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The production of an inducible antisense alternative oxidase (*Aox1a*) plant

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Abstract. Plant mitochondria contain an alternative oxidase (AOX) acting as a terminal electron acceptor of the alternative pathway in the electron transport chain. Here we describe the production of inducible antisense *Aox1a* plants of *Arabidopsis thaliana* (L.) Heynh. and the procedures used to determine the resulting alternative pathway activity. The *Arabidopsis Aox1a* cDNA sequence was cloned behind a copper-inducible promoter system in the antisense orientation. *Arabidopsis thaliana* (Columbia) plants were transformed by in-planta vacuum infiltration with *Agrobacterium* containing the antisense construct. Whole-leaf ethanol production was used as a measure to investigate alternative pathway activity in the presence of antimycin A. After 24 h, leaves from the copper-induced, antisense line F1.1 produced up to 8.8 times more ethanol (via aerobic fermentation) than the non-induced and wild-type leaves, indicating effective cytochrome pathway inhibition by antimycin A and a decreased alternative pathway activity in induced F1.1 leaves. Transgene expression studies also revealed no expression in non-induced leaves and up until 24 h post-induction. Copper-induced transgenic leaves were less susceptible to alternative pathway inhibition than non-induced transgenic leaves, as seen via tissue-slice respiratory studies, and mitochondrial respiration, using F1.1 cell cultures, also supported this. These results demonstrate the successful production of a transgenic line of *Arabidopsis* in which the alternative pathway activity can be genetically manipulated with an inducible antisense system.

Key words: Alternative oxidase – Antisense *Aox1a* – *Arabidopsis* (alternative oxidase) – Copper-inducible gene – Electron transport – Mitochondrial respiration

Abbreviations: AOX = alternative oxidase; AP = alternative pathway; CP = cytochrome pathway; FCCP = carbonyl cyanide *p*-(trifluoromethoxy)-phenylhydrazone; mETC = mitochondrial electron transport chain; RT-PCR = reverse transcription polymerase chain reaction; SHAM = salicylhydroxamic acid; WT = wild type

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Introduction

Plant mitochondria possess two terminal oxidases, the alternative oxidase (AOX) and cytochrome oxidase, within the electron transport chain. The AOX is currently under investigation so that its function in the plant may be known. Increased expression of the *Aox* gene in plant tissues, coupled with increased alternative pathway (AP) activity has been observed (i) during the development of thermogenesis (Rhoads and McIntosh 1992), (ii) during fruit ripening (Cruz-Hernandez and Gomez-Lim 1995) and (iii) during times when strict adenylate control over the mitochondrial electron transport chain (mETC) limits electron flow (Vanlerberghe et al. 1995). It has also been suggested that the AOX may be involved in reducing superoxide and oxygen radical production, by the mETC, during plant stress (Wagner 1995; Wagner and Krab 1995; Popov et al. 1997; Purvis 1997; Wagner and Moore 1997; Millenaar et al. 1998; Maxwell et al. 1999). The AOX enzyme, as well as the activity of the AP, has previously been shown to be induced upon applying different types of stress to plants, e.g. chilling, etiolation, exogenous chemicals and ageing (for a review, see Vanlerberghe and McIntosh 1997).

Investigation of the AP's role in plant stress response has been facilitated by use of antisense technology. The *Aox* gene is one of the few, single polypeptide, nuclear-encoded components of the mETC. The absence of AOX in certain plant tissues, along with the increased expression of *Aox* during stress, indicates that the AP is an important pathway; however, it is not essential for growth. Vanlerberghe et al. (1994) successfully produced tobacco plants expressing antisense and sense *Aox1* transcripts. These transgenic tobacco plants and cultures contained altered levels of AOX and provided an opportunity to further study electron flux via the AP. The AP is known to operate in parallel with the cytochrome pathway (CP), competing with it for electrons (Wilson 1988; Hoefnagel et al. 1995). Respiratory studies using transgenic tobacco cultures have shown that while the overall respiratory rates were constant

among the wild type (WT), over-expressor and antisense cell lines, the antisense line showed reduced AP capacity (Vanlerberghe et al. 1994). These antisense *Aox* cells also showed increased reactive oxygen species in the presence of the CP inhibitor antimycin A when compared to WT cells (Maxwell et al. 1999) indicating that the AOX enzyme may be important for reducing production of reactive oxygen species during periods of oxidative stress.

We were interested in the production of an inducible antisense plant which would provide temporal control over *Aox1a* gene expression. This inducible transgenic material would allow the investigation of mETC behavior with minimal effects from long-term *Aox* silencing. Secondary effects in constitutive antisense tissue may be a concern, considering past studies have shown the restriction of one pathway affects the expression and function of other mETC component(s) (Vanlerberghe and McIntosh 1994; Saisho et al. 1997; Wagner and Wagner 1997). By using inducible antisense plants and cell cultures, studies could occur when alterations to the mETC were immediate, and the results could be compared (both induced and non-induced) with the relevant WT controls.

The most accurate approach to study mETC function under ideal conditions with altered levels of a single mETC component is by using plants of the same age and developmental stage in which one has altered AOX activity by non-stressful induction. Studies involving inducible transgenic plants have adequate controls that make it possible to show, by a direct link, the alteration of the target component (e.g. AOX) affecting the expression of other mETC components. Given the complexity of mETC interactions, an inducible system provides a clearer picture of the relationships between these components. With this in mind we have successfully produced an inducible *Aox* antisense plant and will be able to study it under ideal conditions and its response to stress, in the induced and non-induced state.

Materials and methods

Construct production and plant transformation

The copper-inducible system was kindly provided by Dr. P.H. Reynolds (The Horticulture and Food Research Institute of NZ Ltd.) and is well documented in Mett et al. (1993). The *Aox1a* cDNA sequence of *Arabidopsis thaliana* (L.) Heynh. was subcloned into an *Xba* I site of pPMB7066 such that it was in an antisense orientation, downstream of a copper-regulated promoter (MRE). The plasmid pPMB7066 is a pUC119-based plasmid containing an "expression cassette" made up of the metallo-regulatory element (MRE) fused to the −90-bp 35S promoter. Analysis of the orientation of the *Aox1a* cDNA was performed using diagnostic digestion. Once the *Aox1a* sequence was inserted in an antisense orientation with respect to the chimaeric MRE/P_{nos} promoter, this entire expression cassette was subcloned into the *Not* I site of pPMB765, downstream from the kanamycin resistance marker gene and the activating-copper-element (ACE). It was then introduced into *Agrobacterium tumefaciens* and used to transform *A. thaliana* (Columbia) via in-planta transformation, following the protocol outlined in Bechtold et al. (1993). These plants were allowed to flower and produce seeds that were selectively germinated on media containing kanamycin (50 mg/l). Within 4 d of plating germinants were visible and by day 14 healthy, green germinants were transferred to media without kanamycin.

Analysis of transformed plant lines by polymerase chain reaction (PCR) and reverse transcription (RT) PCR

Leaf material was harvested from putative, antisense plants and WT plants and the genomic DNA was extracted following the method 2 outlined in Rogers et al. (1996) for small-scale extraction of genomic DNA. Total RNA was also extracted from leaf material using a Qiagen RNeasy Plant Mini Kit (cat. #74903). This RNA was amplified in a one-step RT-PCR assay (Boehringer Mannheim) using the primer Araaox2 specific to the 5′ end of *Aox1a* (5′ GCCTCTAGAAGCCCAAAAGCCCCATTG 3′) and one that was specific to the nopaline synthase (nos) terminator sequence within the transgene. A 5-µl sample of the RT-PCR product was removed and used for further amplification with REDTaq DNA polymerase (Sigma) using the nos terminator primer and the primer 2511B (5′ TGTCGCATCAGCAGGAAG 3′) downstream from Araaox2. Genomic DNA from WT and putative antisense plants was also tested via PCR for the presence of the cDNA-sized *Aox1* insert using *Aox1a*-specific primers. The product expected using WT genomic DNA was larger in size than that produced by the cDNA construct (1.6 kb c.f. 1.0 kb, respectively). The presence of the smaller cDNA-sized PCR product was indicative of the presence of the antisense construct.

Ethanol assays

The ethanol assay used by Vanlerberghe et al. (1995) in studying tobacco plant lines was adapted for use with *A. thaliana*. This protocol was preferred over oxygen-electrode analysis of leaf slices, due to the small amounts of tissue required. Leaf material was sprayed with 5 µM $CuSO_4$ solution and leaves were harvested after day 5. Concentrations of ethanol were measured on a spectrophotometer (Aminco DW-2a UV/VIS) and expressed as µmol $(g\ FW)^{-1}$.

Leaf-slice respiratory studies

Plants were sprayed daily with 5 µM $CuSO_4$ to drip point and then drained of excess solution. On day 5, leaves were harvested and sliced finely into a solution of 0.5 mM $CaSO_4$. Slices were gently blotted and weighed before adding to the oxygen-electrode reaction medium (0.3 M sucrose, 10 mM KH_2PO_4, 10 mM Mes, 2 mM $MgCl_2$, pH 7.4). Respiratory rates were measured in an oxygen electrode (Rank Bros., Cambridge, UK) in the presence of the uncoupler carbonyl cyanide *p*-(trifluoromethoxy)-phenylhydrazone (FCCP; 0.02 µM). Inhibitors were used at the concentrations of salicylhydroxamic acid (SHAM; 2 mM) and KCN (0.3 mM). Use of chemical inhibitors can be misleading (for a review, see Day 1996). All inhibitor concentrations were chosen following titration studies. Respiratory data were compared with two controls, the WT and the non-induced transgenic line. All assays were carried out at 25 °C and residual rates were subtracted from all rate values.

Cell culture and respiratory studies on isolated mitochondrial

Cell cultures inoculated on day 0 were used during exponential growth (day 2) for the isolation of mitochondria (following the method described in van Emmerik et al. 1992). Mitochondrial respiration was measured in an oxygen electrode (Rank Bros.) in the presence of the uncoupler FCCP (0.02 µM) with inhibitor concentrations as for leaf slices. These concentrations were chosen

following titration studies. All assays were carried out at 25 °C in standard reaction medium (0.3 M sucrose, 10 mM Tes, 10 mM KH_2PO_4, 2 mM $MgCl_2$ adjusted to pH 7.4) with the addition of 1 mM pyruvate in the case of isolated mitochondria.

Results and discussion

The copper-inducible antisense *Arabidopsis thaliana* plants produced were identified via a number of different screening stages. The initial screen (germination on kanamycin) was followed by PCR analysis of extracted genomic DNA using *Aox1a*-specific primers. The product resulting from amplification of the native gene was approximately 1.6 kb in size while that from amplification of the *Aox1a* transgene was only 1.0 kb. Of the 84 kanamycin-resistant plant lines tested, 33 carried the *Aox1a* transgene (data not shown) and these were targetted for the next stage of screening, where they were tested for ethanol production in the presence of antimycin A, after induction with a $CuSO_4$ (5 μM) foliar spray.

In order to screen for transgenic lines in which the alternative oxidase activity had been significantly reduced, we utilised an ethanol production assay (Vanlerberghe et al. 1995). Antisense tobacco leaves produced ethanol upon incubation in nutrient media containing antimycin A because the tissue underwent substrate metabolism by aerobic fermentation, summarised in the reaction below:

$$\text{Pyruvate} \longrightarrow CO_2 + \text{Acetaldehyde} + \text{NADH} \Leftrightarrow \text{NAD} + \text{Ethanol}$$

The initial reaction is catalysed by pyruvate decarboxylase and the reduction of acetaldehyde is catalysed by alcohol: NAD oxidoreductase. We made use of this observation such that putative antisense *Aox1* leaves, diminished in their ability to use the AP, were identified by their production of ethanol in the presence of the CP inhibitor antimycin A. The concentration and timing of the copper foliar spray to induce the antisense phenotype were assessed from previous studies, using the β-glucuronidase marker gene (*gus*). These previous studies allowed us to verify that the copper-inducible system functioned well in *A. thaliana* and that the day of maximal β-glucuronidase activity was 5 d post-induction (data not shown). This timing was used as a guide for all subsequent inductions. The concentration of 5 μM $CuSO_4$ was non-toxic for the ecotype Columbia (Murphy and Taiz 1995). This was important, considering the work by Padua et al. (1999) showing that the application of a lethal dose (50 μM) of $CuSO_4$ to sycamore cell-suspension cultures stimulated cyanide-insensitive respiration and induced AOX accumulation.

The results presented in Fig. 1 show the ethanol production by leaves from induced plants, floated in nutrient solution containing antimycin A. Those leaves with a lowered level of AOX, due to expression of the transgene after copper induction, were expected to produce proportionally higher amounts of ethanol than the WT leaves that had unaltered AOX levels. The

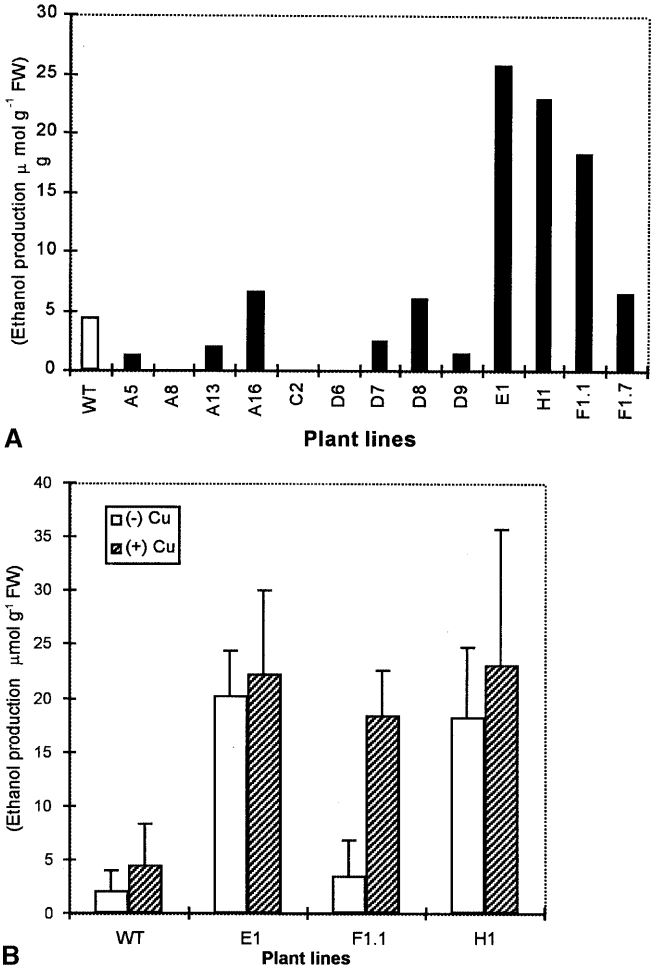

Fig. 1A,B. Ethanol production by detached leaves of *Arabidopsis* after 24 h. Amount of ethanol present in the leaf flotation solution (comprised of nutrient solution and 25 μM antimycin A) after 24 h, expressed as μmol · (g FW)$^{-1}$. **A** Plants had previously been treated with 5.0 μM $CuSO_4$ as a foliar spray and all values (except E1, F1.1 and WT) represent the mean of two assays. The other values represent the average of at least four assays. **B** Comparison of the WT and three highest ethanol producers with and without copper induction (±SD)

results show that a basal level of ethanol produced by the WT *Arabidopsis* leaves was 4.5 μmol · (g FW)$^{-1}$. In contrast, tobacco leaf segments incubated with antimycin A produced low to undetectable levels of ethanol (Vanlerberghe et al. 1995). This variation may reflect the different response to antimycin A poisoning in different plant species as well as the ability of antimycin A to penetrate the different leaf tissue types. From the results it can be seen that several of the trangenic lines (A16, D8, E1, F1.1, F1.7 and H1) had values higher than the WT with three of these more than double the WT ethanol production. Plant lines E1, F1.1 and H1 produced 25.8, 18.4 and 23.0 μmol · (g FW)$^{-1}$ of ethanol respectively within 24 h of incubation with antimycin A, compared to the WT [4.5 μmol · (g FW)$^{-1}$].

Plant lines E1, F1.1 and H1 were re-tested for antimycin A -dependent ethanol production without pretreatment with copper (Fig. 1B). Wild-type leaves were included as a control and showed low-level ethanol

production [2.1–4.5 μmol · (g FW)$^{-1}$]. This showed the application of copper to the WT did not significantly affect AP function. Plant lines E1 and H1 on the other hand, showed high ethanol production with no significant difference between leaves pre-treated with copper and non-treated leaves [20 and 26 μmol · (g FW)$^{-1}$ in E1 and 18 and 23 μmol · (g FW)$^{-1}$ in H1]. This indicated that the either (i) the expression of the antisense construct was not related to the presence of copper in these plant lines, or (ii) there was a mutation elsewhere causing a decrease in AP activity or an increase in electron flow, or (iii) there was a mutation causing increased ethanol production. These plant lines were excluded from further studies.

In contrast, antimycin A -dependent ethanol production by the transgenic line F1.1 was clearly the result of pre-treatment with copper. In the absence of copper pre-treatment the F1.1 line responded as the WT, producing low levels of ethanol [3.5 c.f. 2.1 μmol · (g FW)$^{-1}$, respectively]. This indicated that the copper-induced F1.1 leaves were unable to utilise the AP due to the induced expression of the antisense construct. With diminished AP activity and the CP inhibited by antimycin A, aerobic fermentation was the only path available for the re-cycling of NAD^+ and use of this pathway resulted in ethanol accumulation. The WT leaves were able to utilise the AP as well as potentially inducing *Aox1a* expression in response to the stress caused by antimycin A. The induction of *Aox1a* expression during antimycin A -poisoning has been previously documented (Saisho et al. 1997). These results from the putative antisense plant lines compare favourably with previous work using transgenic tobacco leaves (Vanlerberghe et al. 1995). In tobacco lines containing a constitutive antisense *Aox* gene, leaf material produced approximately 16 μmol ethanol (g FW)$^{-1}$ when under similar conditions. The similar results of ethanol production after 24 h, between the constitutive tobacco and the putative, inducible *A. thaliana* leaves [16 and 18 μmol ethanol (g FW)$^{-1}$, respectively] implied that the induction period in the latter (5 d) was adequate. This meant that if there were any "leakage" by the copper-inducible system the effect of this was negligible on the function of the native AOX in the F1.1 line, and non-induced F1.1 leaves were able to cope with the antimycin A-inhibition of the CP, by utilising the AP as in the case of the WT leaves.

Further assays were also performed in the absence of antimycin A to confirm that the ethanol production was a response to CP inhibition, and was not occurring in the absence of antimycin A (Fig. 2). The similar amounts of ethanol produced by WT leaves, in the absence and presence of antimycin A, indicated that these leaves had enough AOX to maintain electron flow during antimycin A-stress. The plant line F1.1 showed a similar response to WT in the absence of antimycin A, producing ethanol concentrations in the range of 2.4 to 3.0 μmol · (g FW)$^{-1}$.

From the results of Figs. 1 and 2, it is clear that the leaves of the transgenic line F1.1 did not produce ethanol, in the absence of antimycin A or copper induction, beyond the basal level seen in the WT leaves,

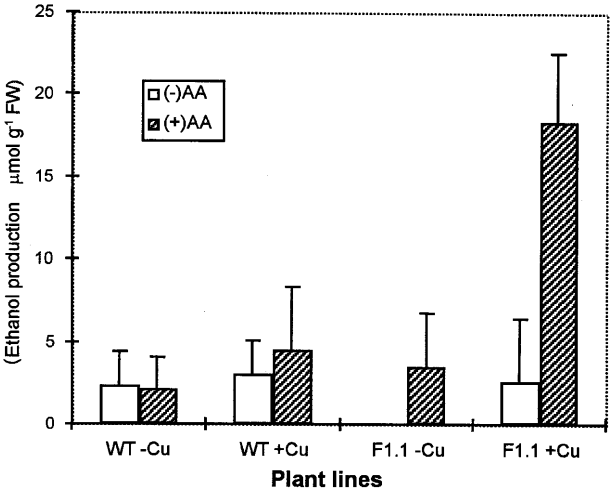

Fig. 2. Ethanol production by detached leaves of *Arabidopsis* after a 24-h incubation in the presence and absence of antimycin A. The amount of ethanol present in the leaf flotation solution expressed as μmol · (g FW)$^{-1}$. Leaf tissue (≥20 mg) that had been treated or not treated with 5.0 μM $CuSO_4$ (+Cu and −Cu, respectively) was floated in 2 ml of nutrient medium with and without 25 μM antimycin A. The incubation media were sampled after 24 h, and assessed for ethanol concentration. Data are the average ± SE of $n \geq 3$ independent experiments. Plant line F1.1 − Cu was not tested for ethanol production in the absence of antimycin A

and that F1.1 showed high production of ethanol [18.4 μmol · (g FW)$^{-1}$]. These results strongly suggest that F1.1 is a plant line with an inducible antisense construct, responsive to low levels of copper. During further incubation the increase in ethanol was accompanied by leaf tissue degradation; however, by 72 h the detached, copper-induced F1.1 leaves had produced three times the amount of ethanol produced by the WT and non-induced F1.1 samples (data not shown).

The expression of the antisense *Aox1a* construct was investigated using RT-PCR analysis. This was carried out on total RNA extracted from F1.1 leaves after foliar induction (2, 4, 12, 24, 48 and 72 h) and compared with WT samples. A primer specific to the nopaline synthase termination signal within the construct was used in combination with a primer specific to the 5′ end of *Aox1a* to determine the expression of the antisense construct. A negative control showed there was no genomic DNA contamination in any of the total RNA samples. The results (Fig. 3) show that expression of the antisense construct was only evident in the transgenic samples taken at 24, 48 and 72 h post-induction with copper and was not present in the earlier F1.1 samples. No transgene expression was seen in the WT samples as expected. These results indicate that little or no transcription of the transgene is occurring in the absence of copper and up until 24 h post-induction.

Respiratory studies on leaf slices were carried out to investigate how the difference in ethanol production (seen in the ethanol assays) and the presence of the transgene (seen with PCR screening) related to the maximal AP and CP activities and inhibitor sensitivities. Leaves were removed from WT and transgenic (induced

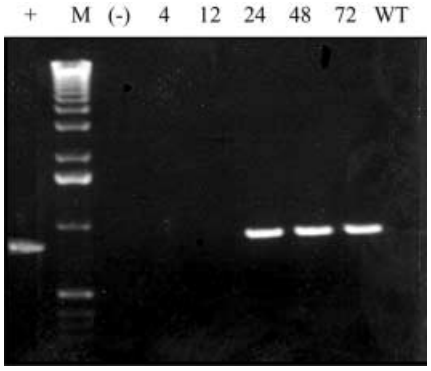

Fig. 3. Transgene expression after copper-induction. Total RNA was extracted from WT and non-induced (–) F1.1 leaf tissue of *Arabidopsis* and from leaves at 4, 12, 24, 48 and 72 h after a foliar spray of 5 μM $CuSO_4$ solution. Each sample (250 ng) was used in RT-PCR with one transgene-specific primer and one *Aox1a* primer. Secondary PCR amplification occurred using the same transgene primer and a different *Aox1a*-specific primer. These were compared with a WT and the positive control (+) of the transgene-construct-carrying plasmid. The transgene product is seen as an obvious band at 825 bp. *M*, marker; (–), unsprayed F1.1 control

and non-induced) plants, sliced thinly and used to measure oxygen consumption rates under maximal and inhibited conditions. The results using the transgenic material are shown in Fig. 4. The uninhibited rates seen with the transgenic F1.1 line were found to be greater than that of the WT, regardless of induction (data not shown). While the inhibition by KCN in WT leaves (55%) was similar, SHAM sensitivity was less (27%) than for the non-induced transgenic leaf slices (38%) and greater than for the induced F1.1 tissue (17%). This may be due to the WT and transgenic plants being at different developmental stages of growth. Transgenic leaf morphology was also noted as being different from that of the WT. While all WT tissue studied was taken from plants that were 6–8 weeks old, determining the physiological age of the F1.1 line was very difficult as it grew in a multi-rosette morphology and plants were propagated by rosette division. The higher rates seen with the transgenic leaf slices may be due to the difference in the age and developmental stage of the plants. Studies investigating the developmental regulation of respiratory activity in other species, such as *Pisum sativum* and *Glycine max*, have found that the uninhibited and the CP and AP rates may vary according to age, and that the gene expression of the different AOX isoforms experiences a shift during post-germinative, cotyledon development (Lennon et al. 1995; Kearns et al. 1992; McCabe et al. 1998). Another contributing factor may be the positional effect of the transgene, as the increased rates were seen in the transgenic line regardless of copper induction. In this study all of the transgenic leaf material was comparable with respect to the date of rosette division.

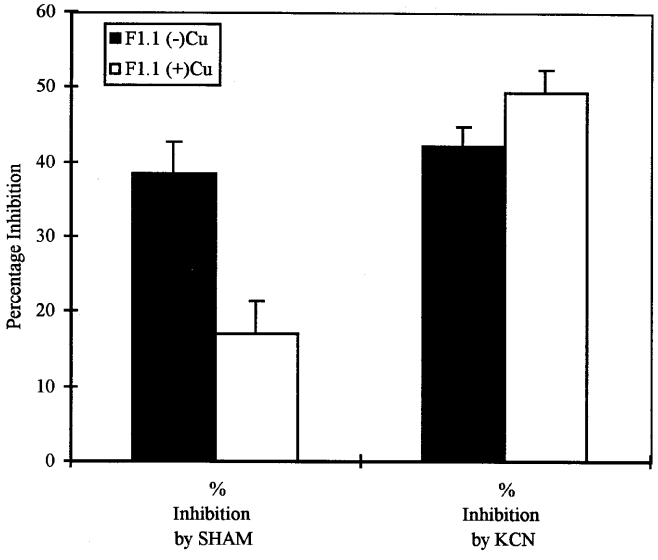

Fig. 4. Leaf-slice respiratory studies. Leaves of *Arabidopsis* were removed from non-induced and copper-sprayed (day 5) F1.1 plants and were finely sliced into 0.5 mM $CaSO_4$ solution. Oxygen consumption was measured in the presence of the uncoupler FCCP (0.02 μM) with and without inhibitors KCN (0.3 mM) and SHAM (2 mM). Inhibition was calculated as a percentage of the maximal, uninhibited rate. Uninhibited rates measured in the presence of the uncoupler FCCP (0.02 μM) were 176, 359 and 334 nmol O_2 min^{-1} $(g\ FW)^{-1}$ for WT, non-induced and induced F1.1 leaf slices, respectively. Data are the average ± SE of $n \geq 4$ independent experiments

The results in Fig. 4 show that the percentage SHAM inhibition for the induced leaves was markedly lower than that seen in the non-induced leaves (17% c.f. 38% with induced and non-induced leaves, respectively). The inhibition by KCN, however, appeared slightly higher in the induced leaves (42% c.f. 49% in F1.1 – Cu and F1.1 + Cu leaves, respectively). These results (Fig. 4) show that the copper-treated leaves had less sensitivity to SHAM than leaves from the same plant line that were not pre-treated with copper, which strongly implies that there was less AP activity in the copper-induced leaves, due to the expression of the antisense transgene. The apparent sensitivity to KCN was marginally greater in the induced antisense leaves. Further investigation is necessary before we can ascertain whether the antisense *Aox1a* plants are utilising a comparatively greater CP component.

To further investigate the results seen in the leaf tissue, mitochondria were isolated from cell cultures. Cell cultures were chosen for the ease of generating material at the same stage of growth. Changes in the oxidative properties of WT mitochondria had also been previously investigated (Davy de Virville et al. 1998). Transgenic and WT leaves were used to make the cell suspension cultures and the growth characteristics of the cultures were compared to ascertain the appropriate day of measurement (data not shown). All cells grew to approximately the same size and shape and did not markedly vary in growth characteristics. These cultures (grown with and without added 5 μM $CuSO_4$) were used to isolate mitochondria from day-2 cells and the results are presented in Table 1. The AP and CP inhibitors used in the mitochondrial respiration studies were *n*-PG and myxothiazol respectively. All rates were maximal, measured in the presence of the uncoupler FCCP and saturating substrates. The non-induced F1.1 and WT

Table 1. Oxygen consumption rates and percentage inhibition results of mitochondria extracted from day-2 cell cultures of WT and F1.1 *Arabidopsis* grown with and without 5 μM $CuSO_4$ as indicated. All rates were measured in the presence of glutamate, malate, succinate (all at 10 mM), NADH (2.5 mM), pyruvate (1 mM), ATP (0.2 mM) and ADP (500 μM), and are expressed in units of nmol O_2 min^{-1} (mg protein)$^{-1}$. Inhibition by *n*-PG (50 μM) or myxothiazol (6 μM) is expressed as a percentage of the uninhibited rate. Data are the average ± SD of $n \geq 4$ independent mitochondrial preparations

Day-2 cell mitochondria	WT + Cu	F1.1 − Cu	F1.1 + Cu
Uninhibited rate	21.2 (9.6)	20.1 (8.6)	39.0 (10.9)
+ *n*-PG rate	17.9 (9.0)	3.5 (0.9)	37.2 (10.6)
% Inhibition by *n*-PG	25.1 (8.7)	75.6 (8.1)	5.9 (2.9)
Uninhibited rate	23.8 (9.3)	20.6 (6.9)	47.7 (10.4)
+ myxothiazol rate	2.5 (0.8)	9.9 (2.1)	2.2 (0.6)
% Inhibition by myxothiazol	93.8 (2.9)	46.0 (4.2)	94.8 (1.3)

uninhibited rates appeared lower than that for the induced F1.1 mitochondria [21, 24 and 48 nmol O_2 min^{-1} (mg protein)$^{-1}$, respectively]. The comparatively higher rates of the F1.1 mitochondria appeared to be copper-dependent and were apparent across a large number of replicates. The reason for this is not known but could relate to secondary effects of the antisense phenotype. This is an area for further investigation.

The results in Table 1 show that the F1.1 + Cu mitochondria were the least sensitive to *n*-PG, seen by the low percentage inhibition by *n*-PG (6%). These mitochondria, isolated from copper-induced antisense *Aox* cells, appeared to use the CP to a greater extent than the non-induced F1.1 mitochondria, as seen by the maximal CP rates following *n*-PG addition. The maximal AP rate (seen after the addition of myxothiazol) in the F1.1 + Cu mitochondria was also low when compared to that seen in the F1.1 − Cu mitochondrial samples. Tha F1.1 + Cu mitochondrial samples exhibited a maximal AP rate of 2.2 nmol O_2 min^{-1} (mg protein)$^{-1}$ while mitochondria isolated from the same cell line, grown without added copper, had a maximal AP rate over 4 times greater [9.9 nmol O_2 min^{-1} (mg protein)$^{-1}$]. The greatest sensitivity to *n*-PG inhibition was also seen with the mitochondria isolated from non-induced F1.1 cells, displaying a high percentage inhibition by *n*-PG (76%). The WT and F1.1 + Cu mitochondria had similar sensitivity to myxothiazol (94% and 95%, respectively); however, the mitochondria isolated from F1.1 − Cu cells appeared far less sensitive (46%). The lower maximal CP rates (after *n*-PG addition) in the F1.1 − Cu mitochondria also implied that there was less usage of the CP than in the mitochondria of either WT or F1.1 + Cu lines, and that the latter had a greater CP capability.

In summarising these results, the transgenic plant line F1.1 showed less SHAM sensitivity after induction with copper. The mitochondria isolated from transgenic F1.1 + Cu cells, displayed less AP activity than those isolated from F1.1 − Cu and WT cells. This indicates the transgene's efficiency at restricting native *Aox* gene expression in a copper-dependent manner. There also appeared to be a greater CP activity and capability in the mitochondria isolated from F1.1 + Cu cells than in those isolated from non-induced F1.1 cells, which indicates the components of the CP may also affected by transgene expression.

Conclusions

We have presented here a method of producing copper-inducible antisense *Aox1a A. thaliana* plants. These plants show increased copper-dependent ethanol production (in the presence of antimycin A) compared to WT plants, as well as copper-dependent, increased transcription of the transgene after 24 h. Leaf-slice studies have also demonstrated that the plant line F1.1 has decreased sensitivity to the AP inhibitor SHAM after copper-induction. Mitochondria isolated from copper-induced transgenic cells support this, showing decreased sensitivity to the AP inhibitor *n*-PG. These mitochondria also displayed lower maximal AP rates and higher maximal CP rates than the mitochondria isolated from non-induced transgenic cells.

To our knowledge this is the first report of an antisense plant dependent upon the down-regulation of gene expression using an inducible promoter. These transgenic plants and cell cultures promise to provide more information on the role of AOX during plant stress, by the manipulation of *Aox1a* expression during stressful conditions. An inducible antisense system offers several advantages over a constitutive one, including the ability to study the role of AOX during stressful conditions with direct comparison using the same non-induced transgenic plant line. These inducible antisense plants may also give information on the turnover of the AOX enzyme. They will allow us to examine the immediate effects of *Aox1a* expression (both increased and decreased) on other members of the *Aox* gene family, as well as the function and expression of other components of the mETC.

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