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Differential Accumulation of Salicylic Acid and Salicylic Acid-Sensitive Catalase in Different Rice Tissues[1]

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We previously proposed that salicylic acid (SA)-sensitive catalases serve as biological targets of SA in plant defense responses. To further examine the role of SA-sensitive catalases, we have analyzed the relationship between SA levels and SA sensitivity of catalases in different rice (*Oryza sativa*) tissues. We show here that, whereas rice shoots contain extremely high levels of free SA, as previously reported (I. Raskin, H. Skubatz, W. Tang, B.J.D. Meeuse [1990] Ann Bot 66: 369–373; P. Silverman, M. Seskar, D. Kanter, P. Schweizer, J.-P. Metraux, I. Raskin [1995] Plant Physiol 108: 633–639), rice roots and cell-suspension cultures have very low SA levels. Catalases from different rice tissues also exhibit differences in sensitivity to SA. Catalase from rice shoots is insensitive to SA, but roots and cell-suspension cultures contain SA-sensitive catalase. The difference in SA sensitivity of catalases from these different tissues correlates with the tissue-specific expression of two catalase genes, *Cat*A and *Cat*B, which encode highly distinctive catalase proteins. *Cat*A, which encodes a catalase with relatively low sequence homology to the tobacco SA-sensitive catalases, is expressed at high levels exclusively in the shoots. On the other hand, in roots and cell-suspension cultures, with northern analysis we detected expression of only the *Cat*B gene, which encodes a catalase with higher sequence homology to tobacco catalases. The role of catalases in mediating some of the SA-induced responses is discussed in light of these results and the recently defined mechanisms of catalase inhibition by SA.

During the past few years extensive studies from several laboratories have provided a mounting body of evidence that SA is an important endogenous signal for activation of certain plant defense mechanisms associated with disease resistance. More than a decade ago, application of exogenous SA or its derivative, acetylSA (aspirin), was shown to induce PR protein synthesis and partial resistance to pathogens such as tobacco mosaic virus in tobacco (White, 1979; Antoniw and White, 1980). More recently, elevated levels of SA have been found to be associated with resistance of infected plants to the invading pathogens in an increasing number of plant species, including tobacco (Malamy et al., 1990, 1992; Enyedi et al., 1992), Arabidopsis (Uknes et al., 1993; Bowling et al., 1994; Dietrich et al., 1994; Greenberg et al., 1994; Weyman et al., 1995), and cucumber (Métraux et al., 1990; Rasmussen et al., 1991). Further evidence for the involvement of SA in the induction of defense responses is provided by transgenic tobacco and Arabidopsis plants that constitutively express the *nah*G gene encoding a salicylate hydroxylase from *Pseudomonas putida* (Gaffney et al., 1993; Delaney et al., 1994). In these transgenic plants there is little or no accumulation of SA after pathogen infection, and their ability to restrict pathogen spread and to establish SAR is correspondingly impaired. Finally, a number of Arabidopsis mutants with defective SA signal transduction show compromised defense responsiveness against pathogen infection (Cao et al., 1994; Delaney et al., 1995; Shah et al., 1997).

During the last several years we have been using a biochemical approach to identify cellular components that directly interact with SA as a first step toward elucidating the perception of the SA signal in plant defense responses. A soluble SA-binding protein has been identified in tobacco leaves (Chen and Klessig, 1991; Chen et al., 1993a). This protein binds only SA and its biologically active analogs, 2,6-dihydroxybenzoic acid, acetylSA, 4-chloroSA, 5-chloroSA, and 3,5-dichloroSA, which are capable of inducing PR genes and enhancing disease resistance. In contrast, those analogs that lack such biological activities, despite their structural similarity to SA, fail to bind to this protein. The binding specificity of the protein is consistent with its functioning as a biological target of SA in the plant's defense response. Upon purifying the SA-binding protein and cloning its gene, Chen et al. (1993b) found that the protein is a catalase with an H_2O_2-degrading activity that is inhibited by SA and its biologically active analogs. More recently, another PR gene and disease resistance inducer, 2,6-dichloroisonicotinic acid (Vernooij et al., 1995), and its biologically active analogs have also been shown to bind to and inhibit in vivo catalase activity in tobacco cell cultures (Conrath et al., 1995). Based on these results, we have proposed that one mode of action of SA in plant defense responses is to bind to and inhibit catalases and consequently elevate cellular levels of H_2O_2 (Chen et al., 1993b, 1995).

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Abbreviations: K_{eq}, equilibrium constant; PR, pathogenesis-related; SA, salicylic acid; SAR, systemic acquired resistance.

Since it was first proposed, this working model has been tested by a number of biochemical and molecular approaches. Several recent reports have indicated that substantial inhibition of catalase activity in leaf extracts requires concentrations of SA far above the level in tissues distant from the sites of infection (Chen et al., 1993b; Bi et al., 1995). Furthermore, whereas H_2O_2 and H_2O_2-inducing chemicals induce PR-1 gene expression in wild-type tobacco, PR-1 induction was strongly suppressed in *NahG* transgenic plants (Bi et al., 1995; Neuenschwander et al., 1995). These results argue against the involvement of catalase inhibition and H_2O_2 accumulation in the activation of PR gene expression and the establishment of SAR by SA. On the other hand, examination of almost 40 phenolic and isonicotinic compounds has demonstrated that the ability to bind to the catalases is strictly correlated with their ability to induce PR genes and enhance disease resistance (Chen et al., 1993a, 1993b; Conrath et al., 1995). Furthermore, we have recently shown that induction of PR gene expression by SA and 2,6-dichloroisonicotinic acid is inhibited by antioxidants (Conrath et al., 1995; Z. Chen, L. Conrath, and D.F. Klessig, unpublished results). These results suggested that SA-binding catalases and reactive O_2 species are involved in some of the action of SA.

More recently, Rüffer et al. (1995) showed that SA binds not only to catalases but also to other iron-containing enzymes such as aconitases, peroxidases, and oxidases. Based on these results, it was suggested that SA specifically inhibits these iron-containing enzymes by chelating the iron ion (Rüffer et al., 1995). However, early studies by Lück (1957) and Itoh et al. (1962) of the effects of carboxylic acid on catalases suggested that high levels of SA inhibited mammalian catalases by promoting the peroxidatic activity rather than by chelating the heme iron of catalase. The same mechanism has been proposed based on kinetic analysis for the inhibition of catalases by other phenolic compounds such as hydroquinone and pyrogallol (for review, see Schonbaum and Chance [1976]).

Recently, Durner and Klessig (1996) reported extensive kinetic and spectral analysis of the interaction of tobacco catalases with SA and concluded that SA inhibits catalases by serving as a one-electron-donating substrate for the peroxidatic activity of catalase, thereby trapping the enzymes in a relatively inactive state. Similar studies have also established that SA, aspirin, and other related phenolic compounds can act as one-electron-donating substrates for myeloperoxidase and horseradish peroxidase (Kettle and Winterbourn, 1991; Durner and Klessig, 1995). Finally, the binding constants of the iron ion for SA (log K_{eq} = 16.36 M^{-1}), 3,4-dihydroxybenzoic acid (log K_{eq} = 19.00 M^{-1}), and 3,4,5-trihydroxybenzoic acid (log K_{eq} = 22.28 M^{-1}) (International Union of Pure and Applied Chemistry, 1979) are not correlated with the ability of these compounds to bind to and inhibit catalases. Although 3,4-dihydroxybenzoic acid and 3,4,5-trihydroxybenzoic acid have higher affinities for the iron ion than SA, neither binds to or inhibits tobacco SA-sensitive catalases (Chen et al., 1993a; Z. Chen, unpublished results). Thus, chelation of iron ions, which might account for inhibition of some iron-containing enzymes by phenolic compounds, appears not to be the direct cause of inhibition of catalases and/or peroxidases by SA.

Because several iron-containing proteins were found to be inhibited by SA, Rüffer et al. (1995) also suggested that SA-sensitive iron-containing enzymes represent nonspecific binding proteins of SA with no physiological function in its action. However, mammalian prostaglandin H synthase and myeloperoxidase are iron-containing proteins, are sensitive to aspirin-like drugs including SA (Chen and Marnett, 1989; Kettle and Winterbourn, 1991), and are well-established biological targets of these drugs, all of which accounts for some, if not all, of their anti-inflammatory effects. In plants, in addition to catalases (Chen et al., 1993b) and ascorbate peroxidases (Durner and Klessig, 1995), aconitase from *Nicotiana plumbaginifolia* is sensitive to SA (Rüffer et al., 1995). Significantly, inhibition of aconitase has recently been shown to induce alternative respiration in tobacco (Vanlerberghe and McIntosh, 1996), as has been observed with SA treatment (Kapulnik et al., 1992). Furthermore, Leslie and Romani (1986, 1988) have shown that SA inhibits iron-containing ACC oxidase, thereby blocking ethylene biosynthesis. Thus, certain iron-containing enzymes may have acquired SA sensitivity so that specific biological processes would be subject to regulation by SA.

To further address whether SA-binding catalases play an important role in the action of SA, we have extended our studies of SA-binding catalases to rice (*Oryza sativa*), which has previously been shown to have unusual features of SA accumulation (Raskin et al., 1990; Silverman et al., 1995). From the present study, we report that different rice tissues have dramatic differences in the level of free SA. The differences in the level of free SA correlate with the presence in these tissues of catalases that exhibit different sensitivity to SA. These results support the proposition that plant SA-sensitive catalases are involved in mediating some of the action of SA. Furthermore, the unusual features of differential accumulation of SA and SA sensitivity of catalase make rice an attractive system for studying the roles of SA-sensitive catalases.

MATERIALS AND METHODS

Rice (*Oryza sativa* cv Cypress) plants were grown in a growth chamber at 25°C with a 16-h photoperiod and a hydroponic system supplemented with 100 $\mu g\ L^{-1}$ silica, as described by Yoshida et al. (1976). All experiments were performed with rice seedlings at the three-leaf stage. Rice cell-suspension cultures were grown at room temperature on a shaker at 140 rpm in the dark in Murashige-Skoog medium supplemented with 1× vitamin solution (Kao/Michayluk, Sigma), 5 mM Mes, 3% Suc, 20 mM L-Pro, 50 mg L^{-1} Trp, 100 mg L^{-1} ascorbic acid, 100 mg L^{-1} citric acid, 2 mg L^{-1} 2,4-D, 0.1 mg L^{-1} NAA, 0.1 mg L^{-1} GA_3, and 0.2 mg L^{-1} kinetin. Cells were maintained by 5-fold dilution with fresh medium every 12 d.

Chemicals

SA, SA analogs, H_2O_2, and other common chemicals were purchased from Sigma or Aldrich. SA and its analogs

were dissolved in water as 100 mM stock solutions and adjusted to pH 6.5 with KOH.

Determination of SA

Free SA was extracted from rice shoots, roots, and cell-suspension cultures with the same procedure used previously for tobacco and Arabidopsis (Bowling et al., 1994). SA separation and quantification were performed by HPLC as described previously (Bowling et al., 1994).

Catalase Assays

For catalase assays soluble proteins from tobacco leaves and rice shoots, roots, and cell-suspension cultures were prepared by homogenizing 0.1 g of tissue with a micropestle in 400 μL of a homogenization buffer containing 20 mM citrate, pH 6.5, 5 mM $MgSO_4$, 1 mM EDTA, 10% glycerol, and 30 μg mL^{-1} PMSF. The homogenate was clarified by centrifugation in a microcentrifuge for 15 min. The resulting supernatant was dialyzed against 2 L of the same buffer for 4 h and then centrifuged for 10 min to remove undissolved proteins prior to catalase assay. All of the preparation steps were performed at 4°C. Protein concentrations were determined according to the method of Bradford (1976) with a protein assay kit (Bio-Rad).

Catalase activity was determined by measuring the H_2O_2-dependent O_2 evolution at room temperature with a commercial O_2 electrode (Hansatech Instruments, Norfolk, UK), as described by Conrath et al. (1995). Assays of specific catalase activity in total soluble proteins from different rice tissues were performed in a 2-mL solution containing 20 mM citrate, pH 6.5, 10 mM H_2O_2, and 5 μg of total soluble proteins.

Recent kinetic and spectral studies have shown that SA inhibits catalases by serving as a one-electron donor for the peroxidatic activity of catalases (Durner and Klessig, 1996); accordingly, the assays for determining the SA sensitivity of catalases from different rice tissues were modified to include in the preincubation mixture a low level of H_2O_2 generated by Glc and Glc oxidase. This assay condition with a low level of H_2O_2 closely mimics the in vivo situation, in which H_2O_2 is continuously being produced by various metabolic pathways, including photorespiration in leaves. Inclusion of a low level of H_2O_2 in the preincubation mixture has also been used for assaying catalase inhibition by other phenolic compounds (Ogura et al., 1950) and 3-aminotriazole (Havir, 1992). Thus, soluble proteins were first preincubated for 1 h in a 100-μL mixture consisting of 20 mM citrate, pH 6.5, 0.05% Glc, 5 milliunits of Glc oxidase, 1 mM SA, and 5 to 50 μg of protein, depending on the tissues. A portion of the mixture (25 μL) was then withdrawn and added to the standard assay mixture containing 20 mM citrate, pH 6.5, 1 mM SA, and 10 mM H_2O_2 for immediate assay of catalase activity. For the control, catalase activity was determined in the absence of SA in the preincubation and assay solutions.

DNA Preparation and Southern Analysis

Rice genomic DNA was extracted from rice shoots by the nucleic acid extraction procedure using hexadecyltrimethylammonium bromide according to the method of Saghai-Maroof et al. (1984). For Southern analysis rice genomic DNA (10 μg) was digested with various restriction enzymes, separated on 0.8% agarose gels, and transferred to nylon membranes using standard procedures (Sambrook et al., 1989). The 810-bp *Xho*I-*Sal*I fragments of the rice *Cat*A cDNA clone and the 651-bp *Hin*dIII-*Hin*dIII fragment of the rice *Cat*B cDNA clone were labeled with [α-^{32}P]dCTP by random priming and used as probes. Hybridization was carried out in 6× SSC, 5× Denhardt's solution, and 10 μg mL^{-1} denatured salmon sperm DNA at 62°C for 24 h. The filters were washed with 0.5× SSC and 0.25% SDS at 62°C for 2 to 3 h.

RNA Preparation and Northern Analysis

Total RNA from rice shoots (blade and sheath), roots, and cell-suspension cultures were prepared according to the method of Hepburn et al. (1983) with slight modifications. Plant materials (1 g) were homogenized in 2 mL of extraction buffer containing 100 mM Tris-HCl, pH 8.4, 4 M urea, 6% *p*-aminobenzoic acid, 2% triisopropylnaphthalene sulfonic acid, 1% SDS, 2 mM EDTA, and 10 mM β-mercaptoethanol. RNA was precipitated with 4 M lithium chloride. For northern analysis total RNA (12 μg) was separated on agarose-formaldehyde gels and blotted to nylon membranes following standard procedures (Sambrook et al., 1989). Blots were hybridized with [α-^{32}P]dCTP-labeled gene-specific probes (the 3′ untranslated 340-bp *Bsm*I-*Not*I fragment of the *Cat*A gene and the 220-bp *Eae*I-*Xho*I fragment of the *Cat*B gene). Hybridization was carried out in 6× SSC, 5× Denhardt's solution, 0.5% SDS, and 50 μg mL^{-1} heparin at 65°C for 16 h. The filters were washed with 1× SSC and 0.1% SDS at 65°C for 2 to 3 h.

Immunoblot Analysis of Catalase Proteins

Soluble proteins from tobacco leaves and rice shoots, roots, and cell-suspension cultures were prepared by homogenizing in 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 12 mM β-mercaptoethanol, and 10 μg mL^{-1} PMSF. After the sample was clarified by centrifugation, the proteins were fractionated by SDS/PAGE on a 10% polyacrylamide gel, and the separated proteins were electrophoretically transferred to a nitrocellulose filter. Immunoblot analysis was performed as previously described with a 1:10,000 dilution of mouse monoclonal antibody 3B6, which was prepared against tobacco SA-binding catalases (Chen et al., 1993a).

RESULTS

SA Levels in Different Tissues

Raskin et al. (1990) and Silverman et al. (1995) first reported that rice shoots contain very high basal levels of free SA (7–30 μg g^{-1} fresh weight). These SA levels are substantially higher than even those found in tobacco tis-

sues undergoing hypersensitive cell death after infection by tobacco mosaic virus (6 $\mu g\ g^{-1}$ fresh weight in tissue 3.5 mm from the lesion center 120 h after inoculation; Enyedi et al., 1992). Furthermore, little or no change in SA levels in rice shoots was found following infections by either bacterial or fungal pathogens (Silverman et al., 1995). These features of SA biosynthesis in rice clearly differ from those found in tobacco, cucumber, or Arabidopsis and, therefore, would argue against a role for SA in disease resistance in rice. However, it was not known whether high basal levels of free SA occur in all rice tissues. To address this question, the basal free SA levels in rice shoots, roots, and cell-suspension cultures were assayed. It was found that, whereas rice shoots contained very high basal levels of SA, as previously reported, the roots and cell-suspension cultures had very low basal levels of SA, comparable to those found in tobacco (Table I). Thus, in rice extremely high basal levels of SA occur only in the photosynthesis-competent green shoots but not in the nongreen tissues such as roots and cell-suspension cultures.

SA Sensitivity of Catalase Activity

A previous survey of different plant species showed that total soluble protein extracts from green rice shoots contain little SA-binding activity or SA-sensitive catalase activity (Sanchez-Casas and Klessig, 1994). Since SA inhibition of catalases was originally believed to be simply caused by SA binding, the assays for catalase inhibition by SA were performed without preincubation with low levels of H_2O_2. In light of the finding that SA and other phenolic compounds inhibit catalases by serving as a one-electron donor for the peroxidatic activity of catalases (Durner and Klessig, 1996), assays for SA inhibition of catalases should include low levels of H_2O_2 in the preincubation mixture to mimic the in vivo conditions. With the modified procedure, however, we still could not detect any significant inhibition by SA of catalase activity from rice shoots (Table I). These results confirmed that the high level of SA is associated with SA-insensitive catalase activity in rice shoots.

Unlike the shoots, rice roots and cell-suspension cultures contain low levels of free SA (Table I). Furthermore, low levels of exogenously supplied SA (100 μM) have been found to protect rice cell-suspension cultures from chitosan-induced cell death and to induce extracellular accumulation of PR proteins (Masuta et al., 1991). To further examine the correlation between the levels of SA and the SA sensitivity of catalases, we analyzed the SA sensitivity of catalase from rice roots and cell-suspension cultures. In contrast to the shoots, catalase activity from these tissues was sensitive to SA (Table I). Several SA analogs with or without the biological activity for induction of PR gene expression and enhanced disease resistance in tobacco were also compared for their ability to inhibit the catalase activity from different rice tissues. The biologically active analogs were as effective as SA at inhibiting catalase from rice roots and cell-suspension cultures but, like SA, were unable to significantly inhibit the catalase prepared from the shoots (Table II). Biologically inactive analogs failed to inhibit catalases from any of the three tissues tested (Table II). Therefore, catalase from rice roots and cell-suspension cultures exhibited the same specificity to SA analogs as tobacco catalases.

In addition to the difference in SA sensitivity, there was a large variation in the specific activity of catalases among rice shoots, roots, and cell-suspension cultures. Rice shoots contained the highest specific activity and roots had about 50% of that found in the shoots, whereas cell-suspension cultures contained relatively low specific activity compared with that of the shoots and roots (Table I).

Catalase Gene Organization

Two cDNA clones of catalase genes (*Cat*A and *Cat*B) have been isolated from rice (Mori et al., 1992; Morita et al., 1994). The difference in SA sensitivity of catalase activity from different rice tissues prompted us to utilize these cloned genes to analyze (a) the size of the catalase gene family, (b) the spatial expression of catalase genes, and (c) the primary structures of catalase proteins in rice.

The size of catalase gene family in rice was assessed by Southern analysis of the genomic DNA cleaved with various restriction enzymes and probed under moderately stringent conditions with a DNA fragment from the cloned *Cat*A or *Cat*B gene. When probed with the *Cat*A gene probe, one or two bands were detected in the genomic DNA digested with these restriction enzymes (Fig. 1A). Similar results were recently reported from Southern analysis of

Table I. *SA levels and SA sensitivity of catalase activity in different rice tissues*

Plant Tissue	Free SA Level	Catalase Activity		Inhibition
		0 mM SA	1 mM SA	
	$\mu g\ g^{-1}$ *fresh wt*	$\mu mol\ O_2\ mg^{-1}$ *protein* min^{-1}		%
Rice				
Shoots	8.95 ± 1.82	15.2 ± 2.1	14.5 ± 1.5	5
Roots	0.07 ± 0.02	6.8 ± 1.2	3.1 ± 0.3	54
Cell-suspension cultures	0.06 ± 0.02	1.2 ± 0.2	0.5 ± 0.1	58
Tobacco leaves[a]	0.05 ± 0.01	2.2 ± 0.3	0.5 ± 0.1	78

[a] The relatively low specific catalase activity in tobacco leaves compared with rice shoots appears to be caused by the extensive cross-linking of proteins in tobacco leaf extracts as detected by immunoblot analysis with monoclonal antibodies prepared against tobacco catalases (Chen et al., 1993a; Sanchez-Casas and Klessig, 1994). No catalase protein cross-linking was detected in extracts prepared from rice shoots, roots, or cell-suspension cultures (data not shown).

Table II. *Specificity of SA sensitivity of catalase activity from different tissues of rice and tobacco*

Catalase Source	Inhibition by SA Analog (1 mM)			
	3-ChloroSA	3,5-DichloroSA	3-HydroxyBA[a]	4-HydroxyBA
	%			
Rice				
Shoots	4	3	2	5
Roots	60	65	6	4
Cell-suspension cultures	56	64	4	4
Tobacco leaves	78	85	5	4

[a] HydroxyBA, Hydroxybenzoic acid.

rice genomic DNA digested with a larger number of restriction enzymes (Higo and Higo, 1996). Detection of one band in the *Hin*dIII- or *Xho*I-digested DNA suggests that there is no closely related *Cat*A gene family member in the rice genome. Since there is no *Eco*RI or *Kpn*I site in the published genomic sequence of the rice *Cat*A gene (Higo and Higo, 1996), detection of two bands in the *Eco*RI- and *Kpn*I-digested DNA could indicate that there are two closely linked *Cat*A genes. We recently isolated several rice *Cat*A genomic clones and found that PCR reactions with *Cat*A-specific primers amplified DNA fragments of identical size from two different regions of the same clones (Z. Chen, unpublished results). Further studies will determine whether one or both of these two closely linked *Cat*A genes are functional. When probed with the *Cat*B gene probe, only one band was consistently detected in the genomic DNA digested with *Hin*dIII, *Kpn*I, and *Xho*I (Fig. 1B). This suggests that *Cat*B is present as a single copy and has no closely related family members. In *Eco*RI-digested DNA, in addition to the major band, a minor band was detected in one of the three blots performed to date. This minor band might have resulted from the star activity of *Eco*RI (Jen-Jacobson et al., 1983). The above results suggest that rice contains very few catalase genes. In fact, all 17 rice catalase cDNA clones in the GenBank database, which were randomly isolated from libraries prepared from various rice tissues (green or etiolated shoots, roots, immature seeds, and callus), can be identified as either the *Cat*A or the *Cat*B gene based on their complete or partial sequences and restriction maps (data not shown).

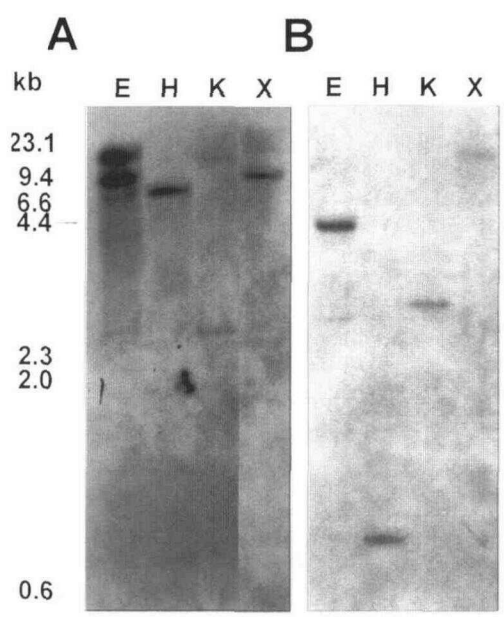

Figure 1. Southern analysis of rice *Cat*A and *Cat*B genes. Genomic DNA of rice shoots digested with *Eco*RI (E), *Hin*dIII (H), *Kpn*I (K), and *Xho*I (X) was hybridized with *Cat*A (A) or *Cat*B (B) gene probes. The *Hin*dIII-digested λDNAs were used as size standards.

Tissue-Specific Catalase Gene Expression

Northern blots with total RNA isolated from different rice tissues were probed with gene-specific probes prepared from the 3′ untranslated ends of the *Cat*A and *Cat*B genes. *Cat*A mRNA was abundant in the shoots (blades and sheath) but almost undetectable in the roots and cell-suspension cultures (Fig. 2A). In contrast, *Cat*B mRNA was present at very high levels in the roots (Fig. 2B). Expression of the *Cat*B gene was also detected at low levels in the shoots and cell-suspension cultures (Fig. 2B). This tissue-specific expression pattern of *Cat*A and *Cat*B is consistent with the frequency at which *Cat*A and *Cat*B cDNA clones have been isolated from libraries prepared from different rice tissues by the Rice Genome Research Program in Tsukuba, Japan. For example, the four catalase clones randomly isolated from a cDNA library prepared from green rice shoots (GenBank accession nos. D46892, D47443, D46901, and D46051) all correspond to the *Cat*A gene. On the other hand, the three clones isolated from a root library

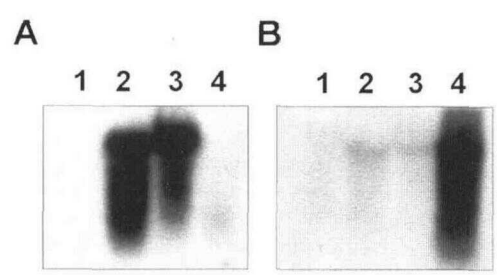

Figure 2. Northern analysis of rice *Cat*A and *Cat*B gene expression. Duplicate blots containing total RNA from cell-suspension cultures (lane 1), leaf blade (lane 2), sheath (lane 3), and roots (lane 4) were probed with gene-specific *Cat*A (A) or *Cat*B (B) probes. The same blots were also probed with an *S*-adenosylmethionine synthetase gene from rice (Van Breusegem et al., 1994) and showed comparable levels of expression among different rice tissues (data not shown).

(GenBank accession nos. D24852, D24082, and D24091) and the one clone from a callus library (GenBank accession no. D10425) all correspond to the *Cat*B gene. In addition, the levels of catalase transcripts were generally consistent with the specific catalase activity found in different rice tissues. Rice shoots and roots abundantly express *Cat*A and *Cat*B genes, respectively, and contain high levels of catalase activity. In contrast, the cell-suspension cultures express the *Cat*B gene at low levels and also exhibit low amounts of catalase activity (Table I; Fig. 2).

Tissue-Specific Distribution of Catalase Proteins

The difference in the SA sensitivity of catalases and the differential expression of catalase genes indicated that different rice tissues contain different catalase isozymes. To confirm this at the protein level, monoclonal antibody 3B6, which was made against tobacco SA-binding catalases (Chen et al., 1993a), was used to detect catalase proteins in the soluble extracts prepared from different rice tissues (Fig. 3). Three other monoclonal antibodies against tobacco catalases (1F5, 2C11, and 7F10; Chen et al., 1993a) did not recognize rice catalases (data not shown). Immunoblot analysis with 3B6 showed that the catalase protein from the shoots exhibited a slightly faster mobility on the gel than catalase from roots or cell-suspension cultures (Fig. 3, lanes 2–4). This difference was verified by analyzing the mixtures of protein extracts prepared from different combinations of rice tissues (Fig. 3, lanes 5–7). These results support the proposition that rice roots and cell-suspension cultures contain the same catalase isozyme, which is distinctive from the enzyme present in the shoots.

Primary Structures of Rice Catalase Proteins

An analysis of the differential expression of catalase genes and the SA sensitivity of catalases from various rice tissues argues that the SA-insensitive catalase in the green shoots is associated with the predominant expression of *Cat*A, whereas the SA-sensitive activity in the roots and cell-suspension cultures is related to the expression of *Cat*B. Thus, it is likely that the *Cat*A gene encodes an SA-insensitive catalase, whereas *Cat*B encodes an SA-sensitive isozyme. Computer analysis of the predicted amino acid sequences of these two isozymes revealed that they are quite different, having only 70% overall sequence identity (Table III). In contrast, catalase isozymes within a plant species, in general, are more similar (e.g. the two cotton catalase proteins share 96% sequence identity, whereas the three tobacco proteins are 77–87% identical; Ni et al., 1990; Table III). Furthermore, CatB protein is structurally more similar than CatA catalase to tobacco catalases (Table III), which are known to be SA-sensitive (Durner and Klessig, 1996). In fact, CatB protein shares higher percentages of sequence identity (or similarity) with all three tobacco catalases than with rice CatA protein (Table III).

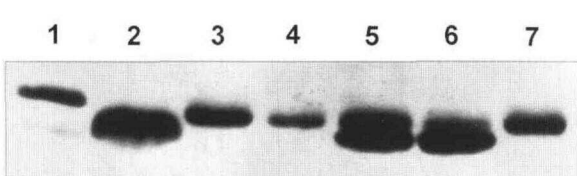

Figure 3. Immunoblot analysis of catalase proteins. Monoclonal antibody 3B6 recognized two catalase isoforms from tobacco leaves (lane 1), which correspond to tobacco Cat1 (higher-molecular-weight form) and Cat2 (lower-molecular-weight form) (Takahashi et al., 1997). The same antibody also detected catalase proteins in rice shoots (lane 2), roots (lane 3), and cell-suspension cultures (lane 4). Catalase from the shoots exhibited a slightly faster mobility than the protein from the roots or cell-suspension cultures. This difference was confirmed by analyzing the mixtures of proteins prepared from the following combinations of different rice tissues: shoots plus roots (lane 5), shoots plus cell-suspension cultures (lane 6), and roots plus cell-suspension cultures (lane 7).

DISCUSSION

Tissue-Specific Expression of Catalase Genes

Comparison of expression profiles of genes encoding different catalase isozymes from a number of plant species have allowed Willekens et al. (1995) to group plant catalases into three different classes. The first class is characterized by high levels of expression in photosynthetic leaf tissues, suggesting that they are involved in the degradation of H_2O_2 produced during photorespiration. This is supported by recent studies showing that antisense or cosuppressed transgenic tobacco plants with severely reduced expression of their *Cat*1 gene, which belongs to class I, exhibited necrotic lesions on their leaves, particularly under relatively high light intensities (Chamnongpol et al., 1996; Takahashi et al., 1997). The second class of catalase genes is expressed in mature plants, with the highest levels present in vascular tissues. The cellular function of this class is unclear. The third class of catalases is highly abundant in seeds and young seedlings and is believed to be involved in degrading H_2O_2 produced during fatty acid β-oxidation in glyoxysomes. According to this classification, rice CatA and CatB belong to class I and class II, respectively. Thus, expression of *Cat*A at high levels in the shoots is related to its presumed primary role of removing H_2O_2 produced during photorespiration. The low levels of *Cat*B expression in the shoots may be restricted to the vascular tissue, as has been shown for other class II catalase genes (e.g. *Cat*2 from *N. plumbaginfolia*, Willekens et al., 1994a, 1994b). Further experiments in rice using promoter-driven reporter gene expression or in situ hybridization will be necessary to provide a more refined picture of the spatial expression patterns of the *Cat*A and *Cat*B genes.

SA Levels and SA Sensitivity of Catalase Activity

Although SA has been widely regarded as an endogenous signal in plant defense responses, its role in induced disease resistance has been extensively characterized and clearly demonstrated in only a few plant species, including tobacco, Arabidopsis, and cucumber. Studies during the last several years of the basal SA levels and the effects of exogenously applied SA have revealed that there might be a substantial difference in SA responsiveness among dif-

Table III. *Amino acid sequence identity (similarity) of catalases from rice and tobacco*

	Rice		Tobacco		
	CatA	*CatB*	*Cat1*	*Cat2*	*Cat3*[a]
			%		
Rice					
CatA	100 (100)	70 (82)	73 (84)	69 (81)	71 (83)
CatB		100 (100)	81 (91)	77 (88)	82 (92)
Tobacco					
Cat1			100 (100)	77 (89)	87 (93)
Cat2				100 (100)	79 (90)
Cat3					100 (100)

[a] Cat3 amino acid sequence is from *N. plumbaginifolia* (Willekens et al. 1994b), since it is not available from *Nicotiana tabacum*. Catalase amino acid sequences were compared using the Genetics Computer Group software package (Version 7, April, 1991, Madison, WI).

ferent plant species. For example, in tobacco the basal levels of SA are very low (<0.05 $\mu g\ g^{-1}$ fresh weight) and only a small increase (1.2- to 4-fold to 0.06–0.2 $\mu g\ g^{-1}$ fresh weight) in the endogenous SA levels is associated with the induction of PR gene expression and the establishment of SAR (Enyedi et al., 1992; Malamy et al., 1992; Vernooij et al., 1994). However, in several plant species, including tomato, potato, and soybean, basal SA levels far exceed the elevated SA levels associated with SAR in tobacco (Raskin et al., 1990). Furthermore, in certain tobacco hybrids (Yalpani et al., 1993) and Arabidopsis mutants (Bowling et al., 1994; Greenberg et al., 1994; Weymann et al., 1995), stunted growth and development of necrotic lesions are associated with elevated SA levels. In contrast, in rice shoots the basal SA levels are at least several-hundred-fold higher than those found in tobacco and Arabidopsis, without apparent deleterious biological effects (Silverman et al., 1995).

A survey of different plant species revealed a large variation in the levels of SA-binding activity and SA sensitivity of catalase activity (Sanchez-Casas and Klessig, 1994). For example, it was found that rice shoots contain SA-insensitive catalase activity and little SA-binding activity (Sanchez-Casas and Klessig, 1994), which parallels the extremely high levels of SA in this tissue. Moreover, the present study showed that there are large differences in both the levels of SA and the SA sensitivity of catalases among different tissues in rice (Table I). Consistent with earlier reports, green rice shoots were found to contain extremely high levels of SA and have SA-insensitive catalase. By contrast, the rice roots and cell-suspension cultures had low levels of free SA and contained SA-sensitive catalase. Thus, there appears to be an inverse correlation between SA levels and the sensitivity of catalase to SA, not only among plant species but also in different rice tissues. This correlation supports the contention that plant SA-sensitive catalases play a role in certain aspects of SA-mediated responses.

It is worthwhile to note that recent kinetic and spectral analyses have indicated that SA inhibits catalases by serving as a one-electron-donating substrate for the peroxidatic activity of catalase and in so doing may be converted to a free radical (Durner and Klessig, 1996). Thus, the difference in SA sensitivity of catalases from different rice tissues may also reflect the difference in their ability to oxidize SA into SA radicals. Since free radicals can initiate chain reactions, the generation of even low levels of SA radicals may have significant biological effects. However, further studies are needed to prove the biological significance of SA radicals generated by catalases.

Structural Basis of SA Sensitivity of Catalases

One interesting finding from this study is the marked difference in SA sensitivity of catalases from different rice tissues (Table I). Since all heme/iron-containing catalases/peroxidases share an identical catalytic mechanism for their peroxidatic activity, it might be expected that they possess a similar chemical capability to use SA as one-electron-donating substrates and, therefore, should exhibit a similar sensitivity to the compound. However, the rate of the peroxidatic reaction may depend on the binding of the substrate to the enzyme. Therefore, the difference in SA sensitivity among different catalases/peroxidases might be accounted for by the difference in binding affinity of the enzymes for this phenolic compound. Indeed, we have observed a strict correlation between the binding affinity of tobacco catalases to a variety of SA analogs and the sensitivity of the enzymes to these compounds (Chen et al., 1993b; Conrath et al., 1995).

Several recent studies have indicated that steric constraint at the active sites may be a key factor limiting the peroxidatic activity of catalases and superoxide dismutases with aliphatic and aromatic substrates. For example, it has recently been shown that site-direct mutagenesis of the lower part of the major substrate channel of yeast catalase A leads to dramatic increases in peroxidatic activity for two-electron oxidation of aliphatic compounds and one-electron oxidation of aromatic compounds (Zamocky et al., 1995). In addition, certain mutations in the gene that encodes copper-zinc superoxide dismutase can enhance peroxidatic activity. This striking increase in peroxidatic activity is probably due to an increase in the openness of the active site, which allows greater access of substrates (Wiedau-Pazos et al., 1996). The enhanced peroxidatic activity of the mutant enzymes may lead to the oxidization of certain organic molecules in the cell to their free radical forms, which in turn might oxidize lipids and other cell components and, consequently, cause apoptotic death of

nerve cells (Marx, 1996; Wiedau-Pazos et al., 1996). Similarly, we have recently discovered that in tobacco cell cultures SA and its biologically active analogs cause lipid peroxidation and, at relatively high levels, induce cell death (Z. Chen, M.D. Anderson, and D.F. Klessig, unpublished results).

In summary, we have found that SA levels vary greatly in different rice tissues. This difference correlates with the difference in the SA sensitivity of their catalases, which results from the tissue-specific expression of two rice catalase genes, *Cat*A and *Cat*B. These unique features of rice with respect to the differential accumulation of SA and SA-sensitive catalases in different tissues may provide an attractive model system for further study of the relationship between SA responsiveness and the SA-binding properties of catalases.

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