

# Partitioning of respiratory electrons in the dark in leaves of transgenic tobacco with modified levels of alternative oxidase

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This study examined tobacco (*Nicotiana tabacum* cv. Petit Havana SR1) leaf respiration in the dark, utilizing both wild-type plants and transgenic plants with increased or decreased levels of alternative oxidase (AOX) protein. AOX represents a non-energy-conserving branch in mitochondrial electron transport. Inhibitor studies showed that the maximum possible flux of electrons to AOX (AOX capacity) correlated with the level of AOX protein present in the different plant lines. A comparison of the plants using online <sup>18</sup>O isotope discrimination was done to determine whether AOX protein level would impact the actual steady-state partitioning of electrons to AOX (AOX engagement). Under a range of pretreatment and measurement conditions, there was little if any effect of AOX protein level on the degree of engagement. This suggests that the metabolic conditions inherent to a particular growth condition and/or the biochemical regulatory properties of AOX itself are the critical factors that control partitioning. Interestingly, we found that measurement temperature and water status are parameters that may have some influence over AOX engagement.

## Introduction

Electron flow from ubiquinol to O<sub>2</sub> via alternative oxidase (AOX) is not coupled to proton translocation and thus represents a non-energy-conserving branch of the mitochondrial electron transport chain (ETC), bypassing two sites of energy conservation associated with the cytochrome (cyt) path (Millenaar and Lambers 2003, Siedow and Umbach 2000, Vanlerberghe and Ordog 2002). Regarding the activity of AOX, particularly in vivo, it is important to distinguish between AOX

capacity and engagement (McDonald et al. 2002). The AOX capacity of a plant cell or tissue is generally measured by the addition of a cyt pathway inhibitor (such as CN) followed by the addition of an AOX inhibitor, such as salicylhydroxamic acid (SHAM). Then, capacity is generally defined as the CN-resistant but SHAM-sensitive O<sub>2</sub> uptake. Capacity is thus a measure of the maximum possible flux of electrons to AOX, a measure that is probably most often dependent upon AOX protein levels but which could be dependent upon other

*Abbreviations* – %A, per cent partitioning to AOX; %RWC, per cent relative water content; AOX, alternative oxidase; CN, cyanide; cyt, cytochrome;  $\delta$ , isotopic composition; *D*, discrimination factor; DMSO, dimethyl sulfoxide; DW, dry weight; ETC, electron transport chain; FTW, fully turgid weight; Fv/Fm, ratio of variable to maximum chlorophyll fluorescence; FW, fresh weight; GDC, glycine decarboxylase; Kn, kanamycin; *O*\*, oxygen concentration; PPF, photosynthetic photon flux density; Q10, proportional change in respiration rate with a 10°C change in temperature; SHAM, salicylhydroxamic acid; WT, wild-type.

limiting components in respiration, particularly when AOX protein levels are high. Alternatively, AOX engagement is a measure of the actual flux of electrons to AOX, in the absence of any inhibitors. Engagement can be measured using an oxygen isotope discrimination technique (Guy et al. 1989, Robinson et al. 1995). This non-invasive method is based on the observation that AOX and cytochrome oxidase discriminate to different extents against heavy  $O_2$  ( $^{18}O^{16}O$ ).

Studies indicate that the level of AOX protein in a tissue can change in response to different growth conditions. For example, increased levels of AOX may be a response to numerous abiotic and biotic stresses (Bartoli et al. 2005, Fung et al. 2004, Noguchi et al. 2005, Parani et al. 2004, Ribas-Carbo et al. 2000, Sieger et al. 2005, Simons et al. 1999, Szal et al. 2003, Takahashi et al. 2003, Vandenabeele et al. 2003). In several instances, it has been further demonstrated that these changes in AOX protein and capacity do not necessarily bring about corresponding changes in AOX engagement, as measured using isotope discrimination (González-Meler et al. 1999, Lennon et al. 1997, Millenaar et al. 2002). Such findings should not, however, be used to indicate that AOX protein level itself has little influence over engagement. This is because such experiments are comparing engagement in tissues adapted to different growth conditions. In this case, it is likely that many metabolic factors (besides the level of AOX protein) are differing between the two tissues, thus confounding the interpretation. As well, other studies do show a positive correlation between changes in AOX protein and changes in engagement or show that the relationship is tissue and/or species-dependent (González-Meler et al. 1999, González-Meler et al. 2001). On balance then, the literature has not adequately resolved the relationship between AOX protein level and engagement.

Studies with isolated mitochondria suggest that the catalytic activity of AOX depends upon specific biochemical regulatory properties of the AOX enzyme. AOX exists in the inner mitochondrial membrane as either a non-covalently or covalently linked homodimer (Umbach and Siedow 1993). The enzyme is inactive when the subunits are covalently linked by a disulphide bond, while reduction of this bond to its component sulfhydryls gives the active form. Reduction is mediated by oxidation of specific TCA cycle substrates, isocitrate and malate (Vanlerberghe et al. 1995). Once reduced, increased AOX activity results from interaction of the regulatory Cys sulfhydryl with particular  $\alpha$ -keto acids (particularly pyruvate) to generate a thiohemiacetal (Millar et al. 1993, Rhoads et al. 1998). Hence, AOX engagement is potentially modulated by both the redox state of the mitochondrion (controlling AOX reduction)

and carbon status (defining the levels of activating  $\alpha$ -keto acids). This represents a feed-forward activation of this ETC component by upstream metabolism (Vanlerberghe et al. 1995).

The importance of the above regulatory mechanisms for AOX engagement and for the ability of AOX to compete with the cytochrome path for electrons has been well documented in isolated mitochondria (Hoefnagel et al. 1995, Ribas-Carbo et al. 1995). However, the in vivo significance of these regulatory mechanisms remains largely unknown. In fact, several studies suggest that these mechanisms of regulation may not be critical in vivo (reviewed in Millenaar and Lambers 2003). In particular, the AOX protein has often (but not always) been shown to be present exclusively in its high activity (reduced) form, and it has been suggested that estimated (but largely unknown) matrix levels of pyruvate are always high enough to fully activate AOX. If this is the case, one might expect that, under a given set of metabolic conditions, AOX engagement might indeed be strongly dependent upon AOX protein level.

Here, we compare AOX engagement between WT plants and transgenic plants with altered levels of AOX protein. Because this comparison is done between plants raised under identical growth conditions, it can more directly assess the influence of AOX protein level itself on AOX engagement. The results show that, at least under the given set of growth and measurement conditions tested, AOX protein level has little impact on engagement, although it does define AOX capacity, as expected. Therefore, the results also imply some further biochemical control over AOX engagement.

## Materials and methods

### Plant material and growth conditions

This study was performed with wild-type (WT) tobacco plants (*Nicotiana tabacum* cv. Petit Havana SR1) and with transgenic tobacco plants previously generated (Vanlerberghe et al. 1994). The transformant S24 has increased levels of leaf mitochondrial AOX protein due to the presence of a constitutively expressed sense construct of the nuclear gene *Aox1*, while transformant AS8 has undetectable levels of leaf AOX protein due to the presence of a constitutively expressed antisense construct (Vanlerberghe et al. 1995).

WT plants and primary transformants (T0 generation) were grown to maturity in a greenhouse and allowed to self-fertilize. Seeds (T1 generation) were collected, surface sterilized and germinated on a modified Murashige and Skoog (MSO) medium (Horsch et al. 1988) under 24 h of fluorescent light. Under these conditions, all

germinants of the WT, AS8 and S24 survived but no WT germinants could survive if the medium was supplemented with 100  $\mu\text{g ml}^{-1}$  active kanamycin (Kn). For AS8, the ratio of Kn resistant to sensitive germinants in the T1 generation was approximately 3:1 (299 resistant and 96 sensitive) indicating that a single copy of the transgene was present in the primary transformant. In S24, the ratio was approximately 15:1 (326 resistant and 23 sensitive) indicating two copies of the transgene in the primary transformant.

All experiments were performed on WT plants and T2 generation transgenic plants resistant to Kn. All seeds were germinated as above (without Kn in the case of WT plants but with Kn in the case of transgenic plants), and after 3 weeks, seedlings were transferred to pots containing a 2:1 gravel : vermiculite (v/v) mix and grown in growth chambers [27/23°C day/night temperature, 14-h photoperiod, 700  $\mu\text{M m}^{-2} \text{s}^{-1}$  photosynthetic photon flux density (PPFD)] for 18–25 days prior to sampling. Plants were irrigated with half-strength Hoagland's solution (Hoagland and Arnon 1950) in the morning and distilled water in the afternoon.

### Respiration and isotope discrimination analyses

Respiration rates and differential uptake of stable oxygen isotopes were simultaneously monitored in the gas-phase using leaf discs sealed within a temperature-controlled stainless steel cuvette at 25°C (except where noted). Typically, for any given 'run', six air samples were taken in sequence over a 30-min period and the  $\text{O}_2$  quantified by gas chromatography (thermal conductivity detector) before passing directly into the inlet of a SIRA-II ratio mass spectrometer (VG Isogas) essentially as previously described (Robinson et al. 1992, 1995). The cuvette was modified for temperature control by insulating it with styrene foam and fixing a copper plate and coil, connected to a water bath, to its base.

Isotope discrimination ( $D$ ) factors, in per mil (‰) units, were calculated using a form of the Rayleigh distillation model. As defined by Guy et al. (1989)

$$D = (1 - \alpha) \times 1000\text{‰} \quad (1)$$

where  $\alpha$  is the fractionation factor, the  $^{18}\text{O}/^{16}\text{O}$  isotope ratio of instantaneous product relative to available substrate ( $R_{\text{product}}/R_{\text{substrate}}$ ). For the progressive consumption of oxygen from air in a closed system, Henry et al. (1999) show that

$$D = \frac{-d \ln(1 + \delta)}{d \ln O^*} \times 1000\text{‰} \quad (2)$$

where  $\delta = \delta^{18}\text{O}/1000$  and  $O^*$  is the concentration of the oxygen substrate.  $D$  is evaluated by unconstrained

linear regression of  $\ln(1 + \delta) \times 1000$  against  $-\ln O^*$  (Henry et al. 1999). Note that  $O^*$  is proportional to the  $\text{O}_2/\text{N}_2$  ratio ( $r$ ), which was obtained from

$$r = \frac{[\text{O}_2 + \text{Ar}]}{[\text{N}_2]} - 0.01275 \quad (3)$$

where  $[\text{O}_2 + \text{Ar}]$  and  $[\text{N}_2]$  are the integrated peak areas off the gas chromatograph (argon co-chromatographs with oxygen), while the constant corrects for the contribution of Ar to the  $\text{O}_2$  peak taking into account the higher thermal conductivity of Ar relative to  $\text{N}_2$ .

Per cent partitioning to AOX (%A) was calculated according to Guy et al. (1989):

$$\%A = \frac{D_{\text{control}} - D_{\text{cyt}}}{D_{\text{AOX}} - D_{\text{cyt}}} \times 100 \quad (4)$$

where  $D_{\text{cyt}}$  and  $D_{\text{AOX}}$  are discrimination factors associated with  $\text{O}_2$  consumption by cyt oxidase and by AOX, respectively, while  $D_{\text{control}}$  is discrimination measured in the absence of inhibitors. To establish  $D_{\text{AOX}}$  and  $D_{\text{cyt}}$ , we pretreated leaf discs for 15 min by soaking in either 1 mM KCN or 10 mM SHAM (in 2% DMSO), respectively. When using CN, filter papers dampened with 1 mM KCN were included in the cuvette and sandwiched between the leaf discs. Afterwards, leaf discs were weighed to obtain the fresh weight (FW) and then oven-dried to determine dry weight (DW). To calculate relative water content (%RWC), we soaked discs in distilled water to a constant, fully turgid weight (FTW) before oven-drying:

$$\%RWC = \frac{FW - DW}{FTW - DW} \times 100 \quad (5)$$

### Other methods

Drought stress was imposed by withholding water for 3 days until plants were wilted. Post-water stress was imposed by soaking leaf discs from the water-stressed plants in distilled water for 15 min. Photoinhibitory stress resulting from a combination of bright light and low temperature was imposed in three ways: (1) by moving plants to another growth chamber for 2.5 h at 10°C and 450  $\mu\text{M m}^{-2} \text{s}^{-1}$  PPFD (slight stress); (2) by placing plants outside in late autumn for 4 h at approximately 10°C under full sun (approximately 1200  $\mu\text{M m}^{-2} \text{s}^{-1}$  PPFD) conditions (moderate stress) and (3) by placing plants outside under the same conditions but for 4 h on each of two consecutive days (severe stress). The ratio of variable-to-maximum chlorophyll fluorescence (an indicator of the quantum efficiency of photosynthesis) was measured on leaf discs following 20-min dark adaptation using a Hansatech PEA fluorimeter.

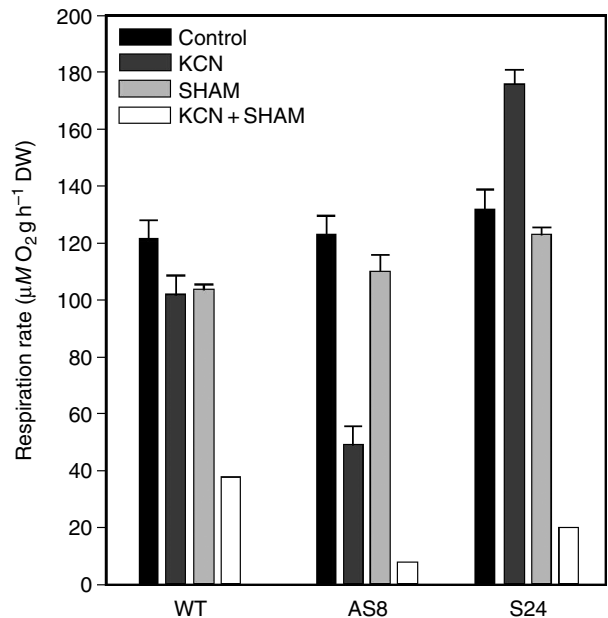
## Statistical analyses

Standard least-squares regression was used to calculate  $D$  for each individual run (Henry et al. 1999, Scott et al. 2004). The coefficient of determination ( $R^2$ ) was never less than 0.988 and exceeded 0.99 in all but four of the 86 runs performed. To calculate appropriately weighted 'mean' discrimination factors across lines or treatments, we combined data from separate runs using multiple linear regression with dummy variables (Scott et al. 2004). For the inhibitor studies, discrimination factors and respiration rates were compared by one-way analysis of variance (ANOVA) followed by means separation using the Tukey test to detect differences between lines within a treatment or between treatments within a line. One-way ANOVA was also used to test for effects of abiotic stress on  $D$ , but in these cases, each treatment by line combination was done only once, and there was no prior need to combine datasets by multiple linear regression. All tests were performed using SIGMASTAT version 3.10 (Systat Software Inc.).

## Results

Previously, we generated transgenic tobacco plants in which AOX levels were modified by constitutively expressed sense or antisense AOX transgenes (Vanlerberghe et al. 1994, 1995). There was no significant difference in the control (uninhibited) respiration rate between leaf discs from WT plants, plants with reduced AOX protein (AS8) and plants with increased AOX protein (S24) (Fig. 1). In the presence of the cytochrome oxidase inhibitor CN, the respiration rate of WT leaves tended to decline slightly, although this was not statistically significant (Fig. 1). However, CN treatment significantly decreased the respiration rate of AS8 leaves ( $P < 0.001$ ) while significantly increasing the respiration rate of S24 leaves ( $P = 0.001$ ). Hence, in the presence of CN, the respiration rate of AS8 was significantly less than that of the WT ( $P < 0.001$ ), and respiration in S24 was significantly greater than in the WT ( $P < 0.001$ ) (Fig. 1). In the presence of the AOX inhibitor SHAM, all three lines exhibited a small inhibition of respiration compared with control, although in no case was this statistically significant (Fig. 1).

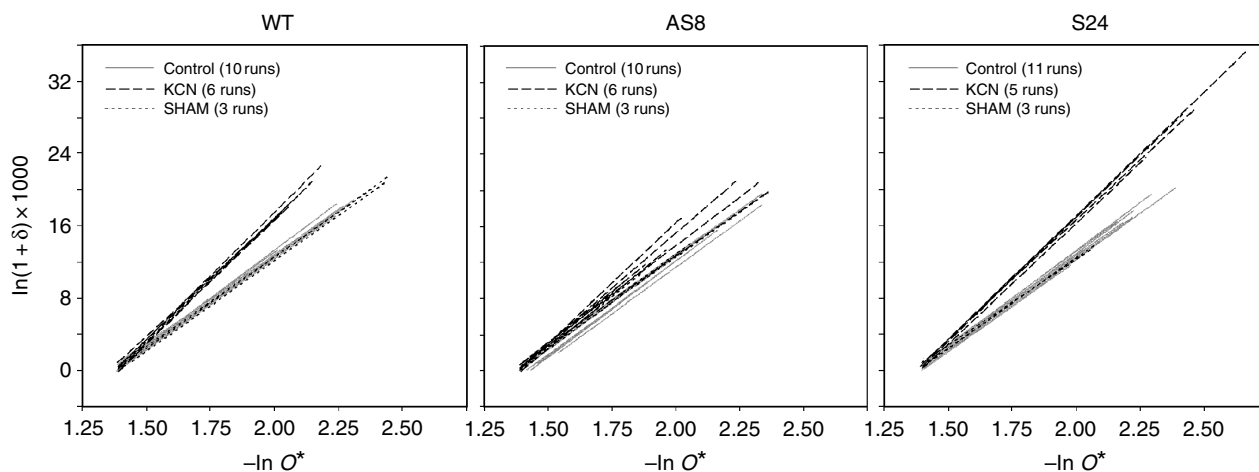
Online  $^{18}\text{O}$  isotope discrimination experiments were used to determine discrimination factors under each of the above conditions (Fig. 2). With the clear exception of AS8 treated with CN, the high degree of overlap of regression lines obtained for any particular treatment by line combination demonstrates excellent reproducibility. From these data, no significant difference was seen between plant lines in the mean discrimination



**Fig. 1.** Dark respiration rates of leaf tissue from the three tobacco lines at 25°C, in the absence of inhibitors (control) or treated with 1 mM KCN and/or 10 mM SHAM. Values are mean rates based on data pooled from several experiments (see Fig. 2 for numbers) except where both inhibitors were used, in which case there was only one experiment per line. Error bars are  $\pm$ SE.

factor for  $\text{O}_2$  consumption by cytochrome oxidase ( $D_{\text{cyt}}$ ; determined in the presence of SHAM) (Table 1). The overall  $D_{\text{cyt}}$  obtained by combining data from all three lines was  $20.01 \pm 0.14\text{‰}$ . Discrimination factors for AOX ( $D_{\text{AOX}}$ ; determined in the presence of CN) were the same in the WT and S24 (Table 1) yielding a combined  $D_{\text{AOX}}$  of  $27.16 \pm 0.13\text{‰}$ . In AS8, discrimination in the presence of CN was significantly lower and more variable than in the WT or S24 (Table 1). Also, a relationship existed between the absolute rate of CN-inhibited respiration in AS8 and the measured discrimination factor. Lower rates of respiration correlated with lower discrimination values (Fig. 3). The discrimination factor for residual respiration ( $D_{\text{residual}}$ ; determined in the presence of CN and SHAM) was similar for all three plant lines. Also, the average  $D_{\text{residual}}$  value obtained by combining the data for WT and S24 ( $22.92 \pm 0.63\text{‰}$ ) was not significantly different from the discrimination factor for AS8 respiration in the presence of CN ( $22.56 \pm 0.38\text{‰}$ ).

The control discrimination factor ( $D_{\text{control}}$ ; measured in the absence of inhibitors) for the WT was not different from the WT  $D_{\text{cyt}}$  value (Table 1) or the overall  $D_{\text{cyt}}$  value obtained by averaging the three plant lines. This indicates that the percentage of control respiration attributed to AOX (%A) in the WT was low (3.7%) and



**Fig. 2.** Isotope discrimination during respiration by tobacco leaves in the absence of inhibitors (control) or treated with 1 mM KCN or 10 mM salicylhydroxamic acid (SHAM) at 25°C. Each regression line represents one run, the slope being proportional to  $D$  (data points omitted for clarity). The number of runs (experiments) plotted for each set of conditions is indicated. To construct these graphs, we expressed  $\delta$  relative to the isotopic composition of the source room air, which was measured prior to each run.

not significantly different from zero (Table 1). In S24, the  $D_{\text{control}}$  was significantly different from the S24  $D_{\text{cyt}}$  value ( $P < 0.001$ ), marginally different from the WT  $D_{\text{cyt}}$  value ( $P = 0.05$ ) and significantly different from the overall  $D_{\text{cyt}}$  value ( $P = 0.02$ ). This indicates that %A in S24 was still low (9.8–14.2%) but was significantly different from zero. In AS8,  $D_{\text{control}}$  was not different from the AS8  $D_{\text{cyt}}$  value but was different from the overall  $D_{\text{cyt}}$  value ( $P = 0.002$ ). Hence, calculation of %A using the overall  $D_{\text{cyt}}$  yielded a value higher than in the WT and significantly different from zero (7.8%), while calculation of %A using the AS8  $D_{\text{cyt}}$  value yielded a number not significantly different from zero (Table 1).

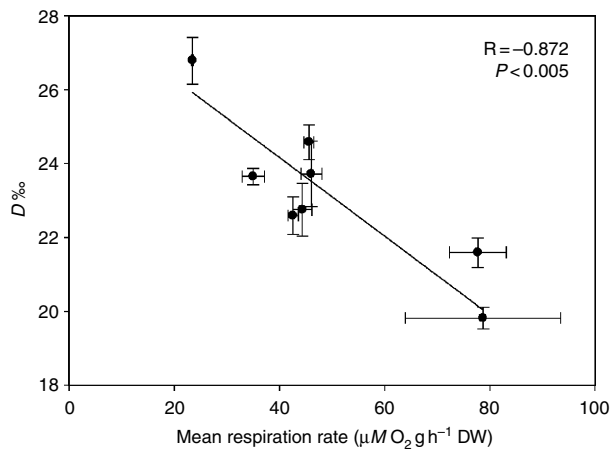
The respiration of WT and S24 plants (grown at 27°C) was evaluated over a range of measurement temperatures (9.7–40°C). Over this range, the respiration rates of WT and S24 leaves were similar to one another and increased linearly with temperature (Fig. 4A). An

exponential fit was not as good but did yield an overall Q10 very close to expectation (2.0). Figure 4B shows the corresponding isotope discrimination results. Both lines displayed similar %A values at any given temperature. ANOVA indicated that temperature effects for S24 and WT were significant ( $P = 0.016$  and  $P = 0.011$ , respectively). Interestingly, both lines showed the highest %A values (21.5–25.8%) at temperatures 5–10°C above the growth temperature. These values assume no effect of temperature on either  $D_{\text{cyt}}$  or  $D_{\text{AOX}}$  in Equation 4. Guy et al. (1989) and González-Meler et al. (1999) found no effects of temperature on  $D_{\text{cyt}}$  in yeast (25–36°C) and  $D_{\text{AOX}}$  in mung bean leaves (10–30°C), respectively. For WT leaves,  $D_{\text{control}}$  at 39.5°C was significantly different from  $D_{\text{cyt}}$  ( $P = 0.006$ ) and from  $D_{\text{control}}$  at 25°C ( $P = 0.014$ ). Means could not be separated for S24.

The respiration of WT and S24 leaves was also evaluated in response to abiotic stress. Neither water stress,

**Table 1.** Mean discrimination ( $D$ ) factors for tobacco leaf obtained by multiple linear regression of the data presented in Fig. 2. The  $D_{\text{control}}$  value (measured in the absence of inhibitors) was used to calculate the percentage of control respiration attributable to AOX (%A). This calculation was done using respective values of  $D_{\text{cyt}}$  and  $D_{\text{AOX}}$  for each line except for AS8 where the combined  $D_{\text{AOX}}$  of wild-type (WT) and S24 (27.16‰) was employed. The probability ( $P$ ) that %A is not different from zero is shown in brackets. NS, not significant; SHAM, salicylhydroxamic acid.

Plant line	$D$ (‰) $\pm$ SE			
	+SHAM ( $D_{\text{cyt}}$ )	+KCN ( $D_{\text{AOX}}$ )	Control ( $D_{\text{control}}$ )	%A
WT	20.00 $\pm$ 0.19	27.26 $\pm$ 0.23	20.27 $\pm$ 0.14	3.7 (NS)
S24	19.65 $\pm$ 0.35	27.09 $\pm$ 0.16	20.71 $\pm$ 0.18	14.2 ( $P < 0.001$ )
AS8	20.31 $\pm$ 0.20	22.56 $\pm$ 0.38	20.57 $\pm$ 0.11	3.8 (NS)



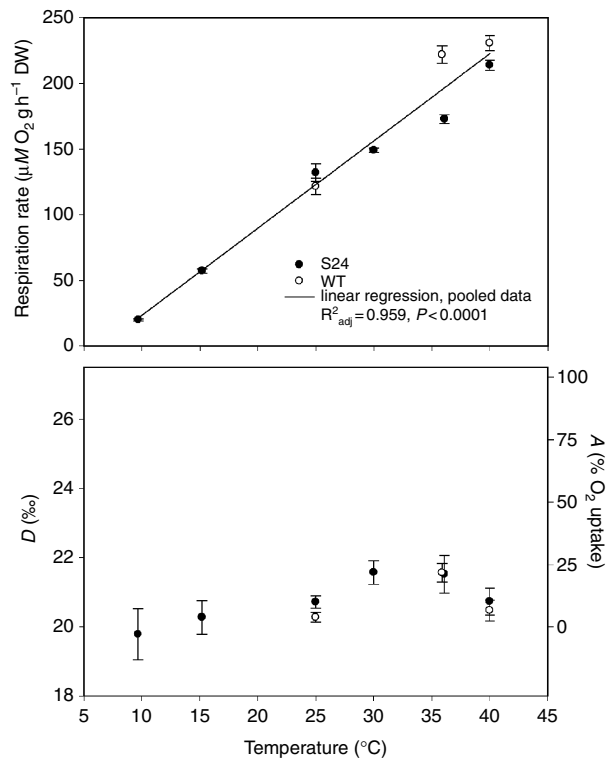
**Fig. 3.** Correlation between discrimination ( $D$ ) and the absolute rate of CN-inhibited respiration in AS8. The trend-line is by geometric mean regression to account for error in both variables (Ricker 1984). Error bars are  $\pm$ SE.

post-water stress or photoinhibitory treatment (slight to severe) was found to dramatically affect isotope discrimination by tobacco leaves (Table 2). However, in WT leaves, both water stress and post-water stress resulted in %A values of approximately 20%, a value that was significantly different than zero (Table 2). Also, %A in water-stressed WT leaves was different than control %A values ( $P = 0.032$ ). In the case of S24, %A was not different than zero under any conditions (Table 2). Nonetheless, the %A value under water stress (21.1%) was similar to that seen in WT leaves.

## Discussion

### $^{18}\text{O}$ isotope discrimination associated with the cyt and alternative pathways

The discrimination factors calculated for respiration in the presence of KCN ( $D_{\text{AOX}}$ ) and in the presence of SHAM ( $D_{\text{cyt}}$ ) fall within the range of values reported in the literature. In general, reported values for  $D_{\text{cyt}}$  (16–21‰) seem more consistent than for  $D_{\text{AOX}}$  (24–32‰) (e.g. González-Meler et al. 1999, Guy et al. 1989, Henry et al. 1999, Ribas-Carbo et al. 1995). This variation may represent real differences between species or tissues, or may, in part, reflect differences in the calculation of  $D$  (Henry et al. 1999). High values for  $D_{\text{AOX}}$  have been associated with green tissues (Robinson et al. 1992), but not consistently so (e.g. Guy et al. 1989, Nagel et al. 2001, Noguchi et al. 2001). Using leaves of the same tobacco cultivar, González-Meler et al. (2001) obtained values of 19.6 and 29.8‰ for  $D_{\text{cyt}}$  and  $D_{\text{AOX}}$ , respectively, whereas, we obtain overall values of 20.0 and 27.2‰. Our  $D_{\text{AOX}}$  is the same as that



**Fig. 4.** Leaf respiration rates (top panel) and isotope discrimination ( $D$ ) (lower panel) as a function of measurement temperature in S24 and wild-type (WT). The calculated partitioning to AOX ( $A$ ; right axis, lower panel) is based on overall best estimates of  $D_{\text{cyt}}$  and  $D_{\text{AOX}}$ . Error bars are  $\pm$ SE.

reported by Henry et al. (1999) for green, 14-day-old soybean cotyledons respiring at a slow rate.

### Effects of AOX protein level on AOX engagement

The respiration data (Fig. 1) clearly indicated the expected differences in AOX capacity between the WT and transgenic plants. That is, S24 plants with increased levels of leaf AOX protein had increased AOX capacity, while AS8 plants with undetectable levels of leaf AOX protein had decreased AOX capacity (Fig. 1; Vanlerberghe et al. 1995). However, when plants were tested under our standard growth and measurement conditions (see *Materials and methods*), each of the plant lines had a similar control (inhibitor)  $\text{O}_2$  discrimination value that indicated a low flux of electrons to AOX (Fig. 2, Table 1). These data indicate that AOX protein level had little if any control over AOX engagement under these conditions.

The CN-inhibited rate of respiration in AS8 was much lower than seen in the WT, consistent with lower levels of AOX in this line. However, the CN-inhibited rate in AS8 was not as low as the residual rate when both inhibitors were present (Fig. 1). These data suggest that

**Table 2.** Oxygen isotope discrimination ( $D$ ) by tobacco leaf when water stressed, upon relief of water stress and while photoinhibited. See *Materials and methods* for a full explanation of the treatments imposed. Per cent relative water content (%RWC) and the ratio of variable-to-maximum chlorophyll fluorescence (Fv/Fm) are shown where relevant.  $D$  was used to calculate the percentage of respiration attributable to AOX (%A) as in Table 1. The probability ( $P$ ) that %A is not different from zero is shown in brackets. <sup>a</sup>Control RWC was  $90 \pm 4\%$ ; <sup>b</sup>Typical control Fv/Fm was 0.80–0.83, regardless of plant line. WT, wild-type.

Treatment	Line	%RWC <sup>a</sup>	Fv/Fm <sup>b</sup> $\pm$ SE	$D$ (‰) $\pm$ SE	%A
Water stress	WT	81.6	$0.794 \pm 0.007$	$21.52 \pm 0.10$	20.9 ( $P = 0.006$ )
	S24	82.3	$0.830 \pm 0.002$	$21.22 \pm 1.06$	21.1 (NS)
Post-water stress	WT	100	–	$21.25 \pm 0.44$	17.2 ( $P = 0.028$ )
	S24	100	–	$20.70 \pm 0.35$	14.1 (NS)
Slight photoinhibition	WT	–	$0.696 \pm 0.019$	$19.73 \pm 0.53$	–3.7 (NS)
Moderate photoinhibition	S24	–	$0.622 \pm 0.060$	$20.34 \pm 0.26$	9.3 (NS)
Severe photoinhibition	S24	–	$0.475 \pm 0.080$	$20.87 \pm 0.71$	16.4 (NS)

AS8 leaves still have some small capacity for AOX respiration despite having no detectable AOX protein (Vanlerberghe et al. 1995). This is also consistent with the observation that these leaves can maintain an apparent low level of AOX engagement in the absence of inhibitors (Table 1). By contrast, the AS8  $D$ -value for AOX (i.e. in the presence of CN), while highly variable, was similar to the residual discrimination (i.e. in the presence of CN and SHAM) (Table 1). This is what one would expect if AOX levels were severely depressed in AS8. The variable nature of the AS8  $D$ -value for AOX could be explained if different AS8 leaf discs in fact maintained variable levels of residual AOX protein. In this case, one would have a variable mixture of residual respiration and residual AOX, resulting in a variable  $D$ -value. However, this hypothesis is not consistent with data showing a negative correlation between the +CN respiration rate of any given set of AS8 leaf discs and the  $D$ -value measured on those same discs (Fig. 3). If higher levels of residual AOX were responsible for the higher +CN respiration rate of some disks, then those same disks should tend to have the higher  $D$ -values. This is clearly not the case. Another possible consideration is that an AOX-lacking leaf might induce residual  $O_2$ -consuming processes that would not normally be seen in an AOX-containing plant and that this confounds  $D$ -values in the presence of some inhibitors or even in the absence of inhibitors. On balance, the results do clearly indicate depressed AOX levels in AS8 compared with the WT, but we cannot exclude the possibility of some (variable) residual AOX.

### Effects of temperature and other abiotic stress on AOX engagement

The experiments done under standard growth and measurement conditions suggested that the higher capacity

for AOX respiration in S24 compared with the other plant lines was not resulting in higher AOX engagement, indicating that protein level has little if any impact on AOX engagement (other than defining the maximum possible activity). To further test this hypothesis, we compared AOX engagement of WT and S24 plants under a range of different experimental conditions that included abiotic stress pretreatments or determination of engagement over a range of measurement temperatures. We hypothesized that a change in AOX engagement (as well as differences in engagement between WT and S24) might become apparent under such fluctuating metabolic conditions.

Temperature is an environmental factor that has been shown to affect the capacity and/or engagement of AOX, although the role of AOX in relation to temperature remains unresolved. González-Meler et al. (1999) examined AOX capacity and engagement in plants under a range of growth and measurement conditions and found that respiratory responses were both tissue and species dependent. Another study found that there was a large increase in AOX engagement in a chilling-sensitive cultivar but not a chilling-resistant cultivar of maize (Ribas-Carbo et al. 2000). Another study has found that AOX and the cyt pathway exhibit similar temperature coefficients (Q<sub>10</sub>) to one another, whether measured in intact tissues or isolated mitochondria of soybean (Atkin et al. 2002). It has also been shown that a shift to lower growth temperature of tobacco suspension cells increases AOX protein and capacity (Vanlerberghe and McIntosh 1992). Such a response was not seen, however, in potato leaves (Svensson et al. 2002). Over a measurement range of 9.7–40°C, we found no obvious difference in the respiratory responses of WT and S24 leaves, despite the differences in AOX capacity. Both plants displayed similar engagement at any given measurement temperature (Fig. 4B;

Table 2). Interestingly, both lines displayed some increase in AOX engagement at measurement temperatures just above the growth temperature, but again, the engagement was similar between the two lines (Fig. 4B, Table 2). Respiration rate in both plants showed a similar dependence on temperature (Fig. 4A).

There is a general appreciation that mitochondrial respiration in the light is necessary for optimal photosynthesis (reviewed in Gardeström et al. 2002, Raghavendra and Padmasree 2003). One important point of interaction is photorespiration, because photorespiratory glycine is oxidized by mitochondrial glycine decarboxylase (GDC). This is the dominant substrate oxidized in mitochondria in the light, and a majority of the reductant produced is thought to be oxidized by the mitochondrial ETC. This may help to prevent over-reduction of redox components in the chloroplast, by allowing chloroplast reductant to support hydroxypyruvate metabolism. In this way, photorespiration may play an important role in the prevention of photoinhibition (Raghavendra and Padmasree 2003). One hypothesis is that AOX supports photosynthesis by supporting GDC activity and preventing photoinhibition. Evidence for AOX involvement in photosynthesis/photorespiration includes: (1) The AOX enzyme is activated by photorespiratory intermediates such as glyoxylate and hydroxypyruvate; (2) GDC and AOX expression may be coordinately regulated; (3) AOX protein levels are generally enhanced by light, and AOX expression is induced during greening of etiolated leaves; (4) During photorespiration, matrix concentrations of NAD(P)H and pyruvate increase, which should favour AOX activation and (5) Chemical inhibition of AOX reduces photosynthetic performance (Gardeström et al. 2002, Raghavendra and Padmasree 2003).

When we subjected WT and S24 plants to photoinhibitory conditions, we saw no change in the partitioning of electrons to AOX in either plant line (Table 2). These results confirm, under different conditions, that increased levels of AOX protein will not necessarily impact partitioning. However, one must remember that these respiratory measurements are still, by necessity, being performed in the dark. We cannot exclude the possibility that AOX is further engaged in the light and that partitioning changes rapidly upon transfer from light to darkness.

Respiratory studies suggest that increased AOX may be a response to numerous abiotic and biotic stresses (Simons and Lambers 1999, see *Introduction*). Interestingly, a few studies have shown that AOX expression or protein level is increased under drought conditions (Bartoli et al. 2005, Rizhsky et al. 2002, Seki et al. 2002). To our knowledge, however, no previous

studies have used isotope discrimination to determine AOX engagement under such conditions. The data in Table 2 suggest that AOX may indeed be more active under drought than well-watered conditions, although this result is based on a limited set of data. However, the absolute level of AOX engagement under drought was similar between the WT and S24, indicating again that increased levels of AOX protein in the transgenic plant will not necessarily alter partitioning.

## Conclusions

The respiration and inhibitor data (Fig. 1) clearly showed that transgenic plants with modified levels of AOX protein had corresponding changes in AOX capacity. However, comparison of WT and transgenic plants using isotope discrimination showed that AOX protein level itself had little if any influence over the partitioning of respiratory electrons to AOX, at least under the tested growth and measurement conditions (Tables 1 and 2, Fig. 4). If changes in AOX protein need not necessarily be associated with corresponding changes in AOX respiration then this further suggests that the metabolic conditions inherent to a particular growth condition and/or the biochemical regulatory properties of the AOX enzyme itself are the critical factors that control partitioning. It is also shown that temperature and leaf water status are parameters that have some influence over the partitioning of electrons to AOX respiration.

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