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Update on Biochemistry

The Activated Oxygen Role of Peroxisomes in Senescence[1]

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Senescence is a genetically regulated oxidative process that involves a general degradation of the cellular structures and enzymes and the mobilization of the products of degradation to other parts of the plant. Senescence is mainly characterized by a cessation of photosynthesis, disintegration of organelle structures, intensive losses of chlorophyll and proteins, and dramatic increases in lipid peroxidation and membrane leakiness (Buchanan-Wollaston, 1997). These latter changes are mainly due to the strong enhancement in the generation of activated oxygen species that takes place in plant tissues during the senescence process (Thompson et al., 1987; Leshem, 1988).

Chloroplasts are one of the earliest sites of catabolism in leaf senescence (Smart, 1994); chlorophyll is degraded and chlorophyll catabolites are transported to the cell vacuole for their disposal (Matile et al., 1996). Under the electron microscope, chloroplasts from senescent leaves show an increase in the number and diameter of osmiophilic plastoglobuli, loosening and disorientation of the grana, and dilation of the thylakoids (Smart, 1994). The formation of plastoglobuli is thought to be linked to the breakdown of thylakoids that accompanies senescence, and they have been reported to contain plastohydroquinones, carotenoids, glycerolipids, and proteins.

Membranes, including thylakoid membranes, are a valuable store of lipid molecules that can be mobilized and metabolized to provide energy for the senescence process (Buchanan-Wolleston, 1997). Galactolipids, which constitute 40 to 50% of the total thylakoid lipid, may be converted to sugars by gluconeogenesis to provide energy for respiration during leaf senescence or to use as building blocks elsewhere in the plant (Landolt and Matile, 1990; Smart, 1994). This breakdown process occurs partially in leaf peroxisomes from senescent leaves, which, like oilseed glyoxysomes, contain the fatty acid β-oxidation and the glyoxylate cycle enzymes (Landolt and Matile, 1990). Levels of key enzymes of the glyoxylate cycle, MS and ICL, increase in senescing leaves (Pistelli et al., 1996, and refs. therein) as a result of increased gene expression (Buchanan-Wallaston, 1997). A metabolic scheme of lipid metabolism during leaf senescence is shown in Figure 1.

Reactions involving oxygen free radicals are an intrinsic feature of plant senescence and they promote the process of oxidative deterioration that contributes to cell death (Thompson et al., 1987). Important factors in plant senescence are lipoxygenase activity, activated oxygen species, and several senescence-promoting compounds such as ethylene and jasmonic acid (Leshem, 1988). Lipoxygenase is an enzyme that catalyzes the direct reaction of polyunsaturated fatty acids with oxygen to give hydroperoxide products and has an important role in the breakdown of membrane lipids induced by senescence. Activated oxygen species are the primary mediators of the oxidative damage in plant senescence, and they mainly include superoxide radicals ($O_2^{\cdot -}$), hydroxyl radicals ($\cdot OH$), singlet oxygen ($^1O_2$), H_2O_2, alkoxyl radicals ($RO\cdot$), peroxyl radicals ($ROO\cdot$), polyunsaturated fatty acids, and semiquinone free radicals. Some of these activated oxygen species, particularly $O_2^{\cdot -}$, are strong oxidizing species that can rapidly attack all types of biomolecules, including DNA, leading to irreparable metabolic dysfunction and cell death (Halliwell and Gutteridge, 1989). However, plants possess enzymatic and nonenzymatic antioxidative defense systems distributed in cell organelles. SODs, catalases, peroxidases, and the ascorbate-glutathione cycle enzymes are examples of antioxidative enzymes. The nonenzymatic antioxidants are mainly ascorbate (vitamin C), glutathione, α-tocopherol (vitamin E), β-carotene, and flavonoids, which are distributed chiefly in chloroplasts but also in other cellular compartments such as mitochondria and peroxisomes. Under normal conditions, the antioxidative defense system of plants provides adequate cellular protection against activated oxygen species, but when the generation of activated oxygen species overcomes the defense provided by the

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Abbreviations: APX, ascorbate peroxidase; CCRase, Cyt *c* reductase; DHAR, dehydroascorbate reductase; GR, glutathione reductase; ICL, isocitrate lyase; MDHAR, monodehydroascorbate reductase; MS, malate synthase; PMP, peroxisomal membrane polypeptide; SOD, superoxide dismutase.

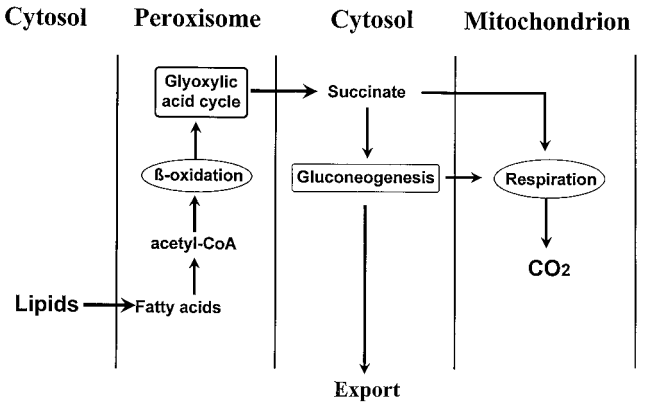

Figure 1. Possible metabolic pathway of lipid breakdown in senescence (redrawn from Smart, 1994).

cellular antioxidant systems, as is the case in senescence, then oxidative stress is produced.

The application of molecular biology techniques to the study of leaf senescence has in recent years permitted the characterization of genes that show increased expression in senescing leaves (Buchanan-Wollaston, 1997). Experiments with transgenic plants and mutants are supplying information about the role played by cytokinins and ethylene in regulating senescence in leaves (Buchanan-Wollaston, 1997). Once the regulatory mechanisms underlying leaf senescence are understood using molecular and genetic approaches, it might be possible to devise ways to manipulate leaf senescence for agricultural applications (Gan and Amasino, 1997).

PEROXISOMES, SODs, AND $O_2\cdot^-$

Peroxisomes can be broadly defined as ubiquitous subcellular organelles bounded by a single membrane that contain as basic enzymatic constituents catalase and H_2O_2-producing flavin oxidases (Huang et al., 1983). These cell organelles were first isolated and biochemically characterized by de Duve at the beginning of the 1960s (del Río et al., 1992, and refs. therein). Initially, the only function described for peroxisomes was the removal by catalase of toxic H_2O_2 generated in the peroxisomal respiration pathway, but in recent years it has become increasingly clear that peroxisomes carry out essential functions in almost all eukaryotic cells (Huang et al., 1983; del Río et al., 1992; van den Bosch et al., 1992). These organelles have an essentially oxidative type of metabolism and have the potential to carry out different metabolic pathways, depending on their source. Glyoxysomes are specialized peroxisomes that occur in the storage tissues of oilseeds and that contain the fatty acid β-oxidation and glyoxylate-cycle enzymes to convert the seed-reserve lipids into sugars, which are used for germination and plant growth (Huang et al., 1983). Leaf peroxisomes are specialized peroxisomes present in photosynthetic tissues that carry out the major reactions of photorespiration (Huang et al., 1983). Another type of specialized peroxisomes are root-nodule peroxisomes from certain tropical legumes, in which the synthesis of allantoin, the major metabolite for nitrogen transport within these plants, is carried out (Huang et al., 1983). The main metabolic processes responsible for the generation of H_2O_2 in different types of peroxisomes are the photorespiration glycolate pathway, the fatty acid β-oxidation, the enzymatic reaction of flavin oxidases, and the disproportionation of $O_2\cdot^-$ (Huang et al., 1983; del Río et al., 1992, 1996).

The family of metalloenzymes known as SODs (EC 1.15.1.1) play an important role in protecting cells against the toxic effects of $O_2\cdot^-$ produced in different cellular loci (Fridovich, 1986; Halliwell and Gutteridge, 1989). SODs are distributed in different cell compartments, mainly chloroplasts, cytosol, mitochondria (Fridovich, 1986; Halliwell and Gutteridge, 1989; del Río et al., 1992; Bowler et al., 1994), and peroxisomes (del Río et al., 1992, and refs. therein). The occurrence of SODs in isolated plant peroxisomes has been reported in at least seven different plant species (del Río et al., 1996), and in four of these plants the presence of SOD has been confirmed by immunoelectron microscopy (Corpas et al., 1998). Results obtained about the presence of SOD in plant peroxisomes have been corroborated for human and animal cells that were found to contain CuZn-SOD in peroxisomes (del Río et al., 1996, and refs. therein).

Peroxisomes, like mitochondria and chloroplasts, produce $O_2\cdot^-$ as a consequence of their normal metabolism. In peroxisomes from pea leaves, at least, there are two sites of $O_2\cdot^-$ generation: one in the organelle matrix, in which the generating system was identified as xanthine oxidase, and another site in the peroxisomal membranes dependent on NADH (del Río et al., 1992). In this production of $O_2\cdot^-$ by leaf peroxisomal membranes, a small electron transport chain similar to that reported in peroxisomal membranes from castor bean endosperm (Fang et al., 1987) appears to be involved. This electron-transport chain is composed of a flavoprotein NADH:ferricyanide reductase of about 32 kD and Cyt b_5 (measured as NADH:CCRase activity) (Fang et al., 1987). Recently, the integral PMPs of pea leaf peroxisomes were identified by SDS-PAGE and three of these membrane polypeptides (with molecular masses of 18, 29, and 32 kD) have been characterized and demonstrated to be responsible for $O_2\cdot^-$ generation (López-Huertas et al., 1996, 1997). The 18-kD PMP has been proposed to be Cyt b_5 (López-Huertas et al., 1997). The properties of these redox polypeptides and a model to explain the NAD(P)H-dependent production of $O_2\cdot^-$ by peroxisomal membranes is shown in Figure 2. This $O_2\cdot^-$ production appears to be an obligatory consequence of the NADH reoxidation by the peroxisomal electron transport chain, to regenerate NAD^+ to be reutilized in the peroxisomal metabolic processes. Under normal metabolic conditions, the $O_2\cdot^-$ production by peroxisomal membranes is not dangerous to the cell, which is adequately protected against these oxygen radicals. But in plant-stress situations, the release of peroxisomal membrane-generated $O_2\cdot^-$ into the cytosol can be enhanced (del Río et al., 1996).

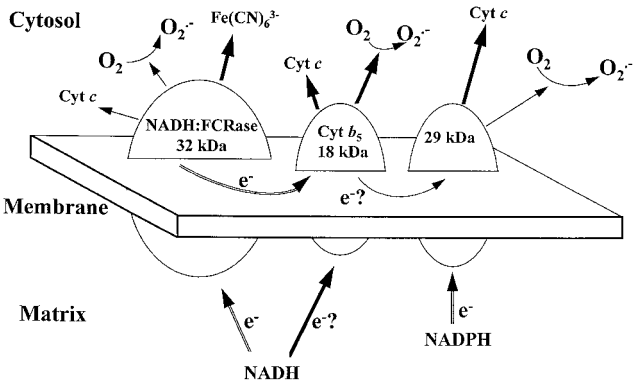

Figure 2. Model illustrating the NAD(P)H-dependent production of $O_2^{\cdot-}$ by peroxisomal membranes from pea leaves (López-Huertas et al., 1996, 1997). The 32-kD PMP is a flavoprotein responsible for the membrane NADH:ferricyanide reductase (FCRase) activity and can reduce O_2 to $O_2^{\cdot-}$ as well as Cyt *c*. The 32-kD polypeptide is part of an electron transport chain system from NADH to Cyt b_5. This Cyt is the major electron donor to Cyt *c* (NADH:CCRase activity) and O_2, being responsible for most of the $O_2^{\cdot-}$ production in peroxisomal membranes. The 32-kD PMP and the 18-kD PMP (probably Cyt b_5) integrate the NADH-dependent $O_2^{\cdot-}$-generating system of peroxisomal membranes. Although the direct reduction of the 18-kD PMP by NADH takes place in vitro, this has not been demonstrated in vivo. The 29-kD PMP has NADPH:CCRase activity but this is lower than the NADH:CCRase activity mentioned above. The 29-kD polypeptide can reduce molecular oxygen to $O_2^{\cdot-}$ using NADPH as electron donor instead of NADH. A possible transfer of electrons from Cyt b_5 to the 29-kD PMP cannot be ruled out, although this still remains to be demonstrated in vivo.

THE ASCORBATE-GLUTATHIONE CYCLE IN PEROXISOMES

The ascorbate-glutathione cycle is an efficient way for plant cells to dispose of H_2O_2 in certain cellular compartments where this metabolite is produced and no catalase is present (Halliwell and Gutteridge, 1989). This cycle makes use of the nonenzymic antioxidants ascorbate and glutathione in a series of reactions catalyzed by four antioxidative enzymes and has been demonstrated in chloroplasts, cytosol, and root nodule mitochondria (Foyer and Mullineaux, 1994). In peroxisomes and mitochondria purified from pea leaves, the presence of all the enzymes of the ascorbate-glutathione cycle was recently reported (Jiménez et al., 1997a). The four enzymes, APX, MDHAR, DHAR, and GR, were present in peroxisomes. Likewise, in intact peroxisomes and mitochondria, the presence of ASC and GSH and their oxidized forms DHA and GSSG, respectively, was found by HPLC analysis (Jiménez et al., 1997a). The intraperoxisomal distribution of the four enzymes was studied by determining enzyme activity latency in intact organelles and by solubilization assays with 0.2 M KCl.

On the basis of the results obtained, a model for the function of the ascorbate-glutathione cycle in leaf peroxisomes is shown in Figure 3. DHAR and GR were found in the soluble fraction of peroxisomes, whereas APX was bound to the external side of the peroxisomal membrane. These results agree with recent findings of an APX isoenzyme in membranes of pumpkin and cotton peroxisomes (Yamaguchi et al., 1995; Bunkelmann and Trelease, 1996). MDHAR was also localized in the peroxisomal membranes. It has been proposed that the *trans*-membrane protein MDHAR can oxidize NADH on the matrix side of the peroxisomal membrane and transfer the reducing equivalents as electrons to the acceptor monodehydroascorbate on the cytosolic side of the membrane (Luster and Donaldson, 1987; Bowditch and Donaldson, 1990). In this process, molecular O_2 could also act as an electron acceptor, with the concomitant formation of $O_2^{\cdot-}$ (López-Huertas et al., 1996).

The evidence of the presence of APX and MDHAR in leaf peroxisomal membranes suggests a dual complementary function in peroxisomal metabolism of these membrane-bound antioxidant enzymes. The first function could be to reoxidize endogenous NADH to maintain a constant supply of NAD^+ for peroxisomal metabolism (Fig. 3), an idea that was originally proposed for the membrane-bound NADH dehydrogenase of glyoxysomes from castor bean endosperm (Fang et al., 1987; Luster and Donaldson, 1987; Bowditch and Donaldson, 1990). A second function of the membrane antioxidant enzymes could be to protect against H_2O_2 leaking from peroxisomes. H_2O_2 can easily permeate the peroxisomal membrane, and an important advantage of the presence of APX in the membrane would be the degradation of leaking H_2O_2, as well as the H_2O_2 that is being continuously formed by disproportionation of the $O_2^{\cdot-}$ generated in the NADH-dependent electron transport system of the peroxisomal membrane (Fig. 3; del Río et al., 1992, 1996; López-Huertas et al., 1996, 1997). This membrane scavenging of H_2O_2 could prevent an increase in the cytosolic H_2O_2 concentration during normal metabolism and under certain plant-stress situations, when the level of H_2O_2 produced in peroxisomes can be substantially enhanced (del Río et al., 1996).

CHANGES IN THE PEROXISOMAL ENZYMES INVOLVED IN THE METABOLISM OF ACTIVATED OXYGEN SPECIES DURING SENESCENCE

When leaves are detached and allowed to senesce in the dark, dramatic changes occur in the enzymes found in peroxisomes purified from leaf homogenates (Pastori and del Río, 1994a, 1994b). Two marker enzymes of photorespiration, glycolate oxidase and hydroxypyruvate reductase, decrease markedly and are hardly detectable when senescence is advanced. The glyoxylate cycle enzymes MS and ICL, which cannot be detected in young leaves, increase dramatically (Pastori and del Río, 1994a, 1997). Xanthine oxidase and urate oxidase, two enzymes involved in the catabolism of purines, also increase. Enhanced activities of xanthine oxidase and urate oxidase will lead to an increase in $O_2^{\cdot-}$ and H_2O_2 production, respectively. The increase in these two enzymes may indicate a role for peroxisomes in the catabolism of purines resulting from RNA degradation during senescence (del Río et al., 1992; Pastori and del Río, 1994a; Corpas et al., 1997).

There are other important changes in the activities of enzymes involved in the metabolism of activated oxygen species in peroxisomes. The Mn-SOD activity that causes

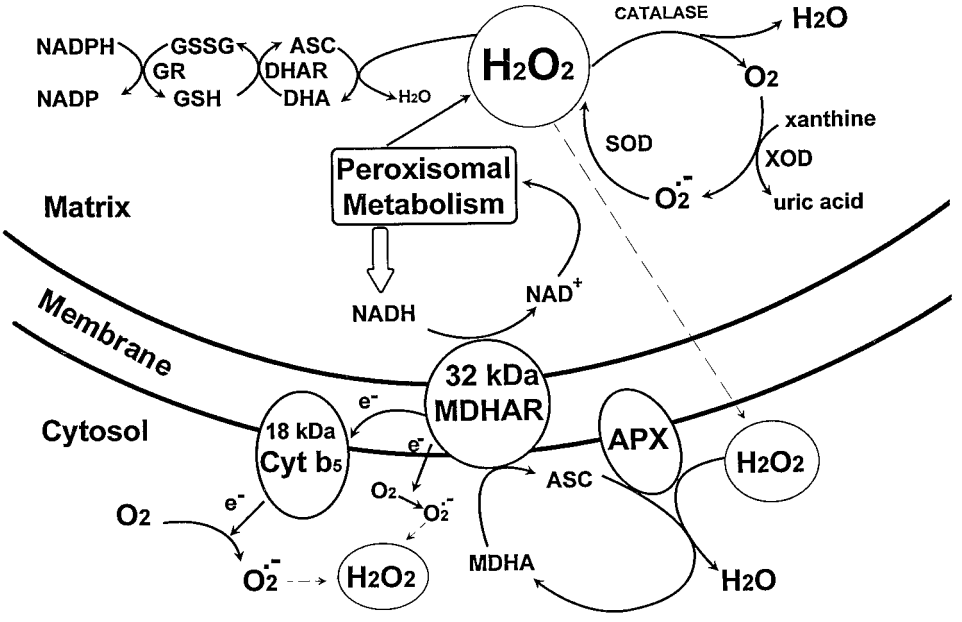

Figure 3. Model proposed for the function of the ascorbate-glutathione cycle in leaf peroxisomes. This model is based on results recently described (Jiménez et al., 1997a) and those previously reported on the characterization of PMPs from pea leaves (López-Huertas et al., 1996, 1997) and the NADH:MDHAR of glyoxysomal membranes from castor bean endosperm (Bowditch and Donaldson, 1990). ASC, ascorbate, reduced form; DHA, ascorbate, oxidized form (dehydroascorbate).

H_2O_2 production also increases. On the other hand, catalase, one of the enzymes responsible for H_2O_2 dissipation, almost completely disappears (Pastori and del Río, 1994a, 1997). The NADH-dependent generation of $O_2\cdot^-$ radicals by peroxisomal membranes and the H_2O_2 concentration in intact peroxisomes, as well as the rate of lipid peroxidation, increases significantly in peroxisomes of senescent leaves (Pastori and del Río, 1994a, 1994b, 1997). Therefore, senescence could stimulate the release of membrane-generated $O_2\cdot^-$ into the cytosol, which could then join the overproduced H_2O_2 that can easily leak out peroxisomes. This could give rise to diverse cellular oxidative stress situations as a result of the formation of the strongly oxidizing (·OH by the metal-catalyzed reaction of H_2O_2 with $O_2\cdot^-$ (Halliwell and Gutteridge, 1989).

A NEW PEROXISOMAL FRACTION INDUCED BY SENESCENCE

Ultrastructural studies of senescent pea leaves show that, whereas chloroplasts are gradually altered and degraded, peroxisomes remain intact, and their population, together with that of mitochondria, increases about 4 and 5 times, respectively, compared with young leaves (Pastori and del Río, 1994a). The proliferation of the peroxisomal population during senescence was first demonstrated in carnation petals and is also found in plants treated with the herbicide isoproturon, with ozone, and with the hypolipidemic drug clofibrate (del Río et al., 1996, and refs. therein).

Senescent pea leaves contain two populations of peroxisomes (Pastori and del Río, 1997). The first population has a density (1.089 g cm^{-3}) similar to that of young leaf peroxisomes, whereas the second and more abundant population has a higher density (1.098 g cm^{-3}). The characteristic glyoxysomal enzymes MS and ICL are found in both populations. When analyzed by electron microscopy, there are clear morphological differences. Peroxisomes from the first population have the typical size but a lower matrix electron density than peroxisomes from young leaves. In contrast, peroxisomes from the higher-density peak are smaller and have a higher matrix electron density (Pastori and del Río, 1997). Senescence apparently induces the appearance of a new peroxisomal population, probably as a result of generalized degradative processes occurring at the final stages of leaf senescence. In animals, several cases of peroxisomal heterogeneity have been reported as a result of different treatments (clofibrate, thyroxine, ischemia-reperfusion injury, cold, etc.; Pastori and del Río, 1997, and refs. therein) but, to our knowledge, this is the first case reported in higher plants of the appearance of qualitatively different peroxisomal populations.

A transition of leaf peroxisomes into glyoxysomes during leaf senescence has been observed by different authors (Landolt and Matile, 1990; Nishimura et al., 1993; Pistelli et al., 1996). The results obtained in peroxisomes from senescent pea leaves on the induction of MS and ICL agree with results reported for leaf peroxisomes from different senescent plants (Pistelli et al., 1996, and refs. therein) and support the idea that leaf senescence is associated with the reverse transition of leaf peroxisomes to glyoxysomes, with the channeling of acetyl-CoA through the glyoxylate pathway. The enhancement by senescence of the fatty acid β-oxidation and the glyoxylate cycle activity of leaf peroxisomes could be a means of converting thylakoidal lipids into sugars to be used as building blocks or for respiration

in younger leaves or storage tissues (Landolt and Matile, 1990; Fig. 1).

PEROXISOMAL SOD ISOZYMES DURING SENESCENCE

The constitutive Mn-SOD activity of leaf peroxisomes increases significantly in senescent leaves and two new CuZn-SODs appear (Pastori and del Río, 1994b, 1997). The constitutive Mn-SOD is present in the lower-density peroxisomal peak, whereas the new CuZn-SODs occur predominantly in the higher-density peroxisomal peak.

These results further support the hypothesis of a senescence-driven transition of leaf peroxisomes to glyoxysomes, since, in addition to the presence of the glyoxylate cycle enzymes MS and ICL in senescent peroxisomes, a new CuZn-SOD that is induced is recognized by an antibody against glyoxysomal CuZn-SOD. The absence of the constitutive Mn-SOD in the second population of peroxisomes of senescent leaves suggests that this new peroxisomal fraction could correspond to the final form of leaf glyoxysomes produced by the senescence-induced transformation of leaf peroxisomes.

EFFECT OF SENESCENCE ON THE PEROXISOMAL ASCORBATE-GLUTATHIONE CYCLE ENZYMES

The ascorbate-glutathione cycle of peroxisomes was also affected by senescence. In dark-induced senescent leaves the peroxisomal APX and MDHAR activities were notably decreased but DHAR was considerably enhanced. In contrast, GR activity was not affected by senescence (Jiménez et al., 1997b). As to the peroxisomal antioxidants, whereas the ascorbate content was only slightly increased by senescence, the total glutathione content augmented about 20 times. The predominant form of glutathione during senescence was GSSG, resulting in a 43-fold increase in the GSSG/GSH ratio in peroxisomes (Jiménez et al., 1997b). We interpret this to mean that the ascorbate-glutathione cycle gains in importance for the elimination of H_2O_2 in peroxisomes as senescence proceeds and catalase disappears.

CONCLUSIONS

Senescence brings about important alterations in the oxidative metabolism, SOD isozymes, and ascorbate-glutathione cycle of peroxisomes, as well as in the quantity and quality of the peroxisomal population. The senescence-induced changes in the activated oxygen metabolism of peroxisomes are mainly characterized by the disappearance of catalase activity and an overproduction of $O_2\cdot^-$ and H_2O_2. This accumulation of activated oxygen species can only be partly counteracted by the peroxisomal ascorbate-glutathione cycle, since this is also negatively affected by senescence. Since $O_2\cdot^-$ radicals have a short half-life under physiological conditions and quickly dismutate into H_2O_2 and O_2, the final result of senescence is a buildup in leaf peroxisomes of the more stable metabolite H_2O_2, which can diffuse into the cytosol. This represents a serious situation not only for peroxisomes but also for other cell organelles such as mitochondria, nuclei, and chloroplasts, because of the possible formation of the strongly oxidizing ·OH by the metal-catalyzed reaction of H_2O_2 with $O_2\cdot^-$. In other words, peroxisomes appear to have an activated oxygen-mediated role in the oxidative reactions characteristic of leaf senescence. However, the senescence-induced H_2O_2 leaking from peroxisomes might also act in the cytosol as a second messenger in cellular signal transduction pathways that lead to specific gene expression (Baeuerle et al., 1996). In this way peroxisomes could have a significant contribution, as a source of activated oxygen transduction signals, in the regulation of genes involved in leaf senescence.

On the other hand, the increase in peroxisomal xanthine oxidase and urate oxidase activities in senescent leaves suggests a function for leaf peroxisomes in the catabolism of purines resulting from senescence-induced nucleic acid degradation. It seems reasonable that an activated oxygen-mediated function similar to that found for peroxisomes from senescent leaves could also be performed by human and animal peroxisomes during the mechanism of aging, a process in which thus far mitochondria have been implicated almost exclusively.

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