several putative effector proteins have been identified. The first, termed salicylic acid-binding protein (SABP), is the cytosolic (peroxisomal) tobacco catalase (CAT) that reversibly binds SA with a  $K_d$  of 14  $\mu$ M (19–22). SA and its functional analogs inhibit CAT's  $H_2O_2$ -degrading activity, whereas biologically inactive analogs do not (21–25). Thus, it was hypothesized that SA-mediated inhibition of CAT might generate the  $H_2O_2$  burst seen during the HR (22). Supporting this possibility, SA was subsequently shown to inhibit cytosolic ascorbate peroxidase, the other major  $H_2O_2$ -scavenging enzyme in plant cells (26, 27). Other studies, however, have suggested that SA functions downstream, rather than upstream, of  $H_2O_2$  in the defense signaling pathway (28, 29). One possible explanation for these conflicting findings is that SA and  $H_2O_2$  are involved in a self-amplifying feedback loop (10, 14, 15). An alternative mechanism through which SA-mediated inhibition of CAT may signal defense responses is via the generation of SA free radicals, which could then activate a lipid peroxidation pathway (24, 25); this possibility remains a topic of debate (11).

A second specific high-affinity  $\approx 25$ -kDa SA-binding protein (SABP2) also has been identified in tobacco cytoplasm by using an SA ligand of higher specific activity than that used to identify SABP/CAT. SABP2 bound SA reversibly with an apparent  $K_d$  of 90 nM and, like CAT, showed specificity for SA and SA analogs capable of triggering SAR (30). The high affinity and specificity of SABP2 suggest that it may be a physiological SA receptor, although its identity is not yet known.

Here we report the presence of a SA-binding protein (SABP3) in the soluble fraction of purified tobacco leaf chloroplasts and identify it as the tobacco chloroplast carbonic anhydrase (CA). Moreover, the recombinant tobacco CA/SABP3 complemented a yeast mutant that was hypersensitive to oxidative stress. A second CA gene was also cloned from tobacco, and the encoded protein

Abbreviations: CA, carbonic anhydrase; CAT, catalase; HR, hypersensitive response; ROS, reactive oxygen species; SA, salicylic acid; SABP, salicylic acid-binding protein; AP, Amer-sham Pharmacia.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF454759).

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was shown to have similar characteristics *in vitro* to CA purified as SABP3. Finally, silencing of CA gene expression in leaves of *Nicotiana benthamiana* suppressed the *Pto:avrPto*-mediated HR. These results not only demonstrate a unique SA-binding activity in chloroplasts but also suggest that CA functions in plant defense, perhaps through antioxidant function.

## Materials and Methods

**Chloroplast Preparation.** Intact chloroplasts were prepared according to Joy and Mills (31) with modifications for large-scale preparation. Briefly, 150 g of tobacco [cv. Xanthi nc (NN)] leaf tissue was pulse homogenized in 500 ml of extraction buffer [50 mM Hepes, pH 7.5/330 mM sorbitol/1 mM EDTA/0.3% polyvinylpyrrolidone/0.1% defatted BSA/8 mM  $\beta$ -mercaptoethanol ( $\beta$ -ME)/1 mM phenylmethylsulfonyl fluoride (PMSF)/1  $\mu$ g/ml of leupeptin], filtered through miracloth, and the filtrate centrifuged for 7 min at  $1,500 \times g$ . The pellet was resuspended in 2.5 ml of wash buffer (20 mM Hepes, pH 7.2/330 mM sorbitol/1 mM EDTA/8 mM  $\beta$ -ME/1 mM PMSF/1  $\mu$ g/ml of leupeptin), and loaded onto a 20-ml 40:80% Percoll step gradient containing 20 mM Hepes, pH 7.2, 330 mM sorbitol, and 1 mM EDTA. The 40 and 80% steps also contained 1.2 and 2.4% polyethyleneglycol ( $M_r$  3,350), 0.4 and 0.8% BSA, and 0.4 and 0.8% Ficoll, respectively. After centrifugation for 6 min at  $4,500 \times g$ , intact chloroplasts were collected from the interface, washed twice with wash buffer (7 min at  $1,500 \times g$ ), and the pellet stored at  $-80^\circ\text{C}$ . Soluble chloroplast proteins were obtained by hypoosmotic lysis of the chloroplasts in lysis buffer (30 mM sodium citrate, pH 6.3/50 mM ammonium sulfate/1 mM EDTA/4 mM DTT/1  $\mu$ g/ml of leupeptin). The lysate was centrifuged for 15 min at  $25,000 \times g$  and the supernatant collected.

**SA Binding.** Standard SA-binding reactions were carried out with 300 nM [ $^3\text{H}$ ]SA (20.5 Ci/mmol; 1 Ci = 37 GBq; NEN) with or without unlabeled SA (10,000-fold molar excess) or its analogs as competitor in 100  $\mu$ l of assay buffer (30 mM sodium citrate, pH 6.3/1 mM EDTA/8 mM  $\beta$ -mercaptoethanol) for 1 hr on ice. Unbound ligand was removed with a 1-ml G-25 Superfine [Amersham Pharmacia (AP)] column by centrifugation for 4 min at  $1,000 \times g$ . Bound [ $^3\text{H}$ ]SA was quantified by scintillation counting 90  $\mu$ l of filtrate. For competition binding studies, 3.7  $\mu$ M [ $^3\text{H}$ ]SA was used. Kinetic values were calculated by using GraphPad (San Diego) PRISM 3.

**SABP3 Purification.** Proteins from chloroplast extracts representing 1.8 kg of leaf tissue were precipitated with 50% ammonium sulfate saturation at  $0^\circ\text{C}$ . The pellet was solubilized in a minimal volume of lysis buffer, exchanged to HA buffer (HAB) (5 mM potassium phosphate, pH 7.1/4 mM DTT), and applied to 196 ml of CHT II hydroxyapatite (Bio-Rad) in a XK 26/40 column (AP). The flowthrough, which contains SABP3, was immediately applied to a 1-ml MonoQ anion exchange column (AP) equilibrated with HAB containing 1  $\mu$ g/ml of leupeptin (HABL). Bound proteins were eluted with a 30-ml gradient from 0 to 200 mM ammonium sulfate in HABL by using FPLC (AP). Fractions with peak SA-binding activity were pooled and precipitated with 50% ammonium sulfate, and the resultant pellet was solubilized in 200  $\mu$ l of HABL. This sample was separated on a 20-ml Superdex 200 gel filtration column (AP) in HABL containing 50 mM ammonium sulfate. The purification process was monitored by subjecting aliquots from each column eluate to mini SDS/PAGE.

**Amino Acid Sequencing.** The amino acid sequence (and matrix-assisted laser desorption ionization–time of flight MS peptide mass fingerprinting) for SABP3 was obtained by submitting the  $\approx 30$ -kDa protein band excised from Coomassie brilliant blue-stained gels to the Iowa State University Protein Facility.

**CA Enzymatic Assays.** CA enzymatic assays were performed and reported essentially as described by Roberts and Spalding by using 100 mM potassium phosphate, pH 7.7, with 10 mM DTT as buffer (32). The reaction was started by addition of 3 ml of  $\text{CO}_2$ -saturated water to the stirring enzyme/buffer mixture, and the time of pH decrease was measured. Units of enzyme activity were calculated as 1 unit =  $(T_b/T_c)-1$ , where  $T_b$  and  $T_c$  represent the time (in sec) it takes for the pH to drop from 7.5 to 7.0 in the blank and the sample reaction, respectively.

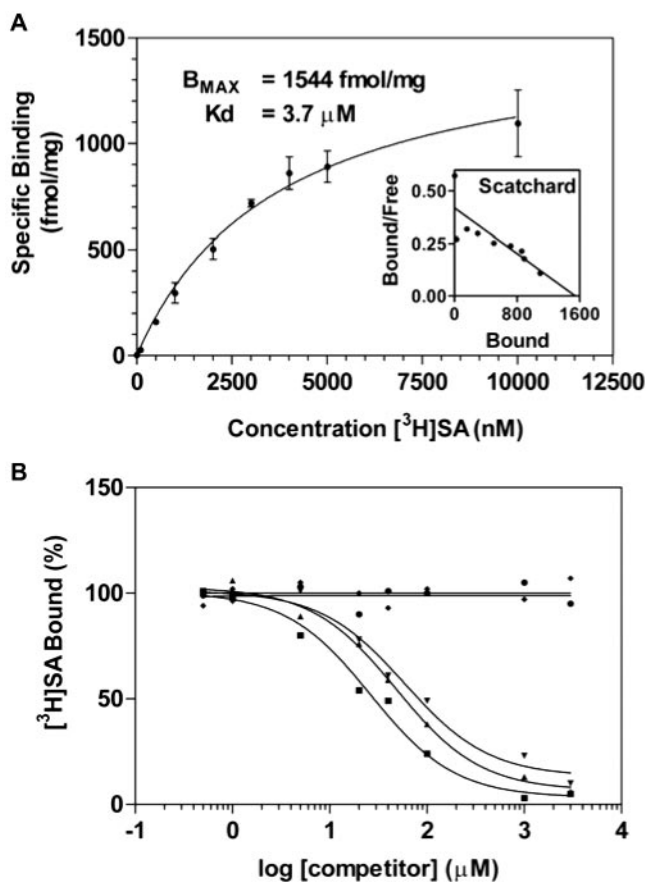
**CA Cloning and Expression.** The tobacco CA cDNAs were PCR amplified from a tobacco cDNA library [cv. Xanthi nc (NN)] by using the primer set CGCCAGCCATATGGAATTGCAATCATCA (forward) and CTCGGCGAATTCGCTTCATACGGAAAGAGA (reverse). The forward primer removed the chloroplast transit peptide as predicted by Majeau and Coleman (33), introducing a methionine and in-frame *NdeI* site upstream of Gln-101 of the full length preprotein. The reverse primer introduced an *EcoRI* site downstream of the native stop codon. The resultant PCR products were cloned directly into pGEM-T (Promega) according to manufacturer's instructions and subsequently into the *NdeI* and *EcoRI* sites of the *Escherichia coli* expression vector pET28a (Novagen), which introduced an N-terminal hexahistidine leader peptide to the coding region. The fusion CA proteins were synthesized in the soluble fraction of *E. coli* strain BL21(DE3) (Novagen) and affinity purified on Ni-NTA agarose (Novagen) in the presence of 8 mM  $\beta$ -mercaptoethanol according to manufacturer's instructions.

**Yeast Complementation.** For expression in yeast, the tobacco CAs were PCR amplified by using primers as above except that the forward primer introduced a *BamHI* site upstream of Gln-101 and the reverse primer an *EcoRI* site downstream of CA's native stop codon. The resultant PCR products were ultimately cloned into the *BamHI* and *EcoRI* sites of the galactose-inducible yeast expression vector pYES2/NT-C (Invitrogen), which introduced an N-terminal hexahistidine leader peptide to the coding region and resulted in vectors pYES-CA1/2.

The yeast (*Saccharomyces cerevisiae*) strains CEN.PK2-1C (MATa; ura3-52; trp 1-289; leu2-3.112; his3 $\Delta$ 1; MAL2-8 $^+$ ; SUC2) and CEN.HE28-1A (CEN.PK; Mat a; ura3-52; his3 $\Delta$ 1; leu2-3.112; trp1-289; YNL036w::KAN) were obtained from the European Saccharomyces Cerevisiae Archives for Functional Analysis. The gene deleted in CEN.HE28-1A, YNL036w, is synonymous with *NCE103*, the yeast CA-like gene. CEN.PK2-1C is the parent strain. CEN.HE28-1A is referred to herein and in the text as  $\Delta nce103$ . *ANCE103* was grown under anaerobic conditions by using the Anaerocult A system (EM Science).

The plasmids pYES-CA1/2 were transformed into CEN.PK2-1C and  $\Delta nce103$  as described for the pYES2/NT-C vector system (Invitrogen). CEN.PKC-1C and  $\Delta nce103$  transformants were selected under aerobic and anaerobic conditions, respectively, at  $30^\circ\text{C}$  on synthetic complete medium lacking uracil (SC-U). Uracil-positive  $\Delta nce103$  transformants were tested for aerobic growth on galactose-containing SC-U, versus glucose-containing SC-U. Complementation by tobacco CA was confirmed under aerobic conditions on yeast peptone adenine medium containing galactose as the primary carbon source.

**Potato Virus X-Based Virus-Induced Gene Silencing.** Construction of the *N. benthamiana* cDNA library in a PVX vector and subsequent screening of 2,400 independent clones for genes whose silencing affected the HR produced by *Agrobacterium tumefaciens*-mediated transient expression of *Pto* and *avrPto* in *N. benthamiana* were carried out on the basis of the methods of Ratcliff *et al.* (34), and Sessa *et al.* (35).



**Fig. 1.** Tobacco leaf chloroplasts contain a specific moderate-affinity SA-binding activity that can discriminate between biologically active and inactive SA analogs. (A) Saturation-binding assays showing saturable specific binding of [<sup>3</sup>H]SA to a soluble chloroplast protein(s). Scatchard analysis of the binding data suggests a single class of binding site.  $K_d$  and  $B_{MAX}$  values were calculated by using single binding-site hyperbolic nonlinear regression analysis. (B) Competition binding assays with SA analogs showing a positive correlation between binding affinity and biological activity. SA (▼) and its active analogs 5-chlorosalicylic acid (■) and 2,6-dihydroxybenzoic acid (▲) showed similar affinity for SABP3 compared with the inactive SA analogs 4-hydroxybenzoic acid (◆) and 4-aminosalicylic acid (●).  $IC_{50}$  values were calculated by using single binding-site competition nonlinear regression analysis. All calculations were made using GraphPad PRISM 3 analysis software.

## Results

**Identification of SA-Binding Activity in Tobacco Chloroplasts.** We have previously identified two cytoplasmic SABPs from tobacco (19, 30). To search for additional SABPs in the plant cell, purified intact chloroplasts were obtained from tobacco leaves by a combination of differential centrifugation and Percoll gradient fractionation (31). Soluble chloroplast proteins were collected and assayed for SA binding in the presence of [<sup>3</sup>H]SA (20.5 Ci/mmol) with or without excess unlabeled SA. A specific moderate affinity

SA-binding activity was observed (Fig. 1A). Nonlinear regression analysis of saturation binding data gave an apparent  $K_d$  of  $3.7 \pm 0.5 \mu\text{M}$  and a  $B_{max}$  of  $1544 \pm 86 \text{ fmol/mg}$  (Fig. 1A). SA-binding activity was reduced between 75 and 100% by boiling, SDS, or proteinase K treatments (data not shown). Thus, we conclude that a protein(s), designated SABP3, is responsible for the SA-binding activity.

To compare SABP3's affinity for biologically active analogs (which induce pathogenesis-related gene expression and enhance resistance) and inactive SA analogs, competition binding assays were performed. SABP3 exhibited a similar binding affinity for the biologically active SA analogs 5-chlorosalicylic acid and 2,6-dihydroxybenzoic acid, whereas the inactive analogs 4-hydroxybenzoic acid and 4-aminosalicylic acid in up to  $\approx 1,000$ -fold molar excess did not compete with [<sup>3</sup>H]SA binding (Fig. 1B). This correlation between binding affinity and biological activity suggests a physiological role for SABP3 as a resistance-associated SA effector protein. Interestingly, under our assay conditions, neither benzothiadiazole in formulation or recrystallized form nor 2,6-dichloroisonicotinic acid was capable of competing with [<sup>3</sup>H]SA in the competition binding assays (data not shown). However, recrystallized benzothiadiazole was visibly insoluble at concentrations of 1 mM or above in our binding reactions.

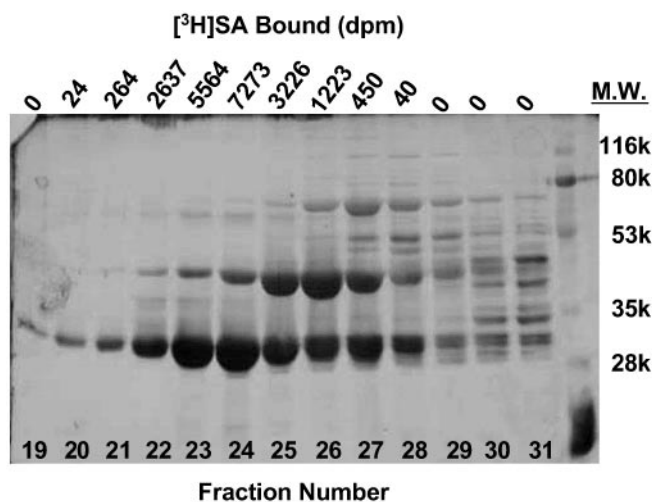
**Purification and Identification of SABP3.** As a first step toward identifying SABP3, a purification strategy that started with soluble proteins extracted from purified tobacco leaf chloroplasts was developed (Table 1). Starting with isolated intact chloroplasts afforded a high initial degree of purification by removal of cytosolic, nuclear, and other organellar proteins. However, because SABP/CAT and SABP2 are present in the cytoplasm (19, 30), the fold purification for this step could not be estimated. The soluble chloroplast extract was fractionated by ammonium sulfate precipitation (0–50%), followed by column chromatographic separations on hydroxyapatite, MonoQ (strong anion exchange), and Superdex 200 (gel filtration) columns. This four-step purification series (excluding chloroplast isolation) yielded a 600-fold purification of specific SA-binding activity (Table 1).

Comparison of the SA-binding activity levels with the SDS/PAGE protein profiles observed in fractions eluted from the Superdex 200 column suggested that SABP3 is a highly purified  $\approx 30$ -kDa protein (Fig. 2). However, its estimated native molecular weight ( $M_r$ ) on this column is approximately 220 kDa (Fig. 3). To investigate the composition of the  $\approx 30$ -kDa band further, aliquots from the Superdex 200 fractions were subjected to SDS/PAGE by using 20-cm gels (data not shown). A single dominant  $\approx 30$ -kDa band and one slightly lower  $M_r$  minor band, most likely a degradation product of the dominant protein (see below), were identified. On the basis of these combined results, we surmised that SABP3 is likely a homomeric protein consisting of six to eight  $\approx 30$ -kDa subunits.

**SABP3 Is the Tobacco Chloroplast CA.** A partial amino acid sequence for SABP3 was determined by sequencing the  $\approx 30$ -kDa protein excised from Coomassie-stained gels. Two peptide sequences (EAVNVSLGNLLTYPFVR and GLMSLPADGSESTAF) were obtained; they showed 100% identity to the tobacco chloroplast CA

**Table 1. Purification of SABP3 from the soluble extract of purified intact tobacco leaf chloroplasts**

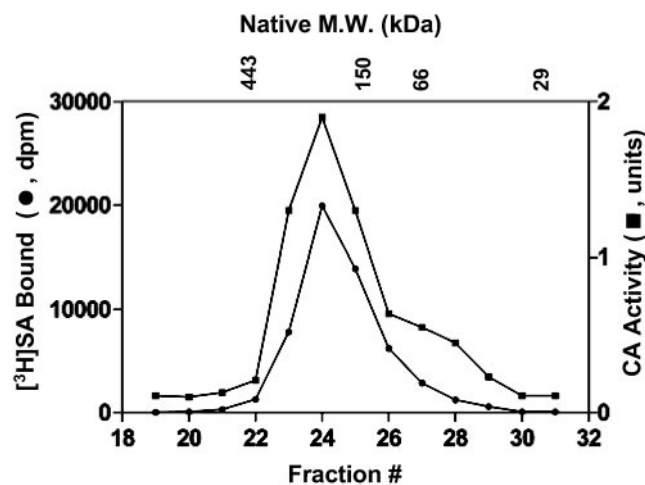
Fraction	SA-binding activity, dpm·hr <sup>-1</sup>	SA-binding activity, %	Protein, $\mu\text{g}$	Protein, %	Specific activity, dpm·hr <sup>-1</sup> · $\mu\text{g}^{-1}$	Purification, fold
Crude chloroplast extract	1,411,425	100	2,701,616	100	0.5	1
50% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	530,530	38	428,380	16	1.2	2.4
Hydroxyapatite	425,733	30	26,639	1	16	32
MonoQ HR 5/5	396,529	28	4,630	0.2	86	172
Superdex 200 HR 10/30	122,208	9	408	0.02	300	600



**Fig. 2.** Superdex 200 fractionation of SABP3 showing a  $\approx$ 30-kDa protein comigrating with SA-binding activity. Fractions (bottom axis) from the final stage of purification (gel filtration on a Superdex 200 column) were visualized on a silver-stained SDS/PAGE gel. Analysis of an 80- $\mu$ l aliquot of each of these fractions for specific SA-binding activity (top axis; dpm) demonstrates that the  $\approx$ 30-kDa protein comigrates with SA-binding activity.

characterized by Majeau and Coleman (here designated CA1) (33). Matrix-assisted laser desorption/ionization–time of flight MS peptide fingerprint analysis of SABP3 tryptic fragments also indicated that this protein is tobacco chloroplast CA. Because no contaminating proteins were detected, the lower  $M_r$  band observed in SDS/PAGE (which would have been present in the band submitted for sequencing) is likely a degradation product of CA. The determination that SABP3 is CA is consistent with our gel filtration and SDS/PAGE data, because plant CAs, including the tobacco chloroplast CA, are homohexamers or homooctamers composed of  $\approx$ 25- to 30-kDa subunits (36, 37).

To test the sequence-based identification of SABP3 as CA, Superdex 200 fractions were assayed for both specific SA-binding and CA enzymatic activities. These two activities copurified (Fig. 3). Interestingly, whereas the traditional CA inhibitors azide and



**Fig. 3.** Coelution of SA-binding and CA enzymatic activities from a Superdex 200 column. Aliquots containing 30- and 2- $\mu$ g protein from each fraction were used to assay specific SA-binding (●, dpm) and CA enzymatic activities (■, relative units), respectively. Molecular weight calibration of the Superdex 200 column (top axis) gave an estimated native molecular mass for SABP3 of approximately 220 kDa.

**Table 2.** Specific SA binding by 10  $\mu$ g of tobacco CA synthesized in *E. coli*

Assay	Bound [ $^3$ H]SA, dpm		
	His <sub>6</sub> -CA1	His <sub>6</sub> -CA2	ADH*
No competitor	2,068	3,721	52
3 mM unlabeled SA	78	93	ND <sup>†</sup>
3 mM unlabeled 5-CSA <sup>‡</sup>	78	92	ND
3 mM unlabeled 4-HBA <sup>§</sup>	1,618	1,610	ND

50  $\mu$ g of alcohol dehydrogenase from yeast was used as carrier protein. Values represent the average of duplicate experiments.

\*ADH, 50  $\mu$ g of alcohol dehydrogenase from yeast.

<sup>†</sup>ND, not determined.

<sup>‡</sup>5-CSA, 5-chlorosalicylic acid.

<sup>§</sup>4-HBA, 4-hydroxybenzoic acid.

acetazolamide completely inhibited the CA enzymatic activity, only azide inhibited SA binding (data not shown). Furthermore, CA enzymatic activity was not inhibited by SA at concentrations up to 3 mM.

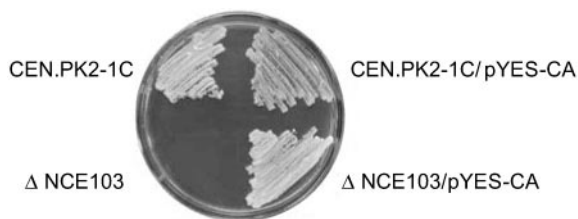
To demonstrate conclusively that SABP3 is CA, a cDNA encoding the mature chloroplast CA, as predicted by Majeau and Coleman (33), was PCR amplified from a tobacco cDNA library [cv. Xanthi nc (NN)] for expression in *E. coli* as an N-terminal hexahistidine fusion protein (His<sub>6</sub>-CA1). PCR amplification based on published CA DNA sequence (33), however, also recovered a second CA gene that we designated CA2. Based on the partial amino acid sequence of the purified protein, which contains a valine at position 184, the CA1 cDNA encodes the isoform of CA that we purified as SABP3, whereas CA2 encodes a second form of the protein, which differs from CA1 at four amino acids in the mature form, including an isoleucine at position 184, and at three amino acids in the chloroplast signal peptide (data not shown). Both mature forms of CA were expressed in *E. coli* and analyzed further.

The size of the affinity-purified fusion proteins on a Superdex 200 column suggested both isoforms were a homohexamer or homooctamer (data not shown). Both His<sub>6</sub>-CA1 and His<sub>6</sub>-CA2 exhibited specific SA-binding and CA enzymatic activities (Tables 2 and 3). Furthermore, both isoforms specifically bound SA and its biologically active analog 5-chlorosalicylic acid with high affinity, but not the inactive SA analog 4-hydroxybenzoic acid, as determined by competition binding assays (Table 2).

**SABP3 Has Antioxidant Properties.** On the basis of CA's ability to reversibly hydrate and dehydrate between carbon dioxide and bicarbonate (36), this enzyme has been hypothesized to play various roles in C<sub>3</sub> plants. However, a definitive role has not been established. In yeast, *Saccharomyces cerevisiae*, a deletion mutation in a CA-like gene, *NCE103*, was recently shown to increase its sensitivity to oxidative stress (38). The deletion strain ( $\Delta$ *NCE103*) was unable to grow under aerobic (ambient oxygen) conditions and was acutely sensitive to H<sub>2</sub>O<sub>2</sub>. Interestingly,  $\Delta$ *NCE103* was complemented for the ability to grow aerobically by the chloroplast CA from *Medicago sativa* (alfalfa), suggesting that one function of plant CA is to provide oxidative stress protection (38). Because the yeast CA-like protein, *NCE103*, does not have CA enzymatic activity, it was suggested that oxidative stress protection and the CA enzymatic activity are independent functions of plant CA.

**Table 3.** Enzymatic activity of tobacco CAs synthesized in *E. coli*

Protein	CA-specific activity, units/ $\mu$ g
His <sub>6</sub> -CA1	0.35
His <sub>6</sub> -CA2	0.33
SABP3/CA1 purified from tobacco	0.95



**Fig. 4.** Complementation of the *S. cerevisiae* CA-like gene deletion strain,  $\Delta NCE103$ , by tobacco chloroplast CA1.  $\Delta NCE103$  shows an oxygen-sensitive growth defect manifested as an inability to grow under aerobic conditions. Plasmid pYES-CA1 provides galactose-inducible expression of a recombinant gene encoding an N-terminal hexahistidine leader peptide fused to mature chloroplast CA1 protein. Yeast were grown under aerobic conditions on yeast peptone adenine medium with galactose at 30°C for 48 hr. Similar results were obtained with pYES-CA2.

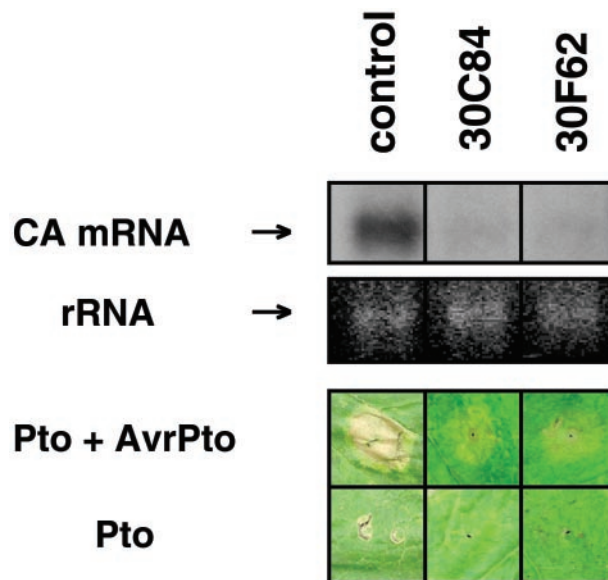
To determine whether the tobacco CA is able to complement  $\Delta NCE103$ , the mature chloroplast CA1 and CA2 cDNAs were cloned into the yeast galactose-inducible expression vector pYES2/NT-C as an N-terminal hexahistidine fusion (pYES-CA1/2). The pYES-CAs were then transformed into both  $\Delta NCE103$  (CEN.HE28-1A) and its parent strain CEN.PK2-1C; transformants were selected for their ability to grow in the absence of uracil under anaerobic conditions. The transformants were then tested for their ability to grow aerobically on inducing medium (galactose-containing), but not on noninducing medium (glucose-containing). Expression of both tobacco CA genes complemented the ability of  $\Delta NCE103$  to grow aerobically, thus SABP3/CA1 and CA2 exhibit antioxidant activity (Fig. 4; pYES-CA2 data not shown).

**Silencing of CA Gene Expression Suppresses the *Pto:avrPto*-Mediated HR in Leaves.** In a large (blind) potato virus X-based virus-induced gene silencing screen for genes whose silencing affects the *Pto:avrPto*-mediated HR in *N. benthamiana* leaves, CA was identified four independent times. In this screen, PVX gene-silenced or control (empty PVX vector) *N. benthamiana* leaves were infiltrated with *A. tumefaciens* strains engineered to transiently express the tomato resistance gene, *Pto*, or both *Pto* and its corresponding *Pseudomonas syringae* avirulence gene, *avrPto*. Coexpression of *Pto* and *avrPto* in *N. benthamiana* leaves triggers HR. In these experiments, silencing of CA (SABP3) suppressed development of HR and led to a complete loss of HR in 45% of the *A. tumefaciens* infiltrations (data not shown). These results were confirmed by another potato virus X-based virus-induced gene silencing experiment in which the silencing of CA at the mRNA level was also monitored (Fig. 5). Suppression of CA mRNA levels correlated with the inhibition of HR, suggesting a physiological role for CA in HR development.

## Discussion

Using a high specific activity radioactive SA ligand, a SA-binding protein, designated SABP3, was identified in the soluble fraction of tobacco chloroplasts. SABP3 binds SA with moderate affinity ( $K_d = 3.7 \mu\text{M}$ ) and exhibits similar affinities for biologically active SA analogs but much lower affinities for inactive analogs. Sequence analysis of the purified protein revealed that it is the tobacco chloroplast CA. Confirming this finding, recombinant tobacco chloroplast CA synthesized in *E. coli* exhibited both CA enzymatic and SA-binding activities.

Interestingly, azide and acetazolamide (classic CA inhibitors) both inhibited the *in vitro* CA enzymatic activity of this enzyme, but only azide inhibited its ability to bind SA. These results, coupled with the finding that SA does not affect CA activity, suggest that the SA binding and CA enzymatic activities are independent. Consistent with this possibility, plant and yeast CAs have been shown to



**Fig. 5.** Silencing of CA gene expression is associated with inhibition of HR. CA expression was reduced in *N. benthamiana* plants by PVX-mediated silencing. Both plants (30C84 and 30F62) showed reduced levels of CA mRNA by Northern analysis (Top) and mounted a weaker HR (Bottom) than control plants after infiltration with *Agrobacterium* strains expressing *Pto* and *avrPto*. *Agrobacterium* carrying only *Pto* was used as a negative control. Ethidium bromide staining of ribosomal RNA (Middle) demonstrated equal loading of RNA to each lane.

contain an antioxidant function that appears to be separate from the protein's CA enzymatic activity (ref. 38; this report; data not shown).

In addition to the form of CA purified as SABP3 and designated CA1, a second gene for the tobacco chloroplast CA was identified and its encoded isoform designated CA2. CA2 was not present in readily detectable levels in the purified SABP3 subjected to peptide sequencing suggesting that CA2 is expressed at much lower levels than CA1 in tobacco leaves. This hypothesis is consistent with the work of Majeau and Coleman (33), who identified by purification and amino acid sequencing only one major form of CA (CA1) in chloroplasts from tobacco.

CA, which catalyzes the reaction  $\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{HCO}_3^- + \text{H}^+$ , occurs in animals as a monomer and in plants as a hexamer or octamer. CA has been shown to play essential roles in various processes in animals, such as facilitating  $\text{CO}_2$  transport out of muscle cells and providing bicarbonate for mitochondrial gluconeogenesis (39). During photosynthesis in  $\text{C}_4$  plants, where it is found mainly in the cytosol, CA is involved in converting  $\text{CO}_2$  into bicarbonate for fixation by the primary carboxylating enzyme phosphoenolpyruvate carboxylase (40). By contrast, the role of CA in  $\text{C}_3$  plants, such as tobacco, where it is localized primarily in the chloroplast stroma, is less clear. On the basis of this location, CA was proposed to expedite diffusion of  $\text{CO}_2$  into the chloroplast, to act as a partner to Rubisco in  $\text{CO}_2$  fixation, and/or to play a role in buffering excessive pH changes resulting from varying light intensities (36). Although there is no convincing evidence to support the latter two hypotheses, CA was shown to facilitate the supply of  $\text{CO}_2$  to Rubisco by maintaining equilibrium between  $\text{HCO}_3^-$  and  $\text{CO}_2$  within the chloroplast (41). Whether this facilitation is biologically important for  $\text{C}_3$  photosynthesis, however, is unclear. No substantial differences in carbon assimilation rates or overall fitness were observed in antisense CA transgenic tobacco, which had as much as 99% reduction in CA activity (41–43).

In addition to reversibly converting  $\text{CO}_2$  to bicarbonate, plant CA also appears to have an antioxidant activity. The hypersensitivity to oxygen of a yeast deletion mutant  $\Delta NCE103$  was recently shown to

be complemented by CA from alfalfa (38). Because the  $\Delta NCE103$ -encoded CA-like protein does not exhibit detectable CA activity, it was proposed that the enzymatic activity and antioxidant functions of the alfalfa CA are independent. We extended these findings by demonstrating that both tobacco CAs also complement the  $\Delta NCE103$  phenotype. Unfortunately, because of SA's high toxicity in yeast, determining whether SA affects the antioxidant function of this enzyme may not be possible until an *in vitro* enzymatic assay for the antioxidant activity of CA is devised.

Along with tobacco chloroplast CA/SABP3, four other tobacco proteins that interact with SA have been identified: CAT (SABP) (19, 20), SABP2 (30), ascorbate peroxidase (APX) (26), and aconitase (44). Interestingly, three of these proteins (CAT, APX, and CA) exhibit antioxidant activity. On the basis of preliminary *in vitro* studies, CA's antioxidant activity is distinct from catalase or peroxidase activities, as determined by the inability of CA to generate oxygen or to use common substrates as an electron donor in the presence of  $H_2O_2$  (data not shown). However, because SA inhibits both CAT and ascorbate peroxidase, it is tempting to speculate that it also alters the antioxidant activity of CA. Possibly, SA's ability to inhibit both cytosolic and chloroplast antioxidant enzymes plays a role in increasing ROS levels, which might activate a positive feedback loop that amplifies SA production and induces defense responses following pathogen infection.

In contrast to this possibility, silencing of CA using the potato virus X-based virus-induced gene silencing system suppressed the HR induced by coexpression of *Pto* and *avrPto*. If elevated levels of ROS *per se* are required for HR development, then reduction of CA antioxidant activity either by silencing or inhibition would be expected to enhance the HR rather than attenuate it as observed in this study. Interestingly, Torres *et al.* (45) reported that suppression of the oxidative burst through mutations in *AtrohD* and

*AtrohF*, genes encoding proposed components of the plant NADPH oxidase, enhanced the HR to *Peronospora parasitica*. They and Delledonne *et al.* (46) suggest that HR is triggered by a finely balanced production of superoxide ( $O_2^-$ ),  $H_2O_2$ , and nitric oxide (NO). In this scenario, NO and  $H_2O_2$  cooperate to trigger cell death (HR), whereas  $O_2^-$  may either contribute to HR by its dismutation to  $H_2O_2$  or antagonize HR formation by converting NO to peroxynitrite ( $ONOO^-$ ), which does not initiate cell death in plants (46). Thus, the effect of altering ROS levels on host cell death (HR) depends on both the resulting relative levels of  $O_2^-$  and  $H_2O_2$ , as well as the corresponding level of NO.

In summary, the discovery that tobacco CAs are SA-binding proteins, have antioxidant activity, and function in the HR in plant disease resistance opens new avenues for the study of SA function in plant defense, as well as CA function in chloroplasts. In particular, determining whether CA has antioxidant activity in plants, the nature of this activity, and subsequently whether this activity is altered by SA may provide insights into how ROS levels are regulated within the chloroplast, as well as the cytoplasm. Interestingly, recent evidence suggests that SA is synthesized in chloroplasts of Arabidopsis (47). Finally, the results presented in this paper further suggest that SA induces its many effects on the physiology of plants (48) by interacting with multiple effector proteins.

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