Leaf age-related changes in respiratory pathways are dependent on complex I activity in *Nicotiana sylvestris*

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The observed decrease in respiration during rosette leaf maturation of *Nicotiana sylvestris* wild-type (WT) plants was shown to be because of a decline in the cytochrome oxidase (COX) pathway activity, measured by $^{18}$O/$^{16}$O oxygen discrimination, while the alternative oxidase pathway (AOX) remained stable. This suggests a higher contribution of the COX pathway to growth respiration than to maintenance respiration. Mitochondrial superoxide dismutase (MnSOD) activity paralleled the decrease in COX activity with leaf age, whereas chloroplastic FeSOD activity increased. Age-dependent respiratory changes were much less apparent in the Cytoplasmic Male Sterile II (CMSII) mitochondrial mutant devoid of respiratory complex I and previously shown to possess increased AOX content and enhanced respiration but lower photosynthesis in mature leaves. Respiration declined less rapidly with leaf age in CMSII than in the WT, and was significantly higher in the mutant when compared with the WT in mature leaves only. In contrast, photosynthesis was lower in the mutant than in the WT at all leaf stages. The higher respiration of mature CMSII leaves was supported exclusively by enhanced COX activity, in association with an increased mitochondrial MnSOD activity. Steady-state levels of AOX1 transcripts increased in maturing WT leaves, and the CMSII mutant had higher amounts of *coxI*, AOX1 and MnSOD transcripts than the WT. Enhanced activity of the proton-pumping COX route in the mutant can be viewed as a compensation for the lack of the first coupling site of the respiratory chain. However, this is not quite sufficient to ensure normal growth rates in the mutant.

Introduction

It has been shown for a long time that respiration decreases with leaf age (reviewed in James 1953). More recently, a decrease in respiratory rate during tissue maturation has been demonstrated in bean leaves (Azcon-Bieto et al. 1983), barley roots (Mc Donnell and Farrar 1993) and *Pinus radiata* shoot buds (Winkler et al. 1994). In addition to the main electron transport chain (ETC) consisting of complexes I–IV, mitochondria in plants and some fungi possess non-proton-pumping
The (18O/16O) oxygen-isotope-fractionation technique between the COX and AOX pathways, as determined by available in the literature on the in vivo partitioning (McCabe et al. 1998). Moreover, very little information is increase during soybean postgerminative development. In contrast, AOX pathway during expansion of bean leaves, without a decline in the in vitro-measured activity of the COX pathway during expansion of pea leaf mitochondria isolated at different plant age, whereas Azcon-Bieto et al. (1983) observed a decline in the in vivo measured activity of the COX pathway by the mutation in CMSII plants have been examined in the greenhouses of the University de les Illes Balears, under natural light. They were watered every 2 days with tap water. Half-strength Hoagland’s solution (Epstein 1972) was applied every 5 days. Three days before respiration and oxygen isotope-fractionation measurements, plants were placed in a growth chamber at 25°C and at a light intensity of 600 μmol m⁻² s⁻¹.

In both conditions, WT and CMS II plants of the same size and with the same number of leaves were compared at two stages of vegetative development: young rosette stage (stage 1), equivalent to 6-week-old WT plants and 8-week-old CMSII plants, and rosette plants just before bolting (stage 2), equivalent to 8-week-old WT plants and 10-week-old CMSII plants. Analyses were carried out on five consecutive leaves at stage 1 and six consecutive leaves at stage 2, from apex (leaf a) to basis (leaf f) (Fig. 1).

Gas exchange experiments
Gas exchange experiments were performed using a LiCor 6400-40 infrared gas analysis system (Li-Cor Corp., Lincoln, NE). Measurements of total leaf respiration (Vᵣ) in the dark and CO₂ assimilation rates (Aᵣ) at saturating illumination (1000 μmol m⁻² s⁻¹) and at 400 μmol mol⁻¹ CO₂ were performed on attached leaves. For each experiment, gas exchange rates were monitored until steady-state rates were attained.
Chlorophyll content measurements

Chl content and Chl a/b ratio were determined spectrophotometrically from 80% acetone extracts using the absorption coefficients of Porra et al. (1989).

Respiratory measurements on intact tissues with a gas phase dual-inlet system

Total dark respiration ($V_t$) and activities of the COX ($v_cyt$) and AOX ($v_{alt}$) pathways were determined using a closed gas phase system connected to a dual-inlet mass spectrometer as previously described (Gaston et al. 2003). Calculations of the oxygen isotopic fractionation and electron partitioning between the two pathways were made as described by Guy et al. (1989) and Ribas-Carbo et al. (1995). Over the course of the experiment, leaf samples consumed a maximum of 10% of the initial oxygen (21%) molar fraction. $r^2$ values of all unconstrained linear regression between $-\ln f$ and $\ln (R/R_o)$ (with at least five data points) were at least 0.995, corresponding to an error in the fraction estimation of less than 0.5%. The entire extraction procedure was performed at -20°C. Supernatants were frozen at -20°C. Samples containing 40 µg protein in non-denaturing Laemmli buffer were separated on 7.5% polyacrylamide gels at 4°C. SOD activities were visualised using the in situ staining technique of Beauchamp and Fridovich (1971). After a 30-min dark incubation with a mixture containing Nitro Blue Tetrazolium, gels were placed in the light for 30 min. Superoxide dismutase activity caused achromatic zones on otherwise uniformly blue stained gels. Relative signal intensities were quantified using ‘Scion Image Beta 4.02 Win’ software (Scion corporation, Frederick, MD).

The individual activities of the COX ($v_{cyt}$) and the AOX pathway ($v_{alt}$) were obtained from multiplying the total oxygen uptake ($V_{tot}$) by the partitioning to each pathway as follows:

$$v_{cyt} = V_{tot}(1 - \tau_a)$$

$$v_{alt} = V_{tot} \times \tau_a$$

RNA isolation and gel blot analysis

Total RNA from leaf tissue pieces was extracted by the Trizol-chloroform procedure (Gibco-BRL, UK). Northern analysis was performed on 10 µg of total RNA as described in Sabar et al. (2000), using the following probes: a 380-bp N. sylvestris sequence, 100% identical to the internal region of NtSR1AOX1 (Sabar et al. 2000), the mitochondrial cox I sequence, as described in Brangeon et al. (2000) and Nicotiana plumbaginifolia MnSOD and FeSOD complementary DNAs, as described in Dutilleul et al. (2003b).

Total protein extraction

Leaf discs were ground in liquid nitrogen and total proteins were extracted in 2× Laemmli (1970) buffer, with or without 30 mM β-mercaptoethanol. The extract was centrifuged for 10 min at 20 000 g to eliminate insoluble material, and protein was determined according to Bradford (1976).

In-gel determination of SOD activity

Leaf discs were ground in liquid nitrogen and extraction buffer: 0.1 M Tris–HCl, pH 8.1, 0.3 M sucrose, 0.5 mM protease inhibitor ‘Pefabloc’ (Fluka) and 30 mM β-mercaptoethanol. Extracts were cleared by centrifugation for 5 min at 20 000 g, the supernatant was kept and debris pelleted by centrifugation (30 min at 150 000 g). The entire extraction procedure was performed at 4°C. Supernatants were frozen at -20°C. Samples containing 40 µg protein in non-denaturing Laemmli buffer were separated on 7.5% polyacrylamide gels at 4°C. SOD activities were visualised using the in situ staining technique of Beauchamp and Fridovich (1971). After a 30-min dark incubation with a mixture containing Nitro Blue Tetrazolium, gels were placed in the light for 30 min. Superoxide dismutase activity caused achromatic zones on otherwise uniformly blue stained gels. Relative signal intensities were quantified using ‘Scion Image Beta 4.02 Win’ software (Scion corporation, Frederick, MD).
Statistical analyses

Analysis of variance was performed with Statistica software (Statsoft Inc., Tulsa, OK) to estimate the statistical significant difference at the P < 0.05 level.

Results

Changes in photosynthetic and respiration rates with leaf age in WT and CMSII

WT and CMSII plants were compared at two different stages of development: young rosette, stage 1 and plants just before bolting, stage 2 (Fig. 1A, B). Data are from five consecutive leaves at stage 1 and six consecutive leaves at stage 2, from apex (leaf a) to basis (leaf f).

In stage 1 plants, no significant differences in chlorophyll content and chlorophyll a/b ratios were observed with leaf age, except in leaf e where they were significantly lower in both genotypes (Table 1, columns 1 and 2). On the basis of chlorophyll content, only leaves e could be considered as senescent. Accordingly, in both genotypes, photosynthesis \((A_n)\) was maximum in leaves b to d and markedly lower in leaves e (Table 1, columns 3 and 4). Photosynthesis was lower in all CMSII leaves compared with the equivalent WT leaves \((P < 0.01)\). Dark respiration decreased with leaf age in both genotypes but this decrease was more important in WT leaves (up to three-fold from the youngest to the oldest) than in CMSII (two-fold decrease) (Table 1, columns 5 and 6). As a result, respiration rates were similar in young CMSII and WT leaves, whereas they were nearly 50% higher in mature CMSII leaves than in equivalent WT leaves.

In stage 2 plants, photosynthesis was lower in mutant leaves than in WT leaves at all stages (data not shown). With respect to respiration, the pattern of decrease with leaf age was similar to stage 1 plants, however, respiration rates did not markedly differ between genotypes, except for leaf f in which respiration was higher in CMSII (Table 1, columns 7 and 8). Thus, a consistent difference between WT and CMSII was observed for \(A_n\) rates, whatever the leaf and plant age, but this was not the case for respiration because differences were much more apparent in mature leaves of young plants. We then focused on stage 1 plants for further analyses.

Changes in respiratory pathway partitioning with leaf age in WT and CMSII

To determine whether the decline in respiration observed with leaf age involved the activity of the COX or the AOX routes, we measured the partitioning of electrons through both pathways using the \(^{18}\text{O}/^{16}\text{O}\) isotope-fractionation technique (Gaston et al. 2003, Ribas-Carbo et al. 1995, 2005), in the culture conditions of the University de les Illes Balears (Table 2). As for the IBP-grown plants, a marked shift in total respiration with leaf ageing was observed in both genotypes, the difference between CMSII and WT values being higher in older leaves. Oxygen uptake of WT stage 1 mature leaves, measured in the presence of either SHAM or KCN, gave discrimination values of 21.3 ± 0.24\(^{\%}\) for COX and 30.1 ± 0.7\(^{\%}\) for AOX, respectively. These values were subsequently used to calculate electron partitioning between the COX and AOX pathways in the absence of inhibitors in WT and CMSII leaves.

In the WT, discrimination factors of uninhibited steady-state respiration increased slightly with leaf age, from 22.51\(^{\%}\) in leaf a to 24.45\(^{\%}\) in leaf e. The electron partitioning through the AOX pathway doubled in the senescent leaf e as compared with the immature leaf a, from 0.17 to 0.38. The activity of the COX pathway was cut by half from the younger to the older leaf, from 2.08 to 1.27 \(\mu\text{mol O}_2 \text{ m}^{-2} \text{s}^{-1}\), with only a limited increase in

Table 1. Total chlorophyll content (Chl, g m\(^{-2}\)), net carbon assimilation \((A_n, \mu\text{mol CO}_2 \text{ m}^{-2} \text{s}^{-1})\), total dark respiration \((V_i, \mu\text{mol CO}_2 \text{ m}^{-2} \text{s}^{-1})\) of wild-type (WT) and Cytoplasmic Male Sterile II (CMSII) leaves. Two developmental stages of plants were studied: young rosette (1) and plants just before bolting (2), as shown in Fig. 1. Data are from five consecutive leaves at stage 1 and six consecutive leaves at stage 2, from apex (leaf a) to basis (leaf f); gas exchange data are expressed in \(\mu\text{mol CO}_2 \text{ m}^{-2} \text{s}^{-1}\) and Chl is expressed in g m\(^{-2}\). Values are means ± standard error, calculated from two to six measurements performed on different plants grown in the Institut de Biotechnologie des Plantes greenhouses.

<table>
<thead>
<tr>
<th>Leaf stage</th>
<th>Chl (1)</th>
<th>A(_n) (1)</th>
<th>V(_i) (1)</th>
<th>V(_i) (2)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>CMS</td>
<td>WT</td>
<td>CMS</td>
</tr>
<tr>
<td>a</td>
<td>0.39 ± 0.03</td>
<td>0.36 ± 0.02</td>
<td>16.4 ± 1.6</td>
<td>11.8 ± 1.1</td>
</tr>
<tr>
<td>b</td>
<td>0.39 ± 0.04</td>
<td>0.37 ± 0.01</td>
<td>17.1 ± 1.1</td>
<td>13.6 ± 0.7</td>
</tr>
<tr>
<td>c</td>
<td>0.38 ± 0.01</td>
<td>0.36 ± 0.01</td>
<td>16.2 ± 1.1</td>
<td>14.7 ± 0.8</td>
</tr>
<tr>
<td>d</td>
<td>0.37 ± 0.01</td>
<td>0.33 ± 0.02</td>
<td>17.8 ± 0.4</td>
<td>10.9 ± 1.5</td>
</tr>
<tr>
<td>e</td>
<td>0.23 ± 0.02</td>
<td>0.21 ± 0.02</td>
<td>8.6 ± 0.6</td>
<td>5.4 ± 0.6</td>
</tr>
<tr>
<td>f</td>
<td>/</td>
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the activity of the AOX route because of the marked decrease in total respiration.

In CMSII plants, COX activity did not decrease markedly between leaf b and leaf d, and as a result it was higher than in the WT plants in mature leaves. In contrast, AOX activity was similar in the two genotypes.

Changes in the expression of AOX transcripts with leaf age

Expression of AOX genes was examined at the different leaf stages. In most species investigated to date, AOX is a multigenic family, comprising at least two subfamilies, termed AOX1, AOX2, which have limited nucleotide homology (Considine et al. 2002, Saisho et al. 1997). These isogenes show different tissue specificities and differential responses to stress (Juszczuk and Rychter 2003). Using a N. sylvestris AOX1 probe homologous to the Nicotiana tabacum AOX1a gene (Sabar et al. 2000) in Northern experiments, we observed the accumulation of two transcripts (Fig. 2A), suggesting either the existence of at least two expressed AOX1 sequences or differential processing of the same transcript. For simplicity, the upper messenger RNA (mRNA) band was named AOX1.1 and the lower mRNA band AOX1.2. The relative abundance of AOX1.1 and AOX1.2 transcripts differed between the genotypes. In the WT, AOX1.1 transcripts accumulated when leaves matured up to stage d, then decreased (Fig. 2A). In CMSII, AOX1.1 mRNAs had the highest expression in leaves a and b (Fig. 2A). AOX1.2 mRNA levels were hardly altered by leaf age but they were much more abundant in CMSII than in the WT. Pooled amounts of both AOX1 transcripts were markedly higher (P < 0.01) in the young leaves of CMSII as compared with WT in leaves a to c (Fig. 2B).

In contrast to AOX1 mRNAs, the accumulation of mitochondrial-encoded coxI transcripts, encoding a complex IV subunit, did not significantly change with leaf age (Fig. 2A, C). However, coxl transcripts were found at higher levels in CMSII leaves b, c than in corresponding WT leaves (Fig. 2C), in good correlation with COX activity (Table 2).

Changes in SOD with leaf age

Plant organelles are major sites of reactive oxygen species (ROS) production under physiological conditions, especially chloroplasts within photosynthetic tissues (Foyer and Noctor 2003). Superoxide generated by electron leakage from ETCs is further dismutated to hydrogen peroxide and water, either non-enzymatically or much more efficiently by the action of mitochondrial (MnSOD) and chloroplastic (FeSOD or Cu/ZnSOD) SOD activities, respectively (Bowler et al. 1994, Kliebenstein et al. 1998, Van Camp et al. 1997). SOD activities were compared during leaf ageing in WT and CMSII stage 1 plants by an in-gel procedure (Beauchamp and Fridovitch 1971) allowing the separation of the activities of the different SOD isoforms from the same plant extract (Fig. 3A). The different isoforms were characterised by inhibitor treatments before staining, KCN inhibiting Cu/ZnSODs and H2O2 inhibiting both FeSOD and Cu/ZnSOD activities.

In N. sylvestris, MnSOD activity slightly decreased with leaf age in both genotypes (Fig. 3A, B). In the WT, MnSOD activities were significantly lower (P < 0.05) in leaves (d, e) as compared with leaf a, whereas in CMSII the activity was significantly lower in leaves (c to e) as compared with leaf a (Fig. 3B). MnSOD activity of leaves (a, b) was higher in CMSII as compared with the WT (P < 0.05). In contrast, FeSOD activity markedly increased with leaf age in the WT but this increase was much less marked in the CMSII plants (Fig. 3A, C). In the WT, FeSOD activities were significantly higher (P < 0.05) in leaves (c to e) as compared with leaf a. In CMSII,
FeSOD activities ($P < 0.05$) increased from leaf a to leaf c, then remained constant in leaves (c to e). FeSOD activities were significantly lower in CMSII compared with WT for leaves (d, e). The ratio of MnSOD/FeSOD activities decreased with age whatever the genotype (Fig. 3D). Cytosolic Cu/ZnSOD activity, identified by organelle fractionation (results not shown), were not present in KCN treatment (Fig. 3A), was detected only in the youngest leaves of both WT and CMSII plants, whereas chloroplastic Cu/ZnSOD activities, identified by organelle fractionation (results not shown), were not present in

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**Fig. 2.** Effect of leaf age on the accumulation of AOX1 and cox1 gene transcripts in wild-type (WT) and Cytoplasmic Male Sterile II (CMSII). Leaf pieces from five consecutive leaves of WT and CMSII stage 1 plants (see Fig. 1A, B) were harvested after 10 h of plant illumination. Total RNA was extracted and subjected to RNA gel blot analysis, using 10 μg RNA from each sample. (A) Gel blot analysis using *Nicotiana sylvestris* AOX1 and mitochondrial cox1 as probes; a–e: leaf stages as in Fig. 1. The two probes were hybridised to the same membrane. Ethidium bromide staining of the 18S ribosomal RNA (rRNA) is given as a loading control. One of three experiments giving similar results is shown. (B, C) Relative abundance of pooled AOX1.1 and AOX1.2 transcripts (B) and cox1 transcripts (C). Northern blot signals were scanned with the Scion imaging software program. Results are expressed as the values of the ratio: integrated density of the signal/integrated density of the 18S rRNA signal (arbitrary units). WT, open columns; CMSII, closed columns. Values are the mean ± standard error from three independent experiments. In the WT, steady-state AOX1 transcripts were in higher abundance ($P < 0.05$) in pooled (c, d) leaves compared with pooled (a, b) leaves. They also accumulated significantly more in CMSII compared with WT for leaves (a–c) ($P < 0.05$). Steady-state cox1 transcripts were in higher abundance in CMSII leaves (b, c) compared with the corresponding WT leaves ($P < 0.05$). a.u., arbitrary units.

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**Fig. 3.** In-gel assay of superoxide dismutase (SOD) activity during leaf ageing. Proteins from five consecutive leaves of stage 1 plants were extracted under non-denaturating conditions and they were separated on a native 7.5% acrylamide gel (40 μg protein per lane). (A) Mitochondrial MnSOD, chloroplastic FeSOD and cytosolic Cu/ZnSOD activities were visualised on the same gel, with (+) or without (−) KCN treatment, as described in the Materials and methods; a–e: leaf stages, from young to old. One representative out of six experiments is shown. A KCN-sensitive Cu/Zn isoform is clearly observed for leaf a in the wild-type (WT), and for leaves (a, b) in Cytoplasmic Male Sterile II (CMSII). (B, C) Histograms of SOD activities pooled from all experiments. Activity signals were scanned with the Scion imaging software program. Results are expressed as the values of the ratio: integrated density of the signal/integrated density of the MnSOD signal of the WT leaf (a) (a.u., arbitrary units). WT, open columns; CMSII, closed columns. Values are the mean ± standard error from six independent experiments. For each genotype, significant differences ($P < 0.05$ level) taking leaf a as the reference are indicated by asterisk (CMSII) or open circle (WT). (D) Histograms of the ratio of MnSOD/FeSOD activities pooled from all experiments. WT, open columns; CMSII, closed columns.
detectable amounts in any of the samples tested. SOD activities were examined in stage 2 plants with similar results (data not shown).

We then examined whether changes in SOD activities were controlled at the transcript level. Northern analyses showed, after a transient increase in b leaves, a marked decline in the accumulation of both MnSOD and FeSOD transcripts with ageing in both genotypes (Fig. 4). Levels of SOD transcripts in leaves (b, c) were higher in CMSII as compared with WT, especially those of MnSOD transcripts ($P < 0.05$), but the difference disappeared with leaf age. Chloroplastic and cytoplasmic Cu/ZnSOD transcripts accumulated at very low levels whatever the leaf stage in both genotypes (data not shown).

**Discussion**

The respiration decrease observed during leaf maturation of *N. sylvestris* rosette plants was because of a significant decline in the COX activity, while the AOX activity showed no marked changes, except in the older leaves which had entered the senescence process (Tables 1, 2). The decrease in COX activity was associated with a decrease in mitochondrial MnSOD activity (Fig. 3). This pattern of age-dependent changes was less marked in CMSII leaves lacking complex I but with high AOX protein content (Gutierrez et al. 1997, Sabar et al. 2000). Indeed, the higher respiration previously described in this mutant (Dutilleul et al. 2003a, Priault et al. 2006a) only becomes significant when mature/senescent leaves are compared. Moreover, the high respiration rate in the mutant was because of enhanced COX when compared with WT, with little differences in AOX activity.

**In N. sylvestris WT, the decrease in respiration with leaf age results from a decrease in COX activity**

In *N. sylvestris* WT plants, the decrease in respiration with leaf age may involve changes in substrate availability, mitochondrial number and metabolic interactions between organelles (Bowsher and Tobin 2001, Tobins and Rogers 1992). Old leaves will present a lower growth respiration rate as their growth decrease, while their maintenance respiration might be enhanced as their size increases (Lambers et al. 1983, Loomis and Amthor 1999, Plaxton and Podesta 2006). Therefore, as the leaf ages, the growth component of respiration will decrease and the participation of each respiratory pathway might change. Very recently, it was observed that the COX and AOX pathways contribute differently to growth and maintenance respiration in *Arabidopsis thaliana* (Florez-Sarasa et al. this issue), with the COX contributing more than 80% to growth respiration. These differential contributions to growth and maintenance respiration might explain why the decline in respiration with leaf age in *N. sylvestris* is caused by a decrease in the activity of the COX pathway. The AOX pathway is only induced in the older leaves analysed that have entered the senescence process.

**Control of superoxide production by mitochondrial and chloroplastic ETC during ageing**

As in animals, ETCs of plant organelles generate superoxide by electron leakage, mostly via photosystem I in the chloroplasts (Asada and Takahashi 1987) and at complexes I and III in the mitochondria (discussed in Møller 2001). As it is generally assumed that under physiological conditions, superoxide production is roughly proportional to electron fluxes, the chloroplastic ETC is believed to generate much more superoxide than the mitochondrial ETC (discussed in Foyer and Noctor 2003). Different types of ROS generated from superoxide are harmful to cell components and have to be detoxified (reviewed in Apel and Hirt 2004). In mitochondria, the first step in ROS avoidance is considered to be played by AOX activity, which may minimise overreduction of the
ETC and thus electron leakage (Møller 2001). However, changes in AOX activity were not observed during N. sylvestris leaf maturation, despite the marked decline in mitochondrial ETC transport and COX activity (Table 2).

The second step in the control of ROS accumulation is the dismutation of superoxide to hydrogen peroxide and water, essentially by the action of mitochondrial (MnSOD) and chloroplastic (FeSOD or Cu/Zn) SOD activities (Bowler et al. 1994, Kliebenstein et al. 1998, Van Camp et al. 1997). As experimental determination of organelle superoxide content is difficult, SOD activities are generally considered to be good markers of superoxide production (discussed in Rhoads et al. 2006). In agreement with the higher activity of the chloroplastic ETC, chloroplastic FeSOD activity has been reported to be markedly higher than mitochondrial MnSOD (reviewed in Van Camp et al. 1997). Therefore, it was interesting to examine SOD activities in the different compartments during N. sylvestris leaf maturation.

Mitochondrial MnSOD activity decreased with leaf age (Fig. 3A, B), not as markedly, however, as total respiration and COX activity (Tables 1, 2). A positive correlation between MnSOD and COX activities has been reported during biotic and abiotic stresses (Bowler et al. 1989). In contrast, although photosynthesis rates are stable, and even decrease in the older leaves analysed (leaves e) which have entered the senescence process (Table 1), chloroplastic FeSOD increase markedly with leaf ageing in N. sylvestris. There are two forms of chloroplastic SOD in tobacco species but it is generally thought that the FeSOD is the main form associated with photosynthesis of mature leaves, the Cu/ZnSOD form being detected only in immature leaves (Van Camp et al. 1997). We were not able to detect chloroplastic Cu/ZnSOD activities under our experimental conditions. The balance of hydrogen peroxide generation by each subcellular compartment seems to be very different between immature and mature/presenescent N. sylvestris leaves. Although an exact quantification is difficult, FeSOD activity was close to MnSOD activity in young leaves, and it was more than two-fold higher in mature leaves (Fig. 3D), thereby showing a good correlation with the much higher electron transfer rate that exists in chloroplasts as compared with mitochondrial ETC. AOX and MnSOD activities may cooperate to control superoxide accumulation in mitochondria of ageing leaves and no excess of hydrogen peroxide may be expected. Hydrogen peroxide is considered as a second messenger involved in signalling the expression of many genes during ageing or environmental stress (Mittler 2002) but our results do not provide evidence for any signalling of the senescence process by plant mitochondria. Thus, the situation might be different in photosynthetic plant cells than in animal cells, in which ageing is associated to increased ROS production by the mitochondria, which damage mitochondrial components (the ‘free radical theory of ageing’, Cadenas and Davies 2000). In photosynthetic cells, ROS-associated senescence signalling might derive from the chloroplast because of the increased activity of FeSOD (Fig. 3A, C) generating excess amounts of hydrogen peroxide.

Changes in ROS detoxification mechanisms of ageing leaves are controlled in part at the level of mRNA accumulation. Indeed, marked changes in AOX1 transcript accumulation were observed with leaf age. The steady-state amounts of the higher molecular weight species, termed AOX1.1, increased as the leaf matured, before decreasing with further ageing (Fig. 2A). Increased AOX expression with leaf age has already been reported in potato (Svensson and Rasmusson 2001) and AOX capacity has been reported to increase with age in pea leaves (Lennon et al. 1995). However, as only limited changes in global AOX activity were observed in N. sylvestris leaves with ageing (Table 2), posttranscriptional regulatory mechanisms must also intervene, or other AOX isoforms might be implicated. On the other hand, no significant changes in the levels of mitochondrial coxl transcripts coding for a subunit of complex IV could be observed (Fig. 2), suggesting that the two-fold decrease in COX activity with leaf age is essentially because of posttranscriptional events.

Regarding SOD enzymes, we observed a marked decrease in the accumulation of MnSOD transcripts with leaf maturation (Fig. 4), suggesting that the decline in MnSOD activity with leaf age is partly controlled at the transcriptional level. In contrast, despite the clear augmentation in FeSOD activity, FeSOD transcript levels decreased with leaf age. Taken together, these results show that the observed changes in mitochondrial and chloroplastic enzymes during ageing of N. sylvestris leaves depend on both transcriptional and posttranscriptional processes.

**Leaf maturation-related mitochondrial ETC changes are dependent on Complex I activity**

The importance of complex I in age-dependent changes of leaf respiration is demonstrated in this study by the use of the mitochondrial mutant CMSII devoid of respiratory complex I (Gutierrez et al. 1997, Pineau et al. 2005, Sabar et al. 2000), in which the decreases in total respiration and COX activities during leaf maturation are much attenuated (Tables 1, 2). It is interesting to note that in contrast to the age-dependent activation of respiration in CMSII, photosynthesis is lower in the mutant than in the WT whatever the leaf age (Table 1), emphasising the importance of mitochondrial metabolism for chloroplast
function, as already reported (Gardeström 1996, Hoefnagel et al. 1998, Raghavendra and Padmasree 2003). Mitochondrial-driven chloroplast redox changes might contribute to the lower photosynthesis in CMSII, as suggested by Dutilleul et al. (2003a, 2005). However, lower net carbon assimilation in CMSII is not apparent under low irradiance (Priault et al. 2006a), and another cause of the low net carbon assimilation may be the lower CO₂ internal conductance correlated with a higher photorespiration recently reported in CMSII plants by Priault et al. (2006b).

The increased respiration in CMSII as compared with the WT (Table 1) was entirely because of higher COX activity (Table 2), correlating with higher levels of coxI transcripts (Fig. 2). The partitioning of electrons to the AOX pathway in immature and young mature leaves was actually slightly lower in the mutant than in the WT, despite its high AOX RNA and protein amounts (Dutilleul et al. 2003b, Gutierres et al. 1997, Sabar et al. 2000). The redox state of the AOX homodimer has been shown to be a major point of control of enzyme activity in higher plants (Lennon et al. 1995, Millar et al. 1996, Umbach and Siedow 1993, Umbach et al. 1994). However, the AOX redox state is essentially the same in N. sylvestris CMSII and WT leaves (G. Vidal et al., unpublished data), and the low activity relative to protein content in the mutant is likely to be caused by metabolic changes resulting from the observed increased amounts of organic acids and pyridine nucleotides (Dutilleul et al. 2005).

Whatever the regulatory mechanisms involved, these results show that plant cells possess highly efficient control mechanisms to avoid AOX engagement when it is not necessary or deleterious to energetic metabolism. Instead, the CMSII mutant is devoid of proton-pumping complex I and therefore is entirely dependent on the activity of complexes III and IV in the COX pathway for ATP production via the respiratory chain. As growth is lower in CMSII (De Paepe et al. 1990, Li et al. 1988), it may be hypothesised that the leaf ageing process is delayed to allow a sufficient development. Indeed, at the adult stage, leaf size is similar in WT and CMSII, although height and flower dimensions are reduced (De Paepe et al. 1990, Dutilleul et al. 2003a). This would also explain why growth respiration, mediated by COX activity (Florez-Sarasa et al. this issue), is maintained up to a later stage of development in CMSII leaves than in WT.

In contrast to AOX activity, MnSOD activity is higher in CMSII than in the WT whatever the leaf age (Fig. 3A, B), strongly suggesting an increased generation of hydrogen peroxide in mutant mitochondria, which is possibly related with the higher COX activity. Overproduction of ROS and/or associated redox changes might signal the expression of genes encoding intra- and extramitochondrial antioxidant enzymes, as previously suggested (Dutilleul et al. 2003b, Foyer and Noctor 2003). In particular, both AOX1 and MnSOD transcripts, which are induced by hydrogen peroxide (Wagner 1995) are in higher abundance in CMSII. Higher peroxide generation by the mitochondrial ETC might also be involved in the better resistance of CMSII to both biotic and abiotic stresses (Dutilleul et al. 2003b).

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