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17. S. M. Russell *et al.*, unpublished data.
18. The γ_c rabbit antiserum, R878, raised to a peptide corresponding to the eight COOH-terminal amino acids of γ_c (NH_2-Cys-Tyr-Thr-Leu-Lys-Pro-Glu-Thr-COOH) that had been coupled to keyhole lympet hemocyanin, was purified with the peptide coupled to EAH-Sepharose beads (Pharmacia). The rabbit antiserum to the IL-4R (P7) was generated with recombinant soluble human IL-4R and can neutralize IL-4 binding. M56 monoclonal antibody to human IL-4R was prepared by immunizing mice with soluble IL-4R; it cannot neutralize IL-4 binding.
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36. The cells were cotransfected with pCD*neo* and pME18S–IL-4R, with or without pME18S-γ_c with calcium phosphate precipitation. Transfected cells were selected in geneticin (500 μg/ml), and expression was confirmed by flow cytometric analysis for the IL-4R and by immunoblotting for γ_c. Although good γ_c expression was achieved, the expression of the human IL-4R on these cells was very low, so murine IL-4 was used to stimulate the cells.
37. We thank S. Gillis and P. Beckmann for providing the P7 and M56 antibodies to the IL-4R, G. Tosato for the normal EBV cell lines, and J. H. Pierce, A. M. Weissman, and J. P. Siegel for helpful discussions.

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Active Oxygen Species in the Induction of Plant Systemic Acquired Resistance by Salicylic Acid

Zhixiang Chen, Herman Silva, Daniel F. Klessig*

A complementary DNA encoding a salicylic acid (SA)–binding protein has been cloned. Its properties suggest involvement in SA-mediated induction of systemic acquired resistance (SAR) in plants. The sequence of the protein is similar to that of catalases and the protein exhibits catalase activity. Salicylic acid specifically inhibited the catalase activity in vitro and induced an increase in H_2O_2 concentrations in vivo. H_2O_2 or compounds, such as SA, that inhibit catalases or enhance the generation of H_2O_2, induced expression of defense-related genes associated with SAR. Thus, the action of SA in SAR is likely mediated by elevated amounts of H_2O_2.

Infection of plants, particularly by necrotizing pathogens, leads to enhanced resistance to subsequent attacks by the same or even unrelated pathogens (*1*). This phenomenon is referred to as systemic acquired resistance (SAR). Because SAR provides long-term (weeks to months) protection throughout the plant (systemic) against a broad range of unrelated pathogens (*2*), its modulation through chemical or genetic engineering means holds considerable promise for reducing crop loss.

Development of SAR correlates with the systemic expression of a number of plant defense-related genes, including five or more families of pathogenesis-related (PR) genes (*3*). Some of the PR proteins inhibit pathogen growth in vitro (*4*); others confer partial resistance to fungal infection when constitutively produced in transgenic tobacco plants (*5*).

Evidence indicates that salicylic acid (SA) is a natural signal molecule for the activation of plant defense responses, including SAR. Application of exogenous SA or its derivative, acetylsalicylic acid (aspirin), induces PR gene expression and enhances resistance to plant diseases (*6*). Increases in the amount of endogenous SA are correlated with expression of PR genes and development of SAR in infected tobacco and cucumber plants (*7*). Also, in transgenic tobacco plants harboring the bacterial *nah*G gene encoding salicylate hydroxylase, which converts SA to catechol, induction of SAR by inoculation with tobacco mosaic virus is blocked (*8*).

We are interested in identifying cellular elements that directly interact with SA in order to understand the mechanism of SA action. We purified a soluble 240- to 280-kD SA-binding protein (SABP) from tobacco leaves (*9*, *10*). This protein is a complex composed of a 57-kD subunit and perhaps one or more additional polypeptides. It has an apparent K_d (dissociation constant) of 14 μM for SA—the concentration of SA found following infection ranges from 2 to 15 μM. Furthermore, the ability of a large number of SA analogs to compete with [^{14}C]SA for binding to the SABP strictly correlates with their ability to induce PR genes and resistance. These results suggest SABP mediates the defense response induced by SA.

Here, we have isolated and characterized the SABP cDNA. A clone (λCK1) encoding the 57-kD subunit of SABP was identified by screening a tobacco cDNA library with an SABP-specific monoclonal antibody (mAb; 3B6) (*11*). The protein encoded by the cloned cDNA reacted with four other SABP-specific mAbs. The amino acid sequence deduced from the cDNA sequence revealed high similarity (60 to 90% identical amino acids) to catalases of other organisms, with highest similarity to the plant catalases (Fig. 1A).

Waksman Institute, Rutgers, The State University of New Jersey, Piscataway, New Jersey 08855.

*To whom correspondence should be addressed.

To confirm that the cDNA encoded the 57-kD subunit of SABP, three tryptic peptides of the purified SABP were sequenced (*12*). All three peptide sequences were similar to the sequence deduced from the cDNA (Fig. 1B). The discrepancy of several amino acid residues between sequences obtained from the peptides and deduced from the cDNA may have resulted from the presence of different isozymes [catalases are encoded by a small gene family in plants (*13*)]. Alternatively, differences between the tobacco cultivars used for purification of SABP (Xanthi nc) and cDNA library screening (SR1) may account for the sequence discrepancies.

To establish that SABP was a catalase, its H_2O_2-degrading activity was measured directly. Highly enriched SABP obtained after four chromatography steps (*10*) exhibited high specific activity (3,000 to 10,000 units per milligram) in a catalase assay. This activity could be immunoprecipitated by SABP-specific mAbs (*14*). The sizes of the SABP complex (240 to 280 kD) and its subunit (57 kD) are also consistent with the structure of known catalases, which are composed of four identical or similar subunits of 50 to 60 kD.

Catalases are present in all aerobic organisms and convert H_2O_2 to H_2O and O_2. The involvement of active oxygen species (AOS) such as H_2O_2 in host defense against microorganisms (*15, 16*) and the discovery that SABP is a catalase suggested that SA may modulate amounts of AOS by influencing the activity of plant catalases. Indeed, in the presence of SA, the catalase activity of the highly enriched SABP was inhibited by 80% (Table 1). Similar inhibition of catalase activity by SA (~70%) was observed with crude extracts (*17*).

Several SA analogs, with varying activity for induction of plant PR genes and disease resistance, were compared for their ability to inhibit the catalase activity of SABP (Table 1). 2,6-Dihydroxybenzoic acid, a highly active inducer of PR genes and resistance, was an effective inhibitor of the catalase activity of SABP. Acetylsalicylic acid, which was less effective in inducing PR gene expression (*17*), was not as good an inhibitor. 2,3-Dihydroxybenzoic acid, which has only weak biological activity, was a poor inhibitor, whereas five other structurally similar but biologically inactive analogs were ineffectual at inhibiting catalase activity. Moreover, the effectiveness of each analog for inhibiting SABP catalase activity correlated with ability of the analog to compete with [^{14}C]SA for binding to SABP. Thus, the binding of SA and its biologically active analogs to SABP was responsible for the inhibition of the catalase activity.

To investigate the in vivo effect of inhibiting catalase activity by SA, we monitored the amount of H_2O_2 in leaves following treatments with SA, 3-hydroxybenzoic acid (a biologically inactive analog of SA), and 3-amino-1,2,4-triazole (3AT; a specific inhibitor of plant and animal catalases). The amounts of H_2O_2 increased in SA- or 3AT-treated tobacco leaves by 50 to 60% over the control levels in water-treated leaves (Fig. 2). In contrast, 3-hydroxybenzoic acid was unable to enhance the amount of H_2O_2, consistent with its ineffectiveness in both binding SABP and inhibiting catalase activity. Thus, elevated H_2O_2, and in turn enhanced oxidative stress, in vivo were consistent with inhibition of catalase activity by SA observed in vitro.

Pathogenesis-related gene expression is both induced by SA and associated with the development of SAR. Therefore, we tested whether SA induces PR genes by inhibiting catalase activity and enhancing H_2O_2 levels. H_2O_2 levels were artificially raised in tobacco by injecting leaves with H_2O_2 or 3AT. Both treatments induced the expression of PR-1 genes (Fig. 3), as did treatment of leaves with two compounds that promote generation of H_2O_2 (glycolate and paraquat). Thus, the SA signal appears to be propagated through H_2O_2, which may act as a secondary messenger to activate defense-related PR genes.

Elevation of H_2O_2 levels may also be responsible for initiating or promoting several other biochemical processes associated with plant disease resistance. Increased lignification of cell walls at sites of infection requires H_2O_2 (*18*), as does oxidative crosslinking of cell wall proteins (*19*). The ability of SA as well to induce this crosslinking (*19*) can be explained by its inhibition of catalase H_2O_2-scavenging activity.

During the rapid and intense oxidative burst associated with pathogen or elicitor treatment in plants (*15*) and with phagocytosis during the immune response in animals (*16*), increased amounts of AOS are thought to be generated by NADPH oxidases located in the plasma membrane. Plants also appear to use AOS for subsequent development of SAR; however, rather than producing H_2O_2 through oxidases,

A

```
          10                  30                  50
SKFRPSSAYDSPFLTTNAGGPVYNNVSSLTVGPRGPVLLEDYHLIEKLATFDRERIPERV

          70                  90                  110
VHARGASAKGFFEVTHDISHLTCADFLRAPGVQTPVICRFSTVVHERGSPESLRDIRGFA

          130                 150                 170
VKFYTREGNFDLVGNNVPVFFNRDAKSFPDTIRALKPNPKSHIQEYWRILDFFSFLPESL

          190                 210                 230
HTFAWFFDDVCLPTDYRHMEGYGVHAYQLINKAGKAHYVKFHWKPTCGVKCMSEEEAIRV

          250                 270                 290
GGTNHSHATKDLYDSIAAGNYPEWKLFIQIMDTEDVDKFDFDPLDVTKTWPEDILPLMPV

          310                 330                 350
GRLVLNRNIDNFFAENEQLAFNPGHIVPGLYYSEDKLLQTRIFAYADTQRHRIGPNYMQL

          370                 390                 410
PVNAPKCAHHNNHRDGAMNFMHRDEEVDYLPSRFDPCRHAEQYPIPSRVLTGRREMCVIE

          430                 450                 470
KENNFKQAGERYRSWEPDRODRYVSKWVEHLSDPRVTYEIRSIWICSLSQADKSCGQKVA

          490
SRLTLKPTM
```

B

Peptide-1	E G N F D L V G N N F P V F F I R
cDNA	- - - - - - - - - - V - - - - N -
Peptide-2	S F T P D R Q E R
cDNA	- W E - - - - D -
Peptide-3	W V E A L S D P R
cDNA	- - - H - - - - -

Fig. 1. Amino acid sequence deduced from nucleotide sequence (GenBank accession number U03473) of the cDNA encoding the 57-kD SABP subunit (**A**) and its comparison with sequences of three tryptic peptides from the purified SABP (**B**). The underlined sequences in (A) correspond to the peptide sequences shown in (B). The cDNA contains an open reading frame of 1467 base pairs encoding a polypeptide of 489 amino acids. The protein is highly similar to plant catalases (to cotton 90% GenBank X52135, to pea 86% GenBank X60169. Sequence comparison suggests that the deduced polypeptide is missing three amino acid residues at the NH_2-terminus.

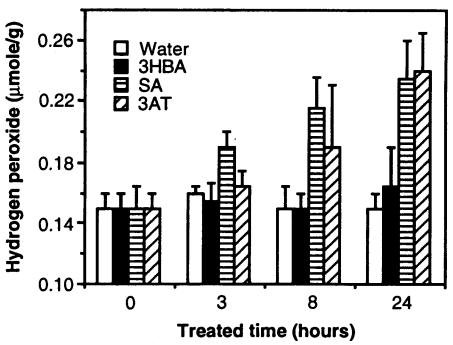

Fig. 2. Time course of H_2O_2 elevation in tobacco leaves treated with water, 3-hydroxybenzoic acid (3HBA; 1 mM), SA (1 mM), or 3AT (2 mM). Leaves were cut and their petioles were submerged in a 100-ml solution containing each of these compounds at the indicated concentrations under 14-hour light conditions. Leaf tissues were frozen in liquid nitrogen and ground for 1.5 min with a polytron in 3 ml of 0.2 N $HClO_4$. Homogenates were centrifuged 10 min in a microfuge, applied (500-μl aliquots) to a 1-ml column of AG1-X8 (Bio-Rad), eluted with 3 × 500 μl of distilled water, and assayed for H_2O_2 (*28*). Values obtained from three independent assays are reported with the sample (n = 3) SD.

Table 1. Inhibition by SA and its analogs of SABP catalase activity and [^{14}C]SA binding. Catalase activity of SABP was assayed over 3 min at room temperature in a 1-ml reaction containing 20 mM citrate, pH 6.5, 5 mM $MgSO_4$, 1 mM H_2O_2, 1 mM SA (or its analogs), and 500 ng of SABP purified through four chromatography steps (*10*). Aliquots (50 μl) were removed from the assay mixture at 30-s intervals to assay for the amount of H_2O_2 remaining (*28*). Rate constants of SABP catalase activity in the presence or absence of SA or its analogs were calculated based on a first-order mechanism.

SA and analogs	Biological activity*	Inhibition (%)	
		Catalase activity	[^{14}C]SA binding†
2-Hydroxybenzoic acid (SA)	++	80 ± 5	89
2,6-Dihydroxybenzoic acid	++	91 ± 3	92
Acetylsalicylic acid	+	53 ± 6	48
2,3-Dihydroxybenzoic acid	±	15 ± 3	9
3-Hydroxybenzoic acid	−	3 ± 1	1
4-Hydroxybenzoic acid	−	4 ± 1	−2
2,4-Dihydroxybenzoic acid	−	5 ± 2	−4
2,5-Dihydroxybenzoic acid	−	3 ± 2	1
3,4-Dihydroxybenzoic acid	−	5 ± 1	0

*Based on ability to induce resistance or PR gene expression, or to inhibit wounding or elicitor-induced synthesis of protease inhibitors (*6*, *17*, *29*). †Previously published data (*10*).

the plant allows accumulation of H_2O_2, a constitutively synthesized by-product of several metabolic pathways (for example, photorespiration and fatty acid β-oxidation), by inhibiting breakdown of H_2O_2 by catalase. Although the increase in amount of H_2O_2 in SA-treated leaves was relatively small (50 to 60%) compared to that during the pathogen- or elicitor-induced oxidative burst in plant cells (two- to fivefold), it persisted for much longer (>24 hours) than the oxidative burst, which usually lasts from minutes to a few hours (*15*). The rapid and transient oxidative burst, produced by cells undergoing pathogen attack, may directly facilitate pathogen killing and rapid induction of early host responses. In contrast, the development of SAR generally requires several days and occurs distant from the primary site of infection. High concentrations of AOS are thus not required to kill pathogens directly, and more modest amounts may be sufficient, if not desirable, for activating plant defense genes.

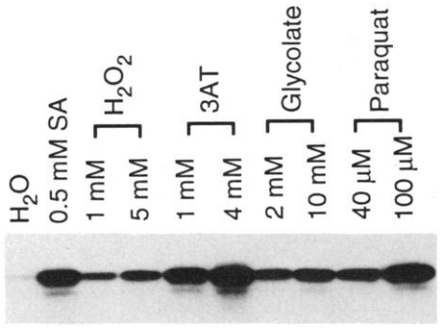

Fig. 3. Induction of PR-1 gene expression by H_2O_2, SA, 3AT, glycolate, and paraquat. Solutions were injected at the indicated concentrations into the extracellular space of leaves of 6-week-old tobacco plants. Leaves were harvested 48 hours later for analysis of PR-1 protein levels by immunoblotting (*10*).

How do AOS activate PR gene expression? In animals, gene activation by AOS is frequently mediated by the transcription factors NF-κB and AP-1 (*20*). NF-κB and AP-1 mediate expression of genes associated with immune responses and cell adaptation to certain adverse conditions such as ultraviolet radiation, respectively (*21*). Thus, AOS may function as secondary messengers for gene activation by different forms of stress in both animals and plants. Similar mechanisms may be involved. PR gene expression is induced by arachidonic acid (*22*) and blocked by the protein kinase C inhibitor H7 [1-(5-isoquiaolinelsulfonyl)-2-methylpiperazine (*23*)]. H_2O_2-induced expression of *c*-fos (a subunit of AP-1) is mediated by arachidonic acid release and involves protein kinase C (*24*).

In addition to tobacco, SABP and SA-inhibitable catalases have been found in other plant species, but not yet in animals (*25*), suggesting that SA-regulated catalases may be unique to plants. Through the binding of SA and the resulting inhibition of its catalase activity, SABP perceives and transduces the SA signal. Thus, it can be considered a receptor, similar to receptor kinases. However, it differs from these membrane-bound receptors not only in that it is soluble, but also in that ligand binding inhibits rather than activates its enzymatic function. Although a number of potential plant receptors or their genes have been identified (*26*) here, we have demonstrated both ligand binding and the mechanism of signal transduction for a purified and cloned plant protein. In addition to inhibition of catalase, SA may have other mechanisms of action in plants, just as one derivative of SA, aspirin, appears to have several modes of action in animal systems (*27*).

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12. Immunoprecipitation with an SABP-specific monoclonal antibody (3B6) was used to purify SABP from about 300 μg of highly purified SABP obtained after four steps of chromatography (*10*). The immunoprecipitated SABP was separated from antibodies by SDS–polyacrylamide gel electrophoresis and submitted to the W. M. Keck Foundation Biotechnology Laboratory (Yale University) for gas-phase sequencing of tryptic peptides.
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Functional Requirement of a Site-Specific Ribose Methylation in Ribosomal RNA

Karen Sirum-Connolly and Thomas L. Mason*

The product of the *PET56* nuclear gene of *Saccharomyces cerevisiae* was shown to be required for ribose methylation at a universally conserved nucleotide in the peptidyl transferase center of the mitochondrial large ribosomal RNA (21*S* rRNA). Cells reduced in this activity were deficient in formation of functional large subunits of the mitochondrial ribosome. The purified Pet56 protein catalyzed the site-specific formation of 2′-*O*-methylguanosine on in vitro transcripts of both mitochondrial 21*S* rRNA and *Escherichia coli* 23*S* rRNA. These results provide evidence for an essential modified nucleotide in rRNA.

Peptidyl transferase is an intrinsic activity of the large ribosomal subunit that catalyzes peptide bond formation in protein synthesis. The sequence and structure of the rRNA in the peptidyl transferase center are highly conserved (*1*), and there is abundant evidence supporting the possibility that rRNA participates in the peptidyl transferase reaction. Most notably, peptidyl transferase activity has been shown to be resistant to protein extraction procedures, consistent with the hypothesis that the rRNA itself is catalytically active (*2*).

The production of mature rRNA involves transcription, nucleolytic processing, and posttranscriptional modification of nucleotides. Although no clear functional role has been demonstrated for any of the modified nucleotides, their importance is suggested by models of the *E. coli* ribosome in which the modifications are clustered around the mRNA-tRNA-peptide complex in the catalytic center of the ribosome (*3*).

K. Sirum-Connolly, Department of Biochemistry and Molecular Biology, University of Massachusetts, Amherst, MA 01003.
T. L. Mason, Department of Biochemistry and Molecular Biology, and the Graduate Program in Molecular and Cellular Biology, University of Massachusetts, Amherst, MA 01003.
*To whom correspondence should be addressed.

Of the 19 identified posttranscriptional modifications in the 23*S* rRNA of *E. coli*, three are ribose methylations at the highly conserved positions Gm2251, Cm2498, and Um2552 in the peptidyl transferase region of domain V (*4*). Mitochondrial large rRNAs are apparently devoid of base methylations but contain as many as three ribose methylations. The mitochondrial large rRNA from hamster contains three ribose-methylated nucleotides at positions corresponding to the universally conserved nucleotides G2251, U2552, and G2553 in the *E. coli* 23*S* rRNA (*5*). The mitochondrial large rRNAs of yeast and *Neurospora* have about two ribose methylations, but the identity of the modified nucleotides has not been established (*6*). The retention of ribose-methylated nucleotides in the otherwise minimally modified mitochondrial rRNAs suggests an essential role for these modifications in ribosome assembly or function, or both. Here we show that the *PET56* gene of *S. cerevisiae* encodes a site-specific rRNA ribose methyltransferase that is required for the formation of functional mitochondrial ribosomes.

The *PET56* gene, required for mitochondrial function, was discovered because it is adjacent to and divergently transcribed from *HIS3*, a histidine biosynthetic gene in the nuclear genome of *S. cerevisiae* (*7*, *8*). Sequence analysis of *PET56* revealed a long open reading frame specifying a basic [isoelectric point (pI) = 10.08], 412–amino acid (46 kD) polypeptide (*9*). The deduced amino acid sequence of Pet56p has 50 to 55% similarity to a 23*S* rRNA ribose methylase from two species of *Streptomyces* (*10*). The *Streptomyces* enzymes form 2′-*O*-methyladenosine at position A1067 of the 23*S* rRNA, rendering the ribosome resistant to the antibiotic thiostrepton, which is synthesized by these organisms (*11*). There was no significant sequence relatedness between Pet56p and several base-modifying methylases (*10*).

Many laboratory yeast strains carry the *his3-Δ200* mutation, a 1036–base pair deletion that removes the entire *HIS3* coding region and part of an AT-rich promoter region that is important for transcription of both *HIS3* and *PET56*. Besides causing histidine auxotrophy, this deletion decreases the transcription of *PET56* by ~80% (*7*). We observed that *his3-Δ200* mutants had a slow-growth phenotype on nonfermentable carbon sources (YPGE) (*12*) at 30°C and were respiration-deficient when grown at 18°C (*13*), presumably because of reduced expression of *PET56*. In addition, the presence of the *his3-Δ200* allele markedly enhanced the leaky respiration-deficient phenotype of a null allele of *MRP49* (*13*), the nuclear gene for a 16-kD protein in the 54*S* subunit of the mitochondrial ribosome (*14*).

We compared the yields of mitochondrial ribosomal subunits obtained by sucrose gradient centrifugation of mitochondrial lysates from the otherwise isogenic *HIS3* and *his3-Δ200* strains. The yield of large subunits from the *his3-Δ200* strain was only 22 to 29% of that from the *HIS3* strain, whereas the yield of small subunits was 82 to 97% (Fig. 1A). Thus, the mutation appears to affect the synthesis or stability of the large subunit. However, Pet56p did not cosedi-