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Sensitivity of Superoxide Dismutase Transcript Levels and Activities to Oxidative Stress Is Lower in Mature-Senescent Than in Young Barley Leaves[1]

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Antioxidant enzyme activities are inducible by oxidative stress and decrease during senescence. To determine if the age-dependent decrease of superoxide dismutase (SOD) activities is due to decreased sensitivity to oxidative stress, we have investigated the changes in steady-state levels of transcripts and activities of mitochondrial Mn-SOD (SOD1), chloroplastic Fe-SOD (SOD2), and cytoplasmic Cu-Zn-SOD (SOD3) in young and mature-senescent detached barley (*Hordeum vulgare* L.) leaves in response to incubation in darkness, growth light (20 W m^{-2}), and photooxidative stress conditions (100 W m^{-2} with 21 or 100% O_2). For a comparison, changes in the mRNA for ribulose bisphosphate carboxylase were also measured. After leaf detachment, the abundance of all three SOD mRNAs increased, then decreased and eventually stabilized after 6 h of incubation. After 20 h of incubation under darkness SOD transcripts decreased in both young and mature-senescent leaves. While under strong photooxidative stress the levels of the three SOD transcripts significantly increased in young leaves; in mature-senescent leaves SOD2 and, to lesser extent, SOD1 and SOD3 transcripts decreased. Generally, SOD activity changes were similar to those of mRNAs. It is proposed that oxidative damage during senescence could be favored by the inability of senescing leaves to modulate the steady-state level of SOD mRNA, and probably those of other antioxidant enzymes, concomitant with the rate of oxyradical formation.

Senescence and oxidative stress syndromes share a number of common symptoms such as net loss of chloroplastic pigments and proteins (Trippi et al., 1989; Casano et al., 1990), lipid peroxidation, and membrane alterations (Thompson et al., 1983; Wise and Naylor, 1987) leading to a progressive decrease in photosynthetic capacity. Moreover, stressful environments, such as drought and high light intensities, which increase the formation of active oxygen species, accelerate senescence (De Luca dOro and Trippi, 1987; Thompson et al., 1987), demonstrating that these toxic compounds may play an important role in the development of senescence.

Oxidative stress arises from deleterious effects of active oxygen species, such as hydrogen peroxide and superoxide anion and hydroxyl radicals, which react with lipids, pigments, proteins, and nucleic acids (Cadenas, 1989, and refs. therein). Oxyradicals are by-products of many biological oxidations, and the electron transport chains of mitochondria and chloroplasts are two well-documented sources of active oxygen compounds (Asada and Takahashi, 1987). To prevent oxidative stress, organisms have evolved a complex antioxidant system composed of nonenzymic and enzymic mechanisms that scavenge active oxygen species (Asada and Takahashi, 1987; Salin, 1988; Bowler et al., 1992). Among antioxidant enzymes, SODs play a key role, since they convert superoxide to hydrogen peroxide and oxygen. SODs are metalloenzymes that generally occur in three different molecular forms containing either Mn, Fe, or Cu-Zn as prosthetic metals (Fridovich, 1986). Subcellular fractionation studies performed in different plant species indicate that in general plants contain mitochondrial Mn-SOD, cytosolic Cu-Zn-SOD, and Fe- and/or Cu-Zn-SOD within chloroplasts (Bowler et al., 1992, and refs. therein). Recently, different types of SODs have also been demonstrated in peroxisomes from pea, watermelon, and carnation (Del Río et al., 1992).

Since active oxygen compounds are continuously formed during the whole of leaf ontogeny, oxidative stress is a priori an ever-present possibility, especially under adverse environmental conditions. However, in young and mature leaves a high steady-state level of antioxidant enzyme activities protects leaves from oxidative damage (Asada and Takahashi, 1987). Besides, tolerance to oxidative stress depends on, at least, inducible high oxygen-detoxifying enzyme activities (Bowler et al., 1992; Gupta et al., 1993). As a plant senesces, activities of all the SOD enzymes, as well as those of the other antioxidant enzymes, decrease (Pauls and Thompson, 1984; Polle et al., 1989; Pastori and Trippi, 1993). At present it is not clear if the decrease in antioxidant activities during senescence is due to a low response to oxidative stress or if oxidative stress is low due to the decreased metabolic activity as tissues age. In *Pinus* needles in early spring, higher SOD mRNA levels in young top shoots than in side shoots could be attributed to the lower photooxidative stress in snow-covered side shoots (Karpinski et al., 1993). In other cases, it

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Abbreviations: *rbc*L, gene for the large subunit of ribulose bisphosphate carboxylase/oxygenase; SOD1, Mn-superoxide dismutase; SOD2, Fe-superoxide dismutase; SOD3, Cu-Zn-superoxide dismutase.

is possible that the sensitivity of the response to photooxidative stress decreases when the leaf becomes old.

The aim of the present work was to determine if during senescence plants lose the capacity to induce antioxidant enzymes at the level of mRNA, especially under adverse conditions. We compared the levels of mitochondrial Mn-SOD (SOD1), chloroplastic Fe-SOD (SOD2), and cytoplasmic Cu-Zn-SOD (SOD3) transcripts and SOD activities in young and mature-senescent barley (*Hordeum vulgare* L.) leaves subjected to dark-induced senescence and photooxidative stress. The results suggest a loss of the capacity for SOD induction as the plant senesces, since even when darkness decreased SOD transcripts and activities in both leaf types, transcripts and enzymic activities increased in young leaves but decreased in mature-senescent leaves after exposure to high light and 100% O_2.

MATERIALS AND METHODS

Plant Materials

Barley (*Hordeum vulgare* L. cv Hassan) was grown on vermiculite in a controlled growth chamber at 23°C under a 16-h photoperiod of 20 W m^{-2} white light as previously described (Cuello et al., 1984; Martín et al., 1986). Under these conditions, the oldest primary leaf expands up to the 9th d. It is mature (fully expanded) between the 9th and 12th d and then begins a slow senescence (Martín et al., 1986). In the present work, we have used primary leaves of 7- and 14-d-old plants as young and mature-senescent leaves, respectively. Senescence acceleration was induced by detachment and incubation on water in darkness. For all treatments, leaves were cut into approximately 1-cm segments immediately after their excision from the plants. Detachment-accelerated senescence was inhibited by 50 μM kinetin treatment in the dark (Martín and Sabater, 1989) and by incubation in the growth light (20 W m^{-2}). Photooxidative stress was induced by incubating detached leaves in higher light (100 W m^{-2}) in the presence of 21 or 100% O_2. All treatments were performed at 23°C and, unless stated otherwise, for 20 h.

RNA Preparation

Ten to 15 g of leaves were ground under liquid nitrogen and RNA preparation was performed by phenol/SDS extraction and selective precipitation using LiCl according to Ausubel et al. (1990). The average yield of total RNA was 0.3 mg from 1 g of leaves.

Labeling of Probes and RNA Analysis

Cloned fragments of tobacco chloroplast DNA pTB18 and pTB29, containing 16S RNA and *rbc*L genes, respectively, were provided by M. Sugiura (Nagoya University Nagoya, Japan), and cDNA clones of mitochondrial SOD1, chloroplastic SOD2, and cytoplasmic SOD3 from *Nicotiana plumbaginifolia* were a gift of M. Van Montagu (State University, Ghent, Belgium). Plasmids were random-prime labeled with digoxigenin according to instructions with the kit purchased from Boehringer Mannheim (Mannheim, Germany). Southern analysis showed no cross-hybridization among SOD probes, as was previously reported (Tsang et al., 1991). By northern analysis (data not shown) we detected in the barley RNA preparation single-band transcripts of 1800, 1050, 830, and 800 nucleotides for *rbc*L, SOD1, SOD2, and SOD3, respectively.

Transcript levels were estimated by dot-blot assays. Fifty to 60 μg of denatured RNA were immobilized on nylon membrane (Zeta-Probe, Bio-Rad) and hybridized to DNA probes according to the instructions of the supplier (Boehringer Mannheim). Washings were performed under low-stringency conditions. Membranes were scanned using a UVP Easy digital image analyzer (Ultra-Violet Limited, Cambridge, UK) to semi-quantify the abundance of transcripts, which was expressed on a chloroplast 16S rRNA basis.

Preparation of Crude Extracts and Assay of SOD Activity

Leaves (2 g) were homogenized in 50 mM K-phosphate buffer, pH 7.0, containing 2 mM EDTA, 1 mM mercaptoethanol, and 1% water-insoluble PVP and then pelleted at 22,000*g* for 20 min. For separation of SOD isozymes, aliquots of supernatants from each treatment (70 μg of protein) were electrophoresed on a 10% nondenaturing acrylamide gel. SOD isozymes were detected in the gel by the photochemical nitroblue tetrazolium staining method according to Beauchamp and Fridovich (1971). The different types of SOD were distinguished by sensitivity to inhibition by 2 mM KCN and 5 mM H_2O_2. Mn-SOD is resistant to CN^- and H_2O_2, Fe-SOD is resistant to CN^- but inhibited by H_2O_2, and Cu-Zn-SOD is inactivated by both inhibitors (Fridovich, 1986). To semi-quantify the different SOD activities, stained gels were scanned using a UVP Easy digital image analyzer. Computed values were expressed as percentages with respect to those of freshly detached leaves scanned from the same electrophoretic separation.

RESULTS

Changes in the Levels of SOD Transcripts after Leaf Cutting and Incubation in the Dark

Cutting and incubation of leaf segments in the dark is a common procedure to accelerate senescence in leaves (Martín et al., 1986). However, mechanical injury also induces a wound-related response in leaves and frequently it is difficult to distinguish wound-dependent from senescence-dependent effects on gene expression in the experimental system of detached leaf segments (Becker and Apel, 1993). Usually, wound-related effects on gene expression take place during the first 1 to 4 h after cutting (Ghosh et al., 1993). To distinguish early from late effects of the combined action of mechanical injury and incubation in the dark on the levels of SOD transcripts, we measured SOD1, SOD2, and SOD3 transcripts at different times in detached mature-senescent leaves incubated in the dark (Fig. 1). We also measured the abundance of *rbc*L mRNA to compare the behavior of this well-documented developmentally regulated and light-regulated gene with those of SOD genes. The results of a limited number of measurements carried out in detached young leaves are also included in Figure 1.

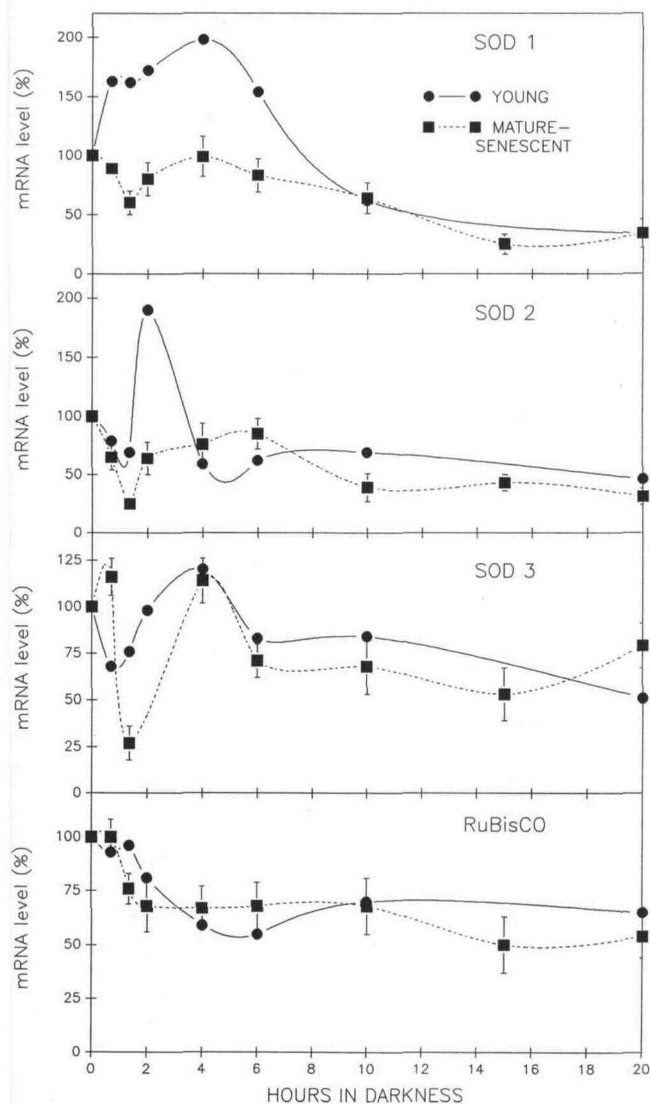

Figure 1. Effect of detachment on the levels of transcripts of SOD1, SOD2, and SOD3 genes in young (7 d old) and mature-senescent (14 d old) barley leaves. At different times of incubation in the dark at 23°C, total RNA was extracted and SOD mRNA levels were measured by dot-blot assay, as described in "Materials and Methods." To overcome possible differences among samples in the amount of total RNA loaded, a set of membranes was hybridized with the 16S rRNA probe. Spots were quantified and the levels of SOD transcripts were referred to a standard amount of chloroplast 16S rRNA. Each indicated value for mature-senescent leaves is the mean ± SE of at least three independent experiments (only SE values larger than the symbol sizes are represented). Most indicated values for young leaves correspond to a single experiment.

When compared to the levels of chloroplast rRNAs, there are initial decreases in SOD transcripts (except for the SOD1 transcript in segments of young leaves) around the 1st h of incubation followed by transient increases between 2 and 6 h after incubation (Fig. 1). Then they decreased again and almost stabilized between 8 and 20 h after incubation at levels 40 to 70% of the original levels. In contrast, transcript abundance of chloroplast-encoded *rbc*L genes continuously decreased for the first 4 h of incubation and then stabilized (Fig. 1). Despite the limited number of assays with young leaves, their general behavior seemed not to be age dependent except for SOD1 transcripts. Cutting and incubation in the dark induced rapid and reproducible oscillation of SOD transcript levels before their final stabilization. To avoid uncertainties during the first hours of incubation, in the following experiments we investigated the response of mRNAs and activities after 20 h of different treatments of leaf segments.

Effects of Senescence Treatments and Photooxidative Stress on the Level of SOD Transcripts

As reported for other species (Bowler et al., 1992), three groups of molecular forms of SOD could be detected in barley leaf extracts after PAGE-zymograms (Fig. 2B). The identity of each type of activity could be determined by controls (Fridovich, 1986) of PAGE preparations previously incubated with CN^- or H_2O_2 (experiments not shown). Mn-SOD isozymes showed the lower electrophoretic mobility, Cu-Zn-SOD isozymes comprised a group of bands with middle electrophoretic mobility, and the intense band with higher mobility corresponded to a Fe-SOD. Sometimes, a weak Cu-Zn-SOD band migrated close to the Fe-SOD band.

SOD activities decrease during senescence, but they often increase under stress conditions (Matters and Scandalios, 1986; Bowler et al., 1991). To determine if stress response changes with leaf age we measured levels of SOD transcripts and activities in young and mature-senescent excised barley leaves after 20 h of incubation in the dark, in growth light (20 W m^{-2}), in high light (100 W m^{-2}) (moderate photooxidative stress), and in high light plus 100% O_2 (strong photooxidative stress). Figure 2A shows typical dot-blot results from when the SOD1 probe was used to assay RNA preparations from young and mature-senescent leaf segments incubated for 20 h under different conditions affecting senescence and photooxidative stress. It may be seen that the level

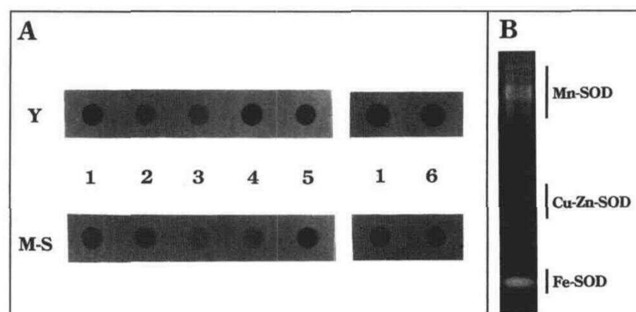

Figure 2. A, Dot-blot assays of SOD1 transcript in RNA preparations from young (Y) and mature-senescent (M-S) barley leaf segments incubated for 0 h (1) or 20 h in the dark (2), in the dark in the presence of 50 μM kinetin (3), under growth light (20 W m^{-2}) (4), under moderate photooxidative stress (100 W m^{-2}) (5), or under strong photooxidative stress (100 W m^{-2} + 100% O_2) (6). B, Native PAGE of SOD isozymes from mature-senescent barley leaf segments. Different types of SOD isozyme, as revealed by differential inhibition with H_2O_2 and CN^- (data not shown), are indicated on the right. For more details see "Materials and Methods."

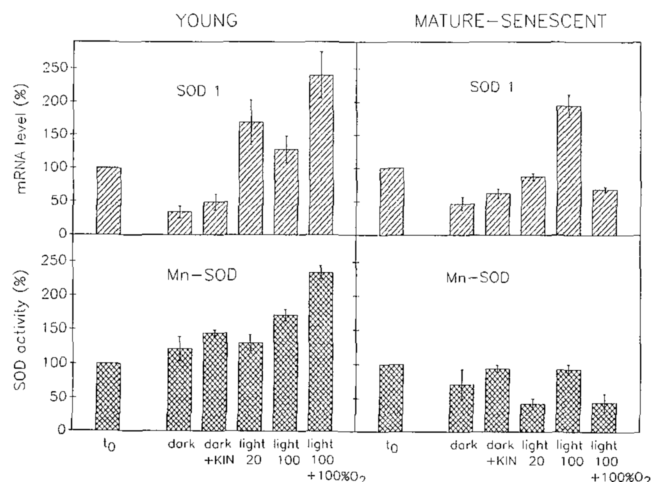

Figure 3. Effects of different treatments of young and mature-senescent leaf segments on SOD1 transcript and Mn-SOD activity levels. After 0 (t_0) or 20 h of incubation in the dark, in the dark in the presence of 50 μM kinetin (KIN), under growth light (20 W m^{-2}), or under moderate photooxidative stress (100 W m^{-2}) or strong photooxidative stress (100 W m^{-2} + 100% O_2), at 23°C, the levels of transcripts of SOD1 and of Mn-SOD activity were measured as described in "Materials and Methods."

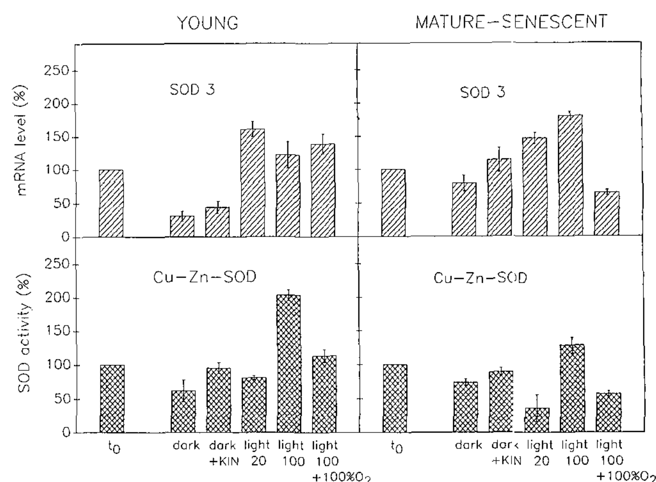

Figure 5. Effects of different treatments of young and mature-senescent leaf segments on SOD3 transcript and Cu-Zn-SOD activity levels. After 0 (t_0) or 20 h of incubation in the dark, in the dark in the presence of 50 μM kinetin (KIN), under growth light (20 W m^{-2}), or under moderate photooxidative stress (100 W m^{-2}) or strong photooxidative stress (100 W m^{-2} + 100% O_2), at 23°C, the levels of transcripts of SOD3 and of Cu-Zn-SOD activity were measured as described in "Materials and Methods."

of SOD1 is strongly affected by the treatment and the age of leaves.

Figures 3 to 5 show detailed quantitative data for transcript levels of SOD1, SOD2, and SOD3, respectively (obtained from assays with different probes), and of probable corresponding SOD activities in young and mature-senescent leaf segments exposed to senescence-accelerating and stress conditions. For comparison, Figure 6 shows data of *rbc*L transcript levels in young and mature-senescent leaf segments exposed to the same conditions

As shown in Figure 1, segments from young and mature-senescent leaves responded similarly to detachment and darkness by reducing the abundance of SOD and *rbc*L transcripts after 20 h of incubation (Figs. 3–6). Since the main source of oxyradicals in the leaf is the photosynthetic electron transport chain (Asada and Takahashi, 1987), one possibility

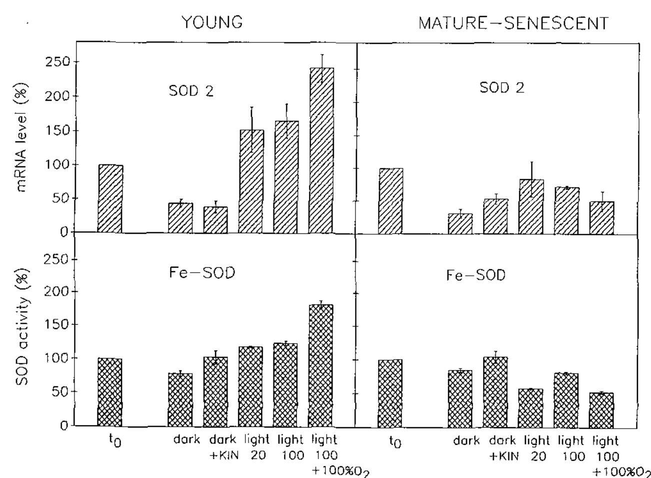

Figure 4. Effects of different treatments of young and mature-senescent leaf segments on SOD2 transcript and Fe-SOD activity levels. After 0 (t_0) or 20 h of incubation in the dark, in the dark in the presence of 50 μM kinetin (KIN), under growth light (20 W m^{-2}), or under moderate photooxidative stress (100 W m^{-2}) or strong photooxidative stress (100 W m^{-2} + 100% O_2), at 23°C, the levels of transcripts of SOD2 and of Fe-SOD activity were measured as described in "Materials and Methods."

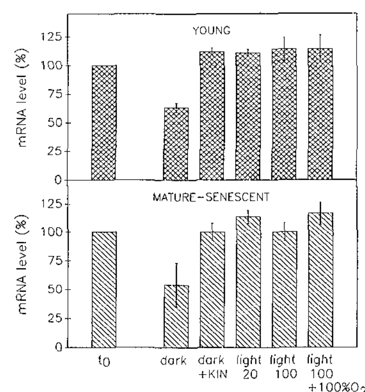

Figure 6. Effects of different treatments of young and mature-senescent leaf segments on *rbc*L transcript levels. After 0 (t_0) or 20 h of incubation in the dark, in the dark in the presence of 50 μM kinetin (KIN), under growth light (20 W m^{-2}), or under moderate photooxidative stress (100 W m^{-2}) or strong photooxidative stress (100 W m^{-2} + 100% O_2), at 23°C, the levels of transcripts of *rbc*L were measured by dot-blot assays. Each indicated value is the mean ± SE of at least five independent experiments. For more details see the legend of Figure 1.

is that the low abundance of SOD transcripts in dark-incubated leaves could be due to a reduced rate of superoxide formation. Alternatively, dark-promoted senescence may have the general effect of lowering SOD mRNAs as well as *rbc*L mRNA. Although the antisenescent plant regulator kinetin was effective in preventing *rbc*L transcript decrease (Fig. 6), as expected (Vera et al., 1990), it did not significantly overcome the dark-induced loss of SOD transcripts (Figs. 3–5). These facts suggest that SOD transcript decreases were more dependent on a reduced rate of superoxide formation in dark-incubated leaves than on a general effect of senescence.

On the other hand, the responses of SOD transcript levels to light treatments of leaf segments were clearly age dependent. Although incubation under a light intensity equal to that under which plants had previously been grown (20 W m^{-2}) significantly increased SOD transcripts in young leaves, it only slightly modified SOD1 and SOD2 levels in mature-senescent leaves, with respect to those of starting material (Figs. 3 and 4, t_0). Growth-light treatment increased levels of SOD3 transcripts by 40% (Fig. 5) in mature-senescent leaves. In fact, in those leaves SOD3 transcripts were barely affected by dark incubation with or without kinetin.

Except for the transcript of SOD1 in mature-senescent leaf segments, which significantly increased, the effects of 100 W m^{-2} light (moderate photooxidative stress conditions) on *rbc*L and SOD transcript abundance were very similar to those of 20 W m^{-2} light (growth light) in both young and mature-senescent leaf segments. Although 100 W m^{-2} is not a very high light intensity in absolute terms, it is sufficient to induce photooxidative stress in low intensity light-adapted leaves (Trippi and De Luca dOro, 1985) and chloroplasts (Casano et al., 1990).

The clearest differences between the responses of young and mature-senescent leaf segments were found under high light intensity and 100% oxygen (strong photooxidative stress). Although young leaves increased the levels of SOD1, SOD2, and SOD3 by factors of 2.5, 2.5, and 1.5, respectively, compared to time 0 values (increases are more pronounced with respect to dark incubations), the abundance of the same transcripts in mature-senescent leaves decreased some 40 to 50% with respect to time 0 (or was barely affected in respect to dark incubation) (Figs. 3–5).

It must to be noted that kinetin and light treatments increased *rbc*L transcript levels (with respect to dark-incubated leaves) in both young and mature-senescent (Fig. 6) barley leaf segments, raising it to values similar to that of 0 time. Also, the strong photooxidative stress did not affect the level of *rbc*L transcript, in spite of the age-dependent and photooxidative stress-induced changes observed in SOD mRNAs.

Effects of Senescence Treatment and Photooxidative Stress on SOD Activities

The effects of different treatments of leaves on SOD activities were not as pronounced as the effects on transcripts (Figs. 3–5). This is not surprising because changes in enzyme synthesis are delayed with respect to changes in mRNA levels, and measured stationary activities depend on rates of synthesis, inactivation, and degradation of the corresponding enzymes. Even so, in general the overall patterns of changes in activities corresponded with those in transcript levels. This is clear for the abundant (Fig. 2) Fe-SOD activity and SOD2 transcript in young leaves (Fig. 4). A small fraction of the low-abundance Cu-Zn-SOD activity (Fig. 2) was not included in the computations (because it co-migrated with the abundant Fe-SOD), which could explain the lower correspondence between SOD2 transcript and Cu-Zn-SOD activity.

As for transcript levels, leaf age markedly influenced the response of SOD activities to photooxidative stress. Young leaves maintained or increased activities under moderate or strong photooxidative stress. However, the three groups of SOD isozymes decreased in mature-senescent leaves exposed to stress treatments, especially under the strong photooxidative conditions (Figs. 3–5). Large relative differences between transcript and activity levels for each SOD (e.g. SOD1 and Mn-SOD in mature-senescent leaves at 100 W m^{-2}) after different treatments of the leaves are more difficult to explain and probably involve differences in the rate of degradation of the enzyme under these treatments.

DISCUSSION

The effect of wounding on protein synthesis is rapid (1–4 h) (Ghosh et al., 1993), whereas common symptoms of senescence (Chl and protein loss) appear only after 20 h of incubation of leaf segments (Martín and Sabater, 1989). Thus, our results suggest that transcript levels after 8-h incubations of leaf segments are more probably related to senescence processes than to the wounding response, independent of the possibility that senescence may be a consequence of the wounding response. Early and transient changes in SOD transcripts may be related to the wounding response. Provided that mechanical injury increases the level of ACC synthase transcript (Liu et al., 1993) and ethylene formation (Smith et al., 1986, and refs. therein), and that this plant regulator induces SOD (Bowler et al., 1989), it is reasonable to attribute the transient increase in SOD transcripts (Fig. 1) to an ethylene increase as well as to oxidative stress generated by wounding.

SOD gene expression seems to be modulated by the rate of oxyradical formation (Bowler et al., 1992), which in our experimental system depended on light intensity and oxygen concentration. Moreover, SOD activities increase during expansion of leaves up to maturity, when they change very little and then decrease (Bowler et al., 1992, and refs. therein). At 7 d from sowing primary barley leaves are still expanding, and at 14 d from sowing leaves slowly begin to senesce (Martín et al., 1986). Assuming that superoxide levels would not be significantly altered by 20 h of incubation under growth light, the increase in SOD transcripts in young leaves exposed to this treatment probably reflects the continuation of leaf development after detachment, since Chl also increased (data not shown). In mature-senescent leaves growth light only delayed detachment-accelerated senescence, so SOD transcript levels changed only slightly (Figs. 3–5). It may be argued that light produced as much photooxidative stress in mature-senescent leaves as in young leaves. The increase in *rbc*L transcripts when mature-senescent leaf seg-

ments were incubated in light suggests that they were fully competent for photosynthesis and to bear photooxidative stress when they received light. Irrespective of the metabolic activity in mature-senescent leaves, the opposite effects of 100% O_2 (supplementing high light) on transcript and activity levels of SOD1, SOD2, and SOD3 in young and mature-senescent leaf segments (but not on *rbc*L transcript levels) clearly indicated that the sensitivity of the expression of SOD genes to photooxidative stress decreased when the leaf aged. Similarly, Pastori and Trippi (1993) found that the activities of several antioxidant enzymes, including SOD, were increased by the effects of incubation with paraquat and H_2O_2 in young leaves but were decreased in senescent leaves of a drought-sensitive maize strain.

Even though treatment with high light intensities with or without levels of O_2 higher than that of the normal atmosphere causes an oxidative stress mainly within chloroplasts, not only the transcript of chloroplastic SOD2 increased but those corresponding to mitochondrial SOD1 and cytoplasmic SOD3 increased as well. As was argued by Tsang et al. (1991), who found that paraquat and light increased the abundance of SOD1, SOD2, and SOD3 mRNA in tobacco leaves, it is possible that although superoxide formation was induced within chloroplasts, the ensuing oxidative stress could affect other compartments of the cell.

Our results suggest that SOD genes are not only stress regulated but also that their expression is modulated by the developmental stage of the organ: they become progressively insensible to induction by photooxidative stress when the leaf ages. Thus, young leaves could minimize the effects of oxidative stress by increasing levels of transcripts of antioxidant enzymes, at least those that dismutate superoxide anion radicals, even under conditions of very high rates of oxyradical formation. Mature-senescent leaves could respond in a similar way only under moderate stress. In several animal systems, the developmentally programmed cell death seems to be mediated by low expression of SOD genes (Kane et al., 1993). Thus, it is tempting to suggest also that when the leaves become old the loss of the SOD genes' capacity to respond to their effectors (compared with the conserved capacity of the *rbc*L gene) may impair cell survival.

Senescence, like other developmental processes, seems to be regulated by differential gene expression (Stoddart and Thomas, 1982), including activation and inactivation of specific genes. At the posttranscriptional level, the net loss of key macromolecules that characterize the senescence syndrome seems to be favored by higher levels of oxyradicals (Trippi et al., 1989). Under natural prooxidative conditions (e.g. high light intensity, water deficiency) senescence is accelerated because senescing cells may not be able to express genes of antioxidant enzymes according to the rate of oxyradical formation.

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