Plant Tissue Culture and Moelcular Markers

Their Role in Improving Crop Productivity

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ALTERNATIVE RESPIRATION MAY HAVE A ROLE IN REDUCING OXIDATIVE STRESS IN PLANTS

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ABSTRACT

The mitochondrial electron transport chain associated with dark respiration in plants branched: the phosphorylating cytochrome-c (cyt-c) pathway and the non-phosphorylating alternative pathway terminating in alternative oxidase (AOX): AOX may have a role in preventing the formation of toxic reactive oxygen species (ROS) in plants. An experiment was conducted to test this hypothesis in wild-type and AOX- antisense soybean plants that contained only 40-45% AOX expression. Incubation of leaf discs with 100 µM paraquat resulted in oxidative stress as inferred from an increased concentration of malondialdehyde (MDA) and a decrease in PS II activity. The paraquat treatment was also associated with decreased rates of dark respiration. Paraquat-induced oxidative damage and inactivation of PS II activity were more severe in AOX-antisense than their wild-type counterparts indicating a possible communication between chloroplast and mitochondria. Treatment of leaf discs with 50 µM antimycin, a known inhibitor of cyt-c pathway that causes electrons to flow through the AOX pathway, caused MDA to accumulate to higher levels in the leaves of the antisense plants than wild-type plants. The resulting effect from the suppression of AOX by antisense technique aggravated the oxidative stress in antisense plants.

Keywords: Alternative respiration, oxidative stress, PS II, antisense plants, paraquat, antimycin A

INTRODUCTION

Alternative pathway in plant mitochondria branches from the classical cytochrome pathway at the ubiquinone site, thereby bypassing two out of the total three phosphorylating sites (Lambers 1980). Therefore, 65% energy of the electron remains uncoupled (Moore and Siedow 1991). The unutilised energy status is dissipated as heat in the alternative pathway. The key

enzyme responsible for this pathway of respiration, uncoupled from ATP synthesis, is alternative oxidase (AOX).

The role of this enzyme is subjected to a great deal of speculation in many plant species. The cyanide resistant and SHAM sensitive alternative respiration is associated with thermogenesis in the flowers of inflorescence of Araceae, Annonaceae and Aristolochiaceae (Raskin *et al.*, 1987). The enzyme appears to lower the production of reactive oxygen species (ROS) in tobacco cells (Maxwell *et al.*,1999; Yip and Vanlerberghe 2001). It is reported that alternative respiration, being an overflow pathway for electrons derived from metabolic oxidation, may prevent over-reduction of the respiratory chain components (Ribas-Carbo *et al.*, 2000). However, the functional role of AOX in cellular metabolism is yet to be proved in many plants.

We address the role of AOX in tissues experiencing oxidative stress. It is hypothesized that an antisense transgenic plant with reduced expression of AOX may not tolerate oxidative stress as effectively as wild-type counterpart. To address this hypothesis, a study was carried out to understand to what extent AOX under-expressed soybean plants can tolerate oxidative stress.

MATERIALS AND METHODS

Plant Material

Soybean (*Glycine max* L.) tissue was previously co-transformed with *Aox*-antisense and hygromycin-resistance genes. Hygromycin–resistant transformants of independent, potentially transformed with *Aox* 2a and *Aox* 2b antisense genes, respectively were already identified and grown to seeds (to generation). For the present study, seedlings were raised in pots under greenhouse conditions with standard agronomic practices.

Identification of Aox-Antisense Sequences by PCR Technique

The DNA was isolated from young leaves of each plant using a highly efficient commercial kit (Extract-N-Amp, Sigma) and tested for the presence of antisense gene using PCR assays developed specifically to amplify and detect *Aox* antisense genes. To distinguish antisense trangenes from their endogenous homologues, one primer of the PCR primer pair was based in the antisense gene promoter (35 S CaMV), which is not normally present in plants.

Isolation of Intact Mitochondria from Leaf Tissues

Mitochondria were isolated from leaves according to the method of Day *et al.*, (1985) from approximately 30 g of tissue. Leaves were disrupted with a Polytron (Kinematica, Kriens, Switzerland) in phosphate buffer (pH 7.5). The homogenate was filtered through 4 layers of miracloth and centrifuged for 5 min at 1100 g. The supernatant was centrifuged for 20 min at 18000 g and the pellet resuspended in 80 ml of wash medium (0.3 M sucrose, 10 mM TES, 1 mM glycine, 0.1% (w/v) BSA, pH 7.5) and centrifuged for 5 min at 1100 g. The supernatant was centrifuged for 20 min at 18000 g and the pellet resuspended in 10 ml of wash medium. Five ml of aliquots were then layered over 27.5 ml solution (0.3 M sucrose, 10 mM TES, 1 mM glycine, 0.1% (w/v) BSA, 28% (v/v) Percoll and a linear gradient of 0-4.4% (w/v) PVP-40, pH 7.5) in a centrifuge

tube, and centrifuged for 40 min at 40000 g. The mitochondria were found as a tight light yellow-brown band near the bottom of the tube. The final mitochondrial pellet was resuspended in approximately 1 ml of wash medium.

Respiration Assays

Oxygen consumption was measured using a Clark type electrode (Rank Bros, Cambridge, UK) connected to a chart recorder. The reaction medium contained 10 mM TES-KOH pH 7.5, 5 mM KH_2PO_4, 10 mM NaCl, 2 mM $MgSO_4$ and 0.1% (w/v) bovine serum albumin, and all measurements were carried out at 25°C. Calibration of the electrode was carried out by the addition of sodium dithionite to remove all oxygen in the electrode chamber and the oxygen concentration was assumed to be 240 µM. Various substrates, inhibitors and effectors were added. The activity of cytochrome oxidase and alternative oxidase mediated respiration rates in wild-type and AOX-antisense soybean plants were assayed in sliced leaf bits. Appropriate inhibitors were added to the assay buffer to arrest any one pathway.

Paraquat and Antimycin A Treatments

To impose oxidative stress in soybean, the leaf discs (4.5 cm^2) were suspended in paraquat solution and exposed to 800 µ mole m^{-2} white light for 5 hours in a growth chamber. The commercially available paraquat (437.5 mg/L), known as Tryquat (Crop care Australia Pvt Ltd, Perth), was diluted in order to get 100 µm active ingredient in the final solution. Another set of leaf discs were incubated with 50 µm antimycin A in order to inhibit the cyt-c pathway.

Western Blotting

The mitochondrial proteins were electrophoretically transferred from polyacrylamide gels on to a Hybond C⁺ membrane according to the method of Towbin *et al.*, (1979) using a Hoefer Semiphor semi-dry blotting apparatus (Amersham Biosciences, Sydney, Australia). The monoclonal AOX primary antibody, diluted in TBS-Tween (150 mM NaCl, 50mM Tris-HCl pH 7.5, 0.1% (v/v) Tween-20), was incubated with membrane at RT for 1 hour. Chemiluminescence was used for detection of horseradish peroxidase-conjugated secondary antibodies and visualized using a LAS 1000 (Fuji). The blots were quantified using the Image Gauge v3.0 software (Fuji) with the control band denoted as 100%; the other bands were calculated relative to that value.

PS II Quantum Measurements

The leaf discs, after respective treatments, were dark adapted for 20 minutes at room temperature and the maximum potential quantum yield of PS II photochemistry (dark Fv/Fm) was determined (Jacob 1995) using chlorophyll fluorescence techniques (PAM 2000, Walz, Germany).

Malondialdehyde (MDA) Assay of Soybean Leaf Extracts

The method of Hodges *et al.*, (1999) was used for accurate measurement of MDA in the leaf discs. Approximately 300 mg of tissue was homogenised in 80% (v/v) ethanol and inert sand in a cold pestle and mortar, followed by centrifugation at 3000 g for 10 min. The supernatants were placed in two different 2

ml cryovials and 1 ml of -TBA solution (20% (w/v) trichloroacetic acid, 0.01% (w/v) butylated hydroxytoluene) was added to one tube and +TBA (0.65% (w/v) thiobarbituric acid, 20% (w/v) trichloroacetic acid, 0.01% (w/v) butylated hydroxytoluene) added to the other tube. The samples were vortexed and heated to 95°C for 25 min, followed by cooling on ice for 5 min. The samples were then spun at 3000 g for 10 min and absorbances of each sample read at 440, 532, and 600 nm using a Cary 300 Bio spectrophotometer (Varian Inc., Australia). The amount of MDA equivalents was calculated from the equation below:

MDA equivalents (n mol ml^{-1}) =

$$(A\text{-}B/157000)10^6$$

$$A = [(A_{532+TBA})-(A_{600+TBA})-(A_{532-TBA}-A_{600-TBA})]$$

$$B = [(A_{440+TBA} - A_{600+TBA})0.0571]$$

The average of the four replications was then used as the concentration of MDA equivalents.

RESULTS AND DISCUSSION

Identification of Soybean AOX-Antisense Plants

Transgenic soybean lines were screened for the presence of *Aox* 2a and 2b antisense gene sequences. DNA was isolated from around 80 transgenic plants and subjected to the PCR analysis with primers based on the 2a and 2b *Aox*-antisense sequences. A total of 18 plants with 2a and 14 plants with 2b *Aox*-antisense sequences were identified. For further experiments involving physiological and biochemical studies, leaf sampling was made from these plants. In the PCR profile, 671 and 500 bp are the 2a and 2b *Aox*-antisense sequences, respectively. The lanes, in which there was a prominent absence of 671 or 500 base-pairs, were considered wild-type or untransformed plants (Fig. not shown). In soybean, AOX is encoded by a family of three genes (Finnegan *et al.*, 1997; Djajanegara *et al.*, 2002).

The expression level of *Aox* gene (2a and 2b) products in wild-type and antisense plants was quantified using the western blot analysis with AOX monoclonal antibody. Taking protein quantity in the wild plant as 100%, there was 55–60% less AOX protein in the antisense plants (Fig. 1). This shows that the antisense technique employed here did not suppress the expression of the genomic copies of the AOX genes fully, but there was partial suppression. For the present study, composite leaf sampling was carried out randomly from many plants among the specific group (2a or 2b antisense plants). In many cases the 'gene knockdown' is not 100%. The activity of the target genes may be somewhere between 50 and 100%. Since an appreciable level of AOX protein translation was inhibited in the antisense plants, the transformed plants were considered AOX under-expressing plants.

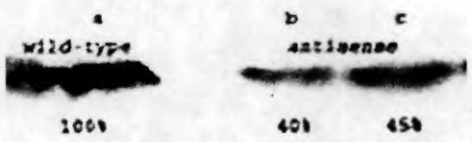


Fig 1. The relative abundance of AOX protein in soybean wild-type (a) and AOX 2a (b) and 2b (c) soybean plants with AOX-antisense genes. The soybean AOX was immunodetected in whole leaf extract by AOX-monoclonal antibody (raised against AOX of *Sauromatum guttatum*). The relative abundance was calculated based on the value for control plant as 100%.

AOX Expression in Wild-Type Plants Experiencing Drought

To demonstrate the expression pattern of AOX protein under abiotic stress condition, one experiment was conducted with well irrigated and drought stressed wild-type plants. The level of water stress (by withholding irrigation) was characterized in terms of light-saturated stomatal conductance (g_s)—well irrigated (g_s > 0.2 mol H_2O m^{-2} s^{-1}), mildly or 50% water stressed (g_s between 0.1 and 0.2 mol H_2O m^{-2} s^{-1}) and severely water stressed (g_s < 0.1 mol H_2O m^{-2} s^{-1}). Mitochondria were isolated from 0, 50 and 100 % water stressed plants and subjected to immunoblot with AOX antibodies. Compared to the unstressed plants, the 50 and 100% water stressed plants recorded 27 and 31% increase in AOX protein abundance, respectively (Fig 2). When probed with AOX antibody, the mitochondria showed two isoforms having the

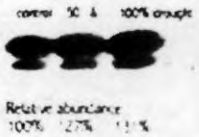


Fig. 2: The alternative oxidase proteins of mitochondrial preparations detected by the AOX monoclonal antibody. Soybean plants were subjected to various levels of drought stress. The lane a, control plants with well irrigated condition, lanes b and c, partial and severe drought imposed plants, respectively. Mitochondrial protein of 40 µg was loaded uniformly in each lane. The relative abundance was calculated based on the value for control plant as 100%.

molecular mass of 34 and 36 kDa (Fig 2). This observation was in corroboration with the findings of Tanudji *et al.*,(1999); Djajanegara *et al.*, (2002). Ribas-Carbo *et al.*, (2005) have recently studied the effect of three different levels of water stress on the activity of AOX. Unlike many other stresses, water stress did not affect the levels of mitochondrial alternative oxidase protein. They have shown that severe water stress caused a significant shift of electrons from the cytochrome to the alternative pathway. The electron partitioning through the alternative pathway increased from 12% under well-watered conditions to nearly 40% under severe water stress. Consequently, the calculated rate of mitochondrial ATP synthesis decreased by 32% under severe water stress. In the present study also we have observed clear over-expression of AOX in soybean wild-type plants experiencing water stress and it was likely that drought-mediated impairment of electron transport through cytochrome pathway led to diversion of more electrons to alternative pathway.

Oxidative Stress in Soybean Leaf Disc

With the exception of a reduction in total respiration in AOX 2b antisense plants there was no difference in cytochrome-c oxidase mediated respiratory oxygen uptake rate between the wild and AOX-antisense plants. Around 50% AOX activity was reduced in antisense plants. After incubating with 100 µM paraquat for 5 hrs, both cyt-c and AOX mediated activities were significantly inhibited in all the leaf samples (Fig 3). Paraquat is known to produce active oxygen species (AOS) and eventually promotes lipid peroxidation of membranes of cellular organelles. It is now widely accepted that most environmental stresses lead to the accumulation of ROS such as $OH^{\bullet}$, H_2O_2 and $O_2^{\bullet-}$ in plant cells (Noctor and Foyer 1998). ROS production through disruption or inhibition of the mitochondrial and chloroplast electron transfer chain (Lam *et al.*, 2001) is a major factor causing the peroxidation of organelle

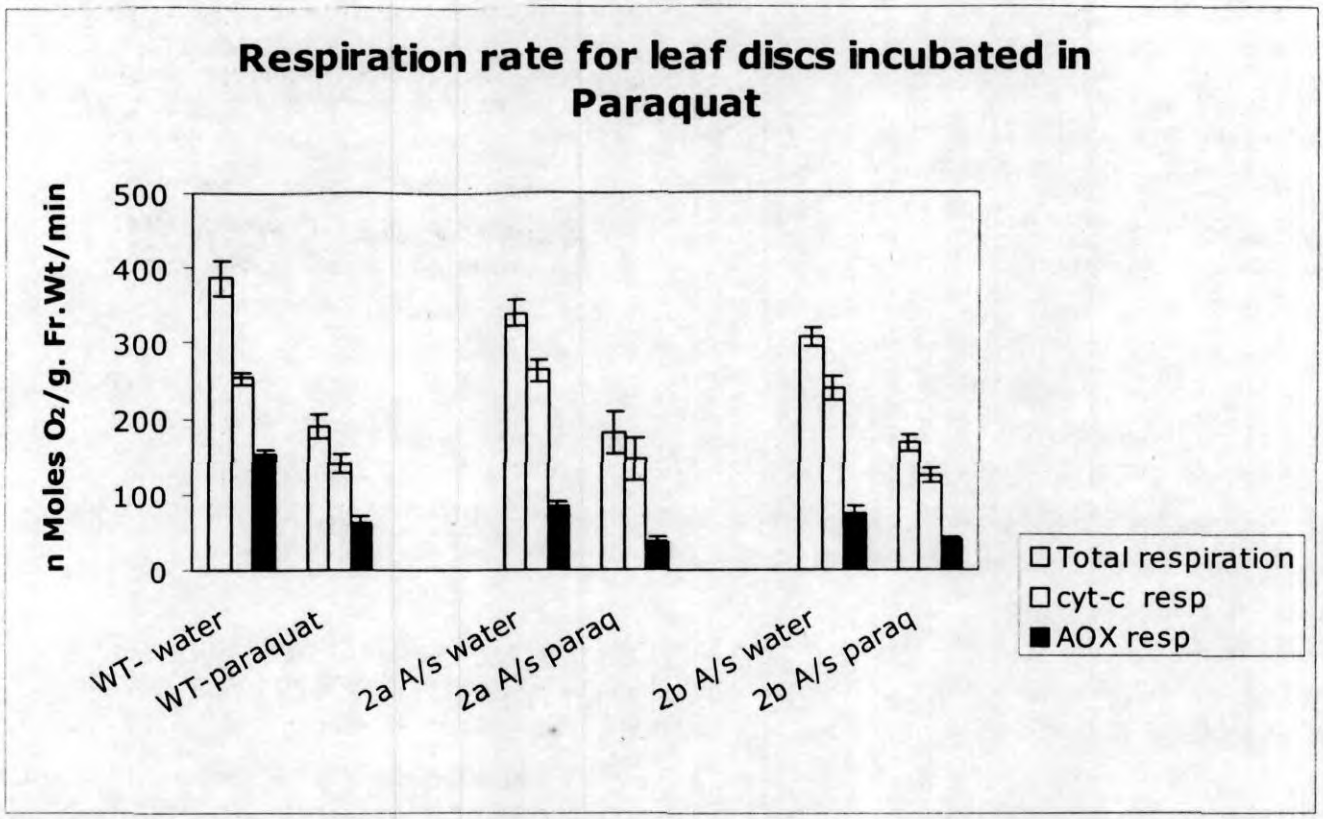
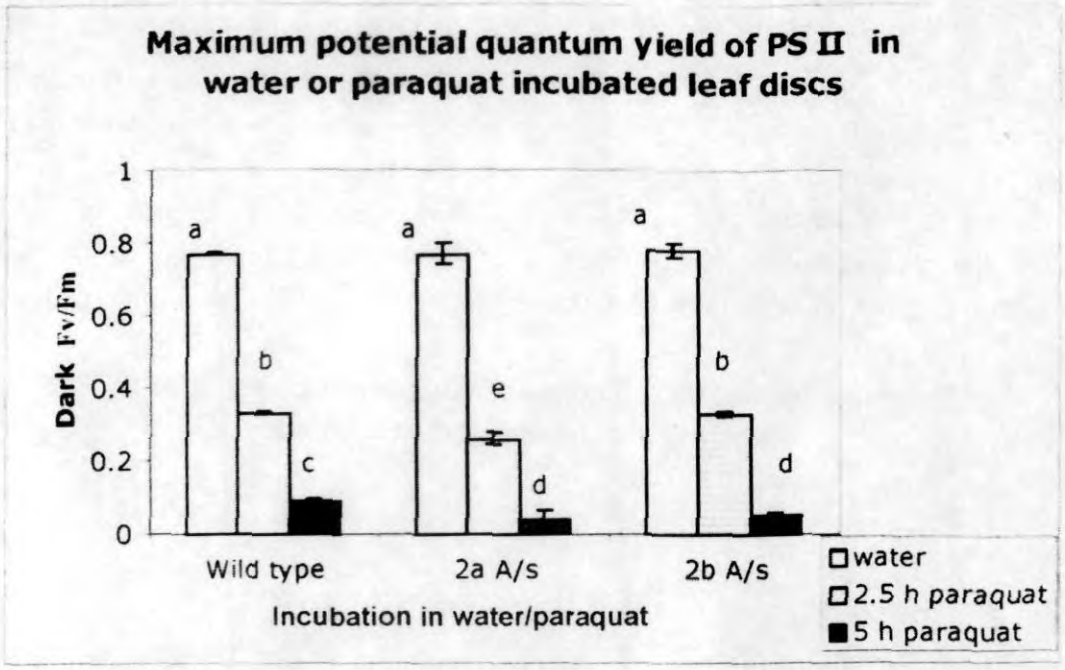


Fig. 3: Rates of respiration in soybean leaf pieces after 5 hours incubation with water or 100 µm paraquat. WT: wild-type, 2a and 2b A/s: soybean plants containing antisense AOX 2a and 2b genes, respectively. For the details regarding the measurement of cyt-c and alternative respiration see materials and methods. Bars corresponding to the mean value of 5 replications with error bars showing standard error.

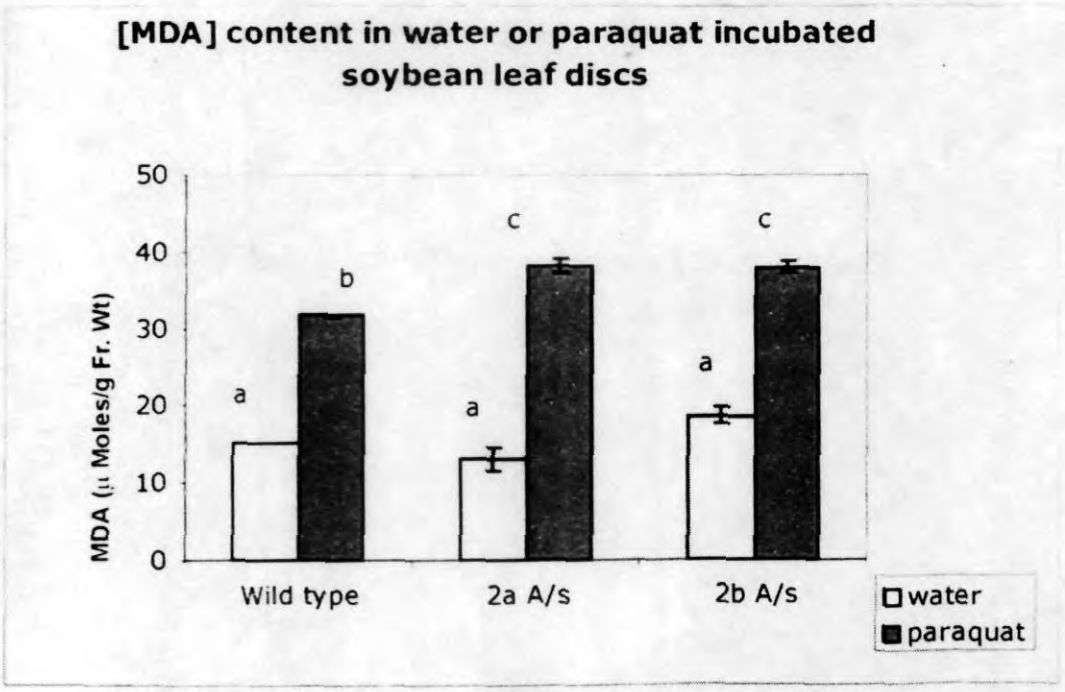
membranes. It has been reported that paraquat severely inhibits the mitochondrial metabolism in pea (Taylor *et al.,* 2002).

The lipid peroxidation product, MDA, was significantly increased in all the paraquat incubated samples. The level of MDA content was significantly higher in antisense plants than the wild-type plants (Fig. 4). This result strongly supports the fact that the plants with reduced AOX content and activity would be more susceptible to oxidative stress. In an experiment, Robson and Vanlerberghe (2002) demonstrated that transgenic tobacco cells lacking alternative oxidase showed increased susceptibility to three 'death inducing' compounds compared to wild-type cells in which AOX was fully active. This was mainly due to the high level of oxidative stress in the transgenic cells. Further they have shown that treatment of AOX lacking cells with an antioxidant compound increased the resistance to 'death inducing' compounds.

The maximum potential quantum yield of PS II also reduced drastically in paraquat incubated leaf discs. The 2a AOX-antisense plants were found to be more vulnerable to photo-oxidative stress than 2b AOX-antisense plants as evidenced from significant reduction in maximum potential quantum yield of PS II. With prolonged incubation in paraquat, both the 2a and 2b AOX-antisense plants were shown to be affected significantly more than their wild-type counterparts (Fig. 4A). This result indicates a possible exchange of intercellular and interorganellar redox equivalents between



(A)



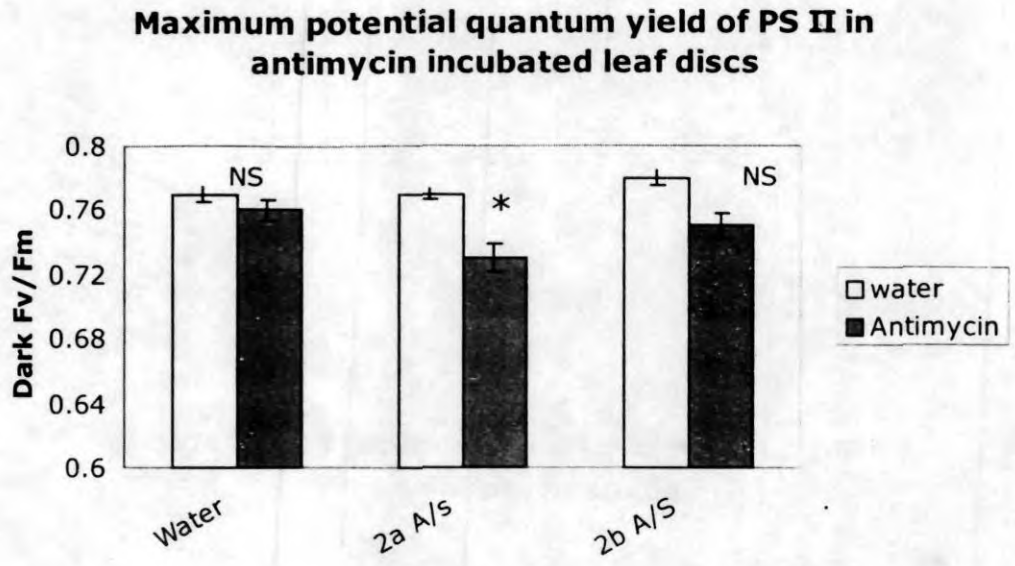
(B)

Fig. 4 A and B: The maximum potential quantum yield of PS II (A) and malondialdehyde content (B) in soybean leaf discs suspended in water or 100 μM paraquat. WT: wild-type, 2a and 2b A/s: soybean plants containing antisense AOX 2a and 2b genes, respectively. Histograms with different letters are significantly different at 5% confidence level.

chloroplast and mitochondria through shuttles (Lis and Atteia 2004). It is known that the major action of paraquat in the presence of light is in chloroplast, and hence paraquat will inhibit PS II activity. Our findings that paraquat induced inhibition of PS II activity was more in the AOX antisense plants indicates the possible movement of reducing equivalents or ROS through chloroplast-mitochondria shuttles within the cell (Padmasree *et al.*, 2002).

Antimycin A is a known inhibitor of cyt-c activity in mitochondria. Incubation of

(A)



(B)

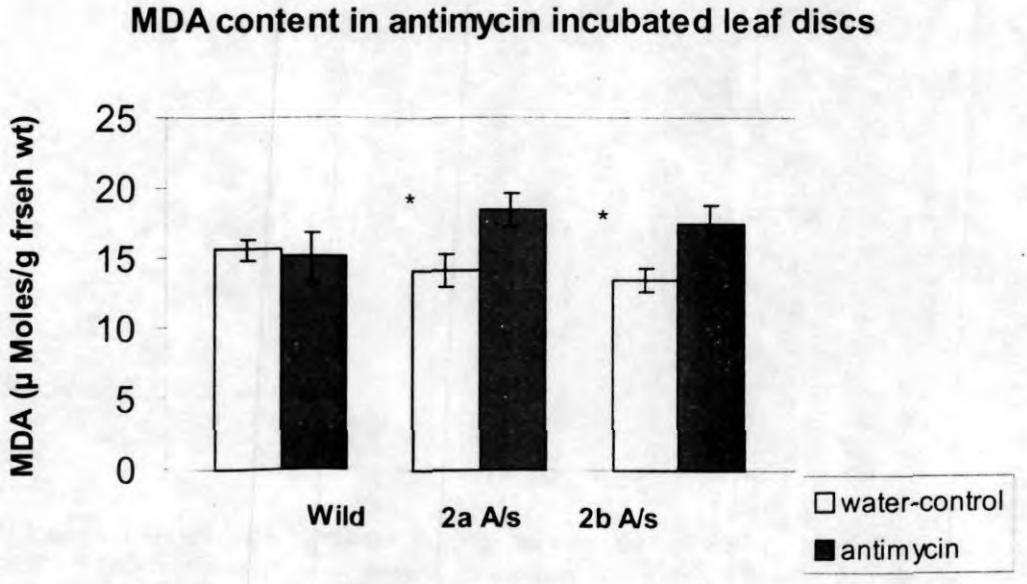


Fig. 5 A and B: The maximum potential quantum yield of PS II (A) and malondialdehyde content (B) in soybean leaf discs suspended in water or 50 µm antimycin-A for 5 hours. Bars marked 2a and 2b A/s represent soybean plants containing AOX 2a or AOX 2b antisense genes. Histograms marked by an asteric are significantly different from their respective water-control at 5% confidence level.

leaf discs with antimycin resulted in significantly more reduction in the PS II activity in 2a AOX-antisense plants than the wild-type (Fig. 5). This also suggests the possible mitochondria-chloroplast communication shuttle. Probably, the 5 hrs incubation period was insufficient to elicit oxidative stress in 2b AOX-antisense plants. The leaf discs from AOX-antisense plants, which were treated with antimycin (cyt-c inhibited), succumbed to stress as evidenced by an increase in MDA content. The wild-type leaf discs incubated with antimycin did not show any stress effect, because there was no difference in MDA contents. However, it has been reported that addition of 25 μM antimycin to soybean suspension cells caused a dramatic increase in intracellular ROS even in wild-type plants (Djajanegara *et al.*, 2002). In the present study, it was likely that electron transport through AOX was not impaired in wild-type plants and therefore there was possibly less generation of free radicals and hence eventually less damage to the membranes. Further the cyt-c activity was inhibited by antimycin and this led to diversion of more electrons through AOX pathway as an 'overflow' mechanism to oxidise the excess NADH. This can lead to stabilization of the ubiquinone pool and prevention of excess ROS generation in mitochondria (Ribas-Carbo *et al.*, 2000) of wild-type plants.

The alternative pathway plays an important role of bypassing the electrons transport when the normal activity of the cyt-c pathway is restricted or impaired by any stress or other cellular parameters which are not conducive for the normal functioning of cyt-c pathway (Wagner and Krab 1995; Yip and Vanlerberghe 2001; Annamalainathan *et al.* 2001). In the case of AOX-antisense plants, the AOX capacity was only around 45% of the wild-type plants and therefore the diversion of additional electrons through alternative pathway is likely to be more than the load it can take. This can lead to increased ROS generation.

Transgenic plants with different levels of AOX expression/suppression is a very convenient system to test the functional role of alternative respiration. The results support the fact that AOX has an important role in plants experiencing abiotic stress. The present work with transgenic soybean plants having reduced AOX expression tested the hypothesis that the alternative respiration functions as a protective mechanism and this appears to be the case. This pathway is probably preventing the over-reduction of electron transport components in mitochondria and thereby avoids the production of excess ROS and thus eventually protects the membranes from oxidative damages. Alternative respiration bypassing two out of the total three phosphorylating sites may be an unsuitable trait for the overall energy metabolism of the cell. However, AOX may have a protective role during stress as found from the present results. Our results strongly indicate that intracellular communication between chloroplast and mitochondria must be much stronger than what we currently believe.

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