Pergamon 0031-9422(95)00674-5 Phytochemistry, Vol. 41, No. 3, pp. 699–702, 1996

0031-9422/96 $15.00 + 0.00

DISTRIBUTION OF CATALASE ISOFORMS IN *NICOTIANA TABACUM*

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(*Received in revised form* 18 *July* 1995)

Key Word Index—*Nicotiana tabacum*; Solanaceae catalase; peroxidatic activity; enhanced-peroxidatic catalase; photorespiration.

Abstract—Total catalase activity per milligram protein averaged three-fold higher in leaves than in green stem or leaf midvein tissue of *Nicotiana tabacum*. The distribution of total activity between low-peroxidatic (LP-CAT) and enhanced-peroxidatic (EP-CAT) isoforms differed in the three tissues. In the leaf 85–90% of the total activity consisted of LP-CAT and EP-CAT was a minor form, whereas in the stem and midvein LP-CAT isoforms were less than 15% of the total and EP-CAT was the predominant isoform. The catalase mRNA levels were higher in the stem than in the leaves, which may result from the inability of the LP-CAT probe to distinguish between the two forms. The EP-CAT of stem was similar to EP-CAT of leaves in subunit size, specific activity, and in its ability to react with antibodies raised to leaf EP-CAT.

INTRODUCTION

Catalase (E.C. 1.11.1.6) converts hydrogen peroxide to oxygen and water. In C_3 plants, large amounts of H_2O_2 are produced by glycolate oxidase (E.C. 1.1.3.1.) during the photorespiration pathway. The role of catalase in regulating the production of photorespiratory CO_2 has been discussed [1].

In addition to the catalatic reaction ($2H_2O_2 \rightarrow O_2 + 2H_2O$), catalase can utilize H_2O_2 to oxidize organic substrates such as ethanol to acetaldehyde ($H_2O_2 + CH_3CH_2OH \rightarrow CH_3CHO + 2H_2O$). This second reaction represents the peroxidatic activity of catalase, and has been extensively investigated for mammalian catalase [2]. Previous work in this laboratory has shown the presence of multiple forms of catalase in leaf extracts of 21-day-old seedlings of *N. sylvestris* which were named, in the order of their elution from a chromatofocusing column by a pH gradient, CAT-1, CAT-2, and CAT-3 [3]. The ratio of peroxidatic to catalatic activity was similar to bovine liver catalase in CAT-1 and CAT-2, but the peroxidatic activity was enhanced about 10-fold in CAT-3 [3]. The CAT-1 and CAT-2 isoforms have been termed low-peroxidatic catalase (LP-CAT) and CAT-3 is called enhanced-peroxidatic catalase (EP-CAT). A form of catalase with enhanced peroxidatic activity has also been demonstrated in leaves of barley, maize, and spinach [4].

The LP-CAT accounts for 85–90% of the total catalatic activity in the mature leaf. In contrast, EP-CAT was not detected in seedlings until 15 days post-germination and increased to a maximum of 10–15% of the total at 21 days post-germination [3]. Another distinction between LP-CAT and EP-CAT is their response upon transfer of seedlings from air to 1% CO_2. The low-peroxidatic forms of catalase showed a 50% drop in activity in 12 h, whereas EP-CAT increased 3-fold in activity over a four day period [5]. In addition, LP-CAT differs from EP-CAT in subunit size (55000 *versus* 53000), and there is no cross reactivity against antibodies raised independently against EP-CAT and LP-CAT [6].

Taken together, the differences in biochemical and physiological properties suggest different roles for the tobacco catalase isoforms. The role of LP-CAT in photorespiration is clear, but the biochemical role of EP-CAT is uncertain although some functions have been proposed. For example, the catalase isoform found in nodes, internodes and stems of maize was the CAT-3 isozyme [7], the same catalase form we showed had enhanced-peroxidatic activity [4], leading to the suggestion that CAT-3 is involved in lignification [7]. More recently one of the three catalase genes investigated in *N. plumbaginifolia* by RNA hybridization was shown to be prevalent in leaf vascular tissue, again suggesting a role in lignification [8], but the biochemical characterization of the isozymes of catalase was not investigated. A study of the effect of liver catalase on the oxidation of coniferyl alcohol by tobacco xylem cell walls concluded that a peroxidatic reaction was not involved [9]. However, an isozyme of catalase showing enhanced-peroxidatic activity was not tested. It is also possible that the primary role

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of EP-CAT is the protection against H_2O_2 produced during lignification [10]. It has also been proposed that the CAT-3 isozyme in maize plays a special role during oxidative stress when H_2O_2 levels are elevated [11].

During a study of catalase activity and steady-state levels of catalase mRNA in tissues from different organs it was observed that the catalase specific activity was higher in leaves than in stem although the levels of mRNA were higher in stem than in leaves. This led to an investigation of the distribution of LP-CAT and EP-CAT isoforms in these tissues.

RESULTS AND DISCUSSION

Comparison of total catalase activity and mRNA levels

Table 1 shows results of a number of typical experiments during a ten month period in which leaf, stem and midvein tissues were assayed for total catalase activity. On average, total units per gram fresh weight were at least 23-fold higher in leaves than in the stem or midvein. Expressed as total units per milligram protein, activity in the leaf was about 3-fold higher than in the stem or midvein. Catalase activity may vary with growth conditions such as light and temperature, but specific activities were consistently higher in the leaf than in the stem. This is not surprising since green stem tissue has fewer chloroplasts and catalase-rich peroxisomes than leaves.

To determine whether the level of catalase activity in various tissues corresponds to the steady state level of mRNA, total RNA was extracted at the same time catalase activity was measured in nearby tissue from the same plant. Catalase activities in the leaf, stem, and green sepal were 3.05, 0.25, and 1.31 μkat mg^{-1} protein, respectively. Northern analysis was performed using radiolabeled CAT-1 (i.e. LP-CAT) clones isolated from cDNA leaf libraries of *N. sylvestris* [12] or *N. tabacum* [13]. Both probes recognized one band at about 1.8 kb corresponding to the predicted subunit size of 55000. Fig. 1 shows that the steady-state levels of LP-CAT mRNA were higher in stem and sepal than in the leaf, although catalase activity was highest in the leaf. Similar results were obtained with the catalase probe whether young or mature leaves were used. However, there was about a ten-fold higher level of LP-CAT mRNA transcripts in woody stems compared to young axillary non-woody stems (data not shown). As expected, the mRNA levels for ribulose bisphosphate carboxylase small subunit were higher in leaf than in stem (Fig. 1).

In maize, a positive correlation between the accumulation of CAT-2 and CAT-3 isozymes and their transcripts was observed in scutellum, root, epicotyl, and leaf [14]. However, the correlation did not always hold and it was shown using gene-specific probes [7] that CAT-1 mRNA was present at all stages of development of the stem even when no CAT-1 protein could be detected. In our studies we found little correlation between catalase activity and the steady-state level of LP-CAT mRNA in different organs.

Table 1. Catalase activity in leaves, stems and leaf midveins of *N. tabacum*

Tissue*	Mean Activity (μkat g^{-1} fr. wt)	Mean Activity (μkat mg^{-1} protein)
Leaf	45.8 ± 17.5† (21.7–54.5)	3.99 ± 1.0 (3.35–5.60)
Stem	1.95 ± 0.7 (0.97–2.56)	1.22 ± 0.4 (0.78–1.68)
Midvein	1.75 ± 0.2 (1.62–1.88)	1.53 ± 0.1 (1.43–1.62)

*Leaf samples (6–12 g), outer stem tissue (19–30 g), and leaf midveins (28–30 g) were taken from mature plants grown in a greenhouse.

†The range of catalase activities are shown in parentheses. n = 7, 5, and 2 for leaf, stem, and midvein, respectively.

Distribution and characterization of catalase isoforms in leaf and stem

Previously we separated by isoelectric focusing but did not characterize the isoforms of catalase present in tobacco leaf extracts [15]. The separation by chromatofocusing of catalase isoforms present in extracts of mature tobacco leaves is shown in Fig. 2A. The peroxidatic as well as catalatic activities were determined in each of the fractions eluting from the column. The enzyme forms eluting from the column between pH 7.7 and about pH 6.3 were CAT-1 and CAT-2, namely the low-peroxidatic forms of catalase. These forms are clearly separated from catalase with enhanced-peroxidatic activity (shaded area) which eluted at about pH 6.0. The low-peroxidatic forms accounted for 88% of the total catalatic activity in the leaves. Similar distribution patterns have been obtained with extracts from leaves of 21-day-old seedlings of *N. sylvestris* (data not shown).

The separation by chromatofocusing of catalase in stem extracts is shown in Fig. 2B. The catalatic activity

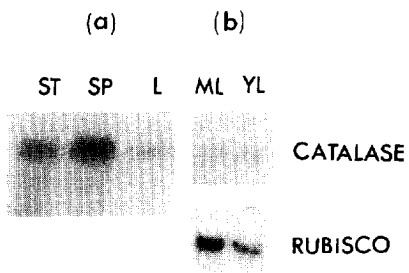

Fig. 1. Steady-state levels of mRNA in leaves and stems of *N. tabacum*. (a) Northern blots of total RNA (15 μg per lane) from stem (ST), sepals (SP) and leaves (L) were hybridized with radiolabelled cDNA of tobacco catalase. (b) Northern blots of total RNA (20 μg per lane) isolated from young (YL) and mature (ML) leaves were hybridized with radiolabelled cDNA of tobacco catalase and the cDNA of Rubisco small subunit (SSu).

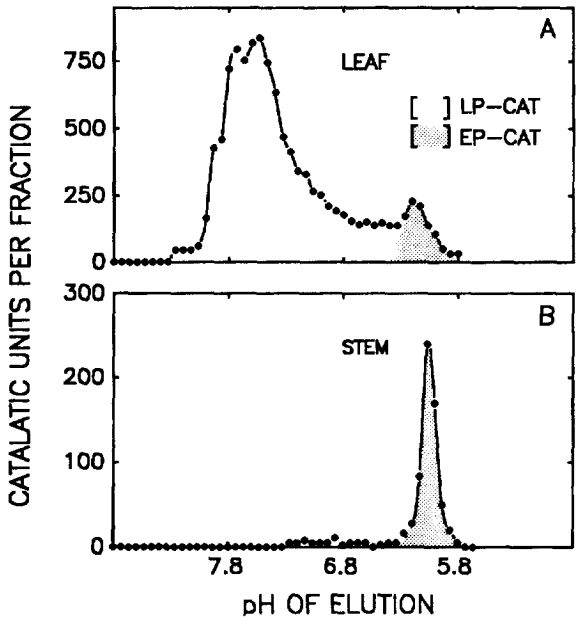

Fig. 2. Separation by chromatofocusing of catalase forms in leaves and stems of *N. tabacum*. For leaves and stems 266 μkat (9.0 g tissue), and 27.5 μkat (18 g tissue) respectively, were chromatofocused from pH 8 to pH 5.

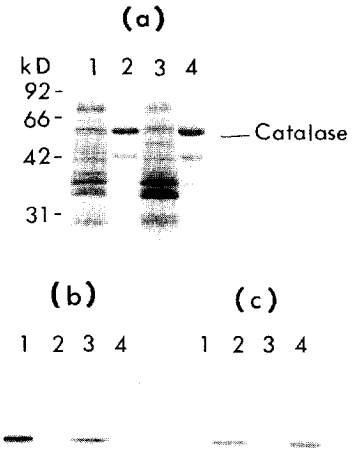

Fig. 3. SDS-PAGE and western blot analysis of leaf and stem catalase previously separated by chromatofocusing. Fractions from chromatofocusing columns were combined into either LP- or EP-CAT fractions as shown in Fig. 2, concentrated and assayed for catalase activity. (a) Sample containing 0.4 μkat of LP- and EP-CAT from leaf (lanes 1 and 2), and LP- and EP-CAT from stem (lanes 3 and 4), added to each lane. After electrophoresis, the gel was stained with Coomassie Blue. For protein blot analysis, total protein applied to lanes 1–4 (in the same order as above) was 5.2, 4.8, 4.8, 5.2 μg, respectively (b, c). After transfer, one membrane was probed with antibodies to LP-CAT (b) and the other to antibody to EP-CAT (c).

profile was characterized by a prominent peak at about pH 6.0 which, based upon determination of the peroxidatic/catalatic activity, is similar to the EP-CAT isoform of leaves. It accounted for 85% of the total activity in stem, whereas in leaves it represented only 12%. The distribution of catalase isoforms in leaf midvein was similar to stem tissue. In sepals, EP-CAT was intermediate to those for stem versus leaf, i.e. 31% of the total (data not shown). This result is not surprising since sepal tissue is leaf-like and vascular tissue was not removed before grinding.

In order to confirm the biochemical identification of the major catalase species in stem, samples of low- and enhanced-peroxidatic catalase from leaf and stem were analyzed by SDS-PAGE and Western blots (Fig. 3). The specific catalatic activity on a protein basis of LP-CAT from leaves of *N. sylvestris* was ten times greater than EP-CAT (910 μkat *versus* 92 μkat per mg protein, respectively [6]). The same relationship also held true for LP-CAT and EP-CAT from stem tissue. The two samples of EP-CAT from the different tissues were similar in subunit size and slightly smaller than LP-CAT (55 300 *versus* 53 300) as was previously observed in leaves [6].

Samples of LP-CAT and EP-CAT from stem were reacted against antibodies produced from LP-CAT and EP-CAT of leaves, and the results are shown in Fig. 3B and 3C, respectively. Stem and leaf LP-CAT showed cross-reactivity as did stem and leaf EP-CAT. However LP-CAT protein did not react with antibodies to EP-CAT nor did EP-CAT protein cross react with LP-CAT antibodies.

These distribution studies indicate that the lack of correlation between mRNA levels and catalase activity might be explained by an increased amount of the low specific activity form (EP-CAT) in the stem and the inability of the catalase probe to discriminate between LP- and EP-CAT, which probably have similar DNA sequences. This view is supported by the high sequence homology found in catalase genes isolated from different plant species and in *Nicotiana* [8, 13]. However, a role for post-transcriptional regulation cannot be ruled out, as has been shown for catalase in maize [11, 16] and cottonseed [17].

EXPERIMENTAL

Plant material. Tobacco plants, *Nicotiana tabacum* cv. Havana Seed, were grown in a greenhouse in commercial potting mixtures and watered periodically with a complete nutrient solution.

Enzyme assays and definition of units. Catalatic activity was assayed by measuring the initial rate of disappearance of H_2O_2 at 240 nm as previously described [3]. One μkat of activity is defined as the amount of enzyme catalyzing the disappearance of one μmol H_2O_2 per sec under standard conditions.

Peroxidatic activity of catalase was measured by monitoring the appearance of acetaldehyde from ethanol utilizing H_2O_2 generated by glucose oxidase [2]. The concn of the dinitrophenylhydrazone derivative of acetaldehyde was determined by HPLC [18]. The reaction

mixture (0.1 ml total volume) consisted of enzyme (20–80 units for low-peroxidatic forms, 1–8 units for enhanced-peroxidatic forms), buffer (10 μmol K phosphate, pH 7.2), glucose (0.9 μmol), glucose oxidase (0.1 munit) and ethanol (10 μl, 95%) added at time zero. At specified time intervals (between 5 and 15 min), 100 μl of 2,4-dinitrophenylhydrazine (0.1% in 2N HCl) was added. The derivative was extracted twice into 0.5 ml portions of chloroform and then evapd to dryness under vacuum. The residue was dissolved in 200 μl acetonitrile. An aliquot, usually 10 μl, was injected into an HPLC for separation on a Spherisorb column (S5ODS2, 4.6 mm × 25 cm) using acetonitrile–H_2O (60:40) as the eluate. A standard curve for peak area vs μmol acetaldehyde was obtained with known amounts of acetaldehyde treated as above. One μkat of peroxidatic activity is defined as the amount of enzyme catalyzing the formation of 1 μmol acetaldehyde per sec.

Preparation of extracts and chromatofocusing. When sampling tobacco leaves, leaf discs or leaf sections were taken from mature leaves ($> 15 \times 25$ cm) avoiding the largest veins. For stem material, samples from the outer stem between mature leaves (consisting of epidermis, cortex and vascular tissue) were collected with a vegetable peeler. The samples were weighed, immediately frozen in liquid N_2, and ground in a mortar. The extraction and preparation of samples for chromatofocusing were as previously described [6]. Chromatofocusing was carried out with a pH gradient from 8 to 5 on a 1.2×21 cm column of PBE 94 polybuffer exchanger (Pharmacia). Fractions for electrophoretic analysis were combined and concentrated on Centricon-10 concentrators (Amicon Corp.).

SDS-PAGE and protein blotting. Conditions for SDS-PAGE employed the Mighty Small apparatus (Hoefer Scientific Instruments), and protein transfer was to a PVDF (Millipore) membrane under conditions previously described [6]. For Western blots, membranes were probed with mouse antibodies prepared against tobacco leaf catalases previously designated CAT-1 (LP-CAT) or CAT-3 (EP-CAT) [6].

RNA analysis. Samples of leaf and stem were collected, frozen in liquid nitrogen and stored at −70°C until analysed. Total RNA was isolated from frozen material using the guanidine thiocyanate method [19], and separated by electrophoresis in 1% agarose gel containing 6.5% formaldehyde before transfer to a nylon membrane (Nytran, Schleicher and Schuell, 0.45 μm) [20]. A parallel gel stained with ethidium bromide and a subsequent hybridization of the blot with a Rubisco small subunit probe served as an internal control for the quantity and quality of RNA. Blots were prehybridized for 1–2 h at 65°C in 0.5 M phosphate buffer (pH 7.2) containing 1% BSA, 7% SDS, 1 mM EDTA [21]. Hybridization of the membrane was performed by adding ^{32}P-labelled cDNA [22] encoding either a partial clone of LP-CAT from *N. sylvestris* [12] or a full-length LP-CAT clone from *N. tabacum* [13], or for the constitutive Rubisco small subunit gene using the same buffer. Filters were washed in several changes of 1× SSC and 1% SDS at room temperature and exposed to X-ray film.

Acknowledgments—We wish to thank Dr Neil Schultes for helpful discussions, Carol Clark for skillful technical assistance, and Arthur Rickel for photographic assistance. Supported in part by the U.S.D.A. National Research Initiative Competitive Grants Office, Grant No. 9201544 to I.Z.

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