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Catalase Is Differentially Expressed in Dividing and Nondividing Protoplasts

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Based on our previous results that peroxidase is induced in dividing tobacco protoplasts but it is not expressed in the nondividing grapevine (*Vitis vinifera* L.) protoplasts during culture (C.I. Siminis, A.K. Kanellis, K.A. Roubelakis-Angelakis [1993] Physiol Plant 87: 263–270), we further tested the hypothesis that oxidative stress may be implicated in the recalcitrance of plant protoplasts. The expression of catalase, a major defense enzyme against cell oxidation, was studied during isolation and culture of mesophyll protoplasts from the recalcitrant grapevine and regenerating tobacco (*Nicotiana tabacum* L.). Incubation of tobacco leaf strips with cell wall-degrading enzymes resulted in a burst of catalase activity and an increase in its immunoreactive protein; in contrast, no such increases were found in grapevine. The cathodic and anodic catalase isoforms consisted exclusively of subunits α and β, respectively, in tobacco, and of subunits β and α, respectively, in grapevine. The catalase specific activity increased only in grapevine protoplasts during culture. The ratio of the enzymatic activities to the catalase immunoreactive protein declined in dividing tobacco protoplasts and remained fairly constant in nondividing tobacco and grapevine protoplasts during culture. Also, in dividing tobacco protoplasts the de novo accumulation of the catalase β subunit gave rise to the acidic isoenzymes, whereas in nondividing tobacco and grapevine protoplasts, after 8 d in culture, only the basic isoenzymes remained due to de novo accumulation of the α subunit. The pattern of catalase expression in proliferating tobacco leaf cells during callogenesis was similar to that in dividing protoplasts. The different responses of catalase expression in dividing and nondividing tobacco and grapevine mesophyll protoplasts may indicate a specificity of catalase related to induction of totipotency.

Plant regeneration from protoplasts and especially from mesophyll cells has many advantages; these protoplasts constitute a stable material that is composed of cells originating from the same tissue and of the same developmental stage (Meyer et al., 1993). The expression of totipotency in cultured protoplasts is a prerequisite for plant regeneration. However, many important agricultural plant species, including *Vitis vinifera*, exhibit recalcitrance during protoplast culture, i.e. the inability to express regenerating potential at the level of either cell division or morphogenesis in vitro. The process(es) and mechanism(s) inducing the onset of the undifferentiated meristematic state in cultured protoplasts are very poorly understood (Roubelakis-Angelakis, 1993). It has been established that changes in gene expression and protein synthesis occur during the isolation and culture of plant protoplasts (Grosset et al., 1990; Meyer et al., 1993; Siminis et al., 1993). No gene directly related to mitosis has been shown to be expressed in protoplasts. Among the 12 genes currently characterized, three, selected by independent screenings, are related to redox reactions (Meyer et al., 1993).

Oxidative stress, induced during protoplast isolation and subsequent culture, may be one of the factors contributing to recalcitrance. Indeed, free radicals are generated during grass protoplast isolation (Ishii, 1987). Lipid peroxidation products, such as lipid peroxides and malondialdehyde, accumulate, and lipoxygenase activity increases during early cereal protoplast isolation (Cutler et al., 1989). Moreover, lipid peroxidation is high in *Vitis* protoplasts concomitant with decreased superoxide dismutase activity during the same period (C.I. Siminis and K.A. Roubelakis-Angelakis, unpublished data). Peroxidase activity is increased in dividing tobacco protoplasts, whereas in nondividing grapevine protoplasts it is not expressed (Siminis et al., 1993). Furthermore, a relationship between oxidative stress and recalcitrance is supported by the positive effect of the antioxidant enzymes glutathione peroxidase and phospholipase A_2 in the proliferation of monocotyledonous protoplasts (Ishii, 1988; Creemers-Molenaar and Van Oort, 1990). Fluorescent compounds with spectral characteristics typical of products associated with oxidative stress increase during the early stages of dedifferentiation (callogenesis) in grapevine nodal stem explants (Benson and Roubelakis-Angelakis, 1993). In the same study, catalase was induced but superoxide dismutase decreased during the 1st week of callus induction.

To understand further the role played by the cellular components of defense mechanisms against oxidative stress in recalcitrance of protoplasts, we now present results on catalase expression in tobacco (*Nicotiana tabacum* L.) and grapevine protoplasts. Catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6), a major enzyme component of cellular mechanisms of defense against oxidative stress, localized mainly in microbodies, catalyzes the conversion of two molecules of H_2O_2 to H_2O and O_2 (Scandalios, 1987). Catalase exists in different forms in various plant species. In maize and cotton,

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Abbreviation: NADH-HPR, NADH-dependent hydroxypyruvate reductase.

microbodies house five isoenzymes of catalase (Ni et al., 1990; Williamson and Scandalios, 1993). These isoenzymes are composed of two different subunits that associate to form tetramers.

Catalase specific activity, isoenzyme profiles, subunit composition, and immunoreactive protein were studied in protoplasts of tobacco, a regenerating plant species, and grapevine, the protoplasts of which are recalcitrant with respect to cell proliferation. We also compared dividing and nondividing tobacco protoplasts. Tobacco protoplasts exhibited very low plating efficiency (nondividing) when the period of incubation with cell wall-hydrolyzing enzymes was increased. This protoplast population was competent to reconstitute cell walls but had a low rate of cell division. The results indicate significant differences between dividing and nondividing protoplasts of tobacco, and some similarities between nondividing tobacco and grapevine protoplasts, with regard to the expression of catalase and the activity characteristics of its isoforms.

MATERIALS AND METHODS

Plant Material, Isolation of Protoplasts, and Callogenesis

Grapevine (*Vitis vinifera* L. cv Sultanina) plants were grown in vitro (Roubelakis-Angelakis and Zivanovitc, 1991), and tobacco (*Nicotiana tabacum* L. cv Xanthi) seedlings were grown in a controlled atmosphere glasshouse. Mesophyll protoplasts were isolated and cultured as already described (Koop and Schweiger, 1985; Katsirdakis and Roubelakis-Angelakis, 1992). The cell wall digestion mixture included 1% Cellulase R-10 and 0.5% Macerozyme R-10 (Yakult Honsha Co., Tokyo, Japan). Grapevine and tobacco mesophyll cell proliferation was induced after cultivation of small leaf discs (2 mm diameter) on solid medium as previously described (Katsirdakis and Roubelakis-Angelakis, 1991).

Protein Extraction and Enzymatic Assays

Leaf tissue and callus cells were ground with a mortar and pestle in liquid nitrogen. Total proteins were extracted from leaf tissue, callus cells, and protoplasts with 0.2 M Tris-HCl, pH 8, 10 mM DTT, 0.5 mM PMSF, 10 μM leupeptin, and 10% (v/v) glycerol in the presence of 20% (w/w fresh weight) polyvinylpolypyrrolidone. The leaf and callus samples and the protoplast samples were homogenized with extraction buffer using a Polytron (Ultra Turrax T25, probe S15N10G) at a speed of 20,000 rpm. The tissue:buffer ratio was 1:4. The homogenate was centrifuged at 40,000g for 30 min at 4°C. The supernatant was divided into aliquots and either used immediately for IEF or frozen at −80°C.

Total protein extracts were used for the enzymatic assays. Catalase activity was determined by measuring the initial rates of H_2O_2 decomposition at 240 nm in a solution containing 1 mL of 30% (v/v) H_2O_2 in 400 mL of 50 mM K-phosphate, pH 7.0 (Havir and McHale, 1987). The assay volume was 1 mL.

NADH-HPR activity was determined by following the oxidation of NADH at 340 nm. The reaction mixture (1 mL) consisted of 25 mM K-phosphate buffer, pH 5.8, 1 mM DTT, 0.2 mM NADH, and 5 mM β-hydroxypyruvate (Huang and Beevers, 1972).

SDS-PAGE, Native IEF, Two-Dimensional Gels, and Catalase Detection

Total protein extracts were subjected to SDS-PAGE using the discontinuous buffer system of Laemmli (1970). Proteins were denatured in SDS sample buffer containing 3% SDS and 5% β-mercaptoethanol (Mattoo et al., 1981). The stacking gel consisted of 4% polyacrylamide and the separating gel was 7.5% (30:0.8, acrylamide:bisacrylamide). Electrophoresis was performed in a Mini-Protean II apparatus (Bio-Rad) at a constant current of 15 mA during stacking and 20 mA during resolving. Native IEF was performed in a vertical mini-slab gel as already described (Robertson et al., 1987). Total protein extracts and proteins from isolated microbodies were subjected to IEF. After IEF separation, a lane was removed and equilibrated with sample buffer for 20 min. Next it was placed on a stacking gel for SDS-PAGE and electrophoresed as described above.

Proteins separated by SDS-PAGE were electrophoretically transferred onto nitrocellulose in 25 mM Tris-192 mM Gly, pH 8.3. After focusing, IEF gels were first equilibrated for 30 min in transfer buffer (25 mM Tris-192 mM Gly, pH 8.3) and subsequently proteins were electrophoretically transferred onto nitrocellulose membranes for 1.5 h at 80 V. Immunodetection of catalase was done as described previously (Kanellis et al., 1989). The antibody used in this study was raised against purified catalase from tomato fruit (Inamine and Baker, 1989). Native IEF gels were stained for catalase activity according to Clare et al. (1984).

In Vivo Labeling and Immunoprecipitation of Catalase

[^{35}S]Met (NEN, DuPont) was added to culture medium (10 μCi/mL culture medium). The labeling period was 6 h. Immunoprecipitation of in vivo-labeled catalase was performed as previously described (Sambrook et al., 1989) after total protein extraction was performed as described above. The immunoconjugates were absorbed to protein A-Sepharose beads for 2 h. Absorbed proteins were eluted by boiling the beads for 5 min in SDS sample buffer. Following SDS-PAGE, radiolabeled catalase was detected by exposing the dried gels to x-ray film (Kodak XAR-5) at −80C.

Protein and Radioactivity Measurements

Protein concentrations were quantified according to Lowry et al. (1951). Radioactivity incorporated into proteins was measured in a liquid scintillation spectrometer (Beckman LS 6000 SE) after 10% (w/v) TCA precipitation of proteins.

Preparation of Microbodies

Microbodies were prepared from freshly isolated mesophyll and callus protoplasts from both grapevine and tobacco. Callus protoplasts were isolated as described for mesophyll protoplasts. Protoplasts (1 g) were suspended in 2 mL of grinding medium, which consisted of 20% (w/v) Suc, 0.01 M KCl, 1 mM EDTA, 1 mM $MgCl_2$, and 0.15 M Tricine, pH 7.5.

The suspension was homogenized with razor blades for 20 s. After filtration through three layers of cheesecloth and centrifugation at 270*g* for 10 min, the supernatant was loaded onto a linear gradient of 30 to 60% (w/w) Suc buffered with Tricine, pH 7.5, and supplemented with 1 mM EDTA. Gradients were centrifuged at 62,000*g* for 5 h in an SW 27 rotor (Beckman). After centrifugation, the gradients were fractionated using an Isco (Lincoln, NE) fractionator. Enzyme activities were measured in 0.5-mL fractions. Catalase and Cyt *c* oxidase activities were determined as described by Havir and McHale (1987) and De Bellis and Nishimura (1991), respectively. Total Chl was determined by the method of Inskeep and Bloom (1985). Concentrations of Suc were determined by refractometry. Fractions exhibiting catalase activity were collected, microbodies were further disrupted by hypotonic shock in extraction buffer (see "Protein Extraction and Enzymatic Assays" above; fraction volume:extraction buffer, 1:5), and protein samples were concentrated by ultrafiltration using Centricon microconcentrators (Amicon, Danvers, MA).

RESULTS

Catalase Isoenzymic and Subunit Composition

Tobacco and grapevine leaf catalase were resolved into at least 12 and 5 isoforms, respectively, after native IEF and subsequent activity staining and immunodetection (Fig. 1). The multiple catalase isoforms from both species were immunologically related (Fig. 1). Tomato anti-catalase serum recognized all the active multiple forms resolved by native IEF.

Immunoblots of leaf proteins resolved by native IEF (first dimension) and SDS-PAGE (second dimension) showed that tobacco and grapevine catalase isoenzymes consisted of two subunits, α, with molecular mass of approximately 57 kD in both plants, and β, with molecular mass of approximately 56 and 55 kD in tobacco and grapevine, respectively, which were associated in an ordered ratio (Fig. 1; see also immunoblots after SDS-PAGE in Figs. 2 and 3). The cathodic (less acidic) and anodic (more acidic) isoforms in tobacco catalase consisted exclusively of subunits α and β, respectively, whereas in grapevine the cathodic and anodic isoforms consisted of subunits β and α, respectively. The intermediate forms were hybrids of the two subunits.

Changes in Catalase Specific Activity and Immunoreactive Protein during Protoplast Isolation and Culture

The incubation period of leaf strips with cell wall-degrading enzymes strongly affected plating efficiency of tobacco protoplasts. Four- and 18-h maceration periods resulted in 80 ± 5% and 5% plating efficiency, respectively (data not shown). Protoplasts in both cases were elongated after 2 to 3 d in culture, suggesting new cell wall formation. In contrast, plating efficiency of grapevine protoplasts remained at a very low percentage despite the duration of the incubation period.

Maceration of tobacco leaf tissue with cell wall hydrolytic enzymes resulted in an increase in catalase specific activity. Enzyme specific activity continued to increase only for the next 24 h in protoplasts isolated after a 4-h maceration period (Fig. 2A). Catalase specific activity decreased constantly during the subsequent culture period (Fig. 2A). This decrease in catalase specific activity was lower in tobacco protoplasts isolated after maceration for 4 h (higher plating efficiency) compared to those isolated after 18 h of maceration (lower plating efficiency). In contrast, catalase specific activity in cultured grapevine protoplasts increased during culture, and this increase was not affected by the maceration period (Fig. 3A). These changes in catalase specific activity were well correlated with the total immunoreactive catalase protein in either species (Figs. 2B and 3B). However, an alteration in subunit composition that was dependent on the duration of the maceration period was observed during protoplast culture. Initially, in tobacco protoplasts the α subunit predominated. After the 6th d in culture, β subunit protein increased in dividing protoplasts and was accompanied by an increase in enzyme specific activity (Fig. 2A; also compare lanes corresponding to 2, 6, and 8 d of culture in Fig. 2B).

In grapevine protoplasts during culture, the increase in catalase specific activity corresponded to the accumulation of

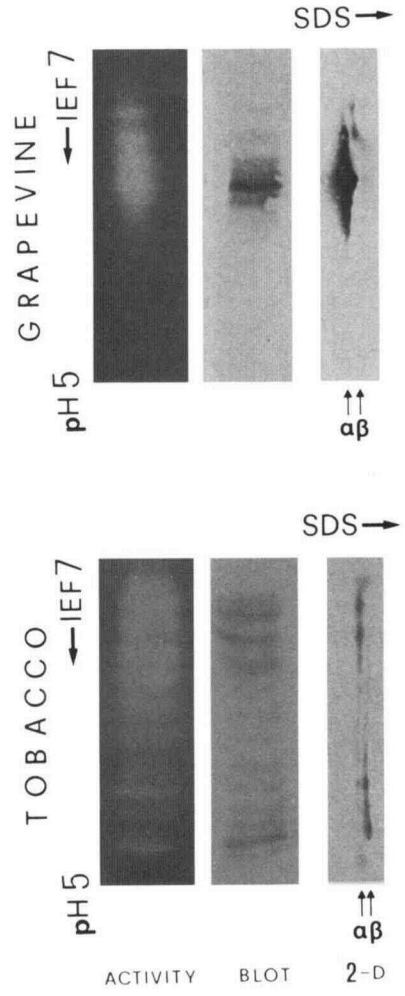

Figure 1. Two-dimensional analysis of catalase isoforms present in tobacco and grapevine leaf tissue analyzed by IEF separation (pH 5–7) and SDS-PAGE and visualized by activity staining and immunodetection with duplicate gels. α and β designate catalase subunits.

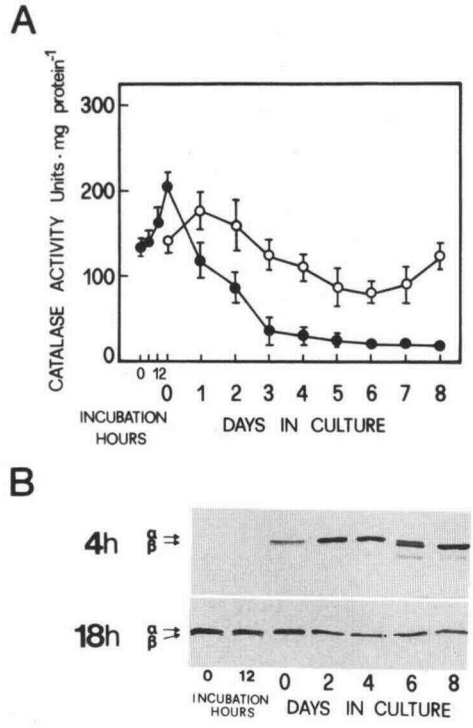

Figure 2. Catalase activity and immunoreactive protein during the maceration period and subsequent culture of dividing and nondividing tobacco protoplasts. A, Time course of catalase activity (units/mg protein ± SE) during the maceration period and the culture of dividing and nondividing tobacco protoplasts isolated after 4 (O) and 18 h (●) of incubation with maceration enzymes. B, Immunoblotting analysis of catalase at the same time periods after SDS-PAGE. α and β designate catalase subunits. Ten and 15 μg of protein were loaded on SDS gels for the immunodetection of catalase from protoplasts isolated after 4- and 18-h maceration periods, respectively.

α subunit independent of the maceration period. The amount of β subunit was fairly constant during the entire culture period but was much lower than the amount of α subunit (Fig. 3B).

To estimate the nature of the enzymatic activity of catalase isoforms in dividing and nondividing tobacco protoplasts, the ratio of the enzymatic activities (Fig. 2A) to the relative densitometric values of catalase immunoreactive protein (Fig. 2B) was calculated over the 8-d protoplast culture period. This ratio declined in dividing tobacco protoplasts, whereas it remained fairly constant in nondividing protoplasts, indicating that the isoenzymes that consisted mainly of β subunit (anodic isoenzymes) exhibited less catalatic activity (data not shown).

Catalase Isoenzymes and Their Subunits during Protoplast Culture

A differential expression of catalase isoenzymes was observed during the 8-d protoplast culture (Figs. 4 and 5). In dividing tobacco protoplasts there was a shift from the cathodic active forms, which predominated during the first days of culture, to anodic forms on the 8th d (Fig. 4A). On the other hand, in nondividing tobacco protoplasts such a shift in the isoenzymic pattern was not observed. Rather, a gradual decrease was detected only in the anodic isoenzymes. The cathodic isoenzymes remained fairly constant during the same period (Fig. 4A). The aforementioned disparities in the staining intensities of catalase isoenzymes corresponded to their immunoreactive isoenzymic profiles during the same culture period, indicating no activation or inhibition of catalase forms (Fig. 4B).

The fate of the isoenzymic pattern of grapevine catalase was similar regardless of the maceration period. The changes in grapevine catalase isoenzymes after 4 h of maceration treatment are shown in Figure 5. The activity-stained and immunoreactive catalase isoenzymes with isoelectric points close to 6 prevailed after 8 d in culture (Fig. 5, A and B).

The shift in isoenzymic pattern of both species reflected a change in the subunit composition as evidenced by two-dimensional analysis and immunodetection (Fig. 6). The dividing and nondividing tobacco protoplasts were characterized by the appearance of β and α catalase subunits, corresponding to anodic and cathodic isoenzymes, respectively, after 8 d in culture (Fig. 6, a and b). In grapevine protoplasts, the predominance of isoenzymes with isoelectric points close to 6 corresponded to the accumulation of the

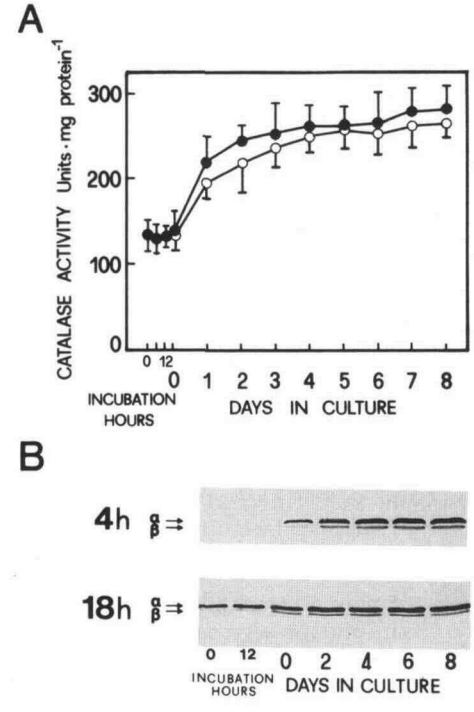

Figure 3. Catalase activity and immunoreactive protein during the isolation and culture of grapevine protoplasts. A, Catalase activity (units/mg protein ± SE) during the maceration period and the subsequent culture of grapevine protoplasts isolated after 4 (O) and 18 h (●) of incubation with maceration enzymes. B, Immunobloting analysis of catalase at the same time periods after SDS-PAGE. α and β designate catalase subunits. Ten micrograms of protein were loaded on SDS gels for the immunodetection of catalase.

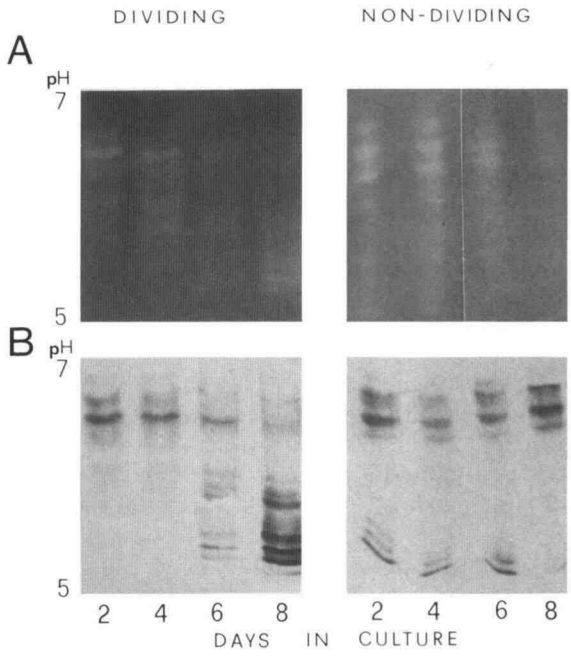

Figure 4. Comparisons of catalase isoform profiles during culture of dividing and nondividing tobacco protoplast. A, IEF gels (pH 5–7) stained for catalase activity (0.3 units/lane). B, Western blot of duplicate IEF gels except that 0.9 units of catalase were applied to each lane. Lanes refer to 2, 4, 6, and 8 d in culture. Differences in bands are not due to difference in migration but to slight distortions that occurred when the 5% gel was placed on the nitrocellulose prior to electroblotting.

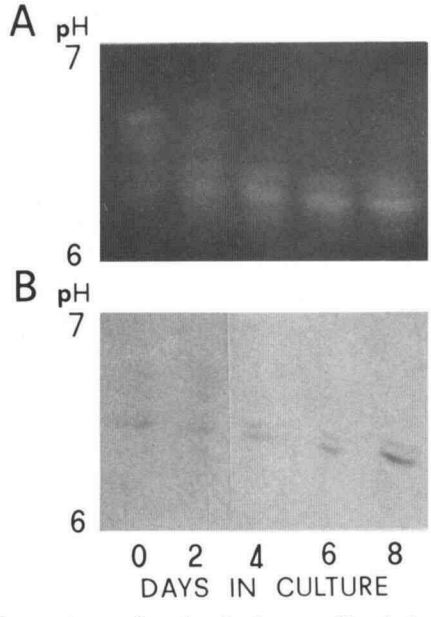

Figure 5. Comparisons of catalase isoform profiles during grapevine protoplast culture. A, IEF gels (pH 5–7) stained for catalase activity (0.3 units/lane). B, Western blot of duplicate IEF gels except that 0.9 units of catalase were applied to each lane. Protein samples were from protoplasts at 0, 2, 4, 6, and 8 d in culture.

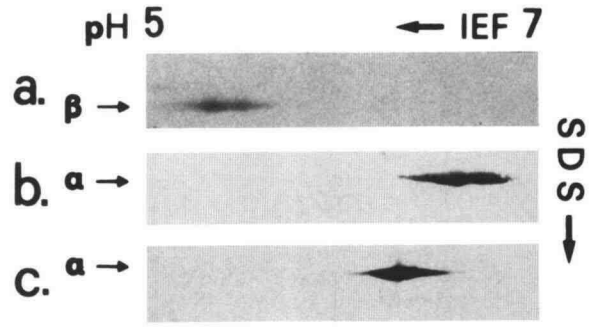

Figure 6. Two-dimensional analysis of catalase isoforms present in dividing (a) and nondividing (b) tobacco protoplasts and in grapevine protoplasts (c) after 8 d in culture. Isoforms were analyzed as in Figure 1.

catalase α subunit (Fig. 6c), showing behavior similar to that of nondividing tobacco protoplasts (Figs. 4–6).

De Novo Catalase Synthesis

As already demonstrated, the catalase subunit pattern showed differential expression in tobacco protoplasts (Fig. 2B) and a rather stable situation in grapevine protoplasts (Fig. 3B). The shift from α to β subunit of catalase in tobacco protoplasts was evident on d 6 of the culture period. The results, however, provided no indication as to whether the accumulation of β subunit and α subunit in tobacco and grapevine protoplasts, respectively, was due to its de novo synthesis during this period.

To answer this question, proteins from dividing tobacco and grapevine protoplasts were labeled with [^{35}S]Met for 6 h on d 0 and 6 of the culture period. It was shown that the shift in tobacco subunits from α to β and the increased synthesis of the latter on d 6 involved de novo synthesis of catalase β subunit. Also, the accumulation of the α subunit in grapevine protoplasts was due to its de novo synthesis (Fig. 7). Taking into account that the first divisions in tobacco protoplasts were noticed after 4 d in culture, the incorporation of the radiolabel into the β subunit and hence the new synthesis of catalase seemed to parallel the tobacco protoplast division process.

Mesophyll Cell Proliferation Induced a Shift in Catalase Synthesis

Based on the observation that the shift and the new synthesis of catalase β subunit correlated with the protoplast division process, we examined the changes in catalase activity and immunoreactive protein during the first 4 weeks of callogenesis in tobacco and grapevine leaf segments. The first visible cell divisions were completed after the 4th d of culture in both plant species (data not shown), as was the case in dividing protoplasts. After 7 d in culture, the α subunit of catalase disappeared, and the β subunit was the only one present during the subsequent culture period in tobacco callus cells (Fig. 8B). Catalase specific activity decreased during the same period after changes in its protein (Fig. 8A). Cell prolif-

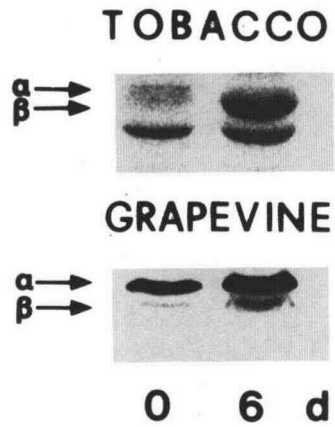

Figure 7. Fluorogram after SDS-PAGE showing the de novo synthesis of catalase polypeptides (subunits α and β) in protoplasts after isolation and after 6 d in cultured, dividing tobacco protoplasts and grapevine protoplasts.

eration induced a differential expression of catalase isoenzymes similar to that observed in dividing protoplasts (data not shown). That is, acidic isoenzymes formed by self-association of β subunits were the only isoenzymes synthesized after 7 d in culture.

In grapevine leaf cells proliferating in vitro, catalase specific activity increased during the 1st week and then gradually decreased, reaching levels similar to those in tobacco callus (Fig. 8A). However, this change in catalase specific activity was correlated with the accumulation of α subunit and the simultaneous disappearance of β subunit (Fig. 8B).

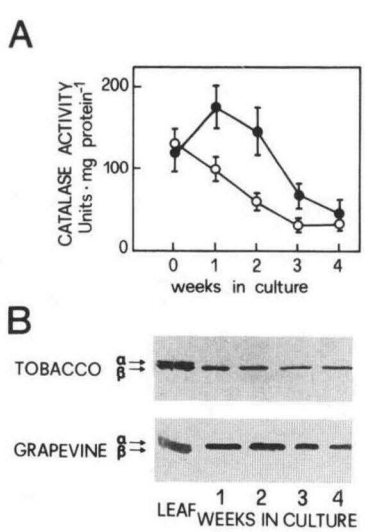

Figure 8. Catalase activity and immunoreactive protein during tobacco and grapevine mesophyll cell proliferation. A, Catalase activity (units/mg protein ± SE) in tobacco (○) and grapevine (●) leaf explants during callogenesis at weekly intervals. B, Immunoreactive protein after SDS-PAGE. Fifteen micrograms of protein were loaded on SDS gels.

Subcellular Localization of Catalase Isoenzymes

To determine the subcellular localization of catalase, organelles were isolated from tobacco and grapevine protoplasts and callus cells. Approximately 95% of protoplasts were disrupted with razor blades as described in "Materials and Methods." Catalase activity on Suc gradients was found at a density of about 1.25 g cm^{-3}. The fractions containing approximately 80 and 70% of total catalase activity in protoplasts from leaf mesophyll and callus, respectively, contained less than 5% of total Cyt *c* oxidase activity and 2% of Chl. Proteins from these microbody preparations were analyzed for catalase isoenzymes (Fig. 9). The results from the protein blots in Figure 9 indicated that the changes in catalase isoenzymic pattern (Figs. 4 and 5) corresponded to catalase of microbody origin. Interestingly, catalase from microbodies isolated from grapevine callus expressed only one isoenzyme, which corresponded to the α subunit (Figs. 8B and 9).

NADH-HPR Specific Activity in Mesophyll and Callus Cells and Protoplasts

The specific activity of NADH-HPR, an enzyme of leaf-type peroxisomes (Huang et al., 1983), was determined during protoplast culture. It was also determined in mesophyll tissue and callus cells. Its specific activity was reduced in dividing protoplasts, whereas it remained fairly constant, despite a temporal fluctuation, in nondividing protoplasts (Table I). The specific activity in tobacco and grapevine callus cells was very low compared to that in leaf tissue.

DISCUSSION

The procedure for the isolation of protoplasts includes the removal of cell walls by cellulase and pectinase; the duration of the enzymatic action drastically affected the fate of the resulting protoplasts. When the digestion period was increased from 4 to 18 h, the plating efficiency decreased from 80 ± 5% to below 5% in tobacco protoplasts. In the recalci-

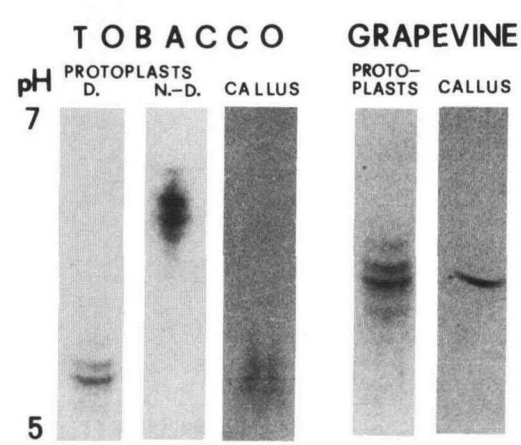

Figure 9. Comparison of catalase isoforms present in isolated microbodies from tobacco dividing (D.) and nondividing (N.-D.) protoplasts, from grapevine protoplasts after 8 d in culture, and from callus cells of both species. The immunodetection of catalase isoforms was performed following IEF (pH 5–7) separation.

Table I. *NADH-HPR specific activity in tobacco and grapevine leaf tissue, protoplasts, and callus cells*

NADH-HPR specific activity was determined in total protein extracts from leaf tissue used for protoplast preparation, protoplasts at various stages of culture after 4 and 18 h of leaf maceration, and subcultured callus cells.

Protein Samples from	NADH-HPR Activity			
	Tobacco		Grapevine	
	4 h	18 h	4 h	18 h
	(units mg^{-1} protein) × 10^2			
Leaf cells	0.42[a]		0.37	
Protoplasts				
0 h	0.41	0.49	0.40	0.50
24 h	0.35	0.68	0.52	0.76
48 h	0.32	0.56	0.71	0.83
96 h	0.24	0.50	0.74	0.84
6 d	0.11	0.42	0.75	0.91
Callus cells	0.06		0.07	

[a] $P < 0.01$.

trant grapevine, the protoplasts did not divide regardless of the duration of action of the cell wall-hydrolyzing enzyme.

Among the factors assessed that may contribute to recalcitrance of protoplasts is oxidative stress (Ishii, 1988; Cutler et al., 1989; Roubelakis-Angelakis, 1993). Wounding, cell wall hydrolytic enzymes, and change of cultured protoplasts and cells from autotrophic to heterotrophic metabolism in the presence of altered hormonal status are some of the factors that induce oxidative stress. Auxins have been shown to stimulate lipid peroxidation (Garcia and Einset, 1989; Maitre and Sen, 1989). Synthetic auxins stimulate the production of ethylene, the biosynthesis of which is dependent on oxygen and free radicals. Auxins enhance the alternate pathway of mitochondrial respiration and stimulate residual oxygen metabolism, which is attributed to increased lipoxygenase activity (Van der Linde, 1990). The components of the culture medium, especially metal cations and FeEDTA, are known to stimulate free radical production and lipid peroxidation in other systems, and they may also participate in the oxidative stress of protoplasts. The absence of basic isoperoxidases and IAA oxidases in grapevine protoplasts (Siminis et al., 1993) may produce an unbalanced hormonal status.

Catalase from mesophyll cells was resolved after native IEF into at least 12 and 5 isoenzymes in tobacco and grapevine, respectively (Fig. 1). Catalase heterogeneity has also been demonstrated in cotton, spinach, wheat, and sunflower (Havir and McHale, 1987, and refs. therein). In *N. tabacum* cv Havana, catalase activity was separated into six bands by native IEF (Zelitch et al., 1991). The difference in the number of multiple forms of catalase between the two varieties of *N. tabacum* can be attributed to the different genetic background of the tobacco varieties or to posttranslational modifications of α and/or β subunits. It is also possible that charge subunits may contribute to this form of multiplicity.

Analysis of catalase (native IEF/SDS-PAGE) revealed two interesting features. First, catalase isoenzymes are formed by the association of two subunits, α and β, which is in agreement with the composition of catalase in cotton (Ni et al., 1990) and maize (Williamson and Scandalios, 1993). The association of the α and β subunits occurred in a controlled order (Fig. 1). Second, tobacco cathodic and anodic isoenzymes were homotetramers of α and β subunits, respectively, whereas in grapevine the cathodic and anodic isoenzymes were homotetramers of β and α subunits, respectively. Whether this disparity in the subunit composition of catalase isoenzymes between the two species has any physiological role remains to be established. However, it should be noted that in protoplasts with low plating efficiency, the isoenzymes with more catalatic activity prevailed (Figs. 4 and 5).

Incubation of tobacco leaf strips with commercial enzyme preparations resulted in a burst of catalase activity, which was more pronounced during the 18-h incubation period, and there was a concomitant increase in its immunoreactive protein (Fig. 2). In contrast, no such increase in catalase activity and protein was observed in grapevine during isolation of protoplasts (Fig. 3). The reason for this differential behavior is not known. In as much as catalase has been shown to be induced by H_2O_2 (Williamson and Scandalios, 1993), a burst in oxidative activity in tobacco cells could lead to induction of catalase activity during protoplast isolation, which is more evident in the 18-h maceration period. The observed disparity in the timing of catalase induction between the two species could be due to the production of a different oxygen-reactive component in grapevine protoplasts. In support of this hypothesis is the rapid occurrence of lipid peroxidation in grapevine protoplasts during the same period (C.I. Siminis and K.A. Roubelakis-Angelakis, unpublished results), followed by the increased level of catalase activity and protein during subsequent culture (Fig. 3).

The length of the maceration period also altered the specific activity of catalase, the structural composition, and the isoenzymic profile in cultured protoplasts (Figs. 2–6). In both the dividing (higher plating efficiency) and nondividing (lower plating efficiency) tobacco protoplasts, levels of catalase activity and protein decreased during the first 6 d in culture (Fig. 2). A correlation was found among protoplast division, the expression of the de novo-synthesized β subunit, and the corresponding anodic isoenzymes, which exhibited less catalatic activity. The persistence of β subunit in tobacco callus (Fig. 8) may indicate a correlation between the expression of the more anodic isoenzymes, composed mainly of the β subunit, and the division process. In contrast, in nondividing protoplasts the α subunit and the more cathodic isoenzymes, with higher catalatic activity, were evident. It is believed that the longer isolation process stimulated a massive production of H_2O_2, which in turn induced the shift to more catalatic isoenzymes to overcome the stress. Although H_2O_2 in low amounts can participate in multiple functions, such as acting as a second messenger (Apostol et al., 1989) and in the metabolism of IAA (Hamilton et al., 1976), higher amounts can be detrimental (Scandalios, 1987). Thus, it is suggested that although the more catalatic isoenzymes persisted, the damage due to oxidative stress was irreversible.

The high levels of catalase activity and protein in grapevine protoplasts, as opposed to tobacco protoplasts, suggests that grapevine protoplasts underwent a pronounced oxidative

stress. Whether this stress is a cause or a consequence of the inability of grapevine protoplasts to divide efficiently and regenerate is unclear. The fact remains, however, that nondividing protoplasts of both species expressed catalase isoenzymes exhibiting higher catalatic activity compared to dividing protoplasts (Fig. 6). Thus, the physiological status of these protoplasts favored the induction of α subunit, which gave rise to more cathodic and more anodic isoenzymes in tobacco and grapevine protoplasts, respectively (Figs. 4–6). No obvious relationship existed between catalase activity and protoplast viability. Nondividing grapevine protoplasts, with high rates of viability, expressed high levels of catalase activity.

Given the higher induction of catalase in grapevine protoplasts, the data from callogenesis suggested that grapevine callus was subjected to a milder oxidative stress. It is worth noting that in both cases the α subunit of catalase predominated. This may imply that the oxygen reactive components are at least one of the factors that control the synthesis of catalase subunits. In addition, since catalase has been shown to be developmentally regulated (Scandalios et al., 1984; Scandalios, 1987; Redinbaugh et al., 1990; Ni and Trelease, 1991), the dedifferentiation process may be another regulating factor.

All catalase isoenzymes were localized in microbodies (Fig. 9); thus, the changes observed are in organellar protein. Such changes in catalase occur during transition of microbodies from one developmental stage to another (Huang et al., 1983; De Bellis and Nishimura, 1991; Ni and Trelease, 1991). Further study of the microbody enzyme expression in both tobacco and grapevine protoplasts and callus could resolve this differential expression of catalase and/or reveal other physiological roles of the enzyme in the dedifferentiation process and the induction of totipotency.

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