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Differential expression of catalase genes in *Nicotiana plumbaginifolia* (L.)

(circadian rhythm/glyoxysomes/oxidative stress/peroxisomes/photorespiration)

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ABSTRACT **We have analyzed the expression of three catalase (Cat; EC 1.11.1.6) genes from *Nicotiana plumbaginifolia* by means of RNA blot and *in situ* hybridizations. Our data demonstrate that the expression of each catalase is associated with a particular H_2O_2-producing process. Cat1 appears to be specifically involved in the scavenging of photorespiratory H_2O_2 and is under control of a circadian rhythm, Cat2 is uniformly expressed in different organs with a cellular preference for vascular tissues, and the expression profile of Cat3 points to a role in glyoxysomal processes. Differential expression of these catalases is also manifested in response to temperature changes. DNA sequence comparison with other dicotyledonous catalases led to the identification of at least three distinct classes, which indicates that the functional organization of catalases is generally conserved in dicotyledonous plants.**

In plants, four different types of peroxisomes have been identified, each type being characterized by their enzymic constituents (1). Leaf peroxisomes contain enzymes involved in photorespiration, such as glycolate oxidase (GO) (2). Glyoxysomes are typified by the presence of enzymes that participate in the degradation of lipids, the most commonly used marker enzymes being malate synthase (MS) and isocitrate lyase (IL) (3). Peroxisomes that lack the enzymes of the glycolate or glyoxylate cycle are referred to as unspecialized peroxisomes. Nodules of leguminous plants may develop an additional type of peroxisome for the production of ureides (1).

Different types of peroxisomes have some enzyme activities in common. The best known example is catalase (EC 1.11.1.6), which removes the H_2O_2 that is produced during various peroxisomal processes (1). In plants, several isoforms of catalase can generally be distinguished, which suggests that catalases are present as a gene family (4). However, it is currently unknown whether the number of catalases is related to the different types of peroxisomes. Studies on maize (4) and cotton (5) have shown that catalases are differentially expressed and are regulated by spatial, developmental, and environmental factors.

Several lines of evidence show that catalases may play a crucial role in plant defense mechanisms. In general, the importance of catalase could reside both in its direct antioxidant activity and its ability to affect signal transduction pathways that entail H_2O_2 as a messenger. For example, catalase levels may in part determine cold sensitivity in maize (6), whereas salicylic acid was shown to specifically bind and inhibit catalase activity, thus suggesting a role for catalase in the plant signal transduction cascade during plant–pathogen interactions (7). To contribute to a better understanding of the role of each catalase in plants, we have performed a detailed analysis on catalase expression in *Nicotiana plumbaginifolia*. Previously, we isolated three different catalase (Cat1, Cat2, and Cat3) cDNAs from *N. plumbaginifolia* that are highly similar in sequence and that all contain a putative targeting sequence for peroxisomes (30). This study addresses the role of each catalase by studying its differential regulation during development and stress conditions. We have focused on alterations in mRNA levels, because the formation of heterotetramers complicates the analysis of catalases at the protein level (8).

MATERIALS AND METHODS

RNA Extraction and Hybridization. RNA from frozen tissue was extracted as described by Logemann *et al.* (9) and analyzed by gel blot and slot blot hybridization using 10 μg and 5 μg of total RNA, respectively. Quantification, blotting, and hybridization were performed according to standard procedures. GO, MS, and IL transcript levels were determined with a GO cDNA from spinach (10) and MS and IL cDNAs from *Brassica* (11) as probes. Because of its low abundance in leaves, autoradiographic exposure times for Cat3, IL, and MS were generally much longer than for Cat1, Cat2, and GO.

Plant Material and Cultivation Conditions. Soil-grown, mature *N. plumbaginifolia* plants were used. For determination of the organ specificity and for analysis of the effect of the different stress and light treatments, plants were precultivated in a greenhouse and, 3 days before experiments, transferred to controlled environment cabinets (Weiss Technik, Lindenstruth, Germany) under photosynthetically active radiation (400–700 nm) at 150 μmol/(m^2·s), 60–70% humidity, and 22°C. Samples were always taken in the light period at least 2 hr after the beginning and 2 hr before the end of the illumination period and consisted of a pool of four leaves from different plants. The cold shock of 6°C and the heat shock of 37°C were applied 2 hr after the beginning of the photoperiod.

Detailed analysis of the circadian rhythm and fumigation with enriched quantities of CO_2 was carried out in Oberschleissheim. *N. plumbaginifolia* plants were cultivated in controlled environment cabinets (Heræus, Balingen, Germany) with a 12-hr light [150 μmol/(m^2·s), 6 a.m. to 6 p.m., 25°C]/12-hr dark (20°C) cycle, 60–70% humidity, and 370 ± 2 ppm CO_2. Two-month-old plants were transferred into walk-in "EPOKA" chambers (12) and were acclimated for 3 days to higher light intensities. Photosynthetically active

Abbreviations: GO, glycolate oxidase; IL, isocitrate lyase; MS, malate synthase.

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radiation (400–700 nm) was about 1020 μmol/(m²·s) at the top of the canopy level; light increased between 6 a.m. and 7 a.m., and decreased between 5 p.m. and 6 p.m.

To follow the circadian regulation of the various catalase transcripts, four different light/dark temperature regimes were applied to the plants: (*i*) regime 1, 12-hr light (25°C)/dark (20°C) periods; (*ii*) regime 2, 12-hr light/dark periods at constant temperature of 23°C; (*iii*) regime 2 followed by a constant illumination period of 60 hr; and (*iv*) regime 2 followed by an inverse 12-hr light/dark regime for 3 days.

Plants, precultivated as described above, were exposed for three consecutive days to 790 ± 2 ppm CO_2. After this fumigation period, untreated plants were added into the chamber and for the next 48 hr, the CO_2 concentration was increased to 1820 ± 5 ppm. Leaf material was collected each day at the same time (noon).

***In Situ* Hybridizations.** Slides were made from leaf, stem, and root material of *N. plumbaginifolia* and hybridized with ^{35}S-labeled antisense RNA probes for Cat1 and Cat2, essentially as described (13). Duplicate sections were hybridized with the sense RNA probes.

Sequence Comparison. Sequence similarity was determined according to the Program Manual for the Genetics Computer Group (Madison, WI) package, version 7 (April 1991), and PAUP version 2.4.1 (29).

RESULTS AND DISCUSSION

Accumulation of Catalase mRNAs in Various Organs. Total mRNA from root, stem, total flower, young leaf, and old leaf of mature *N. plumbaginifolia* plants was isolated and used in gene-specific RNA gel blot hybridizations with fragments of each of the three catalases. As shown in Fig. 1, Cat1 mRNA levels are most abundant in leaves, whereas lower levels can also be detected in flower and stem, but not in root. Cat2 is quite constitutively expressed, with highest levels in stem. Cat3 mRNA is equally expressed in root, stem, and young leaf but accumulates to high levels in flowers.

The accumulation of Cat3 transcripts in flowers points to a possible correlation with glyoxysomal activity. In senescing leaves as well as in cotyledons, peroxisomes were shown to be converted to glyoxysomes, presumably reflecting a switch from photosynthesis to lipid degradation for energy supply (14). These glyoxysomes have also been identified in mature and senescing petals (14, 15).

To further investigate a possible relation between Cat3 expression and glyoxysomes, mRNA abundance of the three catalases and of glyoxysomal marker proteins was determined in various flower organs (Fig. 2*A*). Cat3 mRNA accumulates to similar levels in pistil, stamen, sepal, and half-grown petals but is 4-fold higher in mature and senescing petals; MS and IL display a similar but less pronounced expression pattern. Cat1 and Cat2 mRNA levels in flower organs show no correlation with those encoding the glyoxysomal marker proteins. The low levels of both Cat3 and MS (3) mRNA in leaves, particularly when compared with seeds (Fig. 2*B*), give additional evidence that the main role of Cat3 resides in the removal of glyoxysomal H_2O_2.

Tissue-Specific Expression of Cat1 and Cat2 in Leaf. In photosynthetic tissues, catalase activity is involved in the scavenging of H_2O_2 that is produced during photorespiration in peroxisomes (1). Both Cat1 and Cat2 mRNAs are highly abundant in leaves; to establish whether they accumulate to varying levels in different tissues of the leaf, *in situ* hybridizations were performed under stringent conditions which allow the specific detection of Cat1 and Cat2 mRNAs. As shown in Fig. 3, Cat1 mRNA levels are highest in palisade parenchyma cells; in contrast, Cat2 mRNA is mainly detected in the vascular tissue. These data show that the highest levels of Cat1 mRNA are localized in the cells with the highest photosynthetic activity. Therefore, it appears that Cat1 is specifically involved in the scavenging of photorespiratory H_2O_2. This is in accordance with the expression of Cat1, which, with the exception of senescing petals, is restricted to green organs (Figs. 1 and 2). The latter is not necessarily in contradiction with this function, since the mRNA encoding the small subunit of ribulose-1,5-bisphosphate carboxylase was shown also to accumulate in petals (16).

Effect of Altered Light/Dark Conditions on Catalase Expression. If expression of Cat1 closely reflects photorespiratory activity, alterations in the normal light/dark regime may affect Cat1 mRNA levels. The effect of 3 days of dark treatment on catalase mRNA abundance is represented in Fig. 4*A*. Cat1 mRNA levels show a sharp decline within 24 hr of dark treatment, and normal levels are restored when the plant is returned to light. In contrast, Cat2 and Cat3 mRNAs increase during a prolonged dark period, and this induction continues for at least 24 hr during the following light period. Changes in both Cat1 and Cat3 can be explained in view of a reduction in photosynthesis and an increase in lipid degradation in the dark.

We investigated whether Cat1 would also display a circadian rhythm. The presence of a circadian rhythm in Cat1 expression appeared to be very dependent on the light conditions, a phenomenon also seen in the opening of the

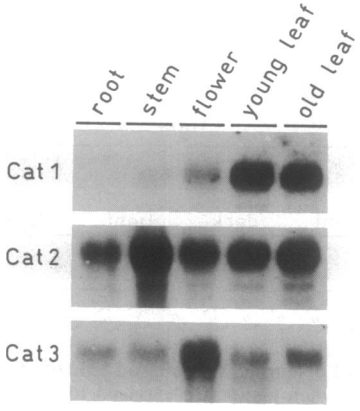

FIG. 1. Accumulation of Cat1, Cat2, and Cat3 transcripts in various organs.

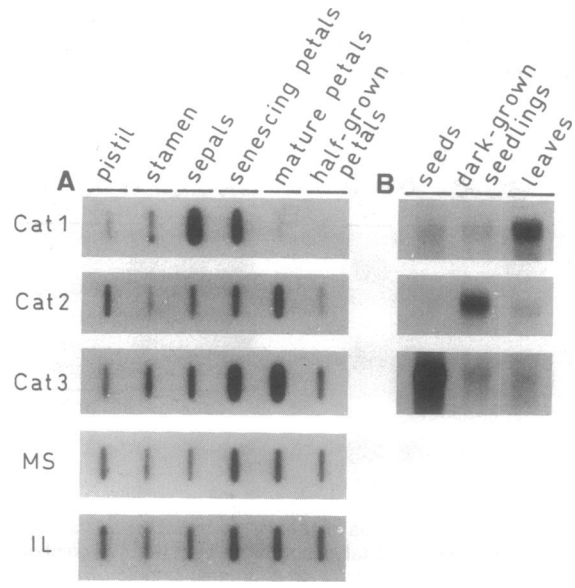

FIG. 2. mRNA abundance of three catalase isozymes, MS, and IL in various flower organs (*A*) and during development (*B*).

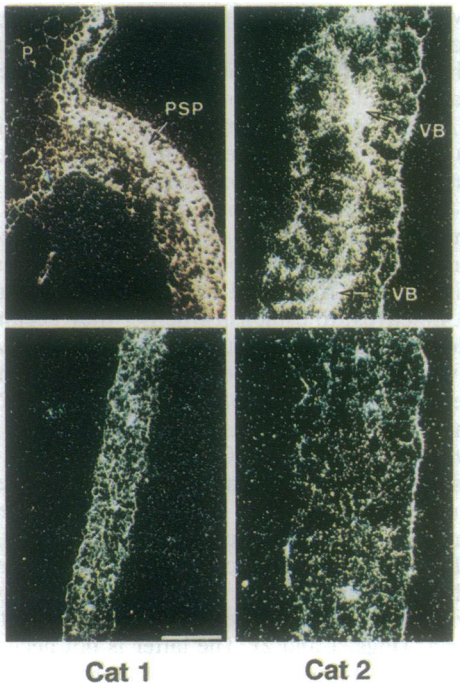

FIG. 3. (*Upper*) Cross sections of leaf hybridized with antisense RNA probes specific for Cat1 (*Left*) and Cat2 (*Right*). (*Lower*) Control hybridizations with the sense RNA probes. P, petiole; PSP, palisade parenchyma; VB, vascular bundle. (Bar = 100 μm for sections hybridized with the Cat2 probe and 260 μm for sections hybridized with the Cat1 probe.)

stomata (17). Under moderate light conditions [≈150 μmol/(m^2·s)] that are characteristic for many growth chambers, Cat1 mRNA levels display no changes between light and dark periods (data not shown). However, when plants are cultivated first in an outside greenhouse in an approximate 12-hr light/12-hr dark rhythm and subsequently exposed for 3 days to growth chamber conditions, daily variations in Cat1 mRNA accumulation occur that closely correlate with the light/dark periods and which persist for at least another 5 days (Fig. 4*B Upper*). Inversion of the light/dark regime causes an induction of Cat1 during the dark period at the time of normal light appearance (Fig. 4*B Lower*), which shows that the daily rhythm is under control of a circadian clock. This dark induction is, however, only transient and disappears within 4 hr in the dark.

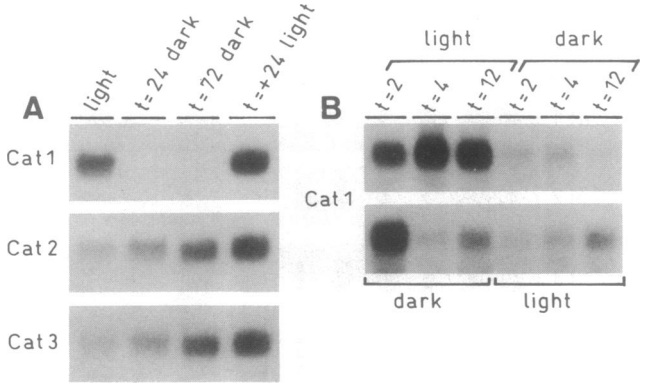

FIG. 4. (*A*) Effect of 3 days of dark treatment on catalase mRNA abundance. Samples were taken before the light was switched off and after 24 and 72 hr of darkness. An additional sample (+) was taken 24 hr after transfer to a normal light/dark regime. (*B*) Cat1 mRNA accumulation during normal (*Upper*) and inverse (*Lower*) light/dark cycles.

To establish the time period required for adaptation to the inverse light/dark regime, plants were exposed to high light irradiation [≈1020 μmol/(m^2·s)] in special environmental chambers. Also under these conditions, a daily rhythm in Cat1 expression is observed, but it is slightly shifted with regard to the time of induction and repression (Fig. 5, *Top*). The reason for this shift is not known, but the increase in Cat1 mRNA levels before the onset of light may be required to ensure sufficient Cat1 enzyme during the first hours of high light; the decrease before dark could be associated with the reduction in light intensity at the end of the day. Temperature changes that go with the light/dark cycle do not influence the circadian rhythm (data not shown) and were omitted in the following experiments with altered light/dark regime.

During the first day of inverse light/dark cycle, Cat1 expression follows accurately the initial/imprinted rhythm, thus demonstrating the control of Cat1 expression by a circadian clock (Fig. 5 *Bottom*). Adaptation occurs during the dark period of the second day, and the new rhythm was installed after 36 hr. Remarkably, when a normal light/dark cycle is altered into continuous light, the circadian rhythm is lost much more rapidly, and Cat1 mRNA levels start to oscillate without a clear pattern (Fig. 5 *Middle*). Cat2 and Cat3 showed no circadian rhythm under any of these conditions (data not shown).

Catalase Accumulation in Response to Increased CO_2. The ratio of photosynthetic carbon reduction versus photorespiration is determined by the competition between CO_2 and O_2 for the ribulose-1,5-bisphosphate carboxylase and oxygenase activities. Leaf internal CO_2 concentration is rate limiting for the carboxylase activity; therefore, photorespiratory activity can be reduced by increasing the atmospheric CO_2 concentration. Catalase expression in conditions of reduced respiratory activity was investigated by exposing plants, precultivated in ambient CO_2 (370 ppm), to 790 (Fig. 6*A*), and 1820 (Fig. 6*C*) ppm CO_2. The effect of 1820 ppm CO_2 on plants preexposed to 790 ppm was also analyzed (Fig. 6*B*). To ensure that CO_2 was rate limiting, experiments were performed under high light [1020 μmol/(m^2·s)]. The leaf internal CO_2 concentration, calculated from the leaf surface gas exchange, was 306, 504, and 1567 ppm at external CO_2 levels of 370, 790, and 1820 ppm, respectively. GO expression is markedly repressed by 1820 ppm CO_2 but not by 790 ppm CO_2, and the decline in GO mRNA levels is attenuated when plants have been preadapted to 790 ppm CO_2, prior to exposure to 1820 ppm CO_2 (Fig. 6). The expression pattern of all three catalases is similar to that of GO. The only difference is that repression of Cat2 is not influenced by preexposure to 790 ppm CO_2. Because MS displays an expression pattern similar to that of GO (data not shown), it is concluded that the different catalases cannot be functionally discriminated by a 2- to 5-fold increase of CO_2 levels.

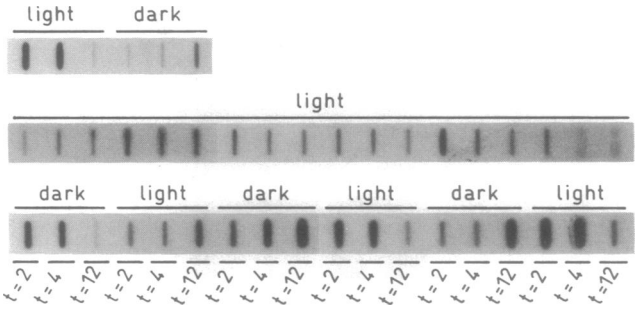

FIG. 5. Effect of normal and altered light/dark cycles on Cat1 transcript levels when plants are exposed to high light irradiation [1020 μmol/(m^2·s)] in environmental cabinets. Conditions: 12 hr light/12 hr dark (23°C) (*Top*); continuous light (*Middle*); inversion of the light/dark regime (*Bottom*).

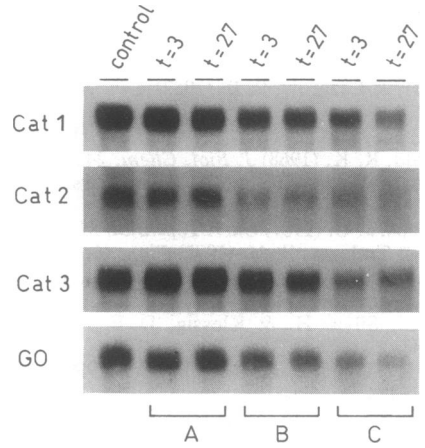

FIG. 6. Effect of elevated CO_2 levels on catalase and GO expression: 790 ± 2 ppm CO_2 (sampling after 3 and 27 hr) (*A*), 1820 ± 5 ppm CO_2 after a 3-day preexposure in 790 ± 2 ppm CO_2 (sampling after 3 and 27 hr) (*B*), and 1820 ± 5 ppm CO_2 (sampling after 3 and 27 hr) (*C*).

Response of Catalases to Temperature. The response of catalases to temperature changes illustrates that the differential expression profile is also manifested during stress conditions. Cat2 and Cat3 are not significantly affected by cold treatment (6°C, 24 hr) but are slightly induced during the recovery period (Fig. 7*A*). In contrast, Cat1 mRNA levels drop drastically within 2 hr at 6°C but are restored to 50% of control after 24 hr at 6°C. The repression of Cat1 is presumably the consequence of a loss of ribulose-1,5-bisphosphate carboxylase activity—and, therefore, of photorespiratory carbon oxidation—that has been observed at low temperature (18). The partial recovery of Cat1 mRNA accumulation after 24 hr could be associated with the synthesis of a new, cold-tolerant isoform of ribulose-1,5-bisphosphate carboxylase, as was reported in rye (19).

Heat shock (37°C, 5 hr) causes a rapid decline in Cat2 mRNA abundance, but initial levels are nearly restored within 2 hr of recovery (Fig. 7*B*). Cat1 mRNA levels decrease in response to heat shock, but the time course of repression is longer than with cold treatment. At high temperature, photosystem II-driven electron transport is inactivated, which may lead to a decline in ribulose-1,5-bisphosphate regeneration and, hence, to a reduction in photorespiratory activity (18). The relatively slow alterations in Cat1 mRNA levels during heat shock and subsequent recovery are in accordance with such an indirect mechanism of inactivation. Cat3 mRNA levels are again not significantly affected.

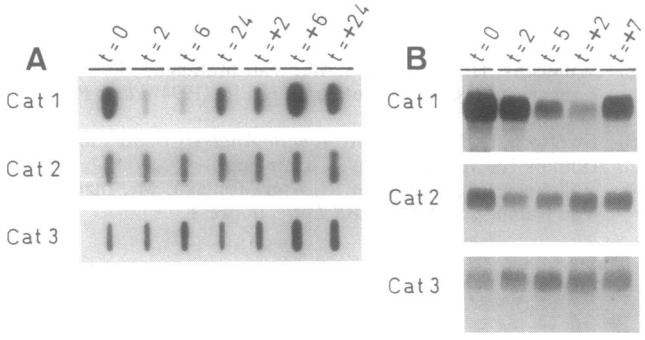

FIG. 7. Changes in catalase mRNA abundance during temperature stress. (*A*) Low temperatures of 6°C were applied for 24 hr (beginning at $t = 0$) to the plants and samples were taken at 0, 2, 6, and 24 hr. In the recovery period (22°C), additional samples (+) were taken after 2, 6, and 24 hr. (*B*) A heat shock of 37°C was applied for 5 hr to the plants and samples were taken at time 0, 2, and 5 hr. In the recovery period (22°C) samples (+) were taken after 2 and 7 hr.

Different Classes of Catalases Can Be Discriminated in Plants. Our data clearly indicate that each catalase isozyme has a specific cellular function in *N. plumbaginifolia*. This is most likely a general feature of catalase gene organization in plants, because differential expression of catalases has also been observed in maize (4). Provided that the catalase gene family is evolutionarily conserved, sequence comparison could lead to the identification of different classes of catalases. We have compared cDNA and protein sequences of plant catalases, but although comparison at the amino acid level clearly distinguished Cat2 from Cat1 and Cat3 (data not shown), only DNA similarity plots appeared to be better suited for classification of Cat1 and Cat3 (Fig. 8).

Dicotyledonous catalases fall into at least three classes. In accordance to our assumption, structurally related catalases from different species seem to have similar functions. One class consists of a cotton catalase from light-grown leaves (20), an *Arabidopsis* catalase from a leaf-specific library (21), and Cat1 from *N. plumbaginifolia*, indicating that this class contains dicot catalases that are involved in photorespiration. Two catalases, one from cotton (22) and Cat3 from *N. plumbaginifolia*, that are both expressed in organs that contain glyoxysomes, belong to a second class. Three legume catalases may also reside in this class, but it is currently not established in which cellular processes these catalases are involved. More distinctly related is another type of catalases represented by Cat2 from *N. plumbaginifolia*, a tomato clone with a similar organ specificity as Cat2 (23), and a catalase from *N. tabacum* (7) and from *Solanum melongena*. A catalase from sweet potato tubers (24) is presented in Fig. 8 as a different branch (according to the Genetics Computer Group program). However, phylogenic analysis (see *Materials and Methods*) indicates that this catalase may be closely related to the Cat2 family. Interestingly, a third type of peroxisomes, unspecialized microbodies, is abundant in the

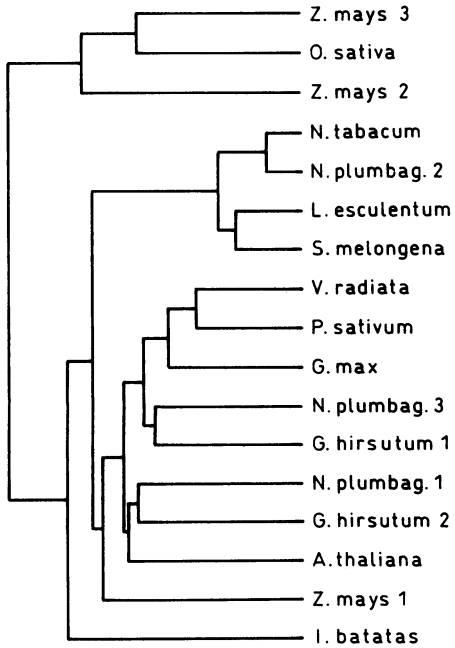

FIG. 8. DNA sequence similarity plot of catalases of *N. plumbaginifolia* (30), *Zea mays* (X12539, X54819, and X12538), *Gossypium hirsutum* (X52135 and X56675), *Oryza sativa* (X61626), *Nicotiana tabacum* (U03473), *Lycopersicon esculentum* (M93719), *Solanum melongena* (X71653), *Vigna radiata* (D13557), *Pisum sativum* (X60169), *Glycine max* (Z12021), *Arabidopsis thaliana* (X64271), and *Ipomoea batatas* (X05549).

tubers from which the sweet potato catalase is extracted. A more detailed expression analysis of the catalase from sweet potato may elucidate whether this catalase is also related in function of the Cat2 class or rather has gained a new function, associated with its localization in tubers.

Monocotyledonous catalases cannot be classified in this system. Expression studies in maize do not support the view that catalase isozymes in monocots are strictly associated with specific types of peroxisomes (25), as seems to be the case in dicot plants.

Conclusions. Based on a detailed analysis of catalase expression in *N. plumbaginifolia*, a classification of dicot catalases is proposed that correlates cellular function with DNA structure. Remarkably, catalases from glyoxysomes, leaf peroxisomes, and unspecialized microbodies fall into distinct groups, suggesting that in dicotyledonous plants, each catalase is associated with a specific type of peroxisome. Our expression data corroborate this view, but conclusive evidence can come only from immunolocalizations.

Together with three other catalases, Cat2 of *N. plumbaginifolia* forms a separate group, but a correlation with a known type of peroxisome is not prominent from its expression. Cat2 is quite uniformly expressed in various organs, with a cellular preference for vascular tissue. In maize, a catalase with enhanced peroxidase activity was localized in vascular tissue and was proposed to play a role in lignification (26). Tobacco also contains a catalase with enhanced peroxidase activity (27), but it is not clear whether it corresponds to Cat2. If Cat2 is truly associated with a specific cellular function such as lignification, it could be localized in a specialized, as yet unidentified, type of peroxisome.

Alternatively, Cat2 may have a housekeeping function, the predominantly vascular expression being the consequence of the high metabolic rate in these cells. Cat2 mRNA levels are induced by root infection of various pathogens in potato (A. Niebel, personal communication). In addition, we observed a rapid increase in Cat2 mRNA levels by UV-B light, ozone, and SO_2 treatment (28), which are stresses that can induce the pathogenesis-related response. These findings become particularly interesting in view of a recent study (7) which shows that salicylic acid can bind and inactivate catalases. Provided that this binding has a biological function and that H_2O_2 acts indeed as a signal molecule in plant–pathogen interactions, Cat2 would be the best candidate to play a role in this process.

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