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The Generation of Active Oxygen Species Differs in Tobacco and Grapevine Mesophyll Protoplasts[1]

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Our previous results have shown that oxidative stress may reduce the regeneration potential of protoplasts, but only protoplasts that are able to supply extracellularly H_2O_2 can actually divide (C.I. Siminis, A.K. Kanellis, K.A. Roubelakis-Angelakis [1993] Physiol Plant 87: 263–270; C.I. Siminis, A.K. Kanellis, K.A. Roubelakis-Angelakis [1994] Plant Physiol 1105: 1375–1383; A. de Marco, K.A. Roubelakis-Angelakis [1996a] Plant Physiol 110: 137–145; A. de Marco, K.A. Roubelakis-Angelakis [1996b] J Plant Physiol 149: 109–114). In the present study we have attempted to break down the oxidative burst response into the individual active oxygen species (AOS) superoxide ($O_2^{\cdot-}$) and H_2O_2, and into individual AOS-generating systems during the isolation of regenerating tobacco (*Nicotiana tabacum* L.) and non-regenerating grape (*Vitis vinifera* L.) mesophyll protoplasts. Wounding leaf tissue or applying purified cellulase did not elicit AOS production. However, the application of non-purified cellulase during maceration induced a burst of $O_2^{\cdot-}$ and H_2O_2 accumulation in tobacco leaf, while in grape significantly lower levels of both AOS accumulated. AOS were also generated when protoplasts isolated with purified cellulase were treated with non-purified cellulase. The response was rapid: after 5 min, AOS began to accumulate in the culture medium, with significant quantitative differences between the two species. In tobacco protoplasts and plasma membrane vesicles, two different AOS synthase activities were revealed, one that showed specificity to NADPH and sensitivity to diphenyleneiodonium (DPI) and was responsible for $O_2^{\cdot-}$ production, and a second NAD(P)H activity that was sensitive to KCN and NaN_3, contributing to the production of both AOS. The first activity probably corresponds to a mammalian-like NADPH oxidase and the second to a NAD(P)H oxidase-peroxidase. In grape, only one AOS-generating activity was detected, which corresponded to a NAD(P)H oxidase-peroxidase responsible for the generation of both AOS.

The active oxygen species (AOS), superoxide ($O_2^{\cdot-}$), H_2O_2, and the hydroxyl radical are inevitably produced in higher plant cells during normal metabolism (Scandalios, 1993). Their accumulation is enhanced by exposure to environmental stresses, chemicals, and microbial factors (Sutherland, 1991; Scandalios, 1993; Baker and Orlandi, 1995). Overproduction of reduced and chemically reactive AOS results in oxidative stress and cellular damage (Scandalios, 1993). On the other hand, AOS also play a positive role in plant growth and development. Some of the better studied downstream responses promoted by H_2O_2 are its participation in the polymerization of lignin, suberin, and possibly other cell wall components, the induction of defense-related genes, the stimulation of phytoalexin production, and the promotion of the hypersensitive response (for review, see Low and Merida, 1996; Lamb and Dixon, 1997, and refs. therein). $O_2^{\cdot-}$ and H_2O_2 also act directly as antimicrobial agents and may play a signaling role in inducing other defense mechanisms (Levine et al., 1994; Jabs et al., 1997).

The mechanism(s) of synthesis of AOS is a topic of active discussion (for review, see Sutherland, 1991; Low and Merida, 1996; Wojtaszek, 1997). The earliest hypothesis on the origin of apoplastic H_2O_2 during the lignification of horseradish roots involved the reduction of O_2 to $O_2^{\cdot-}$ by phenolic and NAD$^{\cdot}$ radicals produced by peroxidase, and the subsequent formation of H_2O_2 by the dismutation of $O_2^{\cdot-}$ (Gross et al., 1977; Elstner and Heupel, 1978; Halliwell, 1978). A second model was proposed for the production of H_2O_2 induced in cultured French bean cells with a *Colletotrichum lindemuthianum* cell wall elicitor and involved an apoplastic peroxidase. The O_2-heme complex of peroxidase is reduced to compound III by reductants exported from the cell. Under the proper conditions, i.e. elevated pH, the complex is hydrolyzed to release H_2O_2 (Bolwell et al., 1995). The third hypothesis on AOS generation, which is gaining increased interest in the literature, involves a transmembrane $O_2^{\cdot-}$ synthase, NAD(P)H oxidase, which transfers electrons from cytoplasmic NAD(P)H to O_2 to form $O_2^{\cdot-}$, which is then dismutated to H_2O_2 (Doke and Miura, 1995; Low and Merida, 1996; Murphy and Auh, 1996; Van Gestelen et al., 1997).

Evidence supports the similarity of NAD(P)H oxidase in plant cells and NADPH oxidase in phagocytic animal cells involved in H_2O_2 production. In particular, antibodies raised against components of the mammalian neutrophil NADPH oxidase cross-react with polypeptides of similar molecular masses in plants (Desikan et al., 1996; Kieffer et al., 1997; Xing et al., 1997). Moreover, the activity of plant NAD(P)H oxidase is inhibited by diphenylene iodonium (DPI), a well-known inhibitor of the mammalian plasma membrane oxidase (Levine et al., 1994; Auh and Murphy, 1995; Jabs et al., 1997). Furthermore, a cDNA from rice (Groom et al., 1996) and a full-length clone of Arabidopsis (Keller et al., 1998) homologous to one integral component of the mammalian NADPH oxidase have recently been characterized.

Plant protoplasts undergo complex metabolic alterations during their isolation and culture. The expression of totipotency in cultured protoplasts is a prerequisite for plant

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regeneration; however, many important agricultural plant genera, including *Vitis*, exhibit recalcitrance. Oxidative stress has been proposed to contribute to the recalcitrance of plant protoplasts (Cutler et al., 1991; Roubelakis-Angelakis, 1993; Siminis et al., 1993, 1994; de Marco and Roubelakis-Angelakis, 1996a, 1996b, 1999). Reduced oxygen species were generated during the enzymic maceration of cell walls during cereal protoplast isolation (Ishii, 1987). Endoxylanase, an enzymic component of some commercial cellulase and pectinase preparations, induced alterations in membrane integrity in tobacco (*Nicotiana tabacum* L.) (Bailey et al., 1992), and caused necrosis in cereal cells (Ishii, 1988) and tobacco protoplasts (Sharon et al., 1993). Cultured grape cells exhibited hypersensitive response-like necrosis after the addition of non-purified cellulase (Calderon et al., 1994). Ishii (1988) suggested that the treatment of cells with xylanase generates $O_2^{\cdot -}$ anions.

In an effort to understand the mechanism(s) governing the regeneration of plant protoplasts, and to identify potential factors contributing to their recalcitrance, we optimized the isolation and culture conditions (Theodoropoulos and Roubelakis-Angelakis, 1990) and studied the biochemical and ultrastructural characteristics of cell wall reconstitution (Katsirdakis and Roubelakis-Angelakis, 1992a), the protein profile, and the activities and profiles of several enzymes contributing to cell defense against oxidative stress (Siminis et al., 1993, 1994; de Marco and Roubelakis-Angelakis, 1996a, 1996b, 1999) between regenerating tobacco and non-regenerating grape protoplasts. In addition, by modifying the procedure of protoplast isolation, we were able to identify two populations of tobacco protoplasts, one that readily regenerated, and one that did not (Siminis et al., 1994). A comparative study indicated a simultaneous higher scavenging activity and H_2O_2 accumulation potential in regenerating tobacco protoplasts (de Marco and Roubelakis-Angelakis, 1996a, 1996b).

In this work we have attempted to reveal the site and mechanism(s) of AOS generation during isolation of protoplasts from two plant species, grape and tobacco. We show that wounding of grape and tobacco leaf tissue alone or the use of purified cellulase for protoplast isolation does not induce AOS production. The addition of non-purified cellulase to leaf strips induced a burst of $O_2^{\cdot -}$ and H_2O_2 accumulation in tobacco leaves, while in grape, significantly lower levels of both AOS accumulated. Protoplasts isolated from tobacco and grape leaves with purified cellulase responded differently to the addition of non-purified cellulase with respect to the kinds and levels of extracellular AOS accumulation. The use of inhibitors revealed the operation of different enzymic systems of AOS production in the two plant species.

MATERIALS AND METHODS

Plant Material

Protoplasts were isolated from fully expanded, but not senescent, leaves of in vitro-grown grape (*Vitis vinifera* L. cv Sultanina) (Roubelakis-Angelakis and Zivanovitc, 1991) and greenhouse-grown tobacco (*Nicotiana tabacum* L. cv Xanthi) plants. Small leaf segments (2 mm) were used in all experiments.

Isolation of Mesophyll Protoplasts

Grape and tobacco mesophyll protoplasts were isolated and cultured as previously described (Koop and Schweiger, 1985; Katsirdakis and Roubelakis-Angelakis, 1992b). The cell wall-hydrolyzing enzymic preparations used were 0.25% (w/v) purified cellulase (Worthington Biochemical, Lakewood, NJ) and 0.5% (w/v) Macerozyme (R-10 Onozuka, Yakult Honsha, Tokyo), and the duration of the maceration period was 4 h. The purified cellulase was free of detectable amounts of xylanase (Fuchs et al., 1989). All treatments were performed in the dark at 25°C.

Elicitation of AOS in Leaf Tissue and Protoplasts

Elicitation of AOS production was studied in leaf tissue and protoplasts. Leaf strips were intensively wounded with a razor blade and floated in the culture medium as previously described for protoplast isolation (Koop and Schweiger, 1985; Katsirdakis and Roubelakis-Angelakis, 1992b). At time zero, two different cellulase preparations with similar enzymic activity were added to leaf segments: 1% (w/v) non-purified cellulase known to contain xylanase (Fuchs et al., 1989) or 0.25% (w/v) purified cellulase. Wounded strips floated in the same medium were used to study AOS production elicited by wounding alone. AOS accumulation in the culture medium was monitored over a period of 16 h.

Protoplasts were cultured as previously described (Katsirdakis and Roubelakis-Angelakis, 1992b). At time zero, 1% (w/v) non-purified cellulase was added and the culture medium was used for determination of AOS accumulation over a period of 4 h.

Plasma Membrane Isolation

Plasma membranes were isolated from control protoplasts and from protoplasts treated with 1% (w/v) non-purified cellulase for 30 min, as previously described (de Marco and Roubelakis-Angelakis, 1996a). Four volumes of the extraction buffer (50 mM Tris-HCl, pH 7.5, 20% [w/v] sorbitol, 1 mM ascorbate, 1 mM EDTA, 10 mM DDT, 10 μM leupeptin, and 0.3% [v/v] Triton X-100) was added to protoplasts and, after intense vortexing, the homogenate was centrifuged for 20 min at 13,000*g*. The resulting supernatant was further centrifuged for 50 min at 85,000*g* to separate the microsomal fraction, which was resuspended in 50 mM Tris-HCl, pH 7.0, 250 mM Suc, 0.5 M KCl, and 10% (v/v) glycerol and re-centrifuged twice to wash away any unspecifically bound enzyme. Plasma membranes were isolated using a two-phase partitioning system, as previously described (de Marco and Roubelakis-Angelakis, 1996a). Total protein of the plasma membranes was measured by the method of Lowry et al. (1951), with BSA as a standard.

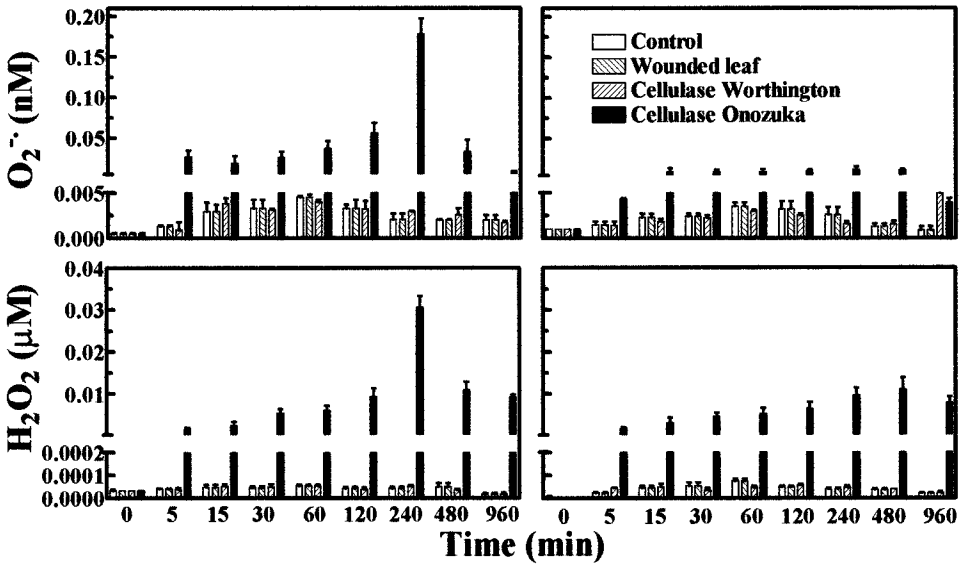

Figure 1. Effect of cellulase preparations and wounding on $O_2^{\cdot-}$ and H_2O_2 accumulation in leaf tissue of tobacco (left) and grape (right). Leaf strips (10 × 5 mm) were treated with 1% non-purified cellulase or 0.25% purified cellulase, or were not treated. Cellulase preparations were added at time zero to the incubation medium in which the leaf strips were floated. Accumulation of $O_2^{\cdot-}$ and H_2O_2 was also determined in leaf strips following intense wounding. Values are means ± SE from seven independent experiments.

Luminol-Dependent Chemiluminescence Assay for H_2O_2

The production of H_2O_2 from leaf cells and protoplasts was determined by the chemiluminescence assay of luminol described by Murphy and Huerta (1990). Leaf segments (50 mg mL^{-1}) were floated in the culture medium as described above, which was used for H_2O_2 assays. The production of H_2O_2 from protoplasts, corresponding to the same fresh weight of leaf tissue, was determined in the culture medium after centrifugation at 1,000 rpm for 15 s. The assay was conducted in a total volume of 2 mL by placing 0.8 mL of reaction buffer containing 10 mM Tris-Mes, pH 7.0, 1 mM $CaCl_2$, 0.1 mM KCl, 0.2 mL of 1 mM luminol solution, 0.1 unit of peroxidase in 20 mM potassium phosphate buffer (pH 7.4), and 1 mL of culture medium in a scintillation vial (Auh and Murphy, 1995). The vial was immediately placed in a scintillation spectrometer (model LS 6000SE, Beckman) and chemiluminescence was detected. Counts were reported every 15 s for 1 min, and the last two values were averaged. A standard curve was produced correlating the chemiluminescence values with standard concentrations of H_2O_2. Proper controls were always used.

Lucigenin-Dependent Chemiluminescence Assay for $O_2^{\cdot-}$

The accumulation of $O_2^{\cdot-}$ was measured by the chemiluminescence of lucigenin, which is specific for $O_2^{\cdot-}$ (Corbisier et al., 1987). Although the validity of lucigenin as a probe for detecting $O_2^{\cdot-}$ has recently been questioned because of its ability to undergo redox cycling (Liochev and Fridovich, 1997), Li et al. (1998) presented a detailed and careful investigation in which the validity of lucigenin as a chemilumigenic probe for detecting $O_2^{\cdot-}$ production by enzymatic and cellular systems was established. The assay was conducted in a total volume of 2 mL by placing 0.2 mL of 1 mM lucigenin in 0.1 M Gly-NaOH buffer (pH 9.0) containing 1 mM EDTA (Auh and Murphy, 1995). All of the other conditions described for H_2O_2 assays were also followed for the $O_2^{\cdot-}$ assays. Counts were reported every 6 s for 30 s, and the last two values were averaged. The xanthine/xanthine oxidase system described by Murphy and Auh (1996) was used to convert chemiluminescence data to production rates: 10^6 cpm was equivalent to 38.21 ± 2.16 pmol $O_2^{\cdot-}$ produced min^{-1} (mean ± SE of three experiments).

Assay for $O_2^{\cdot-}$ Synthase

The assay for $O_2^{\cdot-}$ synthase was also based on the chemiluminescence of lucigenin according to the method of Murphy and Auh (1996) with minor modifications: 1 mL of the reaction mixture containing 100 mM Gly-NaOH, pH 9.0, 1 mM EDTA, 200 μM NADH or 100 μM NADPH, 0.02% (v/v) Triton X-100, and 0.4 mM lucigenin, with or without inhibitors, was measured in a liquid scintillation counter for 60 min. The last count (−5 min) was used for normalization of the samples. At time zero, 5 μg of plasma membrane protein was added and the mixtures were counted for 1 min. The results from experimental treatments were always compared with those from untreated controls counted at the same time. In inhibitor experiments, controls containing similar concentrations of inhibitors were used.

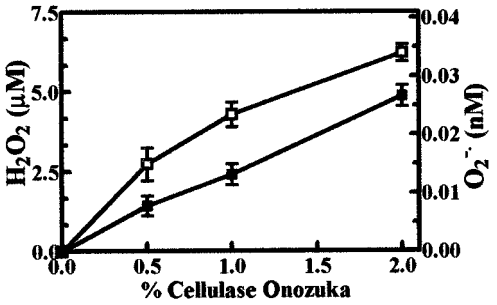

Figure 2. Dose-dependent accumulation of $O_2^{\cdot-}$ (□) and H_2O_2 (■) in the incubation medium of tobacco leaf strips treated with non-purified cellulase. Samples were taken 60 min after cellulase addition. Values are means ± SE from three independent experiments.

RESULTS

Extracellular AOS Accumulation by Leaf Strips

The accumulation of $O_2^{\cdot-}$ and H_2O_2 in the incubation medium of tobacco and grape leaf strips was followed over a 16-h period with the lucigenin- and the luminol-dependent chemiluminescence assays, respectively. $O_2^{\cdot-}$ and H_2O_2 accumulated at low levels in the incubation medium in both systems (Fig. 1). The addition of purified cellulase did not affect AOS accumulation, whereas the addition of non-purified cellulase to the incubation medium resulted in a significant increase in the accumulation of both AOS in tobacco, but only the accumulation of H_2O_2 in grape leaf strips (Fig. 1). In tobacco, both AOS started to accumulate soon after the addition of non-purified cellulase, reached a maximum after 4 h, and decreased thereafter. In grape, the accumulation of $O_2^{\cdot-}$ was very low but, as in tobacco, H_2O_2 started to increase rapidly after the addition of non-purified cellulase; however, the level reached a maximum after 8 h and was 3-fold lower than that of tobacco (Fig. 1). In tobacco the maximum $O_2^{\cdot-}$ and H_2O_2 that accumulated in the culture medium and corresponded to 1 mg fresh weight were 0.180 ± 0.013 nM and 0.030 ± 0.008 μM, whereas in grape the respective values were 0.012 ± 0.002 nM and 0.011 ± 0.003 μM.

No increase in $O_2^{\cdot-}$ or H_2O_2 was found when tobacco and grape leaf strips were either extensively wounded or incubated with purified cellulase, suggesting that wounding and purified cellulase do not induce AOS production (Fig. 1). In both plant species, the accumulation of $O_2^{\cdot-}$ and H_2O_2 was dependent on the amount of non-purified cellulase. This dose response of AOS production by tobacco leaf segments treated with non-purified cellulase is shown in Figure 2. The same effect was found in grape leaves for H_2O_2 production (data not shown).

Extracellular Accumulation of AOS in the Culture Medium of Protoplasts

Freshly isolated protoplasts from tobacco leaves using purified cellulase contained 2.4 nM $O_2^{\cdot-}$ and 40 nM H_2O_2 per 10^5 protoplasts. The respective values for grape protoplasts were 0.9 and 6 nM. These protoplasts were cultured at a density of 10^5 protoplasts/mL. The culture medium of freshly isolated tobacco protoplasts contained 0.36 and 6.6 nM $O_2^{\cdot-}$ and H_2O_2, respectively, and that of freshly isolated grape protoplasts 0.12 and 2.5 nM, respectively (Fig. 3). The levels of both AOS were elevated, possibly due to isolation procedures such as physical pressure (Yahraus et al., 1995) and washing (Qian et al., 1993). Non-purified cellulase was added to these protoplasts, and AOS accumulation in the culture medium was monitored over a 4-h period. The detected levels of the two AOS in the culture medium were at the nanomolar and micromolar range for $O_2^{\cdot-}$ and H_2O_2, respectively.

As shown in Figure 3, a rapid accumulation of both AOS in the culture medium of tobacco protoplasts was found soon after the treatment. $O_2^{\cdot-}$ accumulation in the culture medium showed a 6-fold increase, reached a maximum after 15 min, and decreased slowly thereafter. H_2O_2, which was very low at time zero, increased dramatically (330-fold increase), reaching a maximum 2 h after treatment. In

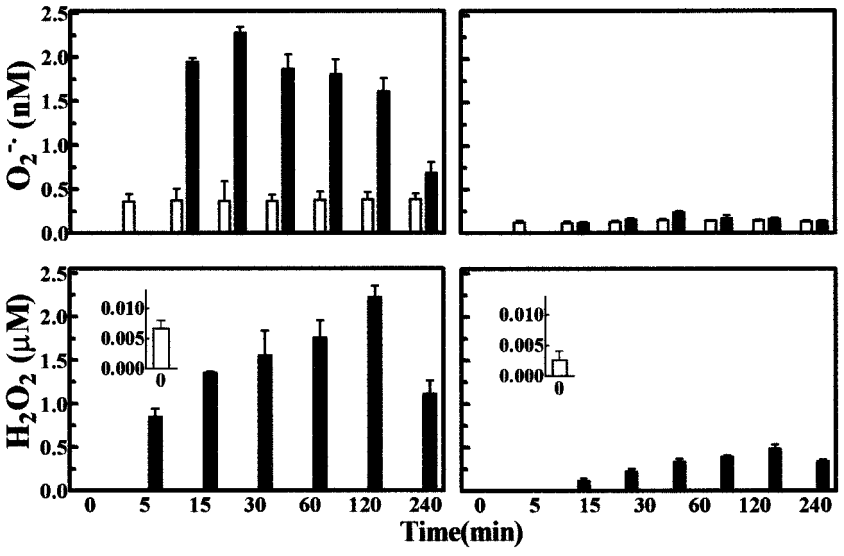

Figure 3. Generation of AOS in tobacco (left) and grape (right) mesophyll protoplasts treated with non-purified cellulase. Protoplasts were isolated after 4 h of maceration with 0.25% purified cellulase and 0.5% Macerozyme. Then, non-purified cellulase was added to protoplasts (10^5 mL^{-1}) at time zero, and the culture medium was assayed for $O_2^{\cdot-}$ and H_2O_2. Values are means ± SE from five independent experiments. White bars, Untreated protoplasts; black bars, treated protoplasts.

grape protoplasts, the addition of non-purified cellulase resulted in a 2- and a 185-fold increase in the extracellular level of $O_2^{\cdot-}$ and H_2O_2, respectively, compared with the controls, reaching a maximum 2 h after treatment (Fig. 3). The maximum extracellular concentrations in the culture medium of tobacco and grape protoplasts were 2.3 and 0.23 nM for $O_2^{\cdot-}$ and 2.22 and 0.48 μM for H_2O_2, respectively.

DDC (*N,N*-diethyldithiocarbamate), an inhibitor of Cu/Zn-SOD (Heikkila et al., 1976), was added to tobacco and grape protoplasts treated with non-purified cellulase to determine whether the inhibition of SOD would result in the accumulation of $O_2^{\cdot-}$ and inhibit the accumulation of H_2O_2 in the culture medium, which would indicate that H_2O_2 was formed by the dismutation of $O_2^{\cdot-}$ by SOD. No such effect was observed in either system. The addition of exogenous SOD to protoplasts strongly diminished the accumulation of $O_2^{\cdot-}$ caused by non-purified cellulase and stimulated H_2O_2 production in tobacco and grape protoplasts (Fig. 4). Furthermore, the addition of catalase to protoplasts resulted in the reduction of H_2O_2 accumulation, supporting the specificity of the method used (Fig. 4).

Inhibitors of the mammalian neutrophil NADPH oxidase (DPI, quinacrine, imidazole, and pyridine) and inhibitors of peroxidase (KCN and NaN_3) were used and their effect on extracellular accumulation of AOS was tested. DPI and quinacrine directly inhibit NADPH oxidase by binding to the flavoprotein component of the oxidase complex (Cross and Jones, 1986, 1991). In tobacco protoplasts, 25 μM DPI and 0.5 mM quinacrine inhibited the production of $O_2^{\cdot-}$ stimulated by non-purified cellulase by 50% and 69%, respectively (Fig. 4). Both imidazole (10 mM) and pyridine (20 mM), which bind to the Cyt *b* component of neutrophil oxidase (Iizuka et al., 1985), inhibited $O_2^{\cdot-}$ production by about 55% and 65%, respectively (Fig. 4). None of the four inhibitors affected H_2O_2 production (Fig. 4).

The addition of peroxidase inhibitors to tobacco protoplasts resulted in reduced accumulation of both AOS: 50 μM KCN and 5 mM NaN_3 decreased $O_2^{\cdot-}$ accumulation by about 40% and 35%, respectively, while both inhibitors completely eradicated H_2O_2 production, giving 100% inhibition (Fig. 4). In grape protoplasts, AOS production was not affected by the first class of inhibitors, but was strongly reduced by both KCN and NaN_3; $O_2^{\cdot-}$ production was inhibited by about 55% and 70% by the addition of KCN and NaN_3, respectively, and H_2O_2 production was completely inhibited by both agents (Fig. 4).

The above data indicate that different systems are responsible for AOS generation elicited by non-purified cellulase in each plant species. In tobacco, a mammalian-like oxidase seems to be responsible for $O_2^{\cdot-}$ production, since its inhibitors were able to reduce the accumulation of $O_2^{\cdot-}$, but this enzyme had no effect on H_2O_2 generation. A peroxidase-like activity may contribute to H_2O_2 generation and may also have a role in $O_2^{\cdot-}$ production. These two enzymes probably either operate in tandem or have different kinetics. In grape protoplasts, only peroxidase activity was identified with the use of inhibitors, and was responsible for the generation of both AOS (Fig. 4).

$O_2^{\cdot-}$ Synthases

In an effort to further identify the nature of the $O_2^{\cdot-}$-generating system in tobacco and grape, plasma membranes were isolated from protoplasts and assayed for NAD(P)H $O_2^{\cdot-}$ synthase activity (Table I). The enzyme was assayed from control protoplasts and from protoplasts treated with 1% non-purified cellulase for 30 min, when $O_2^{\cdot-}$ and H_2O_2 had already started to accumulate (Fig. 3). The $O_2^{\cdot-}$ synthase activity was strongly dependent on the presence of Triton X-100 when either NADPH or NADH was used as a substrate. The latency, given as 1 − (activity without Triton)/(maximum activity with Triton), was 1.0 ± 0.02 (an average from six independent plasma membrane preparations). The strongly stimulating effect of Triton X-100 on $O_2^{\cdot-}$ synthesis in plasma membrane vesicles is consistent with the idea that the oxidizing sites of $O_2^{\cdot-}$ synthase(s) are on the cytoplasmic side of the vesicles, unavailable to the NADPH or NADH in the absence of the detergent (Murphy and Auh, 1996).

In tobacco plasma membrane preparations, the $O_2^{\cdot-}$-generating activity was higher than in grape no matter which substrate used (Table I). $O_2^{\cdot-}$ production from to-

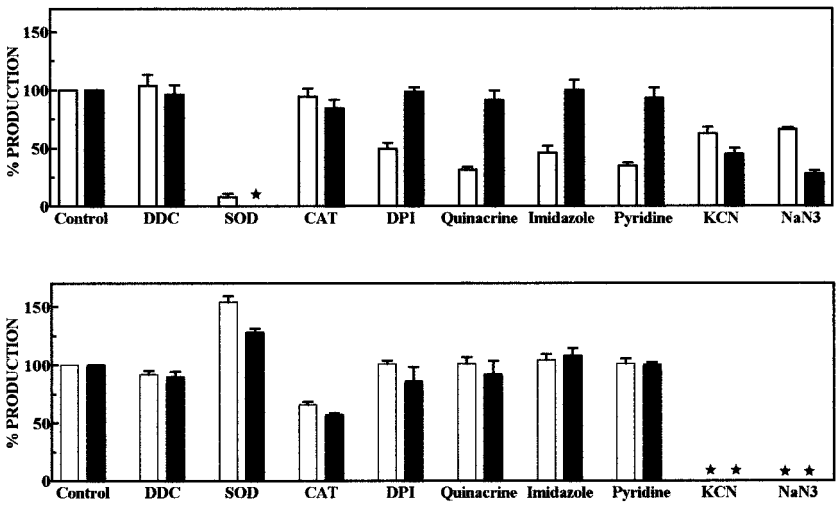

Figure 4. Effects of inhibitors, exogenous SOD, and catalase on the production of $O_2^{\cdot-}$ (top) and H_2O_2 (bottom) by tobacco (white bars) and grape (black bars) protoplasts treated with non-purified or purified cellulase. Inhibitors were added to protoplasts 10 min before the addition of cellulase. Samples (culture medium) were taken 30 min after treatment and assayed for $O_2^{\cdot-}$ and H_2O_2. Values are relative to control (100%). Background activities (without protoplasts but with inhibitor) were subtracted. The concentration of the inhibitors was 10 μM DDC, 25 units/mL SOD, 100 units/mL CAT, 25 μM DPI, 0.5 mM quinacrine, 10 mM imidazole, 20 mM pyridine, 50 μM KCN, and 5 mM NaN_3. ★, Negative values. Means ± SE were calculated from six independent experiments.

bacco plasma membrane preparations showed differences in substrate (NADPH or NADH) affinity, although both obeyed Michaelis-Menten kinetics (data not shown). The K_m was 7.9 ± 2.7 μM for NADPH and 102.5 ± 6.1 μM for NADH. In grape, the respective values for the K_m were 32.6 ± 7.8 and 43.1 ± 8.3 μM (Table I). Plasma membranes prepared from protoplasts treated with non-purified cellulase had the same specific activity as the control preparations in both plant species (Table I).

Various inhibitors were tested for revealing the possible relationship of tobacco and grape $O_2^{\cdot-}$ synthases to other characterized oxidases. The plasma membrane vesicles used in the inhibitor studies were isolated from protoplasts treated with non-purified cellulase. In the presence of 100 μM NADPH, 25 μM DPI reduced the $O_2^{\cdot-}$ synthase activity by 50% in tobacco but had no effect in grape (Fig. 5). Imidazole (10 mM), another inhibitor of the mammalian NADPH oxidase, resulted in about 75% inhibition in tobacco plasma membrane preparations (Fig. 5). With 200 μM NADH, neither DPI nor imidazole affected $O_2^{\cdot-}$ production in either plant species. In tobacco, $O_2^{\cdot-}$ production was reduced by about 55% and 70% by 50 μM KCN and 5 mM NaN_3, respectively, when NADPH was used, and by 40% and 70%, respectively, when NADH was used as the substrate. In grape, an approximately 40% inhibition was caused by both KCN and NaN_3 when NADPH was used, and 75% and 80% inhibition, respectively, when NADH was used as the substrate (Fig. 5).

In tobacco plasma membrane preparations, two different $O_2^{\cdot-}$ synthase activities were found: one that exhibited specificity for NADPH and sensitivity to DPI and imidazole, and a second that could use either NADPH or NADH and was sensitive to KCN and NaN_3. The first activity fits the profile of a mammalian-like oxidase (Doke and Miura, 1995; Murphy and Auh, 1996; Van Gestelen et al., 1997) and the second that of a a NAD(P)H oxidase-peroxidase (Askerlund et al., 1987; Vera-Estrella et al., 1992; Bolwell et al., 1995; Bestwick et al., 1997). In grape only the second activity was detected.

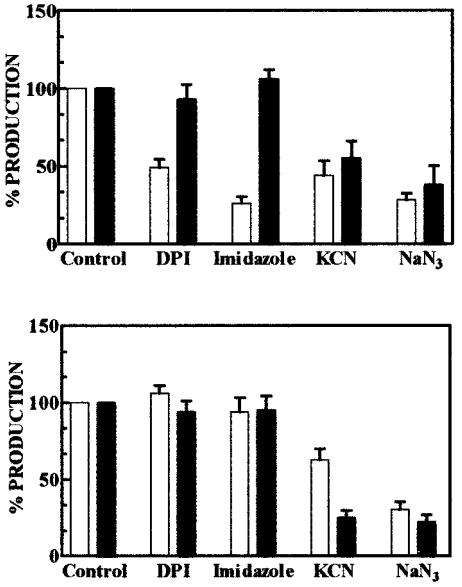

Figure 5. Effect of inhibitors on $O_2^{\cdot-}$ synthase activity of plasma membrane preparations from tobacco (white bars) and grape (black bars) protoplasts. All assays contained 5 μg of plasma membrane protein, plus 100 mM Gly-NaOH, pH 9.0, 1 mM EDTA, 0.02% (v/v) Triton X-100, 0.4 mM lucigenin, and either 100 μM NADPH (top) or 200 μM NADH (bottom). Values are relative to control (100%). Background activities (without enzyme but with inhibitor) were subtracted. The concentration of the inhibitors was 25 μM DPI, 10 mM imidazole, 50 μM KCN, and 5 mM NaN_3. Means ± SE were calculated using five independent plasma membrane preparations.

Table I. *$O_2^{\cdot-}$-synthase activity in plasma membrane preparations from tobacco and grape leaf protoplasts treated with non-purified cellulase or untreated*

All the assays for synthase activity contained 100 mM Gly-NaOH, pH 9.0, 1 mM EDTA, 0.02% (v/v) Triton X-100, 0.4 mM lucigenin, 100 μM NADPH or 200 μM NADH, and 5 μg of plasma membrane protein. K_m was calculated from curves fitted to the Michaelis-Menten equation by the nonlinear regression procedure (data not shown). Background activities (without enzyme) were subtracted. Means ± SE were calculated using four independent plasma membrane preparations.

Species and Treatment	NADPH	NADH
	μmol $O_2^{\cdot-}$ mg^{-1} protein min^{-1}	
Tobacco[a]		
Control	163.5 ± 4.9	110.2 ± 2.6
Non-purified cellulase	150.0 ± 11.8	97.1 ± 15.4
Grape[b]		
Control	44.2 ± 4.6	65.3 ± 8.4
Non-purified cellulase	38.0 ± 6.9	55.8 ± 10.5

[a] K_m NADPH = 7.9 ± 2.7 μM; K_m NADH = 102.5 ± 6.1 μM. [b] K_m NADPH = 32.6 ± 7.8 μM; K_m NADH = 43.1 ± 8.3 μM.

DISCUSSION

The expression of totipotency in cultured protoplasts seems to involve at least two developmental pathways: the suppression of events leading to cell death and the induction of cell elongation and cell division. Results from previous comparative work using regenerating tobacco and non-regenerating grape protoplasts indicated that H_2O_2 participates in the peroxidase-mediated intramolecular isotyrosine ether cross-linking of reconstituted cell walls (Hahne and Hoffmann, 1984; Gaspar et al., 1989; Iiyama et al., 1994; de Marco and Roubelakis-Angelakis, 1996a, 1996b, 1997). Total peroxidase was significantly higher in regenerating than in non-regenerating protoplasts during culture, comprising expression of specific isoenzymes (Siminis et al., 1993), whereas NADH peroxidase activity, which has been proposed to generate H_2O_2 (Gross et al., 1977; Elstner and Heupel, 1978), was not detectable at all in non-regenerating tobacco protoplasts (Siminis et al., 1993; de Marco and Roubelakis-Angelakis, 1996a). Protoplasts survived but lost their dividing potential when peroxidase activity was inhibited by cyanide or DTT and when exogenous catalase was added to the culture medium, suggesting that division is dependent on modification of cell wall plasticity (de Marco and Roubelakis-Angelakis, 1996a, 1996b).

On the other hand, oxidative stress can induce cell death. The H_2O_2-scavenging activity of catalase was higher in regenerating (tobacco) than in non-regenerating (grape) protoplasts, with differences in the expression of specific subunits (Siminis et al., 1994; de Marco and Roubelakis-Angelakis, 1996a). Ascorbate peroxidase activity and transcript were present only in regenerating tobacco protoplasts, which did not survive when ascorbate peroxidase activity was inhibited (de Marco and Roubelakis-Angelakis, 1996b). The activities of antioxidant enzymes of the Halliwell-Asada pathway were significantly lower in non-regenerating protoplasts (de Marco and Roubelakis-Angelakis, 1996a, 1999).

In this work we have attempted to break down the oxidative burst response into individual AOS species and individual AOS-generating systems in leaf strips, protoplasts, and plasma membrane vesicles from tobacco and grape. $O_2^{\cdot-}$ can be generated by the plasma membrane mammalian-like NAD(P)H oxidase (Askerlund et al., 1987; Vera-Estrella et al., 1992; Bolwell et al., 1995). This enzyme in mammals is composed of membrane-bound and cytosolic proteins. In the center of the NADPH oxidase lies the heterodimeric NADPH-binding flavocytochrome b_{558}, which consists of the glycosylated transmembrane protein $gp91^{phox}$ and the nonglycosylated $p22^{phox}$ subunit. The flavocytochrome contains the entire electron transport chain from NADPH to O_2. Upon activation, the cytosolic proteins $p47^{phox}$ and $p67^{phox}$ become phosphorylated and translocate, together with $p40^{phox}$ and $p21^{rac}$, to the membrane to form the active NADPH oxidase complex (Segal and Abo, 1993). The generated $O_2^{\cdot-}$ can then form H_2O_2 by spontaneous or SOD-mediated dismutation (Doke, 1983; Levine et al., 1994; Auh and Murphy, 1995; Doke and Miura, 1995; Murphy and Auh, 1996) and/or by the action of extracellular peroxidases (Askerlund et al., 1987; Vera-Estrella et al., 1992; Bolwell et al., 1995, 1998; Bestwick et al., 1997).

AOS generation by tobacco and grape leaf strips was not induced by extensive wounding and/or addition of purified cellulase (Fig. 1); the addition of non-purified cellulase known to contain, among other things, xylanase and pectine lyase (Fuchs et al., 1989) resulted in a dose-dependent (Fig. 2), significant increase in both AOS in tobacco leaf strips (Fig. 1), whereas in grape the response was much less. The addition of non-purified cellulase to freshly isolated (with purified cellulase) protoplasts resulted in a 6- and 330-fold and 2- and 185-fold increase of $O_2^{\cdot-}$ and H_2O_2 in tobacco and grape protoplasts, respectively (Fig. 3). H_2O_2 was the building force of the oxidative burst, which agrees with other studies (Bolwell et al., 1995; Desikan et al., 1996). On the other hand, Doke (1983) and Auh and Murphy (1995) characterized $O_2^{\cdot-}$ as the major AOS in potato and rose, respectively, whereas in tomato suspension cells both AOS were detected after elicitation (Vera Estrella et al., 1992).

$O_2^{\cdot-}$ and H_2O_2 accumulation in tobacco and grape protoplasts stimulated by non-purified cellulase was not affected by DDC (Fig. 4), a chelator of Cu ions and an inhibitor of Cu/Zn-SOD (Heikkila et al., 1976), indicating that there is no extracellular SOD activity that could mask $O_2^{\cdot-}$ detection or be involved in H_2O_2 production through enzymic dismutation of $O_2^{\cdot-}$. Also, diamine or/and polyamine oxidases do not contribute to H_2O_2 production in tobacco protoplasts (de Marco and Roubelakis-Angelakis, 1996a). The hypothesis that phytotoxic factors from cell walls elicit AOS generation during protoplast isolation (Hahne and Lorz, 1988) is not supported by our results, because: (a) AOS accumulation occurred in protoplasts, which lack cell walls; (b) the generated AOS by leaf strips treated with non-purified cellulase were significantly lower than the AOS generated by protoplasts; and (c) no AOS were generated by leaf strips treated with purified cellulase (Figs. 1 and 3).

The induced $O_2^{\cdot-}$ generation by tobacco protoplasts was sensitive to four inhibitors of the mammalian neutrophil NADPH oxidase, whereas none of them inhibited $O_2^{\cdot-}$ production in grape protoplasts treated with non-purified cellulase (Fig. 4). In contrast, KCN and NaN_3, inhibitors of plant peroxidases, both affected $O_2^{\cdot-}$ production and completely inhibited H_2O_2 generation in both plant species (Fig. 4). These results, along with those from isolated plasma membranes (Table I; Fig. 5), support the idea that in tobacco two different $O_2^{\cdot-}$ synthase activities are operating; one with specificity for NADPH and sensitivity to DPI and imidazole, which fits the profile of the mammalian-like oxidase, and a NAD(P)H oxidase-peroxidase, which can use either NADPH or NADH and is inhibited by KCN and NaN_3. In grape, only the second activity was detected, and it was responsible for the synthesis of both AOS. Two distinct sources of elicited AOS were also described in tobacco epidermal cells: a flavin-containing oxidase system and a cell wall peroxidase-like activity (Allan and Fluhr, 1997). In rose cells, H_2O_2 is produced by a plasma membrane NAD(P)H oxidase, whereas in bean cells it is derived directly from cell wall peroxidases (Bolwell et al., 1998).

Plasma membranes isolated from protoplasts treated with non-purified cellulase did not exhibit significantly higher $O_2^{\cdot-}$-generating activity compared with control preparations of both species (Table I), nor was the addition of non-purified cellulase to isolated plasma membranes from control protoplasts able to further stimulate $O_2^{\cdot-}$ synthesis. This result is in agreement with the results of Murphy and Auh (1996) using membranes from rose cells treated with *Phytophthora infestans* elicitor, and from results using potato (Doke and Miura, 1995), in which $O_2^{\cdot-}$ synthesis could be stimulated in vitro by *P. infestans* elicitors only in the presence of cytosolic components. The immunological and molecular evidence that the cytosolic proteins p47, p67, and Rac1, which are components of the neutrophil NADPH oxidase, also exist in plant cells and are prerequisites for activation and membrane assembly of the oxidase complex (Tenhaken et al., 1995; Dwyer et al., 1996; Groom et al., 1996; Kieffer et al., 1997; Keller et al., 1998) explain the above results.

The release of both AOS was extracellular (Figs. 1 and 3), which is consistent with results from potato protoplasts (Doke, 1983) and rose cells (Auh and Murphy, 1995), while $O_2^{\cdot-}$ synthesis by plasma membrane vesicles was strongly stimulated by detergent, which indicates a cytoplasmic site of $O_2^{\cdot-}$ generation (Murphy and Auh, 1996). Allan and

Fluhr (1997) suggested that AOS generation takes place within the cell or in the apoplast, depending on the elicitor used. Recently, a homolog of the neutrophil NADPH oxidase gp91^{Phox} subunit gene that encodes for a intrinsic plasma membrane protein was reported in Arabidopsis (Keller et al., 1998), suggesting that the initial products of the NADPH oxidase reaction will accumulate on the external face of the plasma membrane, which is in keeping with the functions of AOS in cell wall cross-linking and intercellular signaling (Lamb and Dixon, 1997).

The elicitor molecule for the induction of AOS generation remains unknown, but several putative receptors have been identified and at least partially characterized. Further interactions implicate the involvement of GTP-binding proteins, ion channels, protein kinases and phosphatases, phospholipases A and C, and possibly cAMP along the signaling pathway leading to the activation of NADPH oxidase (Wojtaszek, 1997, and refs. therein). Differences between tobacco and grape in the component(s) of the enzyme(s) and/or the receptors and the activators of AOS generation and removal, as well as their physiological significance on the complex developmental phenomena of viability and regenerating potentiality of protoplasts remain to be elucidated with the use of currently available immunological and molecular probes.

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