A STUDY OF THE ALTERNATIVE OXIDASE (AOX) PATHWAY IN WILD-TYPE *Arabidopsis thaliana* AND THE PRODUCTION OF AN INDUCIBLE (*aox1*) ANTISENSE PLANT

A thesis submitted for the degree of Doctor of Philosophy
at the University of Adelaide

by

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ABSTRACT

The role of the alternative oxidase (AOX) enzyme in plants is unknown, however current studies suggest that the alternative pathway (AP) may have as many as three roles. It may protect the plant cell from reactive oxygen species (ROS) when their production is increased, for example during plant stress; it may allow for the provision of carbon skeletons for anabolic pathways when the cytochrome pathway (CP) is restricted by high levels of ATP, and it may allow excess metabolic energy to escape as heat which may be used to volatilise pollinator attractants or may maintain plant temperatures amidst detrimental environmental conditions such as snow.

The aim of this project was to examine the AP in *A.thaliana* and to produce an inducible antisense (*aox1*) plant to assist future studies of the role of AOX.

The AOX of *A.thaliana* appears as two protein bands of 64 and 28 kDa in size, on immunoblots. These represent the monomeric and dimeric forms of AOX. This project is the first study of the AP in *A.thaliana* plant with and without stress. Treatment with antimycin A (AA) increased the AP capacity of cell cultures. Salicylic acid (SA) application also increased the capacity of the AP, while cold treatment and cold plus dark treatment had a similar but lesser effect.

The copper-inducible system provided by Dr. Paul H. Reynolds (Hort. and Food Research Institute, New Zealand) was found to function well in *A.thaliana* when used with the marker gene (*gus*).

From these studies days 3 and 4 post-induction were found to be the days of maximal construct expression, and were used as a guide for further studies. *A.thaliana* plants were transformed with antisense *aox1* construct under the control of the copper-inducible system. Resultant plants were successfully screened using kanamycin resistance and PCR analysis. Plants that were shown to carry the construct were tested for copper inducibility, and one plant line (F1.1) was made into a cell suspension culture. Leaf material was tested for copper-inducibility using an ethanol assay. Leaves were floated in medium containing AA and the ethanol concentration in the solution was tested over 72 hours. These tests showed that the copper-induced F1.1 leaves produced up to 8.8 times greater ethanol than the WT due to the expression of the antisense construct expression. F1.1 plants which were not induced were found to behave just as the WT, indicating the tight control of the copper-inducible system. Respiration of F1.1 cell lines, with and without copper were measured. The F1.1 cells grown in the presence of copper were not significantly different in their SHAM and KCN-sensitivities to the WT cells. This was in contrast to the non-induced F1.1 cells which showed less SHAM sensitivity and a small reduction in KCN-sensitivity. This indicated that either the copper-inducible system did not function well in cell cultures or that the *aox1a* cDNA sequence used in the construct was not the *aox* gene expressed in the cell culture system.

This project achieved its aim by successfully producing copper-

inducible antisense *aox* plants which will greatly encourage future

work on the role of AOX.

DECLARATION

This thesis describes my independent research carried out at the department of Botany, University of Adelaide and at CSIRO, division of Horticulture, Adelaide. It contains no material previously accepted for the award of any other degree or diploma in any university and to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text. I consent to this thesis being available for photocopying or loan if it is accepted for the award of the degree.

Felicity Johnson Potter

ACKNOWLEDGMENTS

ABBREVIATIONS

AA   antimycin A

AP   alternative pathway

BCIP   5-bromo-4-chloro-3-indolyl phosphate

bp   base pairs

BME β-mercaptoethanol

BSA   bovine serum albumin

cDNA   complementary DNA

CHCl3   chloroform

CIP   calf intestinal phosphatase

CP   cytochrome pathway

2,4-D   2,4-dichlorophenoxyacetic acid

DTT   dithiothreitol

EDTA   ethylenediaminetetra-acetic acid

EGTA   ethylene glycol-bis(β-aminoethyl Ether)-N,N,N′,N′-tetraac

FCCP   carbonyl cyanide ρ-trifluoromethoxy-phenylhydrazone

gDNA   genomic DNA

X-gluc   5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid

HPLC   high performance liquid chromatography

kb   kilobase pairs

kDa   kilo-Dalton

LB   luria broth

MES   2-[N-morpholino]ethanesulphonic acid

mETC	mitochondrial electron transport chain
mRNA	messenger RNA
MS	Murashige and Skoog
NBT	nitroblue tetrazolium
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pDNA	plasmid DNA
PMSF	phenylmethylsulphonyl fluoride
PVDF	polyvinylidene difluoride
PVP	polyvinylpyrrolidone
SA	salicylic acid
SDS	sodium dodecylsulphate
SHAM	salicylhydroxamic acid
TEMED	N,N,N′,N′-tetramethyl-ethylenediamine
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
UQ	coenzyme Q_{10} (ubiquinone 50)

CHAPTER ONE : LITERATURE REVIEW

1.1 General Introduction

The mitochondrial electron transport chain (mETC) is located in the inner membrane (fig 1.1). It's function is to transfer electrons from reducing agents (such as NAD(P)H and succinate) down an energy gradient to the final acceptor molecule, oxygen. This transport of energy allows ATP synthesis in the following manner. Electron flow is coupled to H^+ translocation across the inner membrane at specific points along the chain and a proton motive force (pmf) is generated. The mETC is energetically linked to the large ATP synthase also present in the inner membrane. These ATP synthases relieve the pmf when ADP and inorganic phosphate (P_i) are available by permitting the movement of H^+ back into the mitochondrial matrix. This movement of H^+ drives the phosphorylation of ADP to produce ATP. In this manner, due to the mETC, reducing agents are oxidised, oxygen is reduced to H_2O and the flow of electrons allows a pmf-driven synthesis of ATP.

In plants the transfer of electrons may also be diverted to an alternative pathway (AP). Both pathways share common entry routes for the electrons provided by the substrates NADH, NADPH and succinate (fig 1.2). However the pathways differ in their terminal oxidases which are either cytochrome oxidase (COX) in the cytochrome pathway (CP) or alternative oxidase (AOX) in the AP.
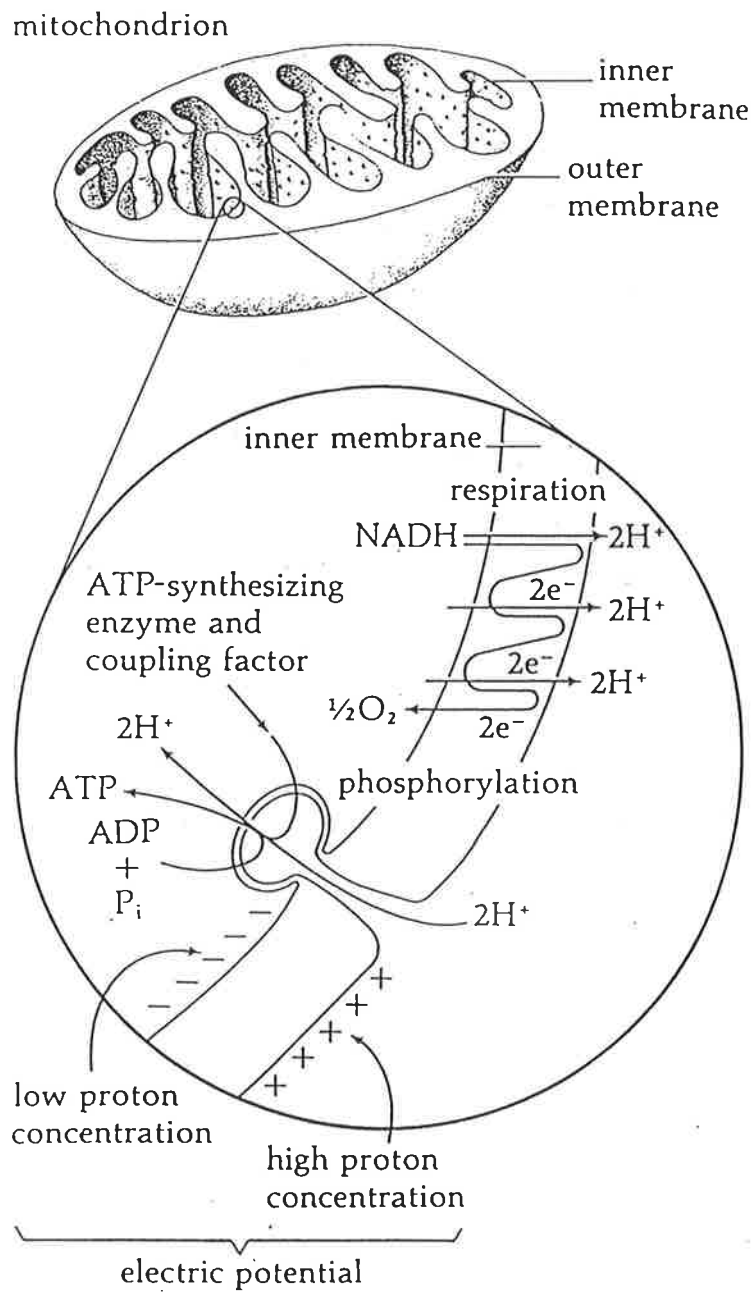
Figure 1.1 : The mETC and its location within the mitochondrion (taken from Devlin and Witham 1983).
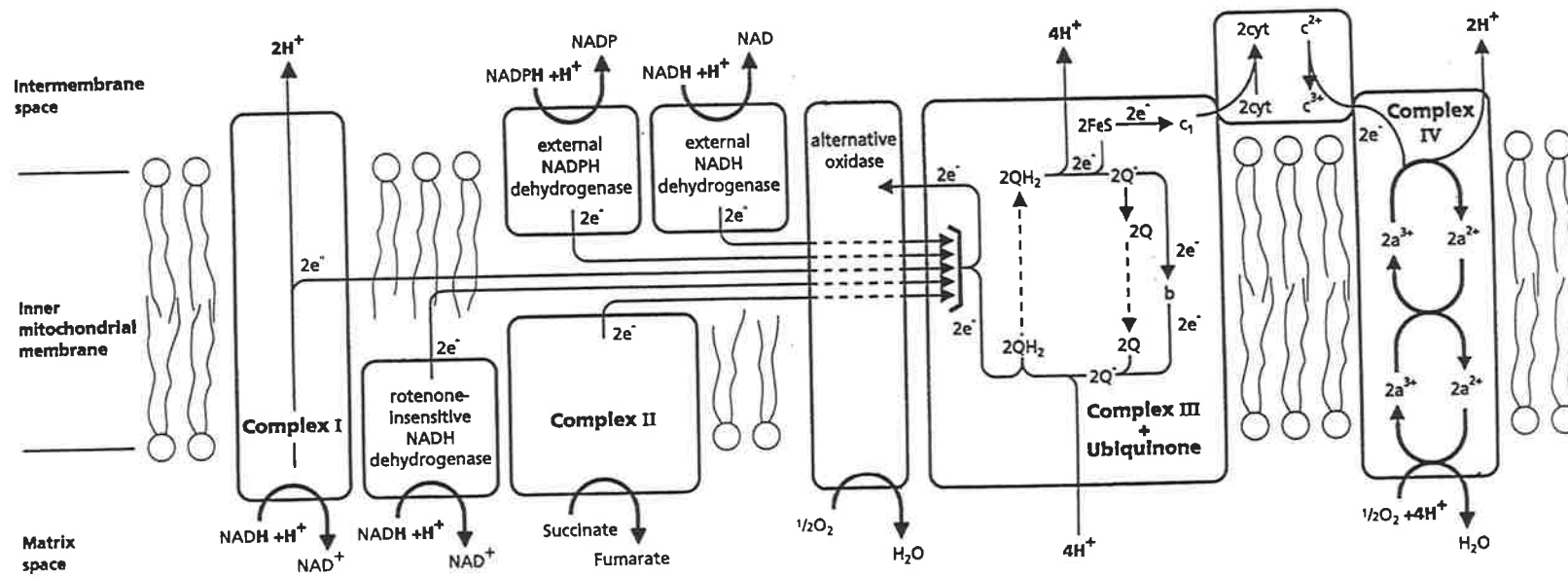
Figure 1.2 : A diagram depicting the mETC of the inner mitochondrial membrane. (Adapted from Dennis et. al. 1997.)

An additional difference is that the CP branches off from the ubiquinone (UQ) pool and continues via complex III (a proton translocator) before terminating at cytochrome oxidase whereas the AP branches off from UQ pool and immediately terminates at the alternative oxidase. This means that the CP translocates more protons per electron transported and hence, produces more ATP for each substrate oxidised. In situations when electrons are flowing through the AP, the energy that would have been coupled to H^+ movement and ATP synthesis, is instead dissipated as heat. As the AP represents a component of the mETC that reduces ATP production from the oxidation of substrates such as NADH, NADPH and succinate, it has been the focus of much research.

1.2 The alternative (cyanide-resistant) pathway and thermogenesis

Attention was initially drawn to the AP after thermogenic (heat producing) flowering plants from the Araceae family were discovered. (While several other species from different families are known to be thermogenic, these are isolated examples in comparison to the Araceae.) A large volume of work on the AP of arum inflorescences was initiated over 200 years ago by Lamarck in 1778, who noted that the spadix of *Arum italicum* became hot and malodorous (Meeuse 1966). The temperature of the spadix tissue, in these and other species within the family, was raised 10 to 15°C above ambient during anthesis (Meeuse 1975) (figure 1.3). The general mechanisms that lead to heat production appeared similar across aroid plant species.

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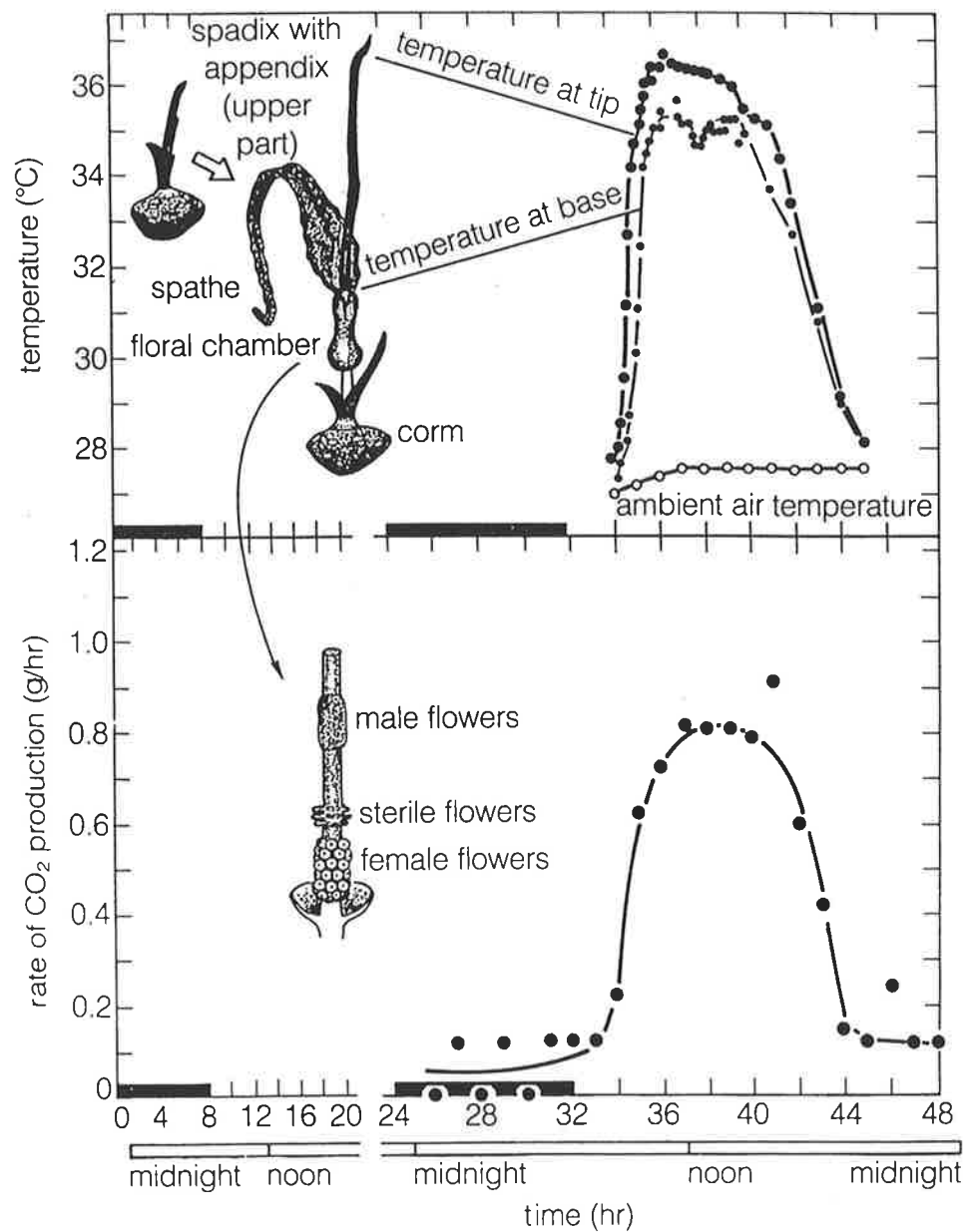
Figure 1.3 : Respiration (rate of CO_2 production) and temperature variations in *Sauromatum guttatum* over time during anthesis. The black bars on the time axes represent the darkness period necessary for initiating anthesis. (Taken from Salisbury and Ross 1992)

Meeuse (1966) showed that it was immediately after anthesis, following the unfolding of the spathe to reveal the spadix, that the temperature of the spadix increased. The heat production served to volatilise plant polyamines, giving off a highly effective insect attractant. At the same time, the mature female flowers on the spadix were receptive to any pollen that might be carried into the inflorescence chamber by an insect pollinator. Insects tended to settle at the bottom of the inflorescence chamber, feeding on nectar. Overnight the male flowers ripened and released pollen which was picked up by the pollinators on their way out, to other inflorescences (figure 1.4). This ensured cross-pollination between plants.

Work by van Herk (1937 and 1939; for a review see Meeuse 1975) showed that the heat producing respiration of *Sauromatum* spadices at anthesis was primarily triggered by the male flowers less than 18 hours before anthesis. He also showed that this respiration was cyanide-insensitive. Cyanide is a potent inhibitor of the cytochrome enzymes (Commoner 1940 ; see Laties review 1982). This indicated that the heat production occurring in the aroid inflorescence could not be explained by the "usual" cytochrome respiratory pathway models and it suggested that there was an alternative enzyme involved in this aroid tissue respiration. It wasn't until 1954 that a technique to isolate mitochondria from another thermogenic Araceae member, *Arum maculatum*, became available (Hackett and Simon 1954).

SPADIX

SPATHE

MALE FLOWERS

STERILE FLOWERS

FEMALE FLOWERS
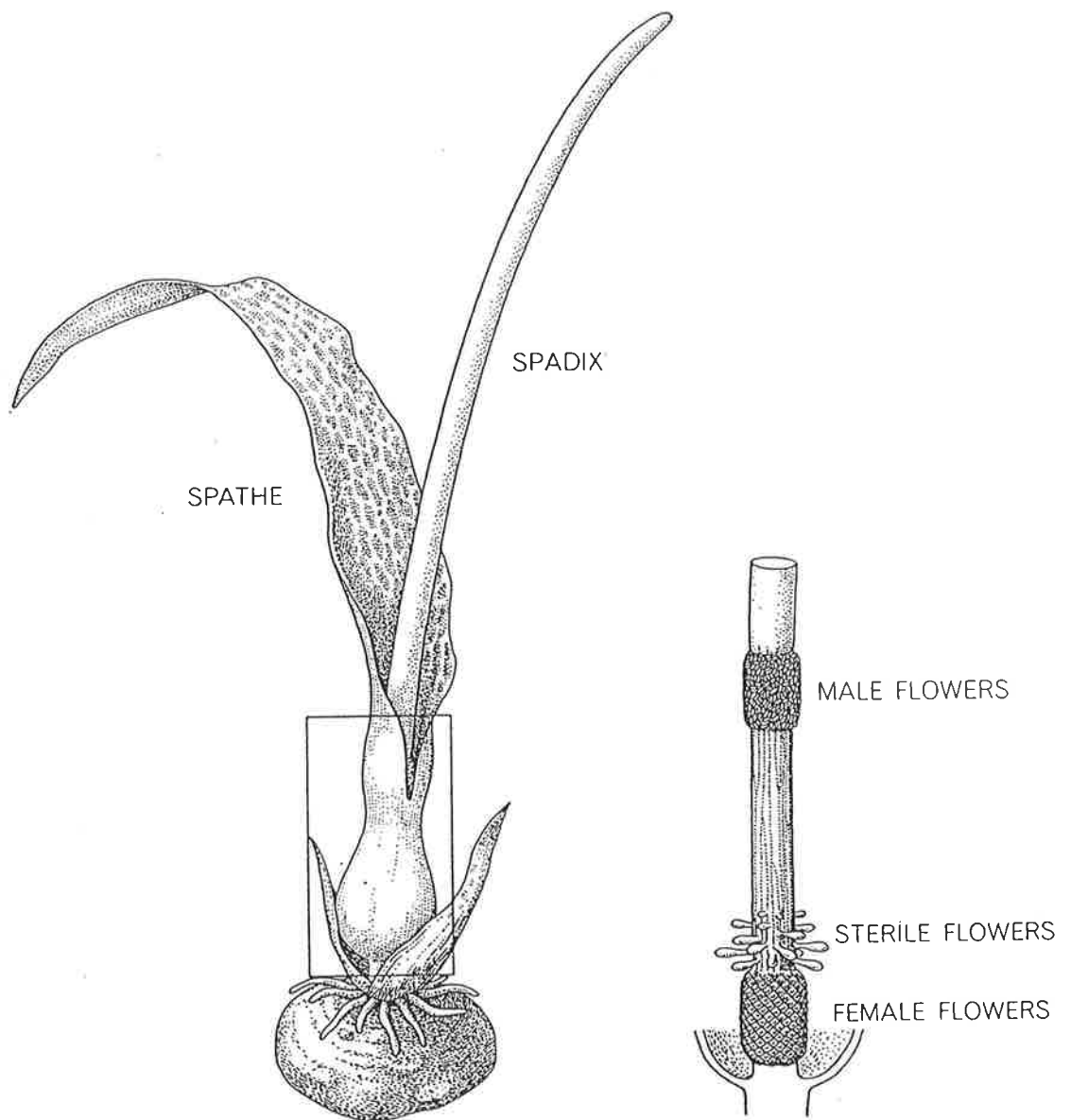
Figure 1.4 : A drawing of the voodoo lily (*Sauromatum guttatum*) showing in more detail the inflorescence region beneath the spathe in the outlined area. (Taken from Meeuse 1966)

In the following year, James and Elliott (1955) established that the cyanide-resistant respiration was occurring in mitochondria isolated from spadices during anthesis because the addition of cyanide to isolated mitochondria oxidising TCA cycle substrates caused no detectable inhibition of oxygen consumption. This suggested that a large component of the oxygen-consuming activity was occurring via a cyanide-resistant pathway. Bendall and Hill (1956) showed that these mitochondria from *Arum* were not unlike other plant mitochondria in that they still contained the cytochrome components essential for CP activity. A year later Yocum and Hackett (1957) proposed a scheme operating in parallel with the conventional terminal oxidase (COX) as the end point for one pathway and an alternative terminal oxidase as the other. This "alternative oxidase" was cyanide-resistant and in *Arum* was responsible for the previous results seen by James and Elliott (1955).

Until this point most of the cyanide-resistant respiration work was performed on aroid tissue, due to the large activity of the AP in this tissue. Once an inhibitor (hydroxamic acid) of the AP was discovered by Schonbaum et. al. (1971) this provided a chemical means to distinguish the AP from the CP activity and opened up the field of AP studies to include many more plant and fungal species (for a review see Henry and Nyns 1975).

The investigation of the AP in thermogenic tissue did not end there. Mitochondria purified from *Sauromatum guttatum* spadices in

more recent years has revealed even greater detail. This work was possible after the production of an antibody to the alternative oxidase and the isolation of a cDNA alternative oxidase clone (Elthon and McIntosh 1987b, Rhoads and McIntosh 1992 respectively) (see section 1.5 for more details). These two major "molecular tools" have allowed a return to the study of the aroid species and an examination of what happens at the molecular level immediately prior, during and after anthesis. Dramatically increased rates of alternative oxidase activity in the spadices of *S. guttatum* were concomittant with increased AOX protein synthesis (Rhoads and McIntosh 1992). Studies have also shown an increase in mRNA and salicylic acid in the days immediately preceding anthesis (Rhoads and McIntosh 1991). They occur well before the time of heat-production and pollination, indicating that they are not in response to the wound-like consequences of anthesis, floral display or pollination. These changes (and the subsequent increase in temperature) occur primarily in the region of the sterile male flowers (figure 1.4) and the heat is believed in part, to come from the energy released during this period of rapid electron flow via the AP.

1.3 Control and partitioning of electron flow.

Since the discovery of cyanide-resistant respiration and the elucidation of the AP, one major focus of research has been to formulate an accurate model of how electron flow is partitioned between the CP and the AP. Once initial studies moved away from

aroid tissue, which had extremely high levels of AP activity, to non-aroid tissue with much lower relative levels, it became obvious that the AP's contribution to respiration was highly variable. The questions still requiring more precise answers were;

- under what conditions is the alternative pathway operating ?

- how is electron flow through either the AP or CP partitioned?, and

- what are the major controlling factors in this partitioning?

Two models, one of which was presented in 1973 by Bahr and Bonner and the other in 1978 by de Troostembergh and Nyns, are diagrammatically represented in figure 1.5. Bahr and Bonner's model led to the prediction of an engagement of the AP once a threshold level of reduced UQ had been reached (eg. 40% in figure 1.5). This lead to the concept of the AP operating as a form of "overflow" mechanism, that is, the AP operated via electron flow which was in excess of that used by the CP. They formulated this model based upon the assumption that until CP saturation, the AP did not compete for electrons. This assumption was based on inhibitor titration experiments using isolated mitochondria (for a review see Day et. al. 1980). Mitochondria were titrated with SHAM in the presence and absence of cyanide. The titration values represented the maximum AP activity at a given SHAM concentration (in the presence of cyanide) and the observed total respiration at a given SHAM concentration (in the absence of cyanide). When these values were plotted against each other a straight line was found, giving an estimation of the

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contribution the AP gave to total respiration. The slope of the line represented the capacity (ρ) to which the AP was operating. If $\rho=1$, the AP was operating at full capacity. If $\rho=0$, the AP gave no significant contribution to the total observed respiration. Bahr and Bonner (1973) used this estimation to show that the CP was not in competition with the AP. If SHAM inhibition had diverted electrons to a less maximally operating CP (as in the case of CP-AP competition) the plot described above would not have been linear. This supported their model depicted in figure 1.5.

de Troostembergh and Nyns (1978) used mathematical equations to argue a kinetic model in which both pathways were in use. They proposed that both pathways could be in operation without either pathway being saturated. In this model there was competition for UQH_2 and the outcome depended upon the relative affinity of each competitor for this substrate. They tested their model using mitochondria isolated from the yeast *Saccharomycopsis lipolytica* and the kinetic data was found to support their model. Acceptance of the de Troostembergh and Nyn's model was slow, however and has only recently been accepted and has implemented the re-evaluation of much of the inhibitor studies that followed the Bahr and Bonner model (Day 1996).
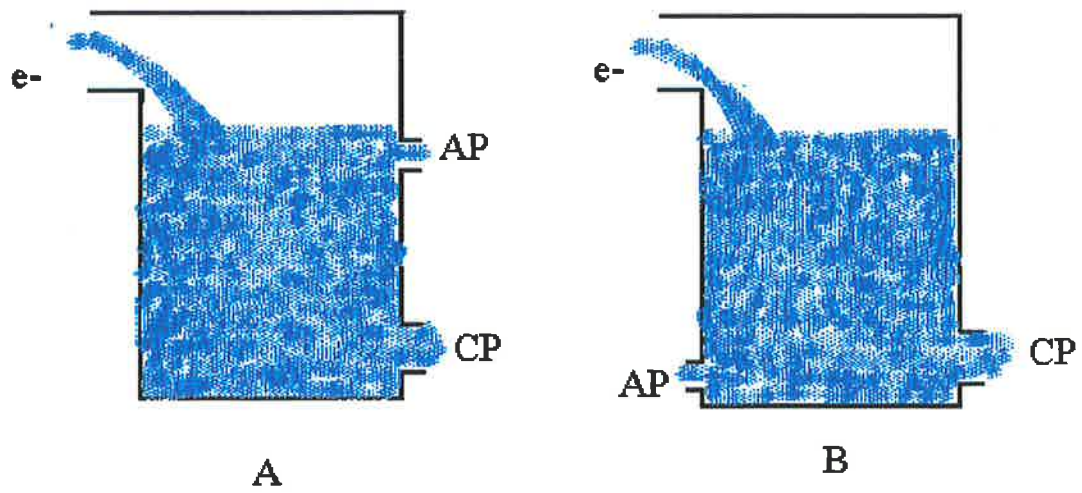
Figure 1.5 : A schematic representation of the two models of electron flow, A. Bahr and Bonner's model, and B. de Troostembergh and Nyn's model. (AP=alternative pathway, CP=cytochrome pathway, e- =electrons)

Since UQ represented the point at which the partitioning between AP and CP occurs, a method of measuring UQ in either oxidised or reduced form was sought. At this stage research was greatly encouraged by the use of cyclic voltammetry to produce an electrode that measured quinone reduction (Moore et. al. 1988, Dry et. al. 1989). Although it could not quantify the actual amount of UQ or UQH_2 present, it allowed the measurement of the ratio of UQH_2 with respect to the total UQ (i.e. UQ_R/UQ_T). This was done using UQ analogues that were less hydrophobic and more mobile in the aqueous media. The UQ analogues were added to the assay's reaction mixture and measurements were made of the quinone analogue's reduced-to-total pool ratio (i.e. UQ $analogue_R$/UQ $analogue_T$). These were believed to reflect those in the UQ pool within the inner membrane. Support for this assumption came from the understanding that there were a large number of sites for Q analogue oxidation/reduction on the inner mitochondrial membrane. Experimental confirmation of this was provided by Ribas-Carbo et. al. (1995a) who found a linear correlation between the actual amount of reduced UQ in mitochondrial membranes (measured using HPLC) and the level of UQ $analogue_R$/UQ $analogue_T$ (measured by cyclic voltametric means).

Previous attempts to measure the engagement of the AP were based upon the assumption that Bahr and Bonner's model was correct, namely that AP activity was strictly controlled by the level of reduced UQ (Dry et. al. 1989 and Wagner et. al. 1992). Dry et. al. (1989) reported

that control of AP engagement seemed to involve a non-linear relationship between the amount of electrons flowing through the mETC and the level of reduced:oxidised UQ. This indicated that engagement of the AP was not a simple reaction of UQ accepting electrons until threshold levels of reduced UQ caused engagement of the alternative pathway. Dry et. al. (1989) found that even after these levels had been reached, engagement of the AP was seen to increase disproportionately with the further reduction of the UQ pool. Another interesting result found by Day et. al. (1991) further complicated the electron partitioning models. Using mitochondria isolated from the cotyledons and leaves of *Glycine max* Day et. al. (1991) showed similar rates of substrate oxidation for external NADH and succinate in the absence of CP inhibitors (e.g. myxothiazol), whilst rates of NADH oxidation in their presence were approximately 50% lower than those with succinate. In all cases, measurements using cyclic voltammetry showed that the levels of reduced UQ were the same. This meant that although both substrates had equally favourable access to the CP via UQH_2, only succinate had good access to the AOX via the same UQ pool. In contrast, mitochondria isolated from the thermogenic tissue *Philodendron selloum*, did not show any discrepancy between external NADH and succinate oxidation via the alternative oxidase in either their respiratory rates or quinone ratios. Day et. al. (1991) surmised that with soybean mitochondria, both substrates (NADH and succinate) competed for the cytochrome chain

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but not for the AP. This suggested separate pools of UQ and separate mETC for different substrates. This discrepancy was explained when the stimulatory effects of pyruvate were discovered by Millar et. al. (1993). Incubation of purified soy bean mitochondria with pyruvate increased the activity of AOX when either NADH (exogenous and endogenous) and succinate was provided as a substrate. Of great interest was the result showing exogenous NADH cyanide-insensitive oxidation (previously 50% the rate of succinate oxidation) was pyruvate-stimulated to a rate equal to that of succinate oxidation. Thus treatment with pyruvate removed the discrepancy between these two substrates in their access to the alternative pathway. Millar et. al. (1993) proposed a conformational change caused by the allosteric binding of pyruvate to the exposed sites of the alternative oxidase. It was suggested that this led to a stimulation of the alternative oxidase activity. The higher rate seen with succinate in the absence of added pyruvate was due to pyruvate being produced intramitochondrially during succinate oxidation. It has been proposed that the malate produced from succinate oxidation can be converted to pyruvate by malic enzyme (Day et. al. 1994).

Eventually the use of inhibitors to measure AP and CP activity was questioned by a number of different research groups and the de Troostemburg and Nyn's model was accepted (see Day 1996 for a review). The use of inhibitors began to be doubted by researchers due to conflicting results and because so many assumptions had to be made

when using inhibitors and comparing results. For example, the inhibitors had to be specific and not affect other key enzymes. They had to accurately reach the site of inhibition and their action on one pathway did not affect the activity of the other. Another incumbrance with inhibitors was that certain ones could not be used when studying purified mitochondria using cyclic voltammetry. This was due to the inhibitor's interaction with the electrode. For all of these reasons, another method of distinguishing the AP and CP without using inhibitors was sought.

In 1989 Guy et. al. (1989) developed the "oxygen isotope discrimination technique". Approximately 0.2% of terrestrial oxygen has a mass of 18 rather than 16. When it was discovered by Guy et. al. (1987) that the two terminal oxidases of mitochondria had different preferences for these oxygen isotopes, this was exploited as a potential method to distinguish the AP and CP pathways without invasive, inhibitory methods. This technique exploits the different affinities the AP and CP have for $^{16}O:^{18}O$ and enables a measurement of the different pathways with respect to the total O_2 consumption (Guy et. al. 1992). Initially discrimination or "D" values were established by measuring each pathway alone, that is by using appropriate inhibitors. The discrimination against ^{18}O was found to be greater in the AP (23.5 to 25.5 ‰) than the CP (17.1 to 19.4‰)(Guy et. al. 1992; Robinson et. al. 1995). To test this difference in D values Guy et. al. compared a number of different organisms, including those that lacked one or

other pathway. The addition of inhibitors (to obtain the D value) had no effect on this value when compared with the D value of the organisms lacking either pathway. Oxygen isotope discrimination has been tested on various species of bacteria, yeasts, diatoms, green algae, fungi and higher plants (Weger et. al. 1990, Guy et. al. 1992, Robinson et. al. 1995, Ribas-Carbo et. al. 1995). Studies with *Glycine max* (Ribas-Carbo et. al. 1995b) confirmed that the AP was able to compete with the CP during state 3 conditions, that is during rapid mETC functioning when ADP concentrations are not limiting. This was only seen once pyruvate, the allosteric activator of AOX, had been added. It was also shown that whilst NADH led to a lower AP activity than succinate, when used as a substrate, upon addition of pyruvate this discrepancy was eliminated. This supported those results of Millar et. al. (1993) previously described, using the oxygen electrode.

The oxygen isotope discrimination technique provides an important method of measuring oxygen consumption during plant respiration in a manner that allows distinction of AP and CP involvement. It can be applied to whole cells or mitochondria in a liquid phase environment. Alternatively, whole tissues of higher plants can be studied using a gas-phase system (Robinson et. al. 1995).

1.3.1. "Electron-sharing" pathways

The focal question of how electrons are "diverted", changed to one of how they are "shared" between the two pathways. Work by Wilson (1988) showed that inhibition of the alternative pathway

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allowed electrons to be re-routed to the cytochrome pathway in purified, mung bean mitochondria. His elegant experiments represented an important step towards understanding the "electron sharing" capacity between the two pathways of the mETC. In 1995 his experiments were repeated in the presence of pyruvate, a putative AOX allosteric activator using *Glycine max*,purified mitochondria (Hoefnagel et al. 1995). It was shown that switching could occur when the CP was not saturated, however this only occurred once AOX was activated by pyruvate. It was noted that in the earlier work, using mung bean, purified mitochondria without the addition of pyruvate (Wilson 1988), intramitochondrial levels of pyruvate may have been already high enough to activate the AOX enzyme (Hoefnagel et. al. 1995).

In summary, the hypothetical schemes for how the AP functions with respect to the CP have varied widely. Initially the AP provided an "overflow" function, that is it was engaged once the CP was saturated or UQH_2 levels were high, then AP engagement beyond this level was believed to be non-linear. And finally, the current "electron sharing" concept exists, where electron flow is able to be shared between the two pathways via path switching, depending upon other factors, such as the substrate in use or the level of pyruvate present.

1.4 Regulation of the AOX activity.

Regulation of AOX activity appears to operate on two main levels summarised as: (a) interaction of glycolytic and TCA pathway intermediates with the enzyme, and

(b) oxidation/reduction of the enzyme. Both of these mechanisms affect enzyme activity in a distinct way.

1.4.1. Regulation of AOX activity by TCA intermediates.

A number of different intermediates were used to investigate the regulation of AP activity in mitochondria isolated from tobacco (Vanlerberghe et. al. 1995). It was found that both the addition of certain organic acids as well as reduction of AOX was required for full AP activation. Only the reduced AOX enzyme could be activated by a limited number of compounds. This was in agreement with the work by Umbach and Siedow (1993) as discussed below (section 1.4.2). The limited number of intermediates found to be effective also represented key branch points in the preceding and subsequent carbon metabolic pathways. The compounds shown to increased the activity of the AP were citrate, iso-citrate, malate and pyruvate. In addition, Day et. al. (1995) reported that glyoxyllate, oxaloacetate, hydroxypyruvate and oxo-2-glutarate were able to stimulate AP activity in *Glycine max* mitochondria.

In order to describe how the allosteric activation of AOX would occur *in vivo*, Day et. al. (1995) suggested that increased pyruvate production could occur via the glycolytic pathway. The resultant build

up of pyruvate would have a feed-forward activation effect on the AP.

As the stimulatory effect of pyruvate was found to be readily

reversible, Day et. al. (1994) maintained that stimulation of the AP

could only occur if the high levels of pyruvate persisted. Vanlerberghe

et. al. (1995) suggested that reduction of the AOX enzyme, first

described by Umbach and Siedow (1993) (see section 1.4.2.) might occur

in vivo via putative agents such as NADPH. They further suggested

that only once this had occurred could the enzyme be subject to the

allosteric control proposed by Millar et. al. (1993).

1.4.2. Regulation of AOX activity by enzymic oxidation/reduction.

Umbach and Siedow (1993) reported the dimeric species of the

AOX protein, describing it as "disulphide-linked". They found that

omission of the reducing agent dithiothreitol (DTT), in protein

loading buffer gave rise to the appearance of a high molecular weight

species (60-70kDa) on immunoblots using purified mitochondria from

Glycine max. The inclusion of DTT caused these high molecular

weight bands to largely disappear and a lower band (32kDa),

corresponding to a monomeric form to become prevalent. They

demonstrated that the monomer represented a second form of the

dimer *in vivo* , namely a non-covalently-linked dimer which was

more active than the disulphide-linked dimer, and they found that the

redox state of the sulphydryl/disulphide system affected the AP

capacity and the stimulatory effects of pyruvate (Umbach and Siedow

1994). This effect of pyruvate was dependent upon the disulphide

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bond being reduced, as discussed below.

Once the results from the genetic analysis of *aox1* in *S.guttatum* (see section 1.5 for more details) and other subsequent species became available, the amino acid sequence was predicted and those residues believed to be involved in disulphide bridges were identified. Using comparative homology to ascertain which amino acid regions were highly conserved across the species known, the cysteine residues believed to be important in the formation of dimeric AOX were predicted. The cysteine residues at positions 56 and 106 were highly conserved across species. Their roles were tested in the plant *S. guttatum* by Rhoads et. al. (1997). They used site-directed mutagenesis to replace one or other cysteine residues of the expressed AOX protein with alanine (using *E.coli* as the host expression system). The protein with Cys-56 replaced was unable to form an intermolecular disulphide linkage, whilst the protein with Cys-106 replaced was still disulphide linked. This showed that Cys-56 was the residue involved in regulatory disulphide bond formation.

Recent work by Umbach and Siedow (1997) using a sulphydryl reagent iodoacetate, which prevents sulphydryl oxidation, showed that iodoacetate stimulated AOX activity in a similar manner to pyruvate. Closer examination found that iodoacetate acted on the same AOX sulphydryl site as pyruvate, however this sulphydryl was different to that which was redox-sensitive (residue Cys-56 described above). Since (*Pichia stipitis* and *Neurospora crassa*) fungal AOX lacked both of these cysteine residues, the fungal enzyme

was examined for dimerisation and activation by pyruvate. It was not surprising that Umbach and Siedow (1997) found no stimulation by pyruvate and no evidence of dimerisation. They postulated that control of alternative oxidase might occur entirely through gene regulation.

Thus the current model shows a disulphide-linked dimer (non-active) being reduced to a more active, non-covalently linked dimer, which now has an accessible cysteine residue. This residue, which is different to that involved in the dimerisation process, can be stimulated by organic acids. This system interacts within a sophisticated model of feed-forward control involving primarily the glycolytic, TCA cycle and mETC to allow AOX to be active when the CP is tightly controlled (Vanlerberghe and McIntosh 1997).

1.5 Molecular studies on AOX

Concommittant with the bioenergetic studies of the AP were studies at the molecular level. The earliest studies occurred in the fungal genes of *Neurospora* where two genes, *aod1* and *aod2* were shown to be involved (Bertrand et. al. 1983). There was a single structural gene named *aod1* and a regulatory gene *aod2*, both of which were nuclear-encoded. This work was closely followed by the identification of plant *aox* genes and cDNA clones. The generation of specific antibodies allowed plant scientists to calculate the molecular weight of the protein and to more closely examine the enzyme's activity.

1.5.1. Cloning of AOX genes

The generation of specific monoclonal and polyclonal antibodies assisted the cloning of *aox* from other plant species. These antibodies were generated from partial purification of the AOX of *S.guttatum* (Elthon and McIntosh 1987b) and allowed the calculation of AOX molecular weight from a number of species, a summary of these is presented in table 1.1. They also permitted the isolation from expression libraries and subsequent sequencing of a number of putative clones encoding AOX of *S.guttatum* and *Solanum tuberosum* (Rhoads and McIntosh 1991; Hiser and McIntosh 1990 respectively).

Table 1.1 : *A summary of all AOX with known size from a number of different plant species.*

plant species	size of AOX (kDa)	reference
S.guttatum	35, 36, 37	Elthon & McIntosh 1987b
G.max	32, 34	Day et. al. 1993
S.tuberosum	36	Hiser & McIntosh 1990
N.tabacum	35	Vanlerberghe et. al. 1994
M.indica	27, 33, 36	Cruz-Hernandez & Gomez-Lim 1995
B.vulgaris	38	Johnson (1993)

The AOX protein sequences of a number of species from plants, fungi and protozoa have also been deduced from either cDNA or genomic clones, including *Solanum tuberosum* (Hiser and McIntosh 1990), *Sauromatum guttatum* (Rhoads and McIntosh 1991), *Hansenula anomala* (Sakajo et. al. 1991), *Arabidopsis thaliana* (Kumar and Soll 1992), *Glycine max* (Whelan et. al. 1993), *Mangifera indica* (Cruz-Hernandez and Gomez-Lim 1995) and more recently *A.thaliana*

(Saisho et. al. 1997). Strong areas of homology occur throughout the nucleic acid sequence across all species. These sequences have provided predicted amino acid sequences containing highly conserved regions (figure 1.6). Areas of high homology are shown in figure 1.6 and are primarily located in exon 3. From the predicted amino acid sequence the secondary structure of the protein has been determined in *G.max* and *S.guttatum* (Siedow et. al. 1992; Rhoads and McIntosh 1991, respectively).

1.5.2. The *aox* gene family

AOX is a nuclear-encoded multigene family. This was shown by the work of Whelan et. al. (1995c) whereby *Glycine max* had three *aox* genes whilst *Nicotiana tabacum* had two. Whelan et. al. (1995c) used a PCR approach to amplify genomic DNA using degenerate primers. The design of these primers came from the amino acid alignment of the AOX sequence from four plant species and the yeast *Hansenula anomala*. The primers were designed according to the regions of absolute homology between species. Whelan et. al. (1995c) have shown that different members of this family are expressed in different tissues. For example, *aox1* of *G. max* is minimally expressed in leaves but strongly expressed in cotyledons, compared to *aox3* that is strongly expressed in leaves but present to a lesser extent in cotyledons.

+ Majority

Majority
```
         MMSR - - AAT - - - - L - - - - PR - - - - - - - - - - AGT - - - A - AATFLHG - P - AG - - - - - - - W - L - - - - - - A - - EEA
              10          20          30          40          50          60          70          80
```
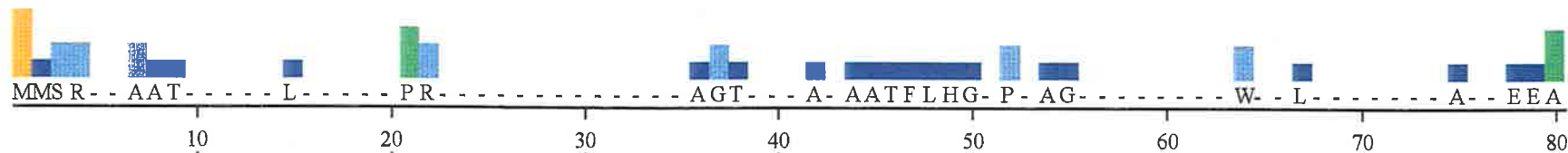
ATHALI	MDTR - - A - - - - - - - - - - - - - PT - - - - - - - - - - - - - - - - - I GGMRF ASTI TLGEKTP MKE - EDA	30
CROSEU	MMSR - - GATRI SRSLI CQI SPRYF SSAAVRGHEPSLGI LTSGGTTTFLHGNPGNGSERTALT - WI KLP MMRARSASTVAT	77
GMAX1	M - - - - - - - - - - - M - - - MMM - - - SR - - - - - - - - - - SGANRVANTAMFVAKG - LSGEVGG - - - LRA - LYGG - GVRSESTLA	46
MINDIC	MLSN - - AGG - AEA	10
NTABAC	MMTR - - GATRMTRTVLGHMGPRYF STAIFR - - - NDAGTGVMSGAAVFMHGVP - ANPSEKAVVTWVRHFP VMGSRSAMSMA	74
OSATIV	MGSR - - AAGSV - - - LLRHLCPR - - - - - - - - - - VSSSTSAAAHAHAQRPPLAGAGGGGVALWARLLST - SAAAAKEET	61
SGUTTA	MISSRLAGTALCRQLSHVPVPQYLPA - - LR - - - PTADT - - - - - ASSLLHRCSAAAPAQRA - GLWPPSWFSPPRHASTLSA	69
ZMAYS	- -	1

+ Majority

Majority
```
         LAQKEKDEKAAG - AAAATGGGDGGA - - - - - - - - - - SYWGVEPSKI TKEDGSEWKWNCFRPWETYKADLSIDLKKHHVPTT
              90         100         110         120         130         140         150         160
```

ATHALI	NQKKTENESTGGDAAGGNNKGDKGI AS - - - - - - - - - YWGVEPNKI TKEDGSEWKWNCFRPWETYKADI TIDLKKHHVPTT	101
CROSEU	VDQKDKDEKREDKNGVADGENGNKA - - - - - - - VVSYWGVEAPKLTKEDGTVWRWTCFRPWETYKPDTDIELKKHHVPVT	149
GMAX1	LSEKEKIEKKVGLSSAGGNKEEKVIV - - - - - - - - SYWGIQPSKITKKDGTEWKWNCFSPWGTYKADLSIDLEKHMPPTT	117
MINDIC	QVKEQKEEKKDA - - - - - - - - - - - - - - - - - - MVSNYWGISRPKITREDGSEWPWNCFMPWETYRSDLSIDLKKHHVPRT	70
NTABAC	LNDKQHDKKAENGSAAATGGGDGGDEKSVV - - - - SYWGVQPSKVTKEDGTEWKWNCFRPWETYKADLSIDLTKHHAPTT	149
OSATIV	AASKENTGSTAAAKAEATKAAKEGPASATASPVGSSYWGIEASKLASKDGVEWKWSCFRPWETYSPDTTIDLKKHHEPKV	141
SGUTTA	RAQDGGKEKAAGTAGKVPPGEDGGAEKEAVV - - - SYWAVPPSKVSKEDGSEWRWTCFRPWETYQADLSIDLHKHHVPTT	145
ZMAYS	- -	1

+ Majority

Majority:
F L D K V A Y W T V K A L R W P T D I F F Q R R Y G C R A M M L E T V A A V P G M V G G M L L H L K S L R R F E Q S G G W I K A L L E E A E N E R M H L M T F M

	170	180	190	200	210	220	230	240
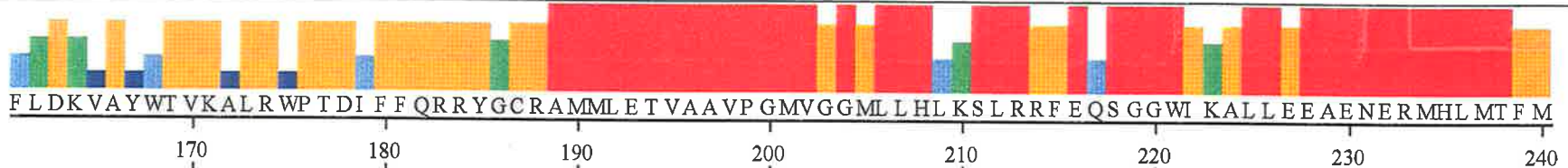
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CROSEU L L D K V A F F T V K A L R W P T D L F F Q R R Y G C R A M M L E T V A A V P G M V G G M L L H C K S L R R F E H S G G W I K A L L E E A E N E R M H L M T F M 229
GMAX1   F L D K M A F W T V K V L R Y P T D V F F Q R R Y G C R A M M L E T V A A V P G M V A G M L L H C K S L R R F E H S G G W F K A L L E E A E N E R M H L M T F M 197
MINDIC F M D K F A - R T V K I L R V P T D I F F Q R R Y G C R A M M L E T V A A V P G M V G G M L L H L K S L R K L E Q S G G W I K A L L E E A E N E R M H L M T M V 149
NTABAC F L D K F A Y W T V K S L R Y P T D I F F Q R R Y G C R A M M L E T V A A V P G M V G G M L L H C K S L R R F E Q S G G W I K T L L D E A E N E R M H L M T F M 229
OSATIV L L D K V A Y W T V K A L R V P T D I F F Q R R Y G C R A M M L E T V A A V P G M V G G M L L H L R S L R R F E H S G G W I R A L L E E A E N E R M H L M T F M 221
SGUTTA I L D K L A L R T V K A L R W P T D I F F Q R R Y A C R A M M L E T V A A V P G M V G G V L L H L K S L R R F E H S G G W I R A L L E E A E N E R M H L M T F M 225
ZMAYS   - - - - - - - - - - - - - - - - - - - A M M L E T V A A V P G M V G G M L L H L R S L R R F E Q S G G W I R A L L E E A E N E R M H L M T F M 52

+ Majority

Majority:
E V A K P R W Y E R A L V L A V Q G V F F N A Y F L G Y L L S P K L A H R V V G Y L E E E A I H S Y T E F L K D L D K G N I E N V P A P A I A I D Y W R L P A D

	250	260	270	280	290	300	310	320
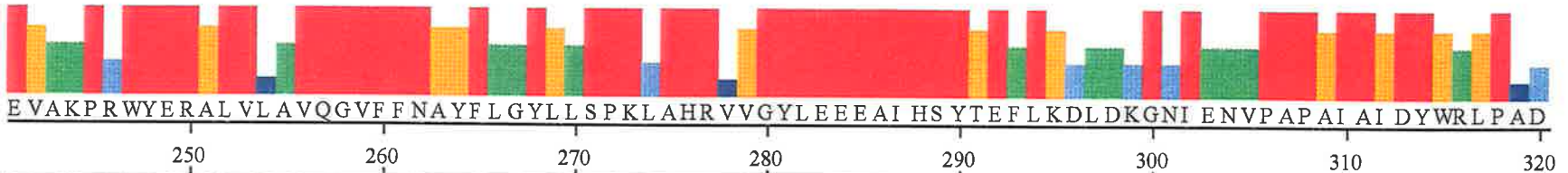
ATHALI E V A K P K W Y E R A L V I T V Q G V F F N A Y F L G Y L I S P K F A H R M V G Y L E E E A I H S Y T E F L K E L D K G N I E N V P A P A I A I D Y W R L P A D 261
CROSEU E V S K P R W Y E R A L V F A V Q G V F F N A Y F L T Y L A S P K L A H R I V G Y L E E E A I H S Y S E F L N E L D K G N I E N V P A P A I A I D Y W Q M P P D 309
GMAX1   E V A K P K W Y E R A L V I T V Q G V F F N A Y F L G Y L L S P K F A H R M F G Y L E E E A I H S Y T E F L K E L D K G N I E N V P A P A I A I D Y W Q L P P G 277
MINDIC E L V Q P K W Y E R L L V L A V Q G V F F N S F F V L Y V L S P K L A H R I V G Y L E E E A I H S Y T E Y L K D I D S G A I K N I P A P A I A I D Y W R L P K D 229
NTABAC E V A K P N W Y E R A L V F A V Q G V F F N A Y F V T Y L L S P K L A H R I V G Y L E E E A I H S Y T E F L K E L D K G N I E N V P A P A I A I D Y C R L P K D 309
OSATIV E V A K P R W Y E R A L V L A V Q G V F F N A Y F L G Y L L S P K L A H R V V G Y L E E E A I H S Y T E Y L K D I E A G K I E N V P A P P I A I D Y W R L P A G 301
SGUTTA E V A Q P R W Y E R A L V L A V Q G V F F N A Y F L G Y L L S P K F A H R V V G Y L E E E A I H S Y T E F L K D I D N G A I Q D C P A P A I A L D Y W R L P Q G 305
ZMAYS   E V A K P R W Y E R A L V I T V Q G V F F N A Y F L G Y L L S P K F A H R V V G Y L E E E A I H S Y T E Y L K D L E A G K I E K R P A P A I A I D Y W R L P A N 132

+ Majority

Majority   A T L R D V V T V V R A D E A H H R D V N H F A S D I H Y Q G L E L K D A P A P L G Y H -

            330   340   350   360

ATHALI	A T L R D V V M V V R A D E A H H R D V N H F A S D I H Y Q G R E L K E A P A P I G Y H	305
CROSEU	S T L R D V V M V V R A D E A L H R D V N H Y A S D I H Y K G L E L K E A A A P L D Y H	353
GMAX1	S T L R D V V M V V R A D E A H H R D V N H F A S D I H Y Q G R E L R E A A A P I G Y H R	322
MINDIC	A T L K D V I T V V R A D E A H H R D V N H F A S D V Q V Q G K E L R D A P A P V G Y H L	274
NTABAC	S T L L D V V L V V R A D E A H H R D V N H F A S D I H Y Q G Q Q L K D S P A P I G Y H	353
OSATIV	A T L K D V V V V V R A D E A H H R D V N H F A S D V H F Q G M D L K D I P A P L D Y H	345
SGUTTA	S T L R D V V T V V R A D E A H H R D V N H F A S D V H Y Q D L E L K T T P A P L G Y H	349
ZMAYS	A T L K D V V T V V R A D E A H H	149

'conserved region': Box residues that match the Consensus exactly.

Four genes in *A.thaliana* have also been identified using a similar PCR-directed approach (Nakazono et. al. 1997). These four genes were labelled *aox1 a, b, c* and *2* and showed varied levels of transcription in different plant tissues. For example, *aox1a* and *c* were present in flowers, buds, stems, rosettes and roots of eight week old plants, whereas *aox1b* was not detected in stems, rosettes and roots nor was *aox2* was detected in flowers or buds. The regulation of gene expression was different in leaf tissue treated with a foliar spray of AA, a CP inhibitor. In young leaves *aox1a* was shown to be induced by AA treatment, while transcription of *aox b* , *c* and *2* was not (Saisho et. al. 1997). This implies that different *aox* genes were tissue specific and may be required at different times and/or with different types of plant stress.

The different AOX proteins of soybean were separated on SDS-polyacrylamide gels via electrophoresis and sequenced (Finnegan et. al. 1997). From the amino acid sequence results the protein monomer seen via immunoblotting could be matched to its gene. This work confirmed that different *aox* genes encoded the separate proteins seen on immunoblots and also showed that the level of transcripts corresponding to the different *aox* genes, varied in different tissue types. Soybean cotyledons showed high levels of *aox3*, moderate levels of *aox2* and low levels of *aox1*. In comparison the roots of soybean displayed higher levels of *aox3* and barely detectable levels of the other two transcript types. They also showed in soybean cotyledons that the

transcripts levels altered throughout a period of light exposure. Transcripts of *aox2* increased with light exposure whilst *aox3* transcripts decreased over the same period (Finnegan et. al. 1997).

1.5.3. Regulation of *aox1* gene expression

Increased expression of the *aox* gene has been shown in four cases; (a) immediately prior to anthesis, (b) after SA treatment, (c) after the application of AA, and (d) during the ripening of fruit. Once the presence of an *aox* gene family was known (see section 1.5.2.) investigation of the different *aox* genes' expression in various plant tissues was initiated, but until this point the main focus of AOX studies was on the change in AP activity or protein levels (Vanlerberghe and McIntosh 1997).

An increase in the level of *aox* transcripts of *S.guttatum* during anthesis was shown by Rhoads and McIntosh (1992) andwhen compared with the level of cytochrome oxidase subunit I and II transcription (Elthon et. al. 1989) it can be seen that cytochrome oxidase transcription decreases before maximal expression of anthesis which indicated that only *aox* was specifically responsive in *S.guttatum*, to the developmental changes with anthesis. Application of SA to *S.guttatum* spadices also increased *aox* expression. SA was believed to "trigger" anthesis in aroid species (see section 1.7.2). This led to the identification of a putative SA-responsive element in the promoter region of this plant, which was similar to that described for the genes encoding the glycine-rich protein 8 (GRP8) and *PR1a* a pathogenesis-related protein (Rhoads and

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McIntosh 1993a).

Changes in *aox* expression in AA-treated tobacco cultures were compared with the expression of *coxI* (Vanlerberghe and McIntosh 1994). Again it appeared that only *aox* expression was affected by the treatment. This increase in expression was further supported in *A.thaliana*. Leaves sprayed with AA showed an increased level of *aox1a* transcription but not *aox1b, c* or *aox2* transcription, as discussed previously (section 1.5.2.), (Saisho et. al. 1997). This indicated that in *A.thaliana* leaf tissue, *aox1a* was specifically induced by AA-treatment.

The expression of *aox* in mango fruit was studied over the ripening period. Total RNA was extracted from the mesocarp of developing, unripe, turning and ripe fruit and was probed using the mango, full-length cDNA sequence (Cruz-Hernandez and Gomez-Lim 1995). The results showed a 1.6kb transcript present to the same degree in the developing, unripe and turning fruit. The ripe fruit showed a large increase in the level of the 1.6kb transcript, indicating that expression of the *aox* gene occurred during the later stages of mango fruit ripening.

1.5.4. Amino acid sequence studies

Analysis of the predicted amino acid sequence of *aox1* has led to the development of two current models of enzyme structure: the "three helical domain model" and the "two helical domain model"(Siedow et. al. 1992). Both of these are based on the number of putative alpha-helices that contain hydrophobic amino acids spanning the inner

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mitochondrial membrane (figure 1.7). Studies by Siedow et. al. (1992) attempted to determine the location and orientation of the alpha-helices within mitoplasts and sub-mitochondrial particles (SMP) using *Glycine max* cotyledonous tissue. Siedow et. al. (1992) used trypsin in a time-dependent study to digest any exposed protein sequences in the mitoplasts and SMP with an inside-out orientation (ISO SMP). These two forms of treated mitochondria allow the selective study of the unobstructed inner membrane surface from a cytosolic and matrix aspect, respectively. Addition of the proteolytic enzyme trypsin would effectively hydrolyse any exposed lysine or arginine residues on the externally-facing surface (mitoplasts) or matrix-facing surface (ISO SMP) of the inner mitochondrial membrane. Electrophoresis of such protease-treated samples and immunoblotting, using antibodies raised against the 35, 36, and 37 kDa set of alternative oxidase peptides showed that there were no vulnerable protease sites facing the cytosol. Degradation of the AOX polypeptide occurred in ISO SMP or mitoplasts treated with lipid-dissolving detergents, eg. Triton X-100. This is supportive of earlier work by Rasmusson et. al. (1990) using *Arum maculatum* mitochondria which suggested the active site of the enzyme was located on the inner, matrix-facing surface of the inner membrane. Both sets of results suggest the two helical domain model for the topology of the alternative oxidase enzyme.
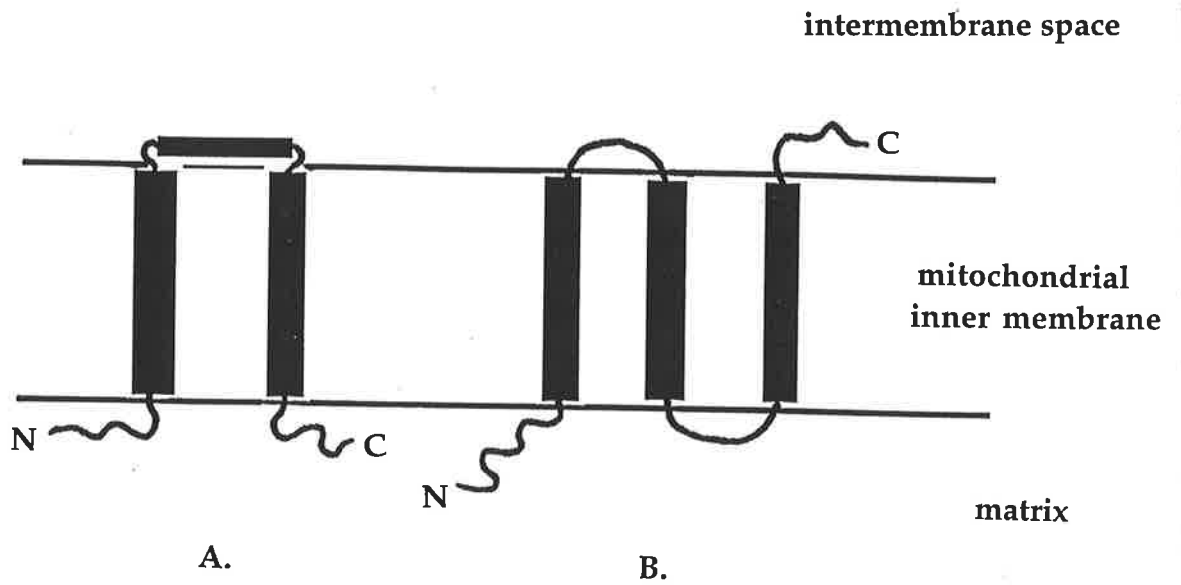
Figure 1.7 : A schematic representation of the **A.** two helical and **B.** three helical topology of alternative oxidase enzyme.

As seen in figure 1.7, this model has significant hydrophilic regions exposed on the matrix side. It has been shown that other membrane-bound (bacterial) proteins may protect their exposed amino acids by close association of the hydrophilic regions with the cell membrane (Akiyama and Ito 1987). Criticism of the three helical model is aimed at the exposed, long amino acid sequences on either side of the inner membrane. These regions should be vulnerable to protease attack and hence would be unlikely to give the results seen by Siedow et. al. (1992). Likewise problems with the two helical domain model exist, primarily due to the small, "interhelical" sequence that contains two lysine and two arginine residues, targetted by trypsin-like proteases. There is no satisfactory explanation why these residues, which would be located on the outer surface of the mitoplasts, were "protected" from digestion in the studies by Siedow et. al. (1992). Although the currently accepted model for the alternative oxidase topology assumes a two helical domain, further studies are required to determine the structure of this enzyme.

1.6 The role of the alternative pathway in plants

In order to understand the role of the AP *in vivo* we must study those situations in which the alternative oxidase protein (AOX) has been induced, and is present and active, to a greater degree than usual. Some fresh plant tissues, such as tobacco leaf, beet root and potato tuber, have no AOX activity nor detectable AOX protein. Once these plants experience ageing or stress, the AOX levels (both activity and

protein concentration) are increased (Liden and Akerlund 1993). These types of changes in activity and protein levels can also vary during the development of a single plant, such as during the annual thermogenesis that occurs in some members of the Araceae family. Here the AP is believed to generate the heat during anthesis that leads to volatilisation of polyamines, which in turn attract pollinators (see section 1.2). Researchers have also described conditions where the AP is able to function at times when the CP is under tight adenylate control. This means that the AP allows continued TCA cycling and the generation of carbon skeletons for other cellular needs (Day et. al. 1995). For example, Azcon-Bieto et. al. (1983) have shown that the AP is more active in situations of high tissue sugar concentration, such as that experienced in leaf cells after periods of photosynthetic activity.

The role of the AP in plants has been examined *in vivo* in at least two cases ;- during thermogenesis and during fruit ripening. Both will be discussed here.

1.6.1. Thermogenesis

The role of the AP in thermogenic aroid species was discussed in detail in section 1.2. There is an additional role believed to occur with the non-aroid species *Symplocarpus foetidus*, "skunk cabbage" (Knutson 1974). The elevated respiration, varying indirectly with ambient temperature, allows heating of the floral appendix and encourages pollinators in a similar manner to the aroid species. However unlike the aroid tissue which holds its elevated temperature

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for several hours, the skunk cabbage can persist for at least 14 days, maintaining temperatures of 15 to 35°C above air temperature (Knutson 1974). The skunk cabbage lives in sub-freezing temperatures in North America, and therefore benefits from maintained heat production that assists the development of floral structures as well as pollination (Meeuse 1975). This plant is easily identified in its environment by its ability to melt the surrounding snow.

1.6.2. Fruit ripening

The role of the AP in the ripening of stored fruit has been investigated in a number of commercially important crops. These include bananas and avocadoes (Theologis and Laties 1978), apples, melons (Passam and Bird 1978), cherimoyas (Solomos and Laties 1976) and others. Certain types of fruit exhibit a pronounced increase in respiration rate during ripening which is termed "climacteric". Also associated with the ripening of stored fruit was a rise in the production of ethylene, which was believed to initiate the respiratory rise (Liebermann 1979). From their work with cherimoya fruit, Solomos and Laties (1976) concluded that the respiratory rise seen at the time of ethylene increase was predominantly cyanide-resistant. This was supported by studies with avocado fruit by Theologis and Laties (1978). Alternative oxidase activity has also been detected in ripening bananas and mangoes (Kumar and Sinha 1992). The increase in *aox1* gene expression was studied in the ripening fruit of *Mangifera indica* (mango) by Cruz-Hernandez and Gomez-Lim (1995). They isolated the

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gene for alternative oxidase from mango leaves and proceeded to demonstrate its increased expression in ripening fruit. Earlier work had shown that alternative oxidase activity increased with ripening alongside an accompanying increase in temperature from 29°C to 38.9°C (Kumar et. al. 1990). This increase in temperature was believed to function in a similar manner to thermogenesis, i.e. volatilising chemicals and giving the fruit their aroma, attracting potential seed dispersors (at least in the case of mangoes). The ripening process of climacteric fruits also produces high levels of ethylene. Ethylene is a known chemical inducer of *aox* expression and is further discussed below.

Thermogenesis presents an *in vivo* role for the AP during pollination, fruit ripening, overcoming sub-optimal temperatures and in facilitating seed dispersal, however AOX protein levels can be induced under a number of other circumstances and may occur in tissues other than the flowers or fruits.

1.7 Induction of the alternative oxidase activity

The alternative pathway appears, by its increased activity, to be required by the plant during other situations. Several exogenous stimuli, such as applied stress and chemical compounds increase the activity of the alternative oxidase. Chemicals such as ethylene, cycloheximide and SA and stresses such as wounding and chilling have been shown to either increase the amount of AOX protein or *aox* transcript levels, as discussed below.

1.7.1. Ethylene

Studies with mitochondria extracted from plant organs treated with ethylene have revealed ethylene to be an inducer of the AP. Potato tubers (*Solanum tuberosum*) that contained little or no alternative oxidase activity were kept in a container with ripening apples (Liden and Akerlund 1993). These tubers experienced the ethylene released from the ripening fruit during the incubation. The result was a dramatic increase in the activity of the AP compared with untreated potatoes. Ethylene also plays a role in initiating the respiratory rise seen in ripening climacteric fruits as previously described (see section 1.6).

1.7.2. Wounding, ageing and salicylic acid (SA)

Several authors have reported an increased AP activity resulting from the "ageing" of plant tissue (Johnson 1993, Shingles et. al. 1982, Van Steveninck 1975, Kahl 1974). Similar increases in the AP have been achieved by the application of exogenous SA to the spadices of *Sauromatum guttatum* (Elthon and McIntosh 1987a) or to sliced *Beta vulgaris* root tissue (Johnson 1993). In both cases there was increased expression of the AOX monomers recognised by antibodies specific for AOX. This was found to correlate with an increased level of activity. These results together with those with tobacco cell-suspension cultures (Kapulnik et. al. 1992) suggest that salicylates cause an increase in *aox* gene transcription or product translation. The results of further studies using antibiotics such as cycloheximide,

which blocks cytoplasmic translation, suggested that, in *S. guttatum* at least, translation was essential for this induction by SA (Rhoads and McIntosh 1993a).

1.7.3. Chemical inhibitors

Vanlerberghe and McIntosh (1994) showed that incubation of tobacco cell suspension cultures with the CP inhibitor antimycin A led to increased levels of *aox1* transcripts and increased alternative oxidase activity. This implied strongly that cells unable to utilise their CP could be induced to over-express the AP component to survive. They also compared the levels of transcripts among *aox1*, *cc-1* (cytochrome c) and *cox1* (subunit 1 of cytochrome oxidase) and concluded that only the *aox1* transcripts showed significant variation, namely a marked increased from the day of antimycin A addition. However, the result of other studies using cycloheximide and other inhibitors by Gallerani and Romani (1996) does not support the view of AA-mediated induction. They believe that much of the AP induction previously reported may be due to the decreased AOX removal or degradation, rather than its increased synthesis. Their conclusions were based upon work with pear cell cultures and past studies with cotyledons of *Cucumis sativus* using cycloheximide, actinomycin-D and D-MDMP. Results showed the increased AP activity correlated with a persistence of AOX protein, rather than *de novo* synthesis.

1.7.4. Chilling

Alternative pathway involvement and/or induction in short-term chilling responses has been studied in a wide variety of plants, such as maize, tobacco, cucumber, rape, potato and soybean (Thomashow 1990). In each case there is an apparent increase in AP capacity following chilling treatment. Vanlerberghe and McIntosh (1992) used tobacco cell suspension cultures to show that this increase in AP capacity was related to an increase in the amount of AOX protein. Cells were grown at 30°C then chilled at 18°C for 24 hours. This treatment resulted in a five-fold increase in AP capacity and a large amount of protein expression as detected by immunoblotting. In order to confirm *de novo* protein synthesis, they incubated the cells with either cycloheximide or chloramphenicol. Chilling failed to show increased amounts of AOX when cycloheximide was present. Cycloheximide also interfered with the concommittant increase in AP capacity, confirming that the increased AP capacity and protein levels upon chilling were a result of induction of AOX synthesis.

In summary, it has been shown that "ageing" alone increases the alternative pathway activity while ethylene, chilling and salicylates further increase this response. The causal effect of salicylates is currently under debate and may be due to an induction of *aox* gene expression or by diminished AOX protein removal.

1.8 Current work with transgenic *aox1* plants

Transgenic cell lines have been produced in *N.tabacum* which show either increased (over-expressors or sense plants) or decreased amounts (antisense plants) of both transcript and protein (Hiser et. al. 1996, Vanlerberghe et. al. 1994).

The work with potato overexpressors has shown that increased levels of transcripts (*aox1*) and protein in leaf and tuber gives rise to an overall higher AP capacity compared with wild type plants (Hiser et. al. 1996).

(Vanlerberghe et. al. 1995)

The work with tobacco has shown similar results. Sense plants show increased protein levels in both leaf and root compared with wildtype tissue, but no significant increase in the mitochondrially-encoded coxII protein levels. Antisense plants show an absence of AOX in leaf tissue when grown at 28°C then transferred to 10°C for 24h compared with wildtype grown at 28°C with and without transfer to 10°C for 24h. Wildtype tobacco leaf tissue has low levels of detectable protein, which is increased slightly via treatment at 10°C.

When studying tobacco cell cultures made from these plants, the AP capacity in the antisense cell cultures was lower than the wildtype (Vanlerberghe et. al. 1994). The wildtype AP capacity was, in turn, lower than the sense cell cultures. Overall respiration rates for the tobacco cell cultures remained fairly uniform. Cultured tobacco cells were grown in the presence of antimycin A in an attempt to understand the role of AOX in the feedforward response of the CP

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chain. The growth rates of the antisense and the wildtype lines were similar in the absence of antimycin A, however in its presence the wildtype had little growth and the antisense had no growth with a loss of dry weight over time. The effect of limiting the CP with AA was enough to induce the expression of AOX in the wildtype cells, however the antisense cells were unable to utilise the AP effectively and resorted to aerobic fermentation. This led to an increase in ethanol production.

Guy et. al. (1997) have recently studied the effects of chilling on the activity of the AP in the transgenic tobacco plants. Initial studies with wildtype, over-expressors and antisense plants showed no significant differences in partitioning to the AP using the on-line ^{18}O isotope discrimination technique (for more details on this technique see section 1.3). Partitioning was measured over a range of temperatures (10 to 40°C) and no significant difference was detected between plant lines. Similarly pre-exposure to low temperatures or photoinhibitory conditions showed no effect. This was a surprising result considering that Vanlerberghe and McIntosh (1992) had already shown the increase in AOX levels in tobacco cell suspension cultures chilled at 18°C for 24 hours. These more recent results, obtained using the on-line ^{18}O isotope discrimination technique, suggest that whilst the amount of AOX protein is increased with pre-exposure to low temperatures, it's overall contribution to respiration in relation to the CP is unaltered, and that earlier AP capacity results (Vanlerberghe and

McIntosh 1992) were not significant when considering the partitioning of electron flow through the mETC *in vivo*.

Whilst a great deal of work has been done to elucidate the role of the AP using wild type plants, the growth of plants lacking AOX during times of stress would greatly assist in our understanding of the role of this pathway *in vivo*. Use of inhibitors to effectively remove the AP has in the past been misleading or inconclusive (Day 1996). This was often due to the interaction of the inhibitors with other proteins. Studying the effects of effective "silencing" of the *aox* gene in any plant species, during those times when AOX synthesis is normally induced, would give valuable information about the importance of the AP. This approach would also allow the more accurate examination of the effects of reduced AP activity on the entire mETC conditions and could potentially elucidate other cellualr or organelle components that are involved.

CHAPTER TWO : MATERIALS AND METHODS

2.1. Growth material

2.1.1. Growth media, reagents, chemicals and molecular kits

Growth material for seed germination and plant growth in "soil" was purchased from Nu-Erth Horticultural and Rural Supplies (Adelaide). All reagents and chemicals were purchased from Boehringer Mannheim Australia Pty. Ltd. or Sigma Pty. Ltd., 'ScimaR' Life Sciences Specialties, Promega Corp. and Life Technologies. All molecular kits were purchased from Qiagen Pty. Ltd. or Promega Corp. unless stated otherwise. Precast gels were purchased from Novex Australia Pty. Ltd.

2.1.2. Plant material

Wild type (WT) plants were grown in trays of potting mix, made up of approximately 75% seed-raising mix and 25% fine vermiculite, in a glasshouse between the months of February to October. From October to February plants were kept in growth cabinets with 14h day at 18-24°C. Transgenic plants and WT control plants were germinated and grown on medium (30g sucrose, 0.5g MES, 4.33g MS salts, 8g phytagar, per litre, pH5.7) in magenta boxes, under controlled conditions (14h day, 18-24°C).

2.1.3. Bacterial cultures

Bacterial cultures were grown on either LB (*E.coli*) media (10

g/L bacto-tryptone, 5 g/L bacto-yeast extract, 10 g/L NaCl) or 2YT

(*A.tumefaciens*) media (16 g/L bacto-tryptone, 10 g/L bacto-yeast

extract, NaCl 5 g/L, pH 7.0). The LB media contained 1%(w/v) bacto-

tryptone, 0.5%(w/v) bacto-yeast extract, 0.5%(w/v) NaCl, 0.2%(w/v)

maltose, 10mM $MgSO_4$ with or without 1.5%(w/v) agar. The YM

media contained mannitol, K_2HPO_4, yeast extract, $MgSO_4.7H_2O$, NaCl

with or without 1.5% (w/v) agar. Cultures of *E.coli* were grown for a

maximum of 24h at 37°C with liquid cultures shaken to ensure

adequate aeration. Cultures of *A.tumefaciens* were grown for 24 to 48h

at 28°C.

2.1.4. Bacterial transformation

Competent *A.tumefaciens* cells and most competent *E.coli* cells

were kindly provided by Dr. Ian Dry (CSIRO, Horticulture).

Competent *E.coli* XLBlue cells used in the cloning of PCR products

(chapter 4) and subcloning of the *aox1a* cDNA were made using the

calcium chloride technique (Sambrook et. al. 1989). Liquid cultures

were grown at 37°C until they reached an optical density of 0.5 (A_{600})

and were then chilled to 0°C by incubation on ice for approx. 10 min.

The cultures were centrifuged at 2 700g for 10 min at 4 °C and the pellet

of bacterial cells was drained thoroughly. To each pellet, from approx.

37 mL of initial culture, 10 mL of ice-cold 0.1 mM $CaCl_2$ was added and

the pellet was resuspended and kept on ice. This suspension was

centrifuged (2 700g) at 4 °C for 10 min and the pellet was drained

thoroughly of all supernatant. Each pellet (equivalent to 50 mL of

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original culture) was then resuspended in 2 mL of ice-cold 0.1M $CaCl_2$ and dispensed into aliquots. These were frozen using liquid nitrogen and stored at -80°C.

All bacterial transformations were done using either the heat-shock technique or the electroporation method. The heat shock technique (Sambrook et. al. 1989) involved using 200 μL of the abovementioned, frozen stock of competent cells that had been thawed on ice. To these cells, 50 ng of ligated pDNA was added to a final volume of no more than 210 μL. This was gently mixed and transferred to a 42°C waterbath and incubated for exactly 90s. After incubation the mixture was immediately placed on ice and chilled for 1-2 min. LB (800 μL) was added and the tube was then incubated at 37°C for approx. 45 min before plating out onto selective media.

Electroporation occurred in a chilled cuvette which had a 0.1 cm electrode gap. Competent cells were mixed with 1 μL of ligation solution and this cell/pDNA mixture (41 μL total volume) was carefully transferred into the cuvette. In a Gene-Pulsor electroporator the cuvette was given a single pulse of 1.8 kV, 25 μFD and 200 Ω. The cuvette was rinsed out using 800μL LB and this mixture was incubated at 37°C for one hour before being plated on selective media.

2.2 Seed sterilisation

Seeds were surface sterilised by mixing with 50% bleach solution which contained 0.02% Triton X-100 for 7 min. The seeds were then repeatedly rinsed in sterile deionised water until no odour of bleach was

detected in the water discarded. The seeds were either mixed with 0.1%

agar solution (for sowing into trays for glasshouse raising) or plated out

on to labelled magenta boxes under sterile conditions. The agar

suspension and/or magenta boxes were kept at 4°C for two days, at

which time the seeds had vernalised, and placed in the growth cabinet.

The agar solution was pipetted out onto moistened potting mix and a

gladwrap cover secured over the tray until the first true leaves emerged.

Germination of seeds occurred within 3-10 days of sterilisation.

2.3 Cell suspension cultures

The initial inoculum for *A.thaliana* cell suspension cultures was

made from friable wild-type callus (see method below) mixed in the

presence of sterile, glass marbles to allow breakage of cell clumps.

Glassware was baked for 2h at >180°C before autoclaving and flasks were

autoclaved with MSA media containing MS salts (4.33g/L), 3% sucrose,

B5 vitamins and 2,4-D (0.5 mg/L). Callus for MSA initiation was

crushed into smaller fragments in a sterile petri dish containing

approximately 10mL of MSA medium (3% sucrose, Gambourg B5

vitamins, 0.5 mg/L 2,4-D and 0.05 mg/L kinetin, pH 5.8). These were

kept at 21-23°C at constant orbital shaking of 60 rpm. After 4-5 days this

mixture was transferred to a sterile flask with sterile marbles, and

shaken for another 4 days, at which point the solution was renewed. At

each subculture, the flask was left to sit for 10 min, then the surface

liquid was removed. Fresh MSA solution was added and the flask was

incubated without interruption for another 7 days. By this time the

solution had thickened into a slurry, and could be divided. Cells were grown until they reached logarithmic phase (determined by measurements of fresh weight) at which point they were treated and studied. Cultures continued to be subcultured every 7 to 10 days.

2.4 Callus culture

Arabidopsis thaliana (ecotype Columbia) seeds were obtained from Beth Rosen (Michigan State University, Plant Research Laboratory) and were sterilised and sown onto germination medium as described in section 2.2. All subsequent WT seed stocks were obtained from the resultant plants. Leaf material was harvested and wounded under sterile conditions. These leaf pieces were placed onto callus inducing medium (MS Gambourg's B5 medium with 3% (w/v) glucose, 0.8% (w/v) phytagar, 0.5 mg/L 2,4-D, 0.05 mg/L kinetin, pH 5.7). After 6-8 days callus was visible. After approximately 10 days of callus proliferation, small, friable pieces were removed and used to inoculate MSA medium or used directly in the oxygen electrode assays.

2.5 Vacuum-infiltration of *Arabidopsis thaliana*

Plants of *A.thaliana* were grown under ideal conditions for approximately 6 weeks and were used 4-5 days after removal of the first bolt. The bolting tissue was removed after the primary bolt was 5-15 cm tall. This was to encourage the appearance of secondary inflorescence spikes. Several days prior to infiltration, the plants were well fertilised and watered. After infiltration they were moved to a growth cabinet and

protected from dessication by plastic wrap. Once they had fully recovered (2-4 days) this was removed and plants were treated as normal, allowed to reach seedset and harvested.

The *A.tumefaciens* LBA4404 culture to be used in the infiltration method was verified (by restriction analysis) as containing the desired plasmid construct. This verification was repeated immediately prior to each infiltration. Inoculations from this culture were used to grow 500 mL cultures for two days at 30°C in LB plus 0.2% (w/v) maltose and 10 mM $MgSO_4$ and selective antibiotics (spectinomycin 100 µg/mL or tetracycline 10 µg/mL). The following infiltration method was adapted from the method kindly provided by Dr. Pauline Bariola (Michigan State University, Plant Research Laboratory). The bacterial infiltrate was spun down at 4 000g (Beckman rotor JA-16) and resuspended in one litre of infiltration medium (2.2 g MS salts, 1X Gambourg's B5 vitamins, 5% sucrose, 0.5 g MES, all adjusted to pH 5.7 and sterilised, followed by addition of sterile 0.044 µM benzylaminopurine and 200 µL Silwet L-77 (OSI specialties).

After resuspension, the solution was poured into plastic containers that were placed inside a glass dessicator. The plants to be infiltrated were placed with their pots upside-down in the solution, such that the green tissue was fully immersed without wetting large amounts of the soil. A vacuum pressure of approximately 18 inches Hg was drawn and once obtained, the dessicator tap was closed and the immersed plants kept at this pressure for 5-10 min. This removed much

of the air present in the tissue spaces, made apparent by the visible escape of air bubbles through the bacterial suspension. After this time the vacuum was rapidly released. This caused a rapid influx of bacterial suspension into the tissue spaces, increasing the agrobacterial infection efficiency. The pots were retrieved and drained. If the retrieved plants did not have signs of saturated leaf material and limpness, they were further infiltrated for another 5 min. Infiltrated plants were allowed to recuperate in the growth cabinet as described previously.

2.6 Purification of mitochondria and oxygen electrode assays

Mitochondria were isolated from fresh leaf tissue, using pre-cooled apparatus with procedures performed at 4°C.

2.6.1. Micropreps.

Approximately 1 to 5 g of leaf tissue was harvested and left at 4°C for ten min. Just before grinding, the leaf tissue was cut into smaller pieces with sharp scissors. A small amount of acid-wash sand was added to the mortar and 10 mL (per gram of tissue) of homogenisation buffer (0.4M sucrose, 50 mM Tris, 10 mM KH_2PO_4, 1 mM EGTA, 5 mM beta-mercaptoethanol, 1% (w/v) BSA, 5 mM pyruvate, pH 7.6). The tissue was ground for 15 s and the slurry was poured into a 25 mL syringe barrel lined with miracloth (previously wetted with homogenisation buffer). The syringe piston was used to force as much of the slurry as possible through the miracloth, and this filtrate was collected in eppendorf tubes. After centrifuging for 5 min at 3,000 g using a

microfuge, the supernatant was transferred to new tubes and centrifuged for a further 10 min at 27,300 g. The resulting pellet was resuspended in approximately 250 μL and layered on top of a microgradient. The layers of the microgradient consisted of 250 μL of 50% percoll (in 0.25 M sucrose with 3% PVP), 500 μL of 25% percoll (in 0.25 M sucrose with 1.5% PVP) and finally an upper layer (300 μL) of 13.5% percoll (in 0.25 M sucrose with 0.4% PVP). These tubes were centrifuged for 15 min in a microfuge at 27,300 g. The mitochondrial band was carefully collected from the interface of the 50% and the 25% percoll fractions and washed twice with resuspension medium (0.4 M mannitol, 10 mM KH_2PO_4 and 0.5% (w/v) BSA, pH7.2).

2.6.2. Minipreps.

Fresh leaf tissue (15 - 40 g) was mixed with homogenisation medium (70 mL for 15 g) and homogenised in a mortar and pestle in two stages, i.e. 2X 35 mL batches. The slurry was filtered through miracloth into a beaker (on ice) and then poured into two Oakridge tubes. These were centrifuged for 10 - 15 min at 4 340g (Sorvall SS34), the supernatant carefully poured into two new tubes and centrifuged for 10 min at 20 200g. The pellet was resuspended in resuspension medium (maximum volume 2 mL, pH 7.2) and layered onto cold percoll gradient of 10 mL (each fraction) 50%, 26% and 13.5% percoll. These were centrifuged in a SW28 rotor for 30 min at 48 200g without braking. The band of mitochondria occurred at the interface of 50% and 26% percoll fractions and this band was removed and placed into eppendorf tubes,

where it was quickly and repetitively washed with resuspension medium and centrifuged until the final pellet was firm. It was then resuspended in 150-to-200 µL of resuspension medium.

2.6.3. Protein estimations

All estimations of protein concentration were carried out according to the method described in Lowry et. al. (1951).

2.7 GUS assays

GUS assays were performed in 3 mL capacity multi-well trays (Corning cat. #430262). Leaf tissue was removed from plants grown in magenta boxes and sliced under sterile conditions before adding to substrate solution (2 mM X-gluc, 50 mM H_2PO_4, 0.1% Trition X-100 and 0.1% BME). The entire tray was placed into a dessicator, and a vacuum applied until bubbles were evident on the surface of the solution. This vacuum was held for approximately 5 min, and then released rapidly, causing the leaf tissue to be thoroughly infiltrated with the substrate solution, thereby sinking to the bottom of the well. The tray was sealed with parafilm and incubated at 37°C over night. The tissues were then fixed for 2-3 h in fixative solution (20% ethanol, 5% formamide, 5% acetic acid). The excess pigments were removed by soaking in 50% ethanol solution, until no more colour was visible in the soaking solution. Tissue was kept in absolute alcohol until it was studied under the light microscope.

2.8 Nucleic Acid procedures

2.8.1. PCR assays

All primers were synthesised by Bresatec Inc. (Adelaide, South Australia) excepting HAO-1 and HAO-2 which were kindly supplied by Dr. Jim Whelan (University of Western Australia). Appropriate size and T_m of synthetic oligonucleotides was calculated with the help of the DNA programme OLIGO. Each PCR reaction tube contained 20 pmoles of each primer, 0.5 mmol of each dNTP, 1X *Taq* buffer; 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 2.5mM $MgCl_2$, 50 ng gDNA and 2.5 units of *Taq* Polymerase I. All PCR runs were done using the following cycling conditions; 94°C/2.5', 94°C/1', 50°C/1', 72°C/1'repeat from 94°C/1' for 29 cycles, 72°C/4' on a MJ Research PTC-200 Peltier Thermal unless stated otherwise. A small amount of the resultant PCR products was run on agarose/TBE gels. If one predominant band were present, the remaining volume of PCR product was cleaned up i.e. the removal of nucleotides, *Taq* buffer etc., using a QIAquick PCR purification kit, and this DNA was subsequently subcloned into an appropriate vector. If more than one band was present, the entire reaction mix was run on an agarose/TBE gel and the separate bands were cut out and extracted from the agarose using QIAquick columns.

2.8.2. Cloning of PCR products

The PCR products were cloned using the vector system pGEM-T (Promega Corp.). This system took advantage of the 3' A overhang

occurring from the use of *Taq* Pol I. The pGEM-T vector was provided as a linear plasmid with 3′ T overhangs. PCR products with *Xba* I ends (due to the specific design of th primers) were digested with Xba I and cloned into pGEM vector which had also been previously digested with *Xba* I and treated with calf intestinal phosphatase (CIP) to avoid vector re-ligation. This CIP treatment involved incubating the *Xba* I-cut pGEM for one hour at 37°C with CIP in a buffer containing 50 mM Tris-HCl (pH 8.5) and 100 μM EDTA. The vector was purified after gel electrophoresis using a QIAquick gel extraction kit.

Vector and insert DNA were added in proportion 1:3 with ligation buffer (30 mM Tris-HCL, pH7.8, 10 mM MgCl$_2$, 10 mM DTT and 1 mM ATP) and T4 ligase (3 Weiss units) to a final volume of 10 μL and allowed to ligate overnight at 4°C. Of this reaction mixture, 2 μL were used in the transformation of *E.coli*. The strain (JM109) provided by Promega was used for initial experiments, however strain XLBlue (Stratagene) made competent following the procedure of Sambrook et. al. (1989) proved just as successful. Mixing 2 μL of the ligation mixture with 50 μL of competent cells was followed by a 15 min incubation on ice. Then the tubes were placed in a 42°C water bath for 50 s without mixing. The tubes were returned to ice for 30 min, at which time 950 μL of SOC medium (20 mM glucose, 2% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract and 0.5g/L NaCl) was gently added. The tubes were incubated for 60 min at 37°C and 100 μL were plated onto room temperature LB/ampicillin (100 mg/L) agar plates. Optional screening

for both Ampicillin resistance and lack of ß-galactosidase activity did not occur, as colony numbers that grew on ampicillin were manageable, and the loss of ß-galactosidase activity was known by experience to be an unreliable indicator.

The resulting colonies were streaked on LB/amp plates and single colonies were taken from these to grow up larger amounts of the putative transformants for plasmid DNA isolation.

2.8.3. Isolation of plasmid DNA

All *E.coli* plasmid DNA (pDNA) isolated from 3 mL LB/amp overnight. cultures was isolated using the Lysis-Boiling method (Sambrook et. al. 1989). This gave pDNA that could be used for restriction digests, although RNAase (1 mg/mL) was required to remove large amounts of RNA carried over via this isolation method. For sequencing purposes it was necessary to clean up this pDNA using QIAquick columns. For isolation of larger amounts of pDNA from 30 mL of overnight culture, the Qiagen Midi-prep kit was used. This gave pDNA of purity that was appropriate for direct sequencing.

Plasmid DNA was isolated from *A.tumefaciens* using a method provided by Dr. Eugene Diatlof (Dept. of Botany, University of Adelaide) with some adaptation. A 5.0 mL culture was pelleted by centrifugation and resuspended in solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris pH 8.0). Lysozyme (10 mg/mL) was added followed by a 10 min incubation at room temperature. A solution containing 1% SDS and 0.2 M NaOH was then added (300 µL) and the mixed incubated at room

temperature for another 10 min. After this 45 µL of chloroform:phenol:iso-amyl alcohol (24:1:1) was added and well mixed. A final addition of 3 M $NaCH_3COOH$ (225 µL) was mixed in and the entire mixture left at -20°C for 20 min. Centrifugation at 15 800g in a microfuge gave a clear supernatant which was carefully transferred to a fresh tube. The DNA was precipitated by adding 500 µL of isopropyl alcohol and, after centrifuging for 10 min at 15 800g, the pellet was rinsed twice with 70% ethanol. The pellet was then dried and resuspended in 90 µL deionised H_2O, and then mixed with 90 µL of 4 M LiCl and 20 µL 3 M $NaCH_3COOH$ and placed on ice for 30 min. After spinning down the pellet, the supernatant was precipitated in a fresh tube with 500 µL of 95% ethanol. This pellet was vacuum dried and resuspended in 30 µL deionised H_2O and of this total amount, 10 µL were used for restriction digests.

2.8.4. Isolation of genomic DNA

Plant tissue grown under sterile conditions was used for genomic DNA (gDNA) extraction when this DNA was to be used for PCR. Genomic DNA for all other purposes was extracted from either sterile plant tissue or plants grown under glasshouse conditons, with the leaf tissue rinsed several times in dH_2O. This extraction method is adapted from that used by Roxy Nicholls (pers.comm.) (Michigan State University-Dept. of Energy, Plant Research Laboratories). Approximately 0.1 g of leaf tissue was frozen (liquid N_{l2}) in screw-topped eppendorf tubes, and then ground to a fine powder using an autoclaved

pestle (Adelab plastic pestle). To each tube 500 μL of hot (80°C) extraction buffer (100 mM LiCl, 100 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS, all mixed in a 1:1 ratio with neutralised phenol) was added and vortexed for 60 s. To this mixture 250 μL of $CHCl_3$ was added and vortexed for another 30 s. The tube was then centrifuged in a microfuge for 5 min at 15 800g. The clear supernatant (containing RNA and DNA) was removed and placed in a new tube and an equal volume of 4 M LiCl was added, followed by incubation at -20°C for 1h or greater. The pellet was collected by centrifuging at 15 800g for 15 min. This pellet contained the majority of leaf RNA, and after removal of the supernatant (containing some RNA and the gDNA) the pellet was frozen at -80°C for future use. The supernatant was mixed with two volumes of 95% ethanol and incubated at room temperature for two hours. The sample was then centrifuged at 15 800g at 4°C for 30 min. The pellet was dried and resuspended in TE or dH_2O, followed by extractions once with phenol:$CHCl_3$, then with $CHCl_3$ and finally with 95% ethanol. The pellet was washed twice with 70% ethanol. It was then dried and resuspended in deionised H_2O.

2.8.5. Small scale genomic DNA extraction for PCR analysis

Extraction of genomic DNA for PCR analysis was used in the screening of putative transgenic plants. This was carried out using limited leaf material, generally less than 10mg. The method #2 outlined in Rogers et. al. (1996) was followed. Approximately 5 μL of the final genomic DNA solution was used in a 25 μL PCR reaction

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mix. PCR procedures were as described previously (section 2.8.1) using primers AraAox1 and 2 or primers 2511A and B as indicated.

2.9 Purification of AOX protein from *E.coli*

Overnight bacterial cell cultures were centrifuged (500 g) for 15 min at 4°C and the drained pellet was weighed. For each 1 g of pellet, 3 mL of lysis buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 100 mM NaCl) was added and the pellet resuspended. To this mixture 8 µL of PMSF (50 mM) per gram of pellet, was added, followed by 80 µL of lysozyme stock (10 mg/mL). This was incubated at 4°C with occasional stirring for 20 min. Deoxycholate (4 mg per gram of pellet) was added whilst stirring continuously and the mixture was placed at 37°C and stirred with a glass rod. When viscous, 20 µL of DNAase I (1 mg/mL) per gram of pellet was added and the lysate was placed at room temperature until it was no longer viscous (approx. 30 min). It was then centrifuged (12 000 g) for 15 min at 4°C and the supernatant decanted. The pellet was resuspended in 9 X volume of lysis buffer supplemented with 0.5% Triton X-100 and 10 mM EDTA (pH 8.0) and was left at room temperature for 5 min. This was then centrifuged (12 000 g, 15 min) and the pellet resuspended in 100 µL of H_2O. Polyacrylamide gel electrophoresis of this solution was used to determine the presence and size of the alternative oxidase protein, produced in *E.coli*.

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2.10 Polyacrylamide gel electrophoresis

Protein samples were run on Tris-glycine polyacrylamide gels that were either purchased or poured manually. The latter had a stacking gel which contained 5% bis-acrylamide (from a 30% stock solution of acyrlamide: bis-acrylamide 150:4 in deionised H_2O), 125 mM Tris-HCl (pH 6.8), 0.1% SDS, 0.1% ammonium persulphate and 0.1% (v/v) TEMED. The resolving gel contained 10% bis-acrylamide, 375 mM Tris-HCl (pH 8.8), 0.1% SDS, 0.1% ammonium persulphate and 0.15% TEMED. Protein samples of 25-100 μg (as labelled) were loaded in loading buffer (0.25 M Tris pH6.8, 8% SDS, 40% glycerol, 0.002% bromophenol blue, +/- 0.4 M DTT). Samples mixed with buffer were boiled for two min at 90°C before loading. Electrophoresis occurred at a constant voltage (125V) with current varying from 40-65 mA for 1-2h. The running buffer (0.1 M Tris pH 8.3, 0.1% SDS and 0.1 M tricine).

2.11 Immuno-blotting

After electrophoresis the separated proteins were transferred from the gel matrix to a PVDF membrane using the semi-dry method described in Soole (1989). After transfer the PVDF membrane was briefly washed in deionised water followed by a 15 min wash in TBS (20 mM Tris, 0.5 M NaCl pH 7.5). It was then incubated at room temperature with constant shaking for 45 min in a blocking solution (5 X TBS, 0.3% (w/v) BSA). This was washed off using TTBS (TBS, 0.25% (v/v) Tween-80) with three 5 min washes. The membrane was

incubated overnight in TTBS containing the primary antibody raised against the alternative oxidase from *S.guttatum* (Elthon and McIntosh 1987b). This was washed off with three 5 min washes using TTBS and the membrane was then incubated for 2 h with the secondary antibody, a rabbit anti-mouse antibody conjugated with alkaline phosphatase. The excess secondary antibody was washed off with three 5 min TBS washes. The proteins on the membrane, that were recognised by the primary antibody, were visualised by incubation with the alkaline phosphatase substrates (0.0065% (v/v) NBT, 0.00325% (v/v) BCIP) in a buffered solution (0.1 M Tris (pH9.5), 0.1 M NaCl, 5 mM $MgCl_2$) for 10 min. The colour change reaction was stopped by washing in deionised water.

2.12 Ethanol assay of transgenic leaf material

To determine whether transgenic leaf tissue, verified by PCR analysis, was copper-inducible, each plant line was tested for ethanol production under axenic conditions, in the presence of AA. All solutions were sterile and all procedures were done in a clean-air station. Wildtype and transgenic plants were sprayed with 5.0 μM $CuSO_4$ foliar spray 5 days before harvesting. This meant that on the day of harvesting leaves and beginning the ethanol assay, the antisense transcripts would be maximally expressed. A "booster" spray was repeated 3 days before harvesting. This was to ensure the maintenance of high levels of antisense transcript for the entire length of the assay. Harvested leaves were weighed and then floated, adaxial

side up, on 2 mL of nutrient medium containing 1X MS salts, 1X G5 vitamins, 3% sucrose, 5.0 µM $CuSO_4$ and 25 µM AA in multiwell trays. These trays were shaken gently for 24 to 72h under ideal growth conditions. After each 24h period a 1 mL sample was taken from each well and immediately frozen with liquid nitrogen until assayed for ethanol concentration on a spectrophotometer. Each well had 1 mL of fresh medium replaced and was further incubated. Fresh weights were taken at the commencement of the assay and the end. Leaf samples were also dried down for final dry weight measurement. Once all nutrient samples had been collected, they were thawed and measured for ethanol concentration using the ethanol assay kit by Boehringer-Mannheim (cat # 176 290).

CHAPTER THREE : CHARACTERISATION OF *Arabidopsis thaliana* (ecotype Columbia) WILD TYPE ALTERNATIVE OXIDASE

3.1 Introduction

Characterisation of the alternative pathway (AP) in *Arabidopsis thaliana* has previously been limited to one paper (de Virville et. al. 1994) despite the ease of generating cell suspension cultures and the availability of the *aox* cDNA sequence (Kumar and Soll 1992). Before the current studies involving transgenic *aox1* antisense plants could be initiated, it was necessary to characterise the AP and *aox1* expression in WT plants.

A.thaliana was chosen as the ideal plant for this project because it could be easily transformed and because cell cultures had shown a considerable amount of AOX activity (de Virville et. al. 1994). Purified mitochondria from cell suspension cultures grown under ideal nutrient and temperature conditions showed AP rates of 49 ($\pm$ 4.0) $nmolO_2$/min/mg protein and 10 ($\pm$ 5.0) $nmolO_2$/min/mg protein with succinate and NADH as the substrate respectively. This encouraging result occurred without the stimulatory effect of additional pyruvate, and compared favourably with the highest non-aroid rate known to date i.e. purified mitochondria from the roots of *Glycine max* (98 ($\pm$ 20) $nmolO_2$/min/mg protein and 80 ($\pm$13) $nmolO_2$/min/mg protein using succinate or NADH as substrate in the presence of pyruvate (Day et. al. 1995).

The AP of *A.thaliana* had not been previously characterised. This study looks at the AP during ideal growth conditions and under stress. The activity of the AP and the amount of AOX present as well as its behaviour under reducing conditions were studied. The AP of mitochondrial respiration is believed important due to its marked induction in plants experiencing environmental stress (Day et. al. 1980). This section examines the response of *A.thaliana* to a number of different stress treatments including respiratory inhibition, chilling, aetiolation and application of SA.

1. Respiratory inhibition

Antimycin A is a potent inhibitor of the CP, blocking the transfer of electrons from cytochrome b to cytochrome c_1 within complex III, a UQH_2-cytochrome c reductase complex. Plant cells inhibited with AA may still respire utilising the alternative and/or fermentative pathways. Incubation of tobacco cultures with 2 μM AA has been shown to induce *aox1* expression and increase AP capacity (Vanlerberghe and McIntosh 1994). The response of *A.thaliana* cell cultures to incubation with AA is investigated in this study.

2. Chilling

Enhanced AP activity has been seen during the chilling of tobacco cell cultures (Vanlerberghe and McIntosh 1992). For this reason, as well as similar responses in other plant species (for a summary see Vanlerberghe and McIntosh 1992) chilling was chosen as a stress treatment for *A.thaliana*. Induction of the AP in response to

chilling has been studied in plants such as maize, tobacco, cucumber, rape, potato and soybean. In each case there was an apparent increase in AP capacity. Vanlerberghe and McIntosh (1992) used tobacco cell culture to show both an increase in AP capacity and an increased amount of AOX protein. The AP in chilled cultures (24 hours at 18°C) showed a five fold higher capacity in comparison with non-chilled cultures. Both the increased capacity and the increase in comparative AOX protein, seen via immunoblots, was reversible.

A.thaliana was considered a good candidate for studying the AP during chilling stress because, as with the majority of the plant species tested and listed above, it undergoes cold acclimation. Cold acclimation is the process whereby initial exposure to low, non-freezing temperatures allows a plant to be more resistant to freezing temperatures (Burdon et. al. 1994, O'Kane et. al. 1996). Chilling damage occurs when the membranes become dysfunctional and "leaky". In chloroplasts and mitochondria there is leakage of electrons such that reactive species are formed. This leads to increased lipid peroxidation and decreased membrane function, and ultimately to cell death. Cold acclimated plants can effectively "protect" themselves against such damage by changing the ratio of unsaturated:saturated fatty acids in the lipids of cellular membranes, or by increasing enzymic activity directed against reactive oxygen species such that lipid peroxidation does not occur, or by inducing plant growth factors such as ABA which promote cold acclimation through a combination of

effectors (O'Kane et. al. 1996). Whilst the exact mechanism of cold acclimation is not yet fully understood, the induction of the AP in plants that have been chilled and undergo acclimation is of great interest when examining the role of the AP during stress. Chilling treatment was used as a stress in this study to investigate whether *A.thaliana* responded with an increased AP capacity and AOX protein levels. This may indicate that during the process of cold treatment, the AP is important in reducing the amount of reactive oxygen species (ROS) in the plant's cells and would ascertain the appropriateness of studying the inducible transgenic (*aox1*) plants with this stress.

3. Aetiolation

Another form of stress used in this study was dark treatment, alone and in conjunction with chilling. Studies by Atkin et. al. (1993) using Belgian endive leaf slices had shown that dark grown plants had little AP capacity whereas plants grown in continuous light had significant AP capacity. (The capacity of the AP is a measurement of the cyanide-resistant, SHAM-sensitive respiration rate. This does not necessarily reflect the actual AP activity in the plant tissue.) In terms of AP activity Atkin et. al. (1993) found no difference between the two treatments. Movement of plants into continuous light gave subsequent increases in AP capacity and indicated to Atkin et. al. (1993) that light itself might responsible for the induction of the AP. Aetiolation was used with and without chilling to determine if plants of *A.thaliana* would respond by altering their AP activity compared to

the control (16h light) treatment plants. Research by Mawson in 1994 used guard and mesophyll cell protoplasts of *Vicia faba* to study AP respiration after dark treatment. Dark treated guard cell protoplasts showed a lack of AP activity whereas dark treated mesophyll cells increased their %SHAM sensitivity to 25-30%.

In both of the above cases (Atkin et. al. 1993, Mawson 1994), adenylate control of respiration played a part in influencing the calculated %SHAM sensitivity. This can be seen by the addition of an uncoupler, relieving adenylate control and giving rise to an increased rate. This needs to be considered when studying photosynthetic plant material where rates may be limited by substrate availability (see section 3.2.2).

4. Salicylic acid (SA) treatment

The increased level of SA seen in developing arum inflorescent spikes was the first indication that SA may be involved in the signal transduction pathway leading to thermogenesis (Raskin et. al. 1987). SA is known as an endogenous elicitor, produced in the male floral region, moving from this region to the appendix on the day prior to anthesis (Raskin et. al. 1987). The induction of thermogenesis by SA requires light and results in an accumulation of *aox* transcripts in the appendix tissue of aroid species such as *S.guttatum* (Rhoads and McIntosh 1993a). Concurrent with this transcript accumulation is the increase in AOX protein. As described previously thermogenesis is a heat-producing event in some aroid inflorescences, occurring due to a

massive increase in AP activity. (For more details see section 1.2). SA also has an effect on the AP of non-aroid tissue, such as *Beta vulgaris* roots and tobacco cell cultures, showing induction of AOX protein, AP activity and increased AP capacity (Johnson 1993, Kalpulnik et. al. 1992; Rhoads and McIntosh 1993a; Lennon et. al. 1997).

SA-treatment was used in this project to determine if *A.thaliana* had a similar response, namely that of increasing its level of AOX protein and maximal AP activity. This would indicate that SA is involved in the plant's mechanisms to induce expression of the *aox1a* gene.

3.2 Material and methods

3.2.1 Plant, callus and cell culture material

Whole plants and calli were grown in magenta boxes containing germination media (plus callus-inducing growth factors where appropriate) as outlined in Chapter two. Forty-eight hours prior to sampling, these boxes were placed in darkness at 4°C or 24°C. A control box was also left at 24°C in the light. Plants sprayed with SA (1 mM) were sprayed to drip point and left for three days before leaves were harvested and slices cut into a solution of 0.5 mM $CaSO_4$. Cell suspension cultures were grown under ideal temperature and aeration requirements as described in Chapter two. SA (1 mM) or AA (25 µM) were added at the time of subculturing into new media. Booster additions of each treatment were made the evening of day two,

approximately 12 hours before testing on the oxygen electrode. Mitochondrial experiments were carried out on isolations from fresh, leaf tissue using the protocol described in Chapter two.

3.2.2 Oxygen electrode assays

The AP of *A.thaliana* was characterised by studying fresh leaf tissue, callus tissue derived from leaf material and cell cultures using an oxygen electrode following the method described in Chapter two. Leaf tissue was finely sliced and these slices were gently blotted and weighed before adding to the oxygen electrode. Measurements were made using KCN and SHAM as inhibitors at concentrations sufficient for the maximum effect during the time of the experiment. With all sample sets of tissue slices and calli, FCCP was used to test for its ability to uncouple the CP. In each sample set when FCCP was unable to accelerate CP activity it was assumed that this pathway was already functioning at maximal capacity.

3.2.3 Protein electrophoresis and immunoblot analysis

Protein gel electrophoresis and immunoblotting were carried out using the method described in Chapter two.

3.3 Results and Discussion

Whilst many factors contributed to the suitability of *A.thaliana*, the size of the plant was not ideal for the purification of mitochondria from leaf tissue, hence leaf studies were completed using leaf tissue slices on the oxygen electrode. Where possible purified mitochondria were used but cell suspension cultures and calli were generated when

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fresh, plant tissue was limited.

3.3.1 Effects of dark and dark plus chilling treatments on the AP activity of WT *A.thaliana*.

Table 3.1 shows the oxygen consumption results using WT leaf tissue, where control plants were kept at normal growth cabinet conditions, dark treatment plants were kept at growth cabinet temperatures for 48H without light, and chilled plus dark treatment plants were kept at 4°C for 48H without light. All plants were approximately six weeks old. $\%SHAM_{sens}$ was calculated by the following formula :

$$\%SHAM_{sens} = 100 - \%SHAM_{insens}$$

$$\%SHAM_{insens} = 100 \text{ X } \frac{\{(rate + SHAM) - residual\ rate\}}{\{uninhibited\ rate - residual\ rate\}}$$

Similar calculations were made for KCN_{sens} rates. These rates represent the measurable amount of electron flow deflected from the AP ($\%SHAM_{sens}$) or CP (KCN_{sens}) when chemical inhibitors are added to cells or tissue respiring at a maximal rate.

Table 3.1 : *SHAM- sensitive and KCN- sensitive rates (expressed as a percentage of uninhibited rates) in leaf slices either untreated, dark or chilled plus dark treatment. Standard deviations are given in parentheses. Oxygen consumption rates (nmolO$_2$/min/mg fwt) in the absence of inhibitors (uninhibited rates) are also given for each treatment. (n=the number of different experiments)*

leaf slices	%SHAM$_{sens}$	n	%KCN$_{sens}$	n	Uninhibited rate nmolO$_2$/min/mg (fwt)	n
control	27.7 (8.7)	4	69.9 (8.6)	4	188.8 (104.8)	8
dark treatment	43.8 (17.1)	5	70.8 (7.0)	4	92.7 (60.8)	9
chilled + dark treatment	51.2 (13.7)	4	58.1 (10.2)	5	97.8 (35.7)	9

The results in table 3.1 show that there was little change in the %KCN sensitivity between the non-treated, dark and chilled plus dark treated plants. The percentage SHAM sensitivity, however, was increased in both treatments. Treated plants increased their SHAM sensitivity (%) by up to 1.8 times the sensitivity of control plants. Differences between the dark and chilling plus dark treatments was not significant due to the variation inherent in measuring leaf slices. These results show that during maximal electron flow through the AP (after KCN addition) all tissues regardless of treatment were able to utilise this pathway to the same degree, however when the mETC was uninhibited and SHAM was added, proportionally more electron flow could be diverted from the AP of dark and chilled plus dark treated tissue than in control plants. This implies that these plants had increased their use of the AP, and that dark stress alone was enough to cause this.

Calli were also subjected to the same treatments and the results are presented in table 3.2. All callus material was between 1.5 and 2.5 weeks of age.

Table 3.2 : *SHAM- sensitive and KCN- sensitive respiration rates (expressed as a percentage of uninhibited rates) of callus either untreated or treated by chilling plus darkness or darkness alone. Rates in the absence of inhibitors (uninhibited rates) are also given for each sample. Standard deviations are given in parentheses. (n=the number of different calli groups tested.)*

callus	%SHAM$_{sens}$	n	%KCN$_{sens}$	n	Uninhibited rate nmolO$_2$/min/mg (fwt)	n
control	31.2 (9.3)	6	62.2 (14.4)	8	355.8 (94.9)	11
dark treatment	31.0 (12.4)	4	74.4 (9.1)	5	333.1 (47.4)	7
chilled + dark treatment	42.2 (5.1)	5	72.2 (4.1)	5	311.0 (29.9)	10

In both %KCN sensitivity and %SHAM sensitivity there was no significant trend. All calli from both treatments and control behaved in a similar manner. Darkness and chilling plus darkness treatment had no obvious impact on cellular respiration in comparison with the control cells' response. This may be explained by the absence of green tissue in calli which have their carbohydrate requirements provided by the medium on which they are cultured. Callus tissue growing in this manner is unlikely to experience substrate limitation.

The fluctuations between periods of carbohydrate availability and production in the leaf tissue would be greater than in callus. Any interference with the ability of leaf tissue to photosynthesise is expected to affect respiration due to the sudden decrease in

carbohydrate availability. The cellular demands for energy and carbon skeletons however aren't likely to be greatly reduced by short term dark treatment. This could lead to an imbalance within the cell unless carbohydrate stores are rapidly mobilised. For this reason, when looking at both leaf and callus treatments, substrate limitation becomes a problem only in dark-treated leaf tissue and this is reflected in the uninhibited rates of respiration. Calculations of the actual SHAM-sensitive and KCN-sensitive rates showed that SHAM-sensitive rates in the dark treated tissue were lower than those of the control. This means that while the percentage rate values indicate that leaf tissue showed an increased sensitivity to SHAM after dark treatment, actual rates indicate that control plants were able to partition electron flow through the AP at a greater rate than treated plants. When considering the induction of AP activity and/or protein levels, inhibitor studies alone can be misleading. For a more accurate analysis investigation of purified mitochondria from leaf tissue need to be studied.

3.3.2 Effects of SA and AA on the AP activity of WT *A.thaliana*.

Table 3.3 shows the results of 1 mM salicylate foliar spray treatment of *A.thaliana* plants. Uninhibited rates of the leaf slices indicate that the SA-treated leaves were respiring at an increased rate compared to untreated plants. To test if this was due to the uncoupling effect of SA upon the mitochondria, leaf slices were titrated with increasing concentrations of FCCP. No increase in

respiratory rates was seen with either control or treated leaf slices.
This showed that SA was not having an uncoupling effect on the SA-
treated leaf slices, as the unihibited rate was not increased to the
treated rate upon addition of FCCP. The reason for the increased
uninhibited rate in SA-treated plants is not clear.

Table 3.3: *SHAM- sensitive and KCN- sensitive rates (expressed as a percentage of uninhibited rate) of leaf slices with and without a foliar spray SA-treatment. Standard deviations are given in parentheses. (n=the number of separate experiments.)*

leaf slices	%SHAM$_{sens}$	n	%KCN$_{sens}$	n	Uninhibited rate (nmolO$_2$/min/g(fwt)	n
control	26.6 (3.0)	3	54.7 (13.7)	3	188.2 (12.6)	5
SA-treated	9.1 (5.1)	3	50.3 (6.3)	4	270.8 (46.4)	8

When investigated as a percentage of the uninhibited rate there was
little variation in %KCN sensitivity between treated and untreated
plants. However SA-treated plants were less sensitive to inhibition by
SHAM. This result implies that in the leaves of SA-treated plants less
electron flow is occurring through the AP. This was not expected as
SA has been shown previously to induce the activity of the AP which
would render plants more SHAM-sensitive. Previous studies using
purified beetroot mitochondria from tissue that had been aged for 5
days in the presence of SA showed that there was an increase in
maximal AP activity and AOX protein levels (Johnson 1993). This
increase was over 8-fold and was above that seen in aged (without SA)
tissue (Johnson 1993). Similarly Kalpulnik et. al. (1992) added SA to

tobacco cell cultures and also found an increase in AP capacity. The inductive effects of SA were also shown by Rhoads and McIntosh (1993a) using a different cultivar of tobacco. They found a dramatic accumulation of the AOX protein by 12 hours. This increase was diminished by the addition of actinomycin D one hour before SA addition and was completely blocked by the addition of cycloheximide. This indicated that in order for SA to have an effect *de novo* transcription and translation were required. Lennon et. al. (1997) treated tobacco leaves with 1mM SA and for a period of time and purified and tested the mitochondria from them. They found that AOX protein levels increased 2.5-fold within 2 hours of SA-treatment and reached its maximum of 9-fold after 12 hours. Even three days after treatment the levels of AOX were twice that of the control plants. Corresponding $O^{18}:O^{16}$ oxygen isotope discrimination studies on this material found that although the levels of AOX protein had changed, the electron partitioning between the CP and AP were similar between the control and the treated samples at all times during treatment. This showed that whilst SA was capable of inducing aox gene expression, it was unable to alter electron partitioning.

The results in table 3.3, showing the unexpected decrease in SHAM-sensitivity upon SA treatment, may be explained with regard to electron partitioning. These rates represent the measurable SHAM-sensitivity after inhibition of the electron flow shared by the two mETC pathways. The decreased %SHAM sensitivity seen in the SA-

treated leaves may not just indicate the decreased use of the AP **before** inhibition has taken place, but it may also indicate an increased capacity by the CP to absorb the deflected electrons **after** inhibition by SHAM. In order to accurately distinguish which or whether both of these effects are occurring, a gas-phase system for on-line measurement of $O^{18}:O^{16}$ oxygen isotope discrimination during respiration is required (for more details on this system see Robinson et. al. 1995).

Cell cultures were used to further investigate the effects of SA. In addition to SA treatment, cells were incubated with AA alone. AA, a CP inhibitor, is known to increase *aox1a* transcription in *A.thaliana* (Saisho et. al. 1997). The results presented in table 3.4 show that cells in culture with SA added respond differently to plants treated with a SA foliar spray.

Table 3.4 : *Oxygen consumption of wild-type cell suspension cultures, including a control and two treatments (1 mM salicylic acid and 25 μM A A). All rates are expressed as a % of the uninhibited (cells respiring in the presence of 0.02 μM FCCP) rate. Standard deviations are given in parentheses. (n=the number of separate experiments.)*

cell cultures	%SHAMsens	n	%KCNsens	n	Uninhibited rate $(nmolO_2/min/g(fwt)$	n
control	30.2 (4.9)	5	70.1 (14.7)	6	1264.5 (344.5)	8
SA-treated	15.4 (1.9)	5	49.2 (8.8)	6	1243.5 (416.7)	7
AA-treated	71.4 (1.8)	6	15.9 (2.2)	4	830.3 (104.1)	7

In comparison with untreated cells, SA-treated cells became less cyanide sensitive as well as less SHAM sensitive whilst the overall

uninhibited rates for both remained the same. The percentage of KCN sensitivity in SA-treated cells was only 70.1% of the uninhibited value. This implied that when inhibited by KCN, cells treated with SA had a far greater ability to divert electron flow through the AP than the control cells. However when SHAM was added to uninhibited cells less cellular respiration (in percentage value) was affected in SA treated cells. SA-treated cells showed approximately half as much SHAM sensitivity compared to the control response. This indicated that while there appeared to be an increased AP ability to absorb the diverted flow of electrons in the SA-treated cells, when these cells were presented with SHAM they showed less sensitivity than control cells, namely that a lower proportion of cellular respiration was via the AP. In other words, SA-treated cells appeared to have an increased AP component of cellular respiration, but this pathway did not appear to be in use.

The comparison was also made between SA-treated and AA-treated cells. While the effects of SA or AA upon the AP had not been previously studied in *A.thaliana*, the induction of *aox1a* transcription and AP capacity by AA had been seen in tobacco cultures (Vanlerberghe and McIntosh 1994). More recently it has been investigated by Saisho et. al. (1997) who noted that young *A.thaliana* plants treated with AA had a marked induction of *aox1a* transcripts. This implied that when the CP was incapable of full function during plant growth, the plant responded by increasing the amount of AOX

present in the mitochondrial inner membrane. The AA treatment used in this section investigated whether the respiratory effects seen in tobacco by Vanlerberghe and McIntosh (1994) would also be evident in *A.thaliana*, and it provided further comparison with the other treatments. A comparison between SA-treated cells with cells having high levels of AP capacity (induced by AA) was considered of value in evaluating the effects of SA on *aox* expression.

The results in table 3.4 show that AA-treated cells had a far lower uninhibited rate compared to both control and SA treated cells. This was expected due to the inhibition of the CP by AA. Because AA is rapidly broken down in solution, booster additions were made at approximately 12 hours prior to measurement. This addition, in the case of AA, was expected to affect the uninhibited rates. Rate comparisons are difficult between cultures where a treatment, such as AA, is lethal. During analysis any effects that are seen may be converted back to actual respiratory rates, however these represent a minimal value of the true rate. This is because they are expressed as units per fresh weight, a component of which will be dead cells. A more accurate assessment could be made if two criteria could be fulfilled, namely (a). treated cells could be washed repeatedly before measurement and assumed free of exogenous AA, and (b). a method of discerning live cell:dead cell fresh weight was used and applied to both treated and untreated cells. Until this is possible the results in table 3.4 represent values lower than the true induction of SHAM

sensitivity by AA treatment. However, these results do show that AA treated cells were the least affected by KCN inhibition when compared with SA-treated cells (15.9% c.f. 49.2% KCN sensitivity) and control cells (15.9% c.f. 70.1% KCN sensitivity) and the obvious KCN sensitivity in the AA treated cells (15.9%) indicates that the CP was in use during AA treatment. These results suggest that the treatment with AA allowed the cells to greatly increase their ability to divert electron flow from the CP to the AP when presented with KCN. In comparing SHAM sensitivity, the AA-treated cells were more highly sensitive (71.4%) than the control cells (30.2%) or the SA-treated cells (15.4%). In terms of the actual SHAM-sensitive respiratory rate, that is, taking into account the lower uninhibited rate in the AA treated cells, this rate was well above that of the untreated cells. It suggests that the AA-treated cells were primarily using the AP during cellular respiration and had a lesser ability to absorb electron flow by the CP once SHAM had been added. This supports the recent results of Saisho et. al. (1997) where AA treatment induced *aox1a* transcription in *A.thaliana* and therefore an increase in AOX protein is to be expected.

3.3.3 Protein electrophoresis and immunoblot analysis

Figure 3.1 shows a Coomassie Brilliant blue-stained SDS-PAGE gel of purified *A.thaliana* mitochondrial proteins from leaf tissue. Due to the large number of protein bands no comparative analysis between chilled and control leaf mitochondria was possible.

Figure 3.2 shows an immunoblot of purified mitochondrial proteins from *A.thaliana*. Visible bands have cross-reacted with antibodies raised to *S.guttatum* AOX proteins. Mitochondrial protein isolated from *Arum italicum* spadices was used as a positive control and displayed a prominent band at 37 kDa.
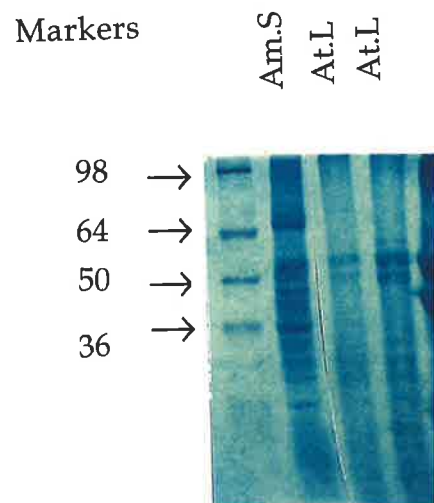
Figure 3.1 : Coomassie Brilliant blue-stained SDS polyacrylamide gel of purified mitochondria from *A.thaliana* leaves (At.L) and *A.maculatum* spadices (Am.S). At.L and Am.S lanes contained approximately 75 and 25 μg of total protein respectively. The positions of molecular mass markers (kDa) are indicated in the left margin.
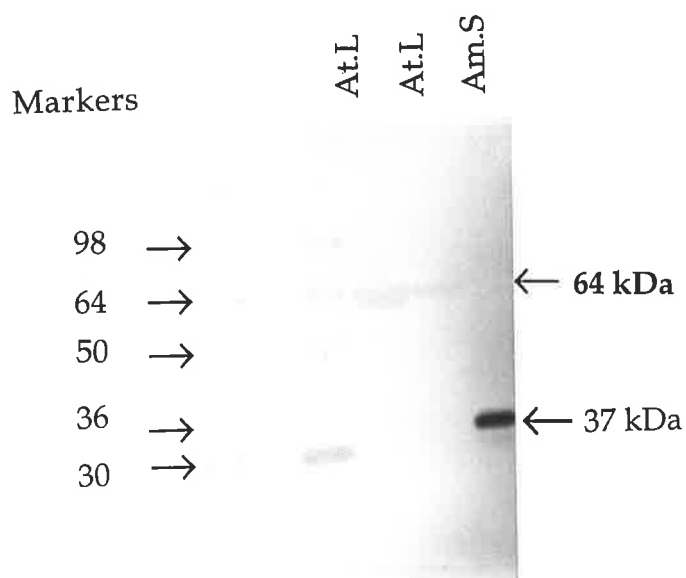
Figure 3.2 : Immunoblot of purified mitochondria from *A.thaliana* leaves (At.L) and *A.maculatum* spadices (Am.S), using antibodies raised against *S.guttatum* AOX protein. At.L and Am.S lanes contained approximately 75 and 25 µg of total protein respectively. Arrows indicate the 64 kDa At.L band and the 37 kDa Am.S band recognised by the *S.guttatum* AOX antibodies. The positions of molecular mass markers (kDa) are indicated in the left margin.

The *A.thaliana* mitochondrial lanes showed a prominent band at approximately 64 kDa and occasionally a fainter band at 28 kDa. This banding pattern which is typical of the dimer and monomeric patterns seen with AOX proteins of other species (Day et. al. 1994; Umbach and Siedow 1993; Elthon and McIntosh 1986), appeared independent of the inclusion of reducing agent DTT in the loading buffer. This was not expected and differed from all other plant species studied. Other investigators have shown with other plant species (e.g. *G.max*, *S.guttatum* and tobacco) that omission of reducing agents in the loading buffer gives rise to the appearance of the dimeric form of AOX (Day et. al. 1994; Umbach and Siedow 1993) (see section 1.5 for more detail). Further investigation using a range of reducing and oxidising agents at increasing concentrations is required.

3.4 Conclusion:

3.4.1 Effects of treatments on cellular respiration

From the oxygen electrode assays using leaf slices, calli and cell cultures the following has been shown;

1. Dark treatment increased the percentage SHAM sensitivity in leaf cells but had no effect on callus material.

2. Foliar spraying with SA decreased SHAM sensitivity in leaf cells **without** affecting KCN sensitivity.

3. Treatment of cell cultures with SA decreased SHAM sensitivity **and** KCN sensitivity.

4. AA treatment of cell culture greatly increased SHAM sensitivity and decreased KCN sensitivity.

These results suggest that electron partitioning to the AP is increased in *A.thaliana* leaf tissue by dark treatment and in cell cultures by the presence of AA. In contrast, treatment of plant tissues with SA had the opposite effect to that expected. Previous research has shown that SA induces AP capacity in *B.vulgaris* and *N.tabacum* (Johnson 1993 and et.al. 1992). The results presented here indicate that in *A.thaliana* plants, SA does not induce AP activity but may in fact suppress AOX activity. Cell cultures treated with SA appeared to have a greater ability than WT to utilise the AP above that used when challenged with KCN. In this case it seemed that these cells had increased amounts of AOX. Protein gels and immunoblots of purified mitochondria from SA-treated plants will allow an investigation of this.

From all of the different types of stress studied here, AA appears to be the most effective AP inducer and may be considered when approaching the study of inducible antisense plant material with and without stress. The plant cell responses to the other forms of stress tested did not appear to involve increased AP activity in the WT tissue. They would still be of interest however in the study of antisense plants with compromised *aox1a* expression.

Future work could include measuring the uninhibited electron partitioning by the CP and AP using oxygen isotope discrimination techniques. This would give a greater understanding of what occurs

without the effects of inhibitors. As well as this, a time study of *aox1a* expression in treated cell cultures and leaf tissue would allow a more accurate appraisal of whether any increase in AP activity is due to *de novo* protein synthesis or due to activation of the AOX protein already present.

3.4.2 Protein electrophoresis and immunoblot analysis

The results obtained via gel electrophoresis and immunoblotting have shown that *A.thaliana* possesses two proteins of size 28 and 64kDa which cross-react with the antibody raised to *S.guttatum* AOX proteins. The larger of the two bands was most prominent and from its size is likely to be the dimeric form of the *A.thaliana* AOX protein. The smaller band appears to be the monomeric form of the AOX. This pattern is similar to that seen with other plant species although unlike these other plant species the proportion of monomer : dimer in *A.thaliana* does not appear to be dependent on the absence or presence of DTT, a reducing agent. No other reducing or oxidising agents were used. This could be an area for further study.

CHAPTER FOUR : GENOMIC SEQUENCE AND GENE COPY NUMBERS FOR ALTERNATIVE OXIDASE

4.1 Introduction

Any studies directed towards "silencing" a gene through an antisense approach requires knowledge of the expression of that gene in the tissue to be studied. At the commencement of this project only one copy of the *aox* gene was believed to exist in *S.guttatum* (Rhoads and McIntosh 1993a) and *A.thaliana* (Kumar and Soll 1992; Whelan and Day, unpublished) from Southern analyses of gDNA, using cDNA *aox* probes. The cDNA sequence from *A.thaliana* was obtained from a cDNA library (Kumar and Soll 1992). Its translated product showed 71% amino acid sequence identity with the AOX sequence from *S.guttatum*. In *A.thaliana* only one AOX protein band was seen via immunblotting using mitochondrial proteins purified from leaves, unlike *S.guttatum* or *G.max*. This implied that there was only one *aox* gene product expressed in *A.thaliana* leaf tissue.

4.1.1. Evidence for more than one *aox* gene

In 1995 evidence was found for more than one *aox* gene in the species *G.max* and *N.tabacum* (Whelan et. al. 1995b; 1995c). This work was undertaken because the multiple protein bands identified on immunoblots, using specific antibodies to AOX (see section 1.5.1), did not appear to be due to either protein modification or different import characteristics (Whelan et. al. 1995a). This implied that these different

protein bands were the protein products of different *aox* genes. Whelan et. al. (1995b;1995c) used a PCR approach to amplify homologous regions of different *aox* genes in *N.tabacum* and *G.max*. Primers were designed from amino acid sequence alignment studies, identifying strictly conserved regions of AOX across a number of species. These primers had a degree of degeneracy and were expected to generate a 120 bp sized DNA PCR product. The resulting product in *G.max* was 170 bp. Sequence analysis of a number of clones carrying this product revealed three different 170 bp sequences, which were named *aox1*, *aox2* and *aox3*. Clone *aox1* showed 100% homology to the *G.max* cDNA sequence. Differences in the sequences of *aox2* and *3* were significant indicating that they were the products from two different genes (Whelan et. al. 1995c). Comparison between *aox1* and the other two clones showed a sequence homology of approximately 64%. Whelan et. al. (1995c) considered this low degree of homology might explain why the other *aox* genes were not detected in earlier Southern analysis using the *aox1* cDNA probe.

Similar studies using *N.tabacum* and the PCR-oriented approach found one extra *aox* gene species. The full-length sequence of this gene was different from the *aox1* already known (Rhoads and McIntosh 1993a) by only 47 bp and was named *aox1b* (Whelan et. al. 1995b). The *aox1* gene sequenced earlier was renamed *aox1a*. This nomenclature was chosen after considering the small degree of sequence variation (47 bp) may have been due to the different tobacco

cultivars used.

At the time of this project it was assumed from previous Southern analysis, using gDNA probed with the *aox* cDNA (Kumar and Soll 1992), that there was only a single copy of *aox* in *A.thaliana* and that this was expressed in leaves. The tissue type chosen for the study of an inducible antisense *aox* plant was leaf tissue and the method of induction chosen was foliar spraying. The copper-inducible system used in this project appeared to be ideal from the results in tobacco (Mett et. al. 1993). It had shown maximal efficiency in tobacco leaves and was expressed to a lesser degree in roots (P. H. Reynolds, pers. comm.). This meant that for successful use in *A.thaliana*, it was also essential to determine whether that *aox* cDNA used in the antisense construct, was specifically expressed in leaf tissue in *A.thaliana*.

This component of the project attempted to answer two questions; (i) how many *aox* genes are in *A.thaliana*, and if there is more than one (ii) what is their sequence relatedness and (iii) are they also expressed in leaf tissue such that they may influence the effectiveness of the construct. The last two questions are interdependent. To have an antisense effect the sequence used in the construct must be of 80% homology or higher (I. B. Dry, pers. comm.). Therefore if the sequence relatedness between *aox* gene copies expressed in leaf tissue is lower than 80%, the influence on the effectiveness of the construct is unlikely to be of concern. To answer

the former question a PCR-orientated approach was used following the methodology used by Whelan et. al. (1995c) in their work with *G.max* and *N.tabacum*. This would potentially amplify and identify any previously unknown *aox* gene family members.

4.2 Materials and Methods

Genomic DNA extractions, PCR analysis cloning and gel electrophoresis was carried out according to the methods outlined in Chapter 2. The following primers were used for the PCR amplification of gDNA; (i) Araaox 1 and 2, which are strictly homologous to the 5′ and 3′ ends of the *A.thaliana aox1* cDNA with addition of the *Xba I* site (underlined) and GCG clamp at the 5′ end, (ii) 2511A which is homologous to a region approximately 560 bp downstream from the 5′ end of the *aox1* cDNA, and (iii) HAO-1 and HAO-2 which are degenerate primers designed from amino acid regions of AOX which were highly conserved across several species. These primers were obtained from Dr. J. Whelan (University of Western Australia).
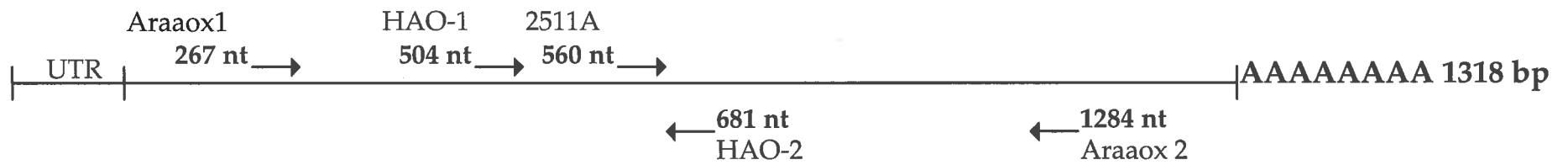
Figure 4.1: A diagram showing the *aox1* mRNA sequence positions of all the PCR primers. (UTR - untranslated region)

Figure 4.1 indicates the priming sites, on the *aox* mRNA sequence, for each of the primers below.

(i) Araaox 1 and 2
Araaox1
5′ GCG <u>TCT AGA</u> CTG GGA GAG AAA ACT CCG 3′

Araaox2
5′ GCC <u>TCT AGA</u> AGC CCA AAA GCC CCA TTG 3′

(ii) 2511A
5′ CTG GCC TAC CGA TTT GTT 3′

(iii) HAO-1 and -2
HAO-1
5′ GA(G/A) GCN (G/T)AN AA(T/C) GA(G/A) (C/A)GN ATG CA(T/C) (T/C)T 3′

HAO-2
5′ GC(C/T) TC(C/T) TC(C/T) TCN A(A/G)(A/G) TAN CCN AC 3′

The PCR products were detected by agarose gel electrophoresis and, after being cut out of the gel, were purified using a Qiaquick gel extraction kit. They were then ligated into the vector pGEM-T and transformed into *E.coli* XL-Blue cells using the heat-shock method outlined in section 2.1.4. Successful transformants were grown on LB with Ampicillin (100 mg/L) and plasmid DNA was isolated using the method outlined in section 2.8.3. Clones containing PCR inserts were identified by diagnostic restriction digests of isolated pDNA. Selected clones were amplified and their pDNA was isolated, purified (using a Qiagen miniprep kit) and then sequenced. Sequencing of pDNA inserts was done by the DNA sequencing Core Facility, Flinders University of South Australia.

4.3 Results and Discussion

4.3.1. Identification of PCR products using primers Araaox1 and 2

Genomic DNA, extracted from *A.thaliana* leaves, was used for PCR using the primers Araaox1 and 2. This resulted in a single DNA product of a size greater than that expected from the cDNA sequence. The expected product from an amplification using the cDNA clone was approximately 1.0 kb. The resulting 1.5 kb product from the gDNA amplification is shown in figure 4.2. This 1.5 kb product was digested with *Xba* I and cloned into the *Xba* I site of the plasmid vector pGEM and transformed into *E.coli*. The pDNA isolated from two potential clones, F1.4 and F12.2, was digested with *Xba* I to confirm the presence of the insert (see figure 4.2). Several digests of the clones were done using other restriction enzymes, to establish whether they were different, however since digestion patterns after electrophoresis on agarose gels were identical (results not shown), only one clone (F1.4) was sequenced. The sequencing results showed a high homology (99.6%) to *aox1* cDNA sequence of *A.thaliana* and to the *aox* tobacco gene. After the use of additional internal primers the entire insert in clone F1.4 was sequenced. This was 1562 bp in length and is shown in Figure 4.3. Comparison of the genomic clone F1.4 with the cDNA sequence (Kumar and Soll 1992) revealed the presence of three introns much like the known plant genes of *S.guttatum* (Rhoads and McIntosh 1993a) and *G.max* (Whelan and Day, unpublished data).
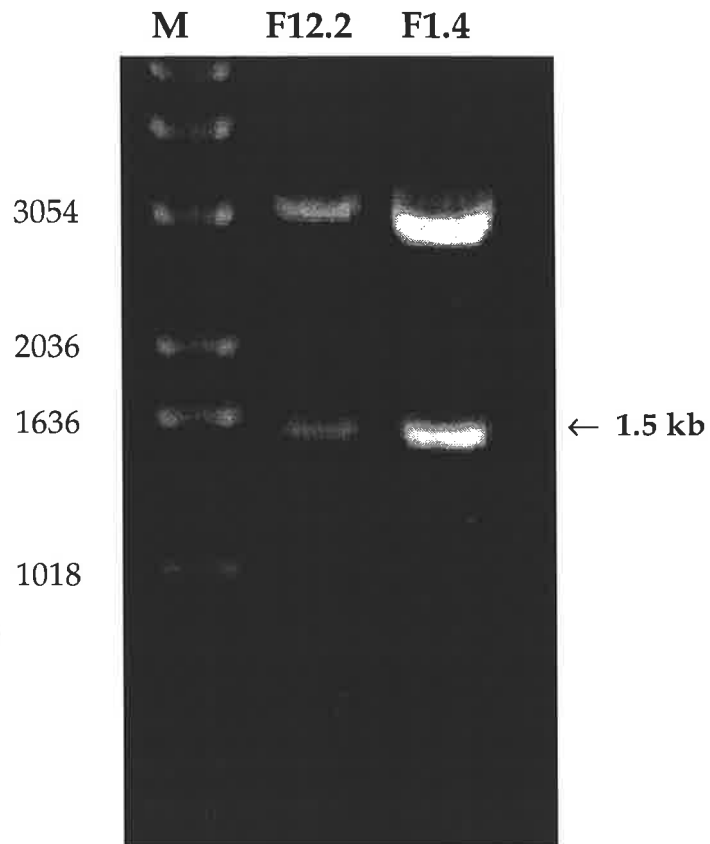
Figure 4.2: Agarose gel (0.8%) of the *Xba* I digestion products of clones **F12.2** and **F1.4**. The arrow indicates the 1.5 kb fragment. The positions of the markers **(M)** are indicated in the left margin.

```
   1    CTGGGAGAGA  AAACTCCGAT  GAAGGAGGAG  GACGCGAATC
  41    AGAAGAAAC   AGAGAACGAA  TCCACCGGTG  GAGACGCCGC
  81    CGGAGGTAAT  AACAAGGGAG  ATAAAGGAAT  CGCGAGCTAT
 121    TGGGGTGTTG  AACCTAATAA  GATTACTAAA  GAAGATGGTT
 161    CTGAATGGAA  GTGGAACTGT  TTCAGG gttacgttatagctagatt
 206    cgtatacagtgttgttcttcgttgatctgtgtaaatttgttgaaatttctttgg
 260    atttttgcag  CCATGGGAAA  CGTATAAAGC  TGATATAACG
 301    ATAGATCTGA  AGAAGCATCA  TGTTCCAACG  ACGTTTCTTG
 341    ATAGAATAGC  TTATTGGACT  GTTAAATCTC  TTCGCTGGCC
 381    TACCGATTTG  TTCTTCCAG gtactgttcttcttcttctcccatgga
 431    aaatttctttacatttttgccaattttatgatatggaaatgatccaaatctgatat
 488    cgtcattgattacgtgtaaccttaaatattttatttcattcttttatagcaacta
 546    gtcaatattttgtttgattattttggaatatatgtgtttattatattaatcacttcg
 602    ttagtgctttggattcttggttaattgttggttaattatcattatgaatgaattatt
 658    ggttaattgttggtctgattgtgatattgtgaattaaaacag  AGGAGATATG
 711    GATGTCGAGC  TATGATGCTT  GAAACTGTAG  CAGCAGTACC
 751    TGGAATGGTT  GGAGGAATGT  TACTACACTG  CAAATCGCTT
 791    CGACGTTTTG  AGCAAAGTGG  AGGATGGATT  AAGGCTCTTC
 831    TTGAGGAAGC  AGAGAATGAG  AGAATGCATC  TTATGACATT
 871    CATGGAAGTC  GCGAAACCGA  AATGGTACGA  GAGAGCGCTC
 911    GTGATCACTG  TGCAAGGAGT  CTTCTTCAAC  GCTTATTTCC
 951    TTGGTTACTT  AATCTCTCCC  AAGTTTGCTC  ATCGTATGGT
 991    TGGGTACCTT  GAAGAAGAAG  CGATCCATTC  TTATACTGAG
1031    TTTCTCAAGG  AACTTGACAA  AGGTAACATT  GAGAATGTTC
1071    CTGCTCCGGC  TATTGCTATT  GATTACTGGA  GGCTTCCTGC
1111    TGATGCGACA  CTTCGTGATG  TTGTGATGGT  TGTTCGTGCT
1151    GACGAGGCTC  ATCACCGTGA  TGTAAACCAT  TTTGCATCT
1191    gtaagtatattatttgcttgagactaaatttctaaagcttaaaatcgtgtg
1242    ttactgatcaattatggttttactttgtag  GATATTCACT  ACCAAGGTCG
1292    TGAACTAAAG  GAAGCTCCAG  CTCCAATTGG  GTATCATTGA
1332    TTCGATTAAA  AGAAGAGCTT  TTTCTCAAGT  TTAAAACTTT
1372    GTTCTAAACA  ATTT aagttcttt  GACTGATATA  CATCATCACC
1406    TCTGCTTAAG  CCATACTTGG  ATTCGGCTTT  CTTTGAATGT
1446    TGCATACGAA  TGTTCTGATT  TCTTCTTTAC  TTTTCCTGTC
1486    AATGGGGCTT TTGGGCT
```

Figure 4.3 : Nucleotide sequence of the F1.4 clone corresponding to the *aox1a* gene of *A.thaliana*. The sequence of F1.4 which corresponds to the *aox1* mRNA is shown in upper case letters and the intervening sequence is shown in lower case letters.

The exon-intron boundaries occurred at nt 453, 582 and 1132 in comparison to the cDNA sequence (Kumar and Soll 1992, accession #M96417). The three introns were labelled A, B and Z and were 83, 300 and 81 nt respectively. There was also a small 9 nt insertion at # 1189 compared to the cDNA sequence. This smaller region did not comply with the typical exon:intron boundaries. A review by Brown (1996) gave the following sequence for the 5′ splice sites in *A. thaliana* ; 5′ CAG:GUAAGU 3′. Similar sites were found at the exon:intron borders of aox1, i.e. for intron A ;5′ CAG:GGUUAC 3′ and intron B ;5′ CAG:GUACUG 3′ and intron Z ;5′ CUG:UAAGUA 3′. The small 9 nt insertion had the boundary of 5′ AGU:UCUUUG which despite showing the characteristic high concentration of uracil, did not conform to the expected sequence. Brown's consensus sequence for the 3′ splice sites was ;$(U)_3(U)_3UUU(U)_5GCAG:GU$

The corresponding sites found in the introns of aox1 were;

intron A; $(U)_3C(U)_3GGA(U)_5GCAG:GC$

intron B; $(U)_2GUGAA(U)_2(A)_4CAGAG:GA$

intron Z; $(U)_2AUGG(U)_4AC(U)_3GUAG:GA$, and in the smaller 9 nt region this was $UC(U)_3GACU:GA$. This showed that in all of the introns, except for the small 9 nt region, the characteristic splice sites could be recognised. Comparison of intron and exon sizes in the *aox* gene of three plant species *S.guttatum*, *G.max* and *A.thaliana* are shown in table 4.1.

Table 4.1 : *A comparison of the length (bp) of exons and introns of the genomic aox sequence in S.guttatum, G.max and A.thaliana. Introns 1, 2 and 3 correspond to A, B and Z in A.thaliana.*

Plant species	exons				introns		
	1	2	3	4	1	2	3
S.guttatum	330	126	486	57	119	78	119
G.max	288	123	489	57	334	1500	140
A.thaliana	453	129	550	186	83	300	81

Table 4.1 indicates that while the number of introns was the same between the three species, the sizes of these introns varied greatly. In contrast to this, the size of the exons was roughly conserved across species with the exception of exon 4 in *A.thaliana* which was greater than three times that of *S.guttatum* or *G.max*.

Sequence similarity with the *aox1* cDNA showed that clone F1.4 contained the gene *aox1* from *A.thaliana* and the sequence was entered in GenBank database (accession #ATU85244).

4.3.2. Identification of PCR products using primers 2511A and Araaox2

Primer 2511A was positioned at approximately 560 nucleotides from the 5′ end of the *aox1* cDNA sequence (see figure 4.1). This region corresponded to a highly conserved amino acid region in AOX across a number of plant species. The primer 2511A was chosen (i) to provide yet another span of a region where potentially different *aox* sequences could be amplified and (ii) because it was expected that smaller, cloned products would be more readily generated from genomic DNA.
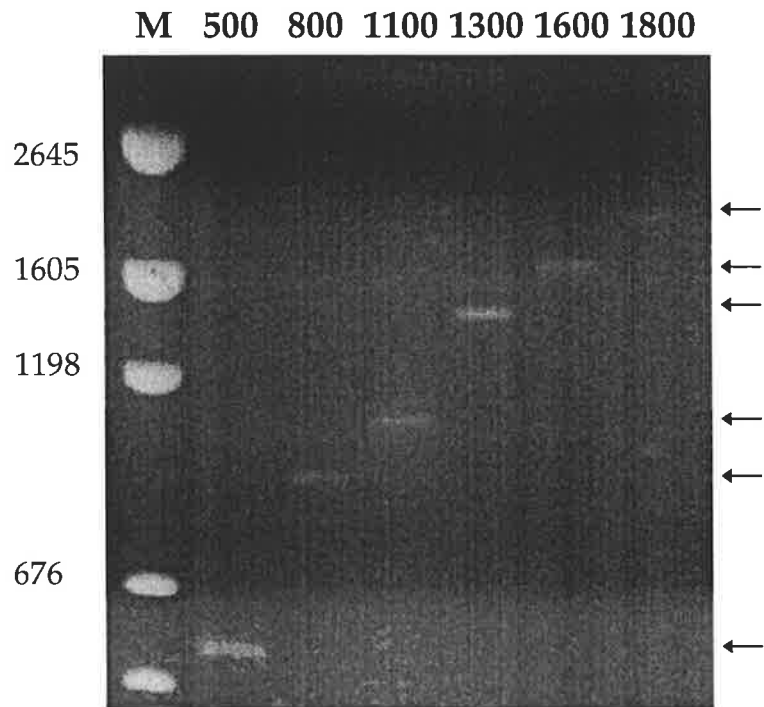
Figure 4.4: Agarose gel (1.0%) indicating with arrows the purified PCR products (500, 800, 1100, 1300, 1600 and 1800 bp) from PCR with *A. thaliana* gDNA using primers 2511A and Araaox2. The positions of the markers **(M)** are indicated in the left margin.

Figure 4.4 shows the products resulting from gDNA amplification by PCR using 2511A and Araaox2 primers. Six PCR products were apparent in approximately equal amounts and were referred to as fragment 500, 800, 1100, 1300, 1600 and 1800 by their approximate length in nucleotides. Attempts to clone these fragments into pGEM-T (see section 4.2) were successful with fragments 500, 800 and 1100 only. The clones carrying the PCR products 500, 800 and 1100 were sequenced and none were found to have any sequence similarity to *aox1*.

Purified PCR products 1300, 1600 and 1800 were sent for sequencing with the primers 2511B and Araaox2, after several unsuccessful attempts to clone them. All sequence information from the three separate submissions were returned with too great a degree of background for accurate sequencing. This suggested that there were mixed species of DNA within the products isolated.

4.3.3. Identification of PCR products using primers Araaox1 and HAO-2

Amplification of gDNA using the primers Araaox1 and HAO-2 was expected to yield PCR products of approximately 800 bp when the gene sequence was known (for results see section 4.3.1.) due to the presence of introns A and B. Figure 4.5 shows the PCR products obtained were around 600 and 1100 bp in length. Attempts to clone these products were successful with the smaller sized product only.
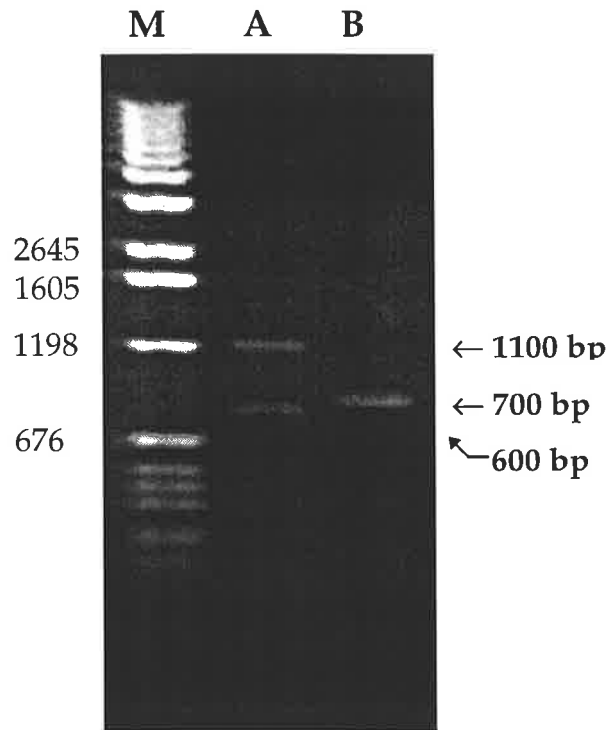
Figure 4.5: Agarose gel (0.8%) showing the PCR products using **(A)** primers Araaox1 and HAO-2, and **(B)** primers HAO-1 and Araaox 2 with *A. thaliana* gDNA. Arrows indicate the approximate size (bp) of the PCR products. The positions of the markers **(M)** are indicated in the left margin.

Clone F21.7 containing the 600 bp product was sequenced and analysed for sequence similarity to the known *aox* sequences. Clone F21.7 showed 88% sequence similarity to the mRNA *aox* sequence of *A.thaliana*. The corresponding region in the genomic sequence contained introns A and B, however both were absent in F21.7. The sequence similarity between F21.7 and the genomic sequence (F1.4) was only 69% which implied that F21.7 was part of an alternative gene.

4.3.4. Identification of PCR products using primers HAO-1 and Araaox2

PCR amplification of *A.thaliana* gDNA using primers HAO-1 and Araaox2 resulted in a single product of approximately 700 bp (Figure 4.5). The expected size for a product using these primers was 780 bp or 1.2 kb with cDNA or gDNA respectively as the template. The larger product expected using gDNA as the template was due to the introns B and Z occurring within the amplified region. Figure 4.5 shows that the PCR product obtained was smaller than expected. It was cloned into an *E.coli* plasmid vector and four clones, carrying inserts of the same size, were obtained. One of these (F22.5) was purified and sequenced. Sequence analysis revealed that clone F22.5 showed greatest sequence similarity (91%) to the *A.thaliana aox1* gene obtained earlier in this project (see section 4.3.1.). Because F22.5 was not identical to these sequences, this indicated the presence of another *aox* gene in *A.thaliana*.

Despite having regions of overlap (due to the combinations of PCR primers used) F21.7 and F22.5 had a low percent sequence similarity (45%). This was expected due to the relatively small region of overlap. Table 4.2 shows the sequence similarity between the three clones. It shows that while F22.5 and F1.4 are similar, F21.7 appears dissimilar to them both. The results of all of the sequence comparisons suggest that all three clone encode different *aox* genes.

Table 4.2 : *A summary of the percent similarity between of each of the three sequences F1.4, 21.7 and 22.5. Values were calculated using the ANGIS Gap/pairwise-comparison programme.*

	F1.4	**F21.7**	**F22.5**
F1.4	100	69.0	90.8
F21.7		100	45.5
F22.5			100

4.3.6. Summary of results

A summary of the results from the sequence analysis of all of the clones is presented in figure 4.6, where regions of sequence similarity between the clones and the mRNA sequence of *aox* are diagrammatically represented. After obtaining the genomic *aox* sequence via clone F1.4 further distinction could be made between the other clones. Clone F22.5 contained the intron Z but did not display 100% homology to the gene *aox*, indicating that it was a different genomic sequence. Clone F21.7 contained a sequence spanning regions in which introns were known to exist in the genomic sequence, however they were not present in F21.7.
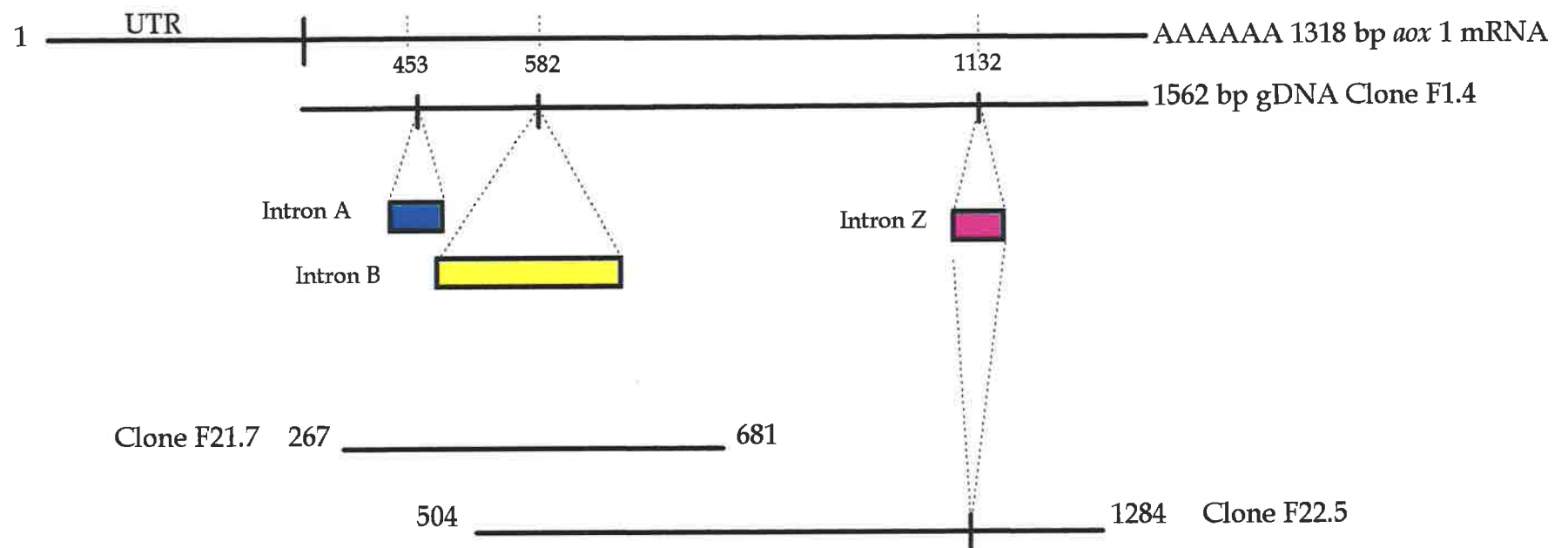
Figure 4.6: A schematic representation of clones F1.4, F21.7 and F22.5 and their position with respect to the *aox*1 mRNA sequence.

It also showed low sequence similarity to both F1.4 and F22.5, therefore F21.7 is also likely to be a different *aox* gene sequence. As F21.7 and 22.5 had no significant overlapping sequences, it was not known whether these clones were from the same gene or indicated two other, separate *aox* genes. Further analysis with new primers designed to the sequences, F21.7 and F22.5, is required.

4.4 Conclusion

While this work was in progress another group completed a study describing the existence of four *aox* genes in *A.thaliana* (Nakazono pers. comm.). These were cloned and compared to the mRNA sequence of Kumar and Soll (1992) and the results published (Saisho et. al. 1997). The gene that corresponded most closely to the mRNA was named *aox1a*. The other genes were called *aox1b, c* and *aox2*. Their gene sequence *aox1a* showed 99% sequence similarity to the F1.4 genomic sequence (see table 4.3 for pairwise comparison details). This suggests that with the exception of several nuceotides, these sequences are of the same *aox* gene. The sequence obtained in this study corresponds with that of Kumar and Soll (1992) 100% sequence similarity.

Table 4.3 : *A summary of the percent similarity between aox sequences recently published by Saisho et. al. (1997), the mRNA sequence (Kumar and Soll 1992) and clone sequences F1.4, 21.7 and 22.5. Values were calculated using the ANGIS Gap/pairwise-comparison programme.*

	aox1a	*aox1b*	*aox1c*	*aox2*	*aox* mRNA
F1.4	99.6	71.2	70.9	57.4	100
F21.7	69.9	69.7	63.3	53.3	87.9
F22.5	87.8	73.3	73.7	50.9	85.4

Interestingly the small 9 nt insert in clone F1.4 was also present in the *aox1a* sequence of Saisho et. al. (1997), although it was 11 bp in length in their sequence. This indicated that this was not anomalous and constituted a fourth intervening sequence. Taking this into account the size of exon 4 decreased to approximately double the size of the corresponding exons in *S.guttatum* and *G.max* (see table 4.1).

The other genes (*aox1b, aox1c* and *aox2*) sequenced by Saisho et. al. (1997) were compared to the clones sequenced in this study (F21.7 and F22.5) to ascertain whether they were the same or represented further *aox* gene family members. These comparisons are presented in table 4.3 and show that F21.7 and F22.5 show greatest similarity to *aox* mRNA and *aox1a* respectively. This suggests that they represent one or more other *aox* genes within *A.thaliana*.

In conclusion the two major objectives of this component of the project were answered. The work presented here, as well as that which was recently published by Saisho et. al. (1997) shows an *aox* gene family of at least four different *aox* genes, with the potential for more in the

clones of F21.7 and F22.5.

With respect to future antisense work, both F21.7 and F22.5 showed a high enough similarity (i.e. over 80%) to present potential problems when trying to reduce AOX activity by gene silencing if they are expressed in the target tissue. It must be noted however that this high similarity calculated with both clones represents only part of an *aox* gene, which overall may not show such high similarity. Work by Saisho et. al. (1997) also confirmed that out of the four *aox* genes they sequenced, the *aox1a* gene was expressed in leaf tissue and was the only gene responsive to treatment with AA, an inhibitor of the CP. If either F21.7 or F22.5 represented a second gene of high similarity to *aox1a*, the probe used by Saisho et. al. (1997) would most likely detect the expression of this second gene. This was not the case and their work confirmed that the cDNA corresponding to *aox1a* was the ideal choice when investigating *aox* expression in *A.thaliana* leaf tissue.

CHAPTER FIVE: INDUCIBLE GUS REPORTER ACTIVITY IN *Arabidopsis thaliana*

5.1 Introduction

Whilst constitutive antisense *aox* plants have previously been made using tobacco (Vanlerberghe et. al. 1994) these had not been used to study the role of the AP during plant stress. The production of inducible antisense *aox* plants would not only allow such studies, but would also give the investigator a degree of control over the timing of antisense expression. Use of inducible antisense plants may also provide more accurate analysis of the AP's role, as it is not yet known whether silencing one component of the mETC alters any of the other components. Inducible systems minimise the potential for long-term alterations in the component profile of the mETC.

The choice of inducible promoter system used in this project was made with consideration of the following criteria:

- There needed to be negligible or no expression of the antisense cDNA without the inducer being present such that the inducible antisense plant changed from a WT phenotype to that of an antisense.
- The method of induction and the inducer needed to be non-toxic to *A.thaliana*.
- The inducer would be easy to apply to either plant or cell cultures.
- There needed to be a method of removing the inducer or adding another component that could neutralise or counteract the inducer.

- Whilst tissue specificity was not strictly required, a maximal expression in leaves would be desirable.

All of the promoter systems investigated at the commencement of this project fell into two categories : (a) promoter-activating systems and (b) promoter-repressing systems. The promoter-repressing systems included the tetracycline-inducible gene expression (see Weinmann et. al. 1994 for more detail) and the IPTG-inducible gene expression (Wilde et. al. 1992) systems, both of which are made up of components which are bacterial in origin. The promoter-activating systems include those promoters that are activated by light (Lam and Chua 1990, Orozco and Ogren 1993, Uozumi et. al. 1994), heat (Volker et. al. 1993), low temperatures (Qoronfleh et. al. 1992), ferritin (Beaumont et. al. 1994), metal ions (Mett et. al. 1993, Inoue et. al. 1992) and nitrate (Rastogi et. al. 1993).

For this study a copper-inducible system that was known to function well in tobacco (Mett et. al. 1993) was used. This system had key components from the yeast copper metallothionein regulatory system of *S.cerevisiae*. Initially, it was necessary to investigate whether this system would work in *A.thaliana* and if so, to discern the day of maximal transcript expression after induction with copper. This was investigated using the copper system with a reporter gene (*gus*). This construct (figure 5.1) was kindly provided by Dr. Paul Reynolds (NZ) and had the copper-inducible system fused to the reporter gene β-glucuronidase (*gus*).
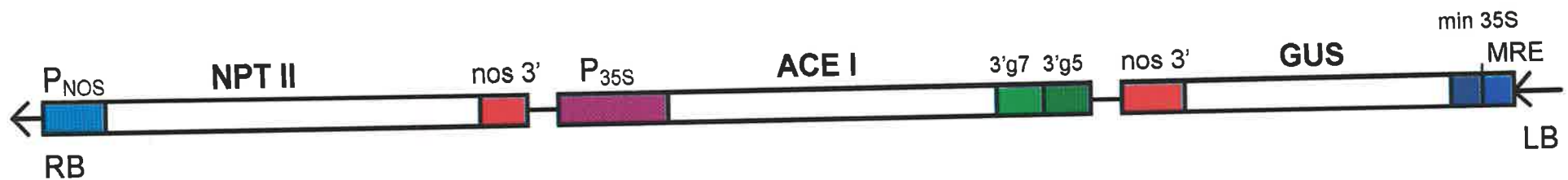
Figure 5.1 : Construct used to test the copper-inducible system (Mett et. al. 1993) in *A.thaliana.*

P_{NOS} = nopaline synthetase promoter
NPT II = neomycin phosphotransferase II selectable marker
nos3' = nopaline synthetase terminator
P_{35S} = CaMV 35S RNA promoter
ACEI = activating copper-metallothionein expression
3'g7 and 3'g5 = gene 7 and gene 5 terminator from *A. tumefaciens*
GUS = β-glucuronidase reporter gene
MRE = copper-metallothionein transcription factor binding site
min35S = 90 bp domain A of the CaMV 35S RNA promoter

5.1.1. The reporter system

The β-glucuronidase gene originates from the bacterium *E.coli* and encodes a polypeptide of 602 amino acids. The active enzyme, which is believed to be a tetramer, is very stable and is active over a broad pH (5.0 - 7.5) and temperature range ($T_{1/2}$ @ 55°C = 2H). Once the substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) had been infiltrated into the plant tissue being studied, *gus* expression and activity was easily assayed. The chemical reaction catalysed by the GUS enzyme causes the reduction of X-Gluc which is detected as localised blue staining.

5.1.2. The copper-inducible reporter system

The copper-inducible reporter system (figure 5.1) is also known as pPMB711 when present with the vector. It carries an npt II gene, conferring resistance to kanamycin, as well as the *ace* gene encoding the activating copper-MT expression transcription factor (ACE1) from yeast (*S.cerevisiae*). Both of these genes were under the control of strong constitutive promoters. The *gus* gene was under the control of a chimaeric promoter made up of the 90 bp CaMV 35S RNA domain A and the ACE1 binding site (also known as the MRE). This allows strong expression of the gene once the ACE1 transcription factor is bound. The ACE1 transcription factor changes conformation at elevated Cu^{2+} levels, and this allows binding to the MRE site/MT gene promoter (Mett et. al. 1993). This means that little or no expression of

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gus should occur in the absence of, or in low amounts of copper.

Although this system has been known to function well in *Nicotiana tabacum* (Mett et. al. 1993) and *Lotus corniculatum* (P. H. Reynolds pers. comm.), it was necessary to test both its effectiveness and the day of maximal *gus* expression in *A.thaliana*. These results would provide a guide for future *aox1* antisense plant work, in determining the appropriate time for testing AOX capacity and *aox1* transcript levels after copper induction.

5.1.3. Inducer levels and mode of induction

The amount of $CuSO_4$ used to induce *A.thaliana* was decided by consideration of : (a) the amount and application method used successfully with tobacco plants, *N.tabacum* (Mett et. al. 1993) and, (b) the results of metal toxicity trials on *A.thaliana* seedlings (Murphy and Taiz 1995).

In the report by Murphy and Taiz (1995) the metal tolerance of a number of *A. thaliana* ecotypes and variants was tested by germinating seeds in their presence and measuring (%) seedling inhibition and root extension (mm). When tested with $CuCl_2$, *A. thaliana* (ecotype Columbia) displayed no apparent inhibition or deleterious effects with concentrations less than 20 μM. This fell well within the optimal $CuSO_4$ concentration range used with the copper-inducible system in *N.tabacum* (Mett et. al. 1993). In testing their system in tobacco, Mettand co-workers used concentration of 0.05, 0.5, 5.0, 15, 30, 50 and 500 μM $CuSO_4$. This was applied to the plant's growth medium. The

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results using concentrations of 0.5, 5.0 and 50 μM $CuSO_4$ were published, showing plants grown for 20 days exhibited toxicity effects after days 20 and 15 with 5.0 and 50 μM $CuSO_4$ respectively. The difference in the activity of GUS between these two application concentrations was not evident in the maximal GUS activity, but rather in the day and duration of maximal activity. Plants grown with 5.0 μM $CuSO_4$ reached a similar maximal GUS activity by days 15 to 20, whereas those grown with 50 μM $CuSO_4$ had obtained this by days 5 to 10. Due to the toxicity effects seen in tobacco with both 5.0 and 50 μM $CuSO_4$, the lower concentration of 0.5 μM $CuSO_4$ was chosen as a foliar spray (Mett et. al. 1993).

5.2 Material and Methods:

Details of all methods are described in Chapter two, but the protocol plan is outlined below.

A clone of *E.coli* (DH5α) carrying the plasmid pPMB711 :pGA643/ACE1.6.1/MT-GUS2 was received from P.H. Reynolds (The Horticulture and Food Research Institutue, New Zealand) and grown on the appropriate selective media (tetracycline 10 mg/L). The pDNA was extracted and screened for the presence of the *gus* construct, using diagnostic digestion with the restriction enzyme *Eco* RI. After confirming a band of approximately 2.5kb, this pDNA was amplified and purified, ready for transformation into *A.tumefaciens*.

The plasmid vector carrying the appropriate GUS system was transformed into *A.tumefaciens* LB4404 by electroporation (see section

2.1.4) The resulting *Agrobacterium* colonies were grown on selective media (tetracycline 10 mg/L), and their plasmid DNA extracted and screened for the presence of the GUS construct as described previously for *E.coli*. A colony carrying the appropriate construct was named PMB711.5 and was cultured and used to vacuum-infiltrate *A.thaliana* plants following the method described in section 2.5.

Plants that survived the infiltration procedure were grown through to seed set (i.e. allowing self-fertilisation). These seeds were harvested and selected on kanamycin (50 mg/L) germination medium as previously described. These plants were grown and allowed to set seed. The seeds harvested from this generation were also selected and once grown to adult size, were destructively harvested and assayed for GUS activity in response to copper induction.

Leaf tissue, harvested under axenic conditions, was assayed for GUS activity (see section 2.7) and viewed under a light microscope (4X magnification). Photographs were taken using an Olympus microscope (type CHK) with filter 80A, and Practika MTL5B camera.

5.3 Results and Discussion

5.3.1. Inducer concentration trials

Previous work with tobacco had shown that foliar spraying of copper was more effective than its application to growth media when testing leaf material (Mett et. al. 1993). Maximal GUS activity in tobacco was seen by day six using a concentration of 0.5 µM $CuSO_4$ with

no apparent toxicity effects (Mett et. al. 1993). Foliar sprays of 0.5, 5.0 and 50 μM were initially chosen for testing with *A.thaliana* to ascertain the most effective inducer concentration. It became apparent from early trials that 50 μM $CuSO_4$ was too toxic for *A.thaliana* leaf tissue. Tissue sprayed with 50 μM $CuSO_4$ developed necrotic areas on the leaf margins (results not shown). This result was supported by those seen in Murphy and Taiz' work (1995) using *A.thaliana* germinants which also indicated that $CuCl_2$ solutions of concentrations above 20 μM were increasingly toxic to the germinants.

The GUS assay system included the use of both positive (tobacco) and negative (*A.thaliana* WT) controls. The positive control used was a constitutive tobacco *gus* transgenic line, kindly provided by Dr. I.B. Dry (CSIRO, Plant Industry, Horticulture unit). The *A.thaliana* WT control was leaf material sprayed with 5.0 μM $CuSO_4$. Use of these controls allowed detection of any variation in blue staining due to the assay procedure, rather than the levels in the plant tissue. In all assays the positive and negative controls gave consistent, invariant results. An example of this is shown in figure 5.3 and discussed in more detail in section 5.3.2.

Initial results with 0.5 μM $CuSO_4$ used as a foliar spray indicated that it was not as effective as 5.0 μM $CuSO_4$. Figure 5.2A shows the staining results for three positive plant lines using 0.5 μM $CuSO_4$ foliar spray. Staining in *A.thaliana* was lower in intensity than that observed with the 5.0 μM $CuSO_4$ application (figure 5.2B).
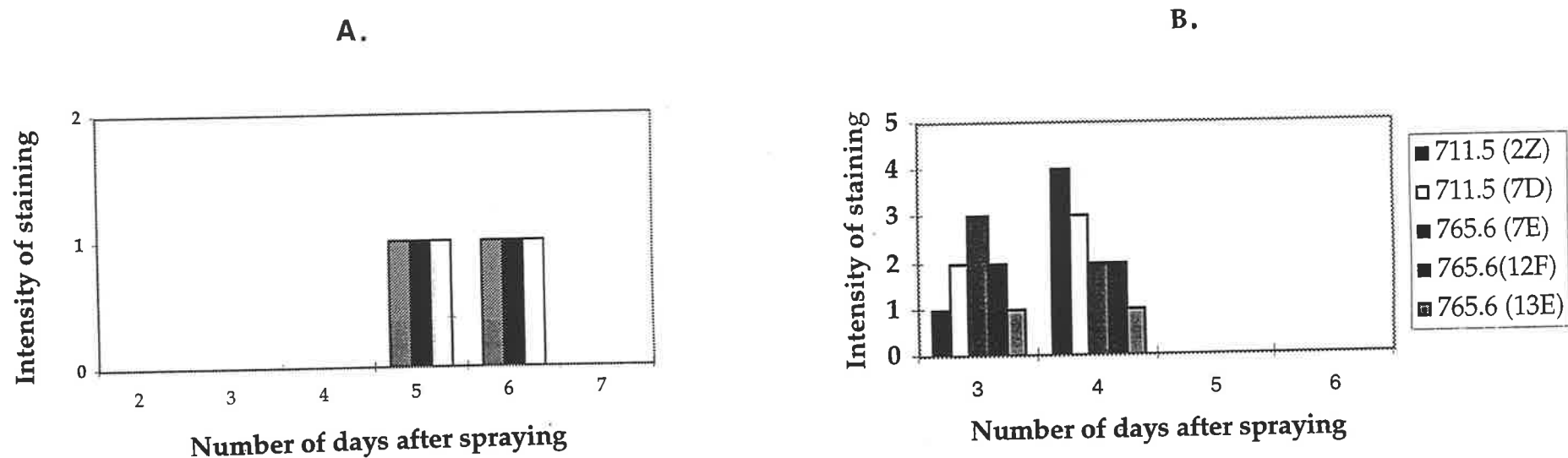
Figure 5.2 : Comparison of maximal *gus* reporter staining results in **A.** three different plant lines (2Z, 7E and 12F) of *A.thaliana* sprayed with 0.5 μM CuSO₄ and **B.** five plant lines (see legend) sprayed with 5.0 μM CuSO₄. Intensity of staining showing 0 - no apparent staining, 1 - low level, 2 - medium level, 3 - high level and 4 - very high level of blue coloured staining in comparison to the controls (see figure 5.3).

As well as this, maximal staining occurred on days 5 and 6 after application. This indicated that, unlike the results found by Mett et. al. (1993) with tobacco, 0.5 µM CuSO$_4$ was not the ideal concentration for foliar induction in *A.thaliana*. It was not expected that *Arabidopsis* and tobacco would behave the same in this regard due to the difference in leaf tissue between the two species. Many factors influence the ability of copper to penetrate leaf tissue as well as its ability to persist within the leaf cells. From these results with *Arabidopsis*, showing a lower GUS stain intensity, it was decided that the 0.5 µM CuSO$_4$ spray would not be used in subsequent experiments.

### 5.3.2. Induction of GUS expression using 5.0 µM CuSO$_4$

The foliar spray concentration of 5.0 µM CuSO$_4$ was found to be more effective with *A. thaliana* than 0.5 µM and was still well within the known range of safe copper concentration for the ecotype (Columbia) used. For this study 5.0 µM CuSO$_4$ is used as a foliar spray on whole plants approximately 5-to-6 weeks of age, applied as a foliar spray. No apparent physiological changes occurred over the study time period (see figure A4 in appendix).

Figure 5.3 shows photographs of the positive and negative controls during identical assay conditions as the test samples seen in figure 5.4. The consistent results with these controls shows that there was little or no variation in staining as a result of the assay procedure. It also showed that the treatment of the WT plant with the same 5.0 µM CuSO$_4$ foliar spray did not give any discernible background.

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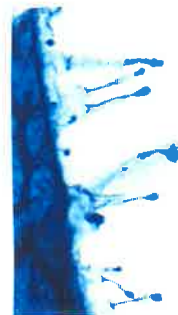
A. tobacco

day 3   day 4   day 5   day 6

B. *A.thaliana* **wild-type**

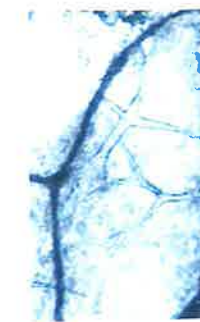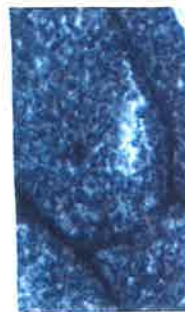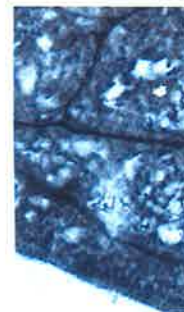**Figure 5.3 :** Photographs of GUS stained leaf tissue from **A.** constitutive (*gus*) tobacco positive control, and **B.** 5.0 µM $CuSO_4$-sprayed (on day 0) WT *A.thaliana*, over a four day sampling period.
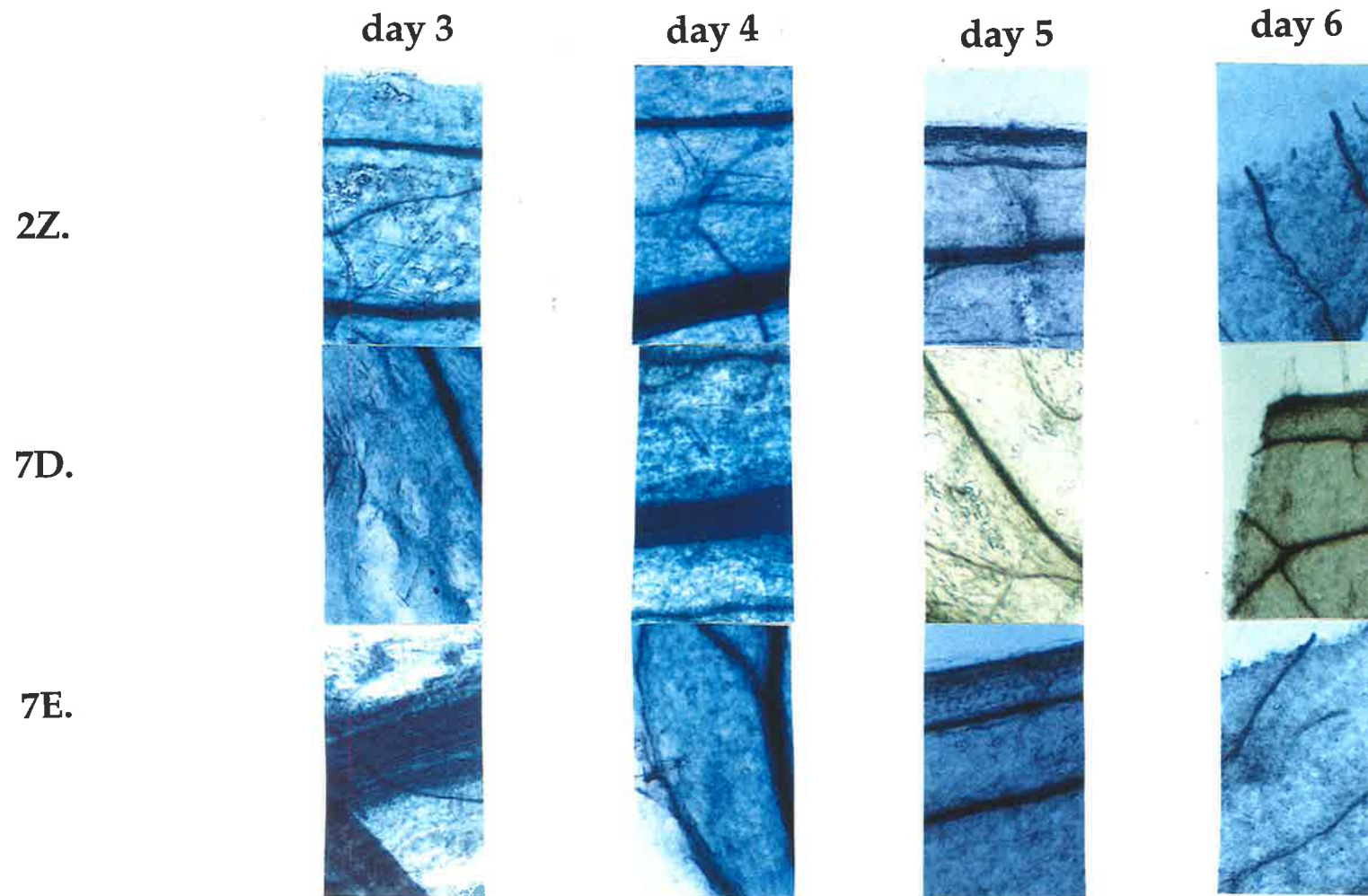
Figure 5.4 : Photographs of GUS stained leaf tissue from three plant lines (2Z, 7D and 7E) over a four day sampling period. Plants were foliar sprayed with 5.0 μM $CuSO_4$ on day 0.

Figure 5.4 shows the test samples of three of the four putative copper-inducible *gus* transgenic plant lines. All of the tissue samples taken from these plant lines indicated that GUS activity, present on day 3, reached a maximum on day 4. Activity rapidly diminished by days 5 and 6 to that of the wild-type levels.

The response of the copper-inducible GUS system in *A.thaliana* was compared to the published data using tobacco (Mett et. al. 1993). In that study tobacco plants, once foliar sprayed, were sampled over a period of 20 days. The response in tobacco occurred over a greater period of time (approximately 18 days) and the maximum was maintained for 4-6 days. Transgenic tobacco plants showed maximum activity at a later stage than the *A.thaliana* plants tested, and were able to maintain this maximum for longer. In accordance with the response in *A.thaliana* the reduction in GUS activity in tobacco appeared to occur rapidly. This overall trend of rapid decrease after maximal expression appeared similar between the two species. This indicates that the response to copper in both species containing the copper-inducible system, is rapid and tightly controlled.

In all of the transformed *A.thaliana* lines tested, the decreased GUS activity occurred on the same day and to a similar degree. Within the sample set sprayed with 5.0 μM $CuSO_4$ there was some variation in the maximal staining/GUS activity, however this maximum occurred on the same day after foliar spraying in all but one plant line.

5.4. Conclusion

From the results it is apparent that the copper-inducible system designed using the yeast $S.cerevisiae$ elements was able to function well in $A.thaliana$. The use of a foliar spray of 5.0 μM $CuSO_4$ was more effective than 0.5 μM $CuSO_4$ or 50 μM $CuSO_4$ and was therefore adopted for subsequent experiements. Maximal activity of GUS appeared to have a narrow window, becoming apparent on day 3, with a maximum on day 4, and then rapidly diminishing to the WT level by days 5 and 6. This means any comparable studies using this system will need to be done approximately 4 days after foliar-induction when 5.0 μM $CuSO_4$ is used.

CHAPTER SIX: PRODUCTION OF AN INDUCIBLE ANTISENSE (*aox1*) PLANT

6.1 Introduction

The use of antisense technology in producing plants with reduced gene expression has been successful in a wide variety of applications and plant species (see table 6.1). Whilst the exact mechanism of silencing is not known, there are many hypotheses (for a review see Meyer and Saedler 1996). One early mechanism of how antisense RNA mediated gene silencing occurs involves RNA/RNA interactions. These interactions between the antisense and native gene were thought to disrupt the native gene expression (Eguchi et. al. 1991). The earliest and most visually spectacular experiments showing a decrease in native gene expression due to antisense RNA were done by van der Krol et. al. (1988) using the chalcone synthase (*chs*) gene in *Petunia hybrida*. They were able to detect changes in CHS activity by altered corolla pigmentation. The wildtype flower pattern was an evenly coloured, purple flower. Transgenic plants carrying the antisense *chs* constructs displayed sectored pigment patterns with white and purple, or an overall reduction in pigment. The work of van der Krol et. al. (1988) demonstrated that there was no correlation between the number of antisense constructs integrated into the plant genome and the resultant reduction in pigmentation or *chs* mRNA levels. Likewise the antisense gene copy number did not relate to the level of antisense RNA.

The effects of the same antisense gene (*chs*) were also compared under the control of either the endogenous, *chs* promoter or the constitutive CaMV promoter 35S (van der Krol et. al. 1990). Transcription of the antisense gene using either of these promoters did not lead to an excess of transcripts when compared to that level seen with the endogenous gene. This led van der Krol et. al. (1990) to propose that the antisense transcript interruption of endogenous gene expression was more than a simple RNA/RNA interaction between the antisense and native genes.

More recent experiments have also shown that "co-suppression" can occur, that is when the same gene is introduced in the correct orientation, silencing of the endogenous gene occurs (refs.). This widened the debate to the mechanism of homology-dependent gene silencing in plants, including antisense genes, sense genes and homologous sequences that naturally occur in the plant genome.

The use of antisense RNA in research

Antisense technology has been applied to a wide range of enzymatic processes within plants with some marked effects. These are listed in table 6.1. As well as the marked pigmentation changes seen in *Petunia hybrida* (van der Krol 1988), antisense technology has altered the storage properties of fruit such as *Lycopersicon esculentum* (tomato) (Picton et. al. 1995; Kramer and Redenbaugh 1994) and has allowed the study of plants with reduced production of the small subunit for RuBisCo (Von Caemmerer et. al. 1997).

Table 6.1 : *Examples of the use of the antisense approach to study and/or improve plants.*

plant species	gene(s) targetted	aims of study	references
tomato	ripening-related genes (including polygalacturonase)	flavour & aroma generation, susceptibility to pathogens, carotenoid biosynthesis improved shelf-life, process characteristics, nutrient content etc., ethylene production	Picton et. al. (1995), Kramer and Redenbaugh (1994)
tomato	polyphenol oxidase (PPO)	PPO role in plant defense	Thipypong and Steffens (1997)
cantaloupe melon	ACC oxidase	ethylene & ripening studies, improved shelf-life & fruit quality	Ayub et. al. (1996)
poplar	cinnamyl alcohol dehydrogenase CAD	lignin biosynthesis & optimising pulp and paper treatment processes	Baucher et. al. (1996)
potato	ADP-glucose pyrophosphorylase AGPase	examine importance of starch synthesis in leaves with respect to sink-source interactions	Leidreiter et. al. (1995)
Flaveria bidentis	RuBisCo	examine relationship between CO_2 assimilation, RuBisCo content & carbon isotope discrimination	Von Caemmerer et. al. (1997)

The antisense RNA approach was chosen for this project because biochemical methods involving the use of chemical inhibitors of the enzyme AOX were in dispute (for a review see Day 1996). The partitioning of electrons between the two pathways, AP and CP, was largely misunderstood and hampered the ability to study the role of

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the AP during stressful conditions. The production of constitutive, antisense *aox* tobacco plants by Vanlerberghe et. al. (1994) provided an opportunity to study the AP's role in plant tissue.

This project deals with an inducible antisense system providing a number of advantages over the constitutive antisense RNA approach. With an inducible antisense plant, the silencing of the *aox1* gene can occur at any point before, after or during stress, depending upon the investigator's goal. In contrast to this, constitutive antisense plants are always reduced in their ability to synthesise AOX. Any immediate adjustments the mitochondrion or plant cell may make, with respect to this reduced level of AOX, have already occurred. It is also likely that long term silencing of one gene causes changes in other subcellular components. These long term adjustments may mask the full response of plant mitochondria to stress. An inducible system allows the study of a phenotypically wild type plant changing to a phenotypically antisense plant at any chosen point of the stress response. Thus, in order to fully examine the role of the AP during plant stress the use of an inducible antisense plant was necessary.

6.2 Materials and Methods

An overview of the protocol used in this project is summarised in the flow chart below (figure 6.1). Each step is numbered and discussed in turn. Plasmid DNA (pPMB7066 and pPMB765) was kindly provided by Dr. Paul H. Reynolds (The Horticulture and Food Research Institute of New Zealand, Palmerston North).
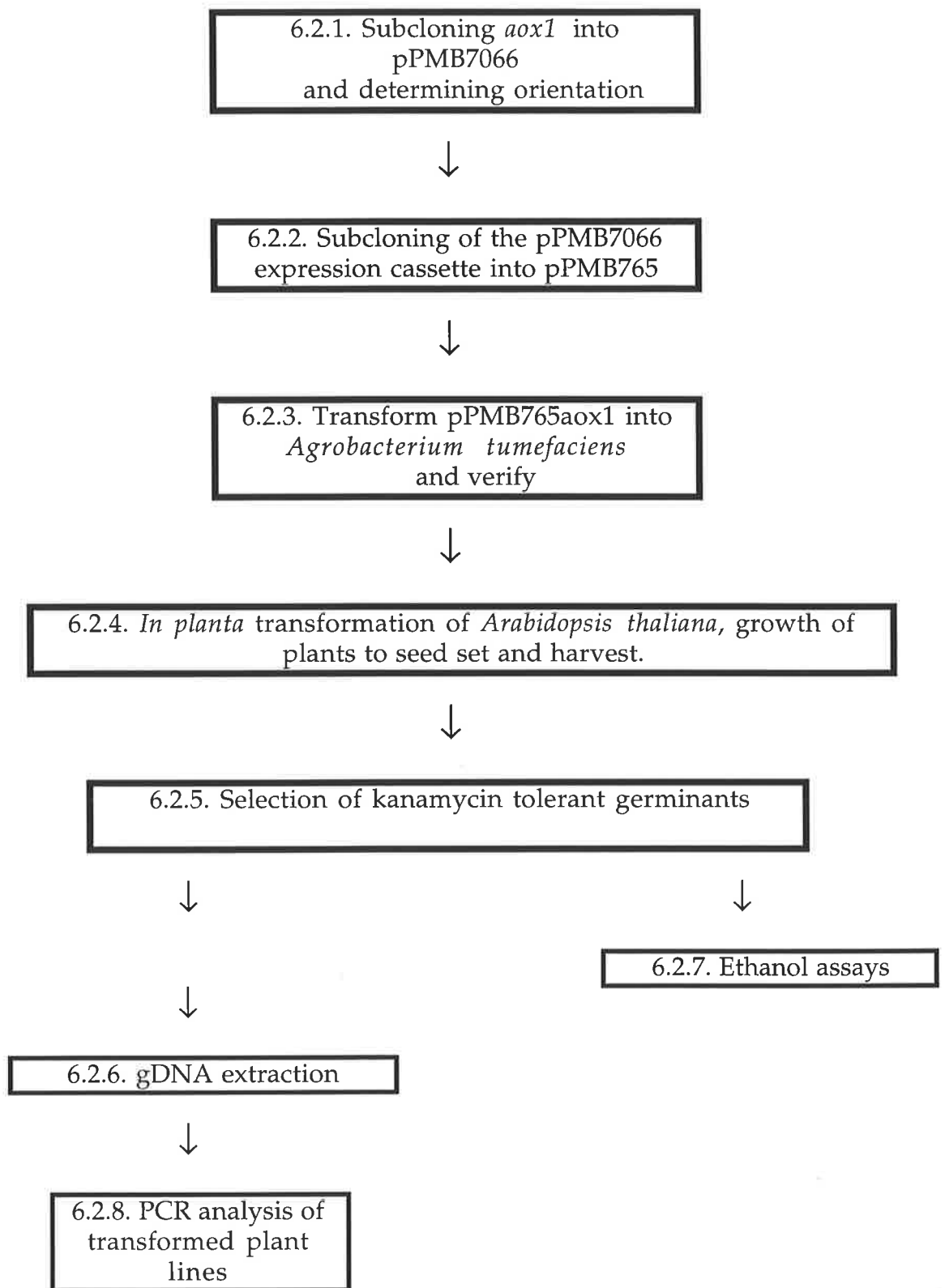
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Figure 6.1 : *An overview of the method used to generate inducible antisense aox1 plants. Each point is discussed in detail in section 6.2.*

6.2.1. Subcloning *aox1* into pPMB7066 and determining orientation

The *aox1* cDNA sequence required subcloning 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 -90bp 35S promoter (figure 6.2). Due to the presence of the 35S promoter there could be some background expression in root tissue in the absence of copper (P. H. Reynolds, pers. comm.). In all other plant tissues, full expression control is expected with no background expression when copper is below inducing levels or absent.

Initially two primers were made which had *Xba* I ends, GCG clamps and were homologous to the 5′ and 3′ ends of the *aox1* cDNA sequence. Their complete sequences were as follows. Those bases underlined represent the *Xba* I ends and the bases in bold are homologous to the 5′ (forward) and 3′(reverse) ends of the *aox1* sequence:

ARAAOX1, primer #1, (forward/+)
5′ GCG <u>TCTAGA</u> **CTG GGA GAG AAA ACT CCG** 3′

ARAAOX2, primer #2, (reverse/-)
5′ GCC <u>TCTAGA</u> **AGC CCA AAA GCC CCA TTG** 3′

The *aox1* sequence was amplified from *E.coli* XL-Blue#6 using these two primers according to the PCR conditions outlined in section 2.8.1 and the above primers, copies of the *aox1* cDNA were amplified.
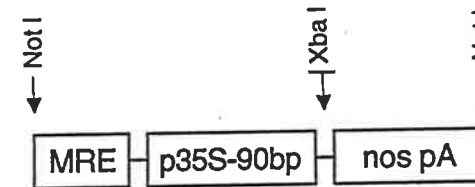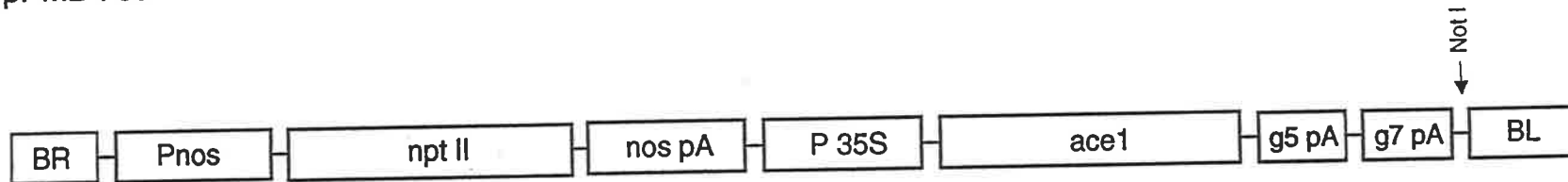
Figure 6.2 : Diagrams representing the two cassettes in vectors **A.** pPMB7066 and **B.** pPMB765. (MRE=copper-metallothionein transcription factor binding site, BR/BL=right and left borders, P_{nos}=nopaline synthetase promoter, npt II=neomycin phosphotransferase II, nos=nopaline synthetase terminator, P_{35S}=CaMV 35S RNA promoter, ace 1=activating copper-metallothionein expression 1 gene and g5 pA and g7 pA=terminators from *A.tumefaciens*.)

The 1.0 kb product was purified away from unincorporated nucleotides using a Qiagen QIAquick nucleotide removal kit, digested with *Xba* I and run on a low melting point agarose gel (Nusieve, FMC Bioproducts). The resultant single band was cut out of the gel and stored at -20°C.

Bacterial cultures (*E.coli* DH5α) containing pPMB7066 were selectively grown on LB agar using ampicillin (100 mg/L). A single colony was used to inoculate LB (plus ampicillin) and after overnight growth a plasmid miniprep was completed, using a QIAfilter plasmid Midi kit. A small amount of the extracted pDNA was diagnostically digested with the restriction enzyme *Not* I, to verify its identity as pPMB7066. The rest was cut using *Xba* I and then incubated with calf intestinal phosphatase to discourage self re-ligation (see section 2.8.2). This pDNA was run on an agarose gel and extracted as above.

The insert (*Xba* I digested PCR product) and the plasmid vector (XbaI digested pPMB7066) were removed from -20°C storage and melted at approximately 57°C. Once melted, the vector (approximately 100 ng) was added to a prewarmed (37°C) tube containing ligation buffer (250 mM Tris-HCl pH7.5, 100 mM MgCl$_2$, 25% (w/v) PEG8000, 5 mM DTT), 5 mM ATP and T4 DNA ligase (approximately 0.01 units). The insert was allowed to slowly reduce in temperature to 37°C whereupon approximately 50 ng was mixed into the prewarmed ligation mixture tube. This was allowed to stay at 37°C for ten minutes, and was then left out overnight in a foam rank, to gradually

reduce down to room temperature. The ligation mix was then used to transform competent DHα cells by electroporation (as described in section 2.1.4) which were incubated with LB at 37°C for one hour before being plated out on selective media (LB agar plus 100 mg/L ampicillin) and grown overnight.

Ten colonies were selected and grown in LB media overnight. Their pDNA was then extracted using the Lysis-boiling plasmid preparation method (section 2.8.3). Purified pDNA was diagnostically digested with *Xba* I to confirm the presence of the insert. The orientation of the insert in pPMB7066 was verified using the restriction enzyme *Kpn* I. This enzyme was chosen because it had a single site in the vector DNA, as well as a single site placed assymetrically in the *aox1* cDNA sequence (#636 on the *aox1* sequence). Out of the ten colonies chosen, one was selected as carrying the antisense *aox1* within the pPMB7066 expression cassette in the correct antisense orientation. This was grown in selective medium to gain greater amount of purified pDNA.

6.2.2. Subcloning of the pPMB7066 expression cassette into pPMB765
(Kumar and Soll 1992)

The *aox1* sequence was inserted in an antisense orientation with respect to the chimaeric MRE/P_{nos} promoter. This expression cassette required removal from pPMB7066 and insertion into the *Not* I site of pPMB765 (figure 6.2) such that the antisense could be in place downstream from the kanamycin resistance marker gene and the

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activating-copper-element (ACE), as described in chapter five. Once this construct was made it could be introduced into the bacterium used to transform the *A.thaliana* plants.

The expression cassette was released from pPMB7066, using the restriction enzyme *Not* I, and purified via agarose gel electrophoresis. The smaller band representing the expression cassette with *aox1* insert was cut out of the agarose gel and extracted using a QIAquick gel extraction kit.

To prepare the second cloning vector pPMB765, bacterial cultures (*E.coli* DH5α) with this plasmid were selectively grown on LB agar (spectinomycin 100 mg/L). A 150 mL LB culture was inoculated with a single colony (plus spectinomycin) and grown overnight. The pPMB765 plasmid vector was isolated and purified from this culture using a QIAfilter plasmid Midi kit. The identity of the extracted pDNA was confirmed by diagnostic digestion with the restriction enzyme *Sal* I. The purified plasmid to be used in the ligation with the expression cassette was cut using *Not* I. This linearised plasmid was incubated with calf intestinal phosphatase and run on an agarose gel. The band on the gel was cut out and the pDNA extracted using a QIAquick gel extraction kit.

The insert (*Not* I cut expression cassette) and the vector (*Not* I cut pPMB765) were ligated in a similar manner to that described in section 6.2.1. Vector and insert were used in a ratio of approximately 2:1. Competent cells of *Agrobacterium tumefaciens* LBA440 (40 µL)

were electroporated in the presence of the ligation mix (5 µL) according to the method outlined in section 2.1.4. These cells were grow overnight at 37°C on selective media (LB agar plus spectinomycin 100 mg/L). The pDNA was extracted from twelve of the resultant colonies following the Lysis-boiling method (section 2.8.3). For diagnostic digestion the enzymes *Bgl* II and *Pst* I were chosen to identify the presence of the *aox1* construct. The clone labelled pPMB765aox#12 (figure 6.3) was used to transform *A.tumefaciens*.

6.2.3. Transformation of *A.tumefaciens* with pPMB765aox1

Plasmid DNA was purified from clone pPMB765aox1#12 using a Qiagen plasmid midiprep and the purified pDNA was used (approximately 100 ng) to transform 40 µL of competent *A.tumefaciens* cells via electroporation (section 2.1.4). The resulting cells were washed out of the electroporation cell with 800 µL LB and allowed to stand for 3 hours at 28°C before plating on LB agar plus spectinomycin (50 mg/L) and incubated for 36 to 48 hours at 28°C. Spectinomycin-resistant colonies were used to inoculate liquid media (LB supplemented with spectinomycin 50 mg/L) and left to grow for 48h. These cultures were used to purify pDNA following the isolation procedure for *Agrobacterium* pDNA as described in section 2.8.3. The presence of the construct was verified using the restriction enzyme *Not* I (figure 6.3) and a single colony selected as PMB765aox1.
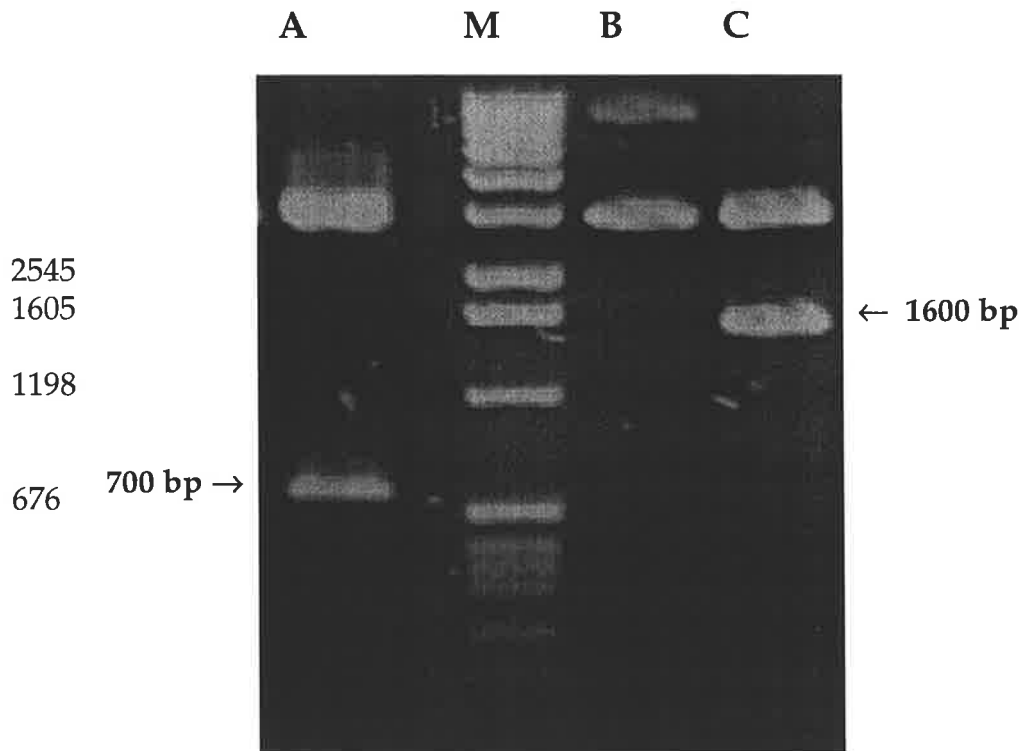
Figure 6.3: Agarose gel (0.8%) showing **(A)** pPMB7066aox#12 digested with *Kpn* I to give a 700 bp product, **(B)** undigested pPMB765aox1 and **(C)** pPMB765aox1 digested with *Not* I to give a 1600 bp product. The positions of the makers **(M)** are indicated in the left margin.

6.2.4. *In planta* transformation of *Arabidopsis thaliana*, growth of plants to seed set and harvest.

The method of choice for introducing the construct into *A.thaliana* was *in planta* transformation. This method involved the growth of large volumes of *A.tumefaciens* carrying pPMB765aox1 and followed the protocol outlined in chapter two (section 2.5). The plant tissue surviving this technique, and the subsequent infection by the bacterial carrier, was grown up and produced self-fertilised flowers and seeds that potentially carried the construct. (The seed harvesting arrangement is shown in appendix, figure A1.)

6.2.5. Selection of kanamycin tolerant germinants

The seeds of the plants infiltrated with *A.tumefaciens* carrying pPMB765aox#12 were collected (see appendix figure A1 displaying collection set-up) and dried further by storage in a dessication jar at room temperature. Seeds were manually extracted from their seed pods and stored in eppendorf tubes until required. Before germinating on selective media containing 50 mg/L kanamycin, 1 X MS salts 3% (w/v) sucrose, 0.05% (w/v) MES, 1 X Gambourg's B5 vitamins and 0.8% (w/v) phytagar, seeds were surface sterilised as described in section 2.2. Containers were incubated at 4°C for two days to vernalise the seeds. Within 4 days of plating, green germinants were visible and within 14 days most seeds had germinated. Germinants were transferred to media without kanamycin. With each seed sterilisation

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and plating, positive and negative controls were included. The positive and negative controls were wild-type seeds plated on non-selective and selective media respectively.

6.2.6. Extraction of gDNA from leaf material

Leaf material was harvested from mature putative antisense plants and wildtype plants and the gDNA was extracted following the method for small scale genomic DNA extraction (section 2.8.5).

6.2.7. Ethanol assays of putative inducible antisense plants

The ethanol assay used by Vanlerberghe et. al. (1995) in studying tobacco plant lines was adapted for use with *A.thaliana*. The protocol followed is outlined in section 2.12 and was a preferred, over oxygen electrode analysis of leaf slices, due to the small amounts of tissue needed. An explanation of ethanol production by AP-impaired leaf tissue is diagrammatically presented in figure 6.4. Putative antisense *aox1* leaf material, unable to use the AP, produces ethanol in the presence of the CP inhibitor AA. This is because the tissue undergoes substrate metabolism by aerobic fermentation, summarised in the reaction below:

$$PYRUVATE \rightarrow CO_2\uparrow + ACETALDEHYDE \overset{+NADH}{\underset{}{\rightleftharpoons}}^{NAD+} ETHANOL$$

The initial reaction is catalysed by pyruvate decarboxylase and the reduction of acetaldehyde is catalysed by alcohol:NAD oxidoreductase. Leaf material was sprayed with 5 μM $CuSO_4$ solution and after day 5 leaves were harvested.
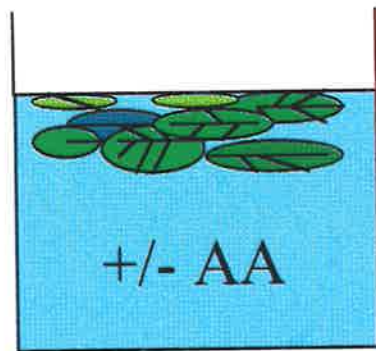
- AA

CP and AP in use

+ AA

CP inhibited
AP in use if available

+/- AA

leaf tissue floating on media +/- AA

wild-type leaves: utilise CP in absence of AA
utilise AP in presence of AA
(native *Aox1* gene expressed)
transgenic leaves;(-Cu) act as wild-type leaves
(native *Aox1* gene expressed)
(+Cu) utilise CP in absence of AA
cannot use either AP or CP in presence of AA, utilise aerobic fermentation and produce EtOH
(native *Aox1* gene and antisense *Aox1* construct expressed)

Figure 6.4 : Interpretive diagram showing leaf tissue responses in flotation studies.

A detailed description of the assay procedure is found in section 2.12. Concentrations of ethanol were measured on a spectrophotometer (Aminco DW-2a UV/VIS) and expressed as μmoles/g(fwt).

6.2.8. PCR analysis of transformed plant lines.

Wild-type and putative antisense plant gDNA was tested via PCR for the presence of the cDNA sized*aox1* insert. The initial set of primers used was ARAAOX1 and ARAAOX2. The WT gDNA was included in each PCR assay as a control.

6.2.9. Generation of transgenic cell suspension cultures.

Once a transgenic plant was confirmed positive by both PCR analysis and the ethanol screening, its leaf material was used to generate callus as described in section 2.4. Friable callus was then used to establish cell cultures (see section 2.3 for details) which were subcultured at regular weekly intervals.

6.2.10. Oxygen electrode analysis of wild-type and transgenic cell cultures.

Cell cultures (WT and transgenic) were subcultured at weekly intervals in the presence or absence of 5 μM $CuSO_4$. Subculturing took place three days prior to analysis, to allow the cells to reach the ideal exponential growth stage. Samples of culture were taken and fresh weights calculated per mL. This fresh weight value was used in the subsequent rate calculations. For each oxygen consumption assay 1 mL of cell culture was diluted with 2 mL fresh culture medium (without

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growth factors). Cells were titrated with FCCP to determine the maximum oxygen consumption rate. Inhibitors were added (2 mM SHAM, 0.3 mM KCN) where indicated, the concentrations and incubation times having been previously calculated from titration assays.

6.3 Results & Discussion

6.3.1. Making the inducible antisense construct

The vector pPMB7066 containing the *Xba* I cloning site was approximately 3.3kb in total. The *aox1* cDNA was amplified using PCR and the *Xba* I ends allowed easy ligation into pPMB7066 (see figure 6.2). The pPMB7066 expression cassette contained the copper-responsive promoter. The orientation of the *aox1* cDNA, with respect to the promoter region, was verified using two *Kpn* I restriction sites. One was assymetrically placed within the inserted cDNA and one was on the right border of the vector pPMB7066. Figure 6.3 indicates the size of the DNA fragment expected with a *Kpn* I digest. When the *aox1* was inserted in the antisense direction the *Kpn* I fragment was approximately 700 bp. Figure 6.4 shows the diagnostic pDNA *Kpn* I digest of a successful clone, indicating the *Kpn* I fragment of the correct size.

The 7066*aox1*-antisense expression cassette was cut out (using *Not* I), isolated and subcloned into the *Not* I site of pPMB765. The resultant hybrid cassette within pPMB765 contained the 7066*aox1*

expression cassette as well as the selective marker for kanamycin resistance and the ACE1 gene to produce the copper-modulated transcription factor. Clones containing this larger, hybrid cassette were verified by a diagnostic digest with *Not* I (see figure 6.3, lanes B and C of uncut and cut pDNA respectively). The successfully transformed *A.tumefaciens* was identified by purifying pDNA, digesting with *Not* I and verifying the presence of the *Not* I 7066*aox1* fragment (results not shown).

6.3.2. Kanamycin selection and growth of putative transgenic seedlings.

After vacuum infiltration of WT plants using the transformed *A.tumefaciens*, plants were grown until seed-set and the potentially transformed seeds were harvested. These seeds were screened on selective (kanamycin 50 mg/L) germination medium. The germination and growth of WT *A.thaliana* was severely restricted in the presence of kanamycin (50 mg/L). Wild-type seeds rarely germinated and if they did the germinant survived 4 to 5 days, turning either dark purple and blanched or completely blanched. (Figure A5 shows the effect of kanamycin on WT seed germination.) Germination of putative transformed seeds on selective media was as rapid as the WT on non-selective media (positive control), however cotyledons of putative transformed plants were a lighter green when compared with the dark, green colour of WT germinants on non-selective media. This indicated that the kanamycin resistance was not

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efficient enough to completely overcome the high concentration of kanamycin in the media. This concentration was reduced from 50 mg/L to 30 mg/L. The putative transgenic germinant had a phenotype that was an intermediate form, between the WT germinant restricted by kanamycin poisoning and the healthy, WT positive control, and demonstrates the limitations of the kanamycin gene expression in the putative transgenic plant. Germinants grown on kanamycin (50 mg/L) were transferred to non-selective media. These plants took several weeks to recover and continue growth, producing dark green tissue. Wild-type seeds which had managed to germinate on 30 mg/L kanamycin media were also transferred, however were unable to recover (results not shown).

The putative transgenic plants showed three general phenotypes (see figure A3) which were either (a) indistinguishable from the WT, (b) noticeably bulkier in appearance to the WT, with multiple, thick bolts and larger, fleshier leaves, or (c) similar in size to the WT but with multiple rosettes forming at the base of the plant such that a vegetative "ball" structure occurred. The latter phenotype, exhibited by the plant F1.1, was termed "tease-ball" (figure A2) due to the ability of these multiple rosettes to be teased apart, using forceps. New plants were generated from the individual rosettes which displayed little or no root structure and were slow to flower. New plants could also be generated from implanting single leaves into germination media devoid of growth factors. Multiple attempts to

generate new WT plants in this way were unsuccessful.

6.3.3. PCR analysis of construct-carrying plant cell lines

Putative transgenic germinants that survived growth on kanamycin media for at least 2 weeks were transferred to non-selective media, allowed to recover and then leaves were harvested for gDNA extraction and PCR screening. Genomic DNA was amplified using PCR and the internal *aox1* primers AraAox1 and AraAox2 (for more detail see section 6.2.1). These results are presented in figure 6.5. Amplification of WT gDNA produced a single band of approximately 1.6 kb as predicted from the genomic sequence (section 4.3.1). Amplification of putative transgenic plant lines gave two fragments of approximately 1.6 and 1.0 kb. The larger band was indicative of the native plant gene and the smaller band was amplified from the *aox1* cDNA insert in the construct. Plants showing the double band pattern were taken to the next stage of screening to ascertain whether the copper-inducibility of the introduced construct was effective. The number of positive PCR results from the 84 different plant lines tested was 33. This meant that the efficiency of transformation was 39.3%.
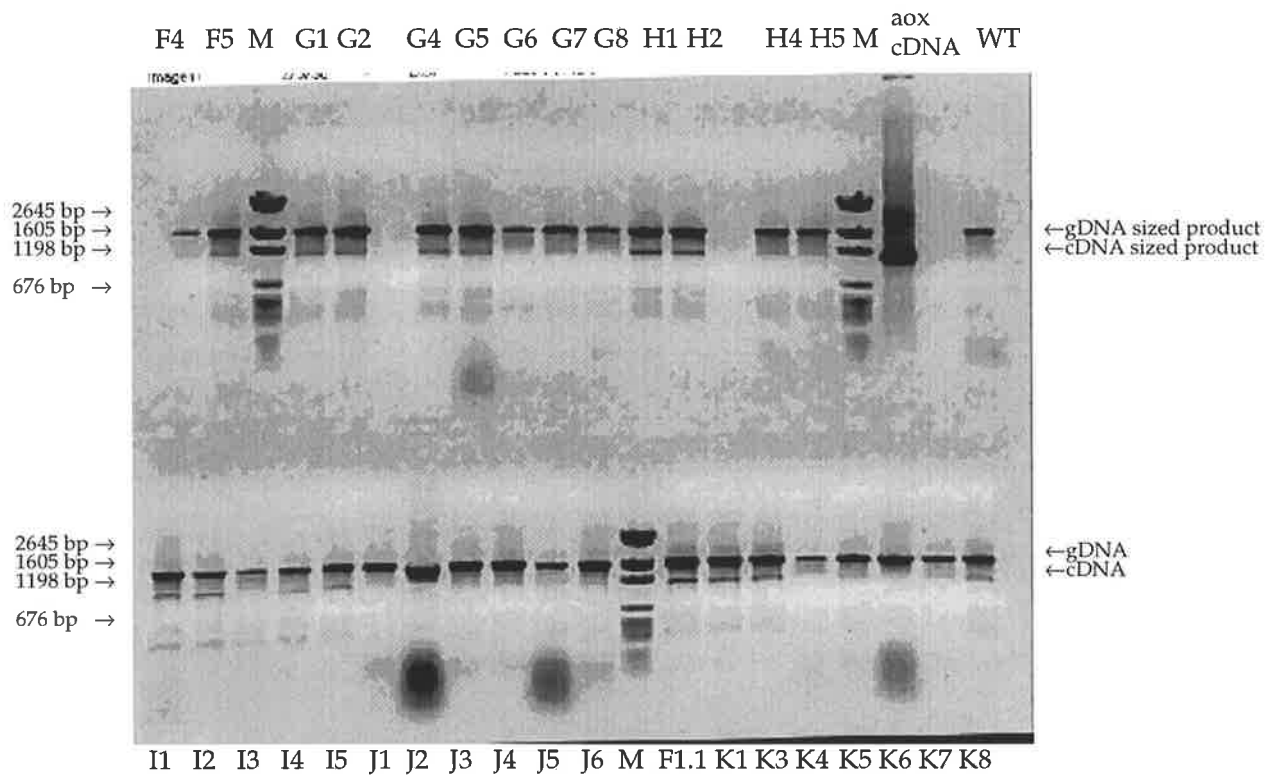
Figure 6.5: Agarose gel (0.7%) of the PCR products (1500 and 1000 bp) resulting from PCR with *A. thaliana* gDNA from putative transformants, using primers Araaox1 and Araaox2. The WT gDNA and *aox*1 cDNA exhibit products of 1500 and 1000 bp respectively, as indicated with arrows. The positions of the markers **(M)** are indicated in the left margin.

6.3.4. Ethanol assays of putative inducible antisense plants

Leaf flotation studies were originally used by Vanlerberghe et. al. (1995) to investigate the consequences of the *aox* antisense plant's inability to use AOX when the CP was limiting respiration. They found that antisense (*aox*) tobacco leaves produced approximately 16 µmol of ethanol per gram of fresh tissue after 24 hours (measured in the flotation solution), compared to wild-type tissue which had low to undetectable levels of ethanol. The leaf flotation experiments were adopted from this work and used in this project as a method of screening plants for copper-inducibility without a large destructive harvest of leaves.

While the ethanol assay was primarily used as a method of detecting the copper-inducibility of the transformants with minimal tissue harvest, it also provided a sensitive method confirming that the application of 5.0 µM $CuSO_4$ solution as a foliar spray did not induce the AP. Wildtype plants were similarly sprayed with the inducer and leaves were harvested after day 5 and tested in the same manner as the putative antisense plant material. The results presented in table 6.3 showed that the presence of copper did not significantly affect the concentration of ethanol produced by the wild-type leaves.

From the plant lines that had been shown positive for the presence of the construct, fifteen of these were induced with 5.0 µM $CuSO_4$ and tested for the production of ethanol after incubation with the CP inhibitor, AA. The rationale for distinguishing the inducible

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antisense plant tissue from the wild-type phenotype is explained in figure 6.8. Plant lines that contained a copper-inducible construct were expected to behave like WT plants (i.e. use their AP and therefore not produce ethanol) when not induced with 5 µM $CuSO_4$ and were expected to behave like antisense plants (i.e. produce ethanol) when induced. The degree of construct efficiency was expected to be reflected in the level of ethanol produced. Out of the plant lines tested two were found to consistently produce higher levels of ethanol (compared to wild-type controls) when induced.

Table 6.2 : *Concentration of ethanol in leaf flotation solution (comprising of nutrient solution and 25 µM AA) after 24 hours, expressed as µmol/g(fwt). Plants had previously been treated with 5.0 µM $CuSO_4$ foliar spray. All values (except F1.1) represent the mean of two assays. The F1.1 value represents the average of four assays and is also presented in table 6.3 along with the wild-type results.*

plant line	[EtOH] (µmol/g(fwt)
F1.1	18.4
F1.7	6.6
A5	1.4
A8	0
A13	2.1
A16	6.7
C2	0
D6	0
D7	2.5
D8	6.1
D9	1.5

Table 6.2 shows the mean of two measurements taken from a number of different putative transgenic plants. The results from one of these lines, F1.1 showed high ethanol production in comparison to

the other plant lines and to the wild-type results, as well as copper-inducibility. These results are presented in Table 6.3.

Table 6.3 : *Concentration of ethanol in leaf flotation solution expressed as $\mu mol/g(fwt)$. Leaf tissue ($\geq$ 20 mg) which had been previously treated with 5.0 μM $CuSO_4$ foliar spray, was floated in 2 mL of nutrient medium containing 25 μM AA, which was sampled after 24 hours. (n=the number of separate experiments.)*

plant line	[EtOH] (µmol/g(fwt)	n	[EtOH] (µmol/g(fwt)	n
	- AA		+ AA	
WT - Cu	2.4 (2.0)	4	2.1 (1.9)	4
WT + Cu	3.0 (2.1)	4	4.5 (3.8)	4
F1.1 + Cu	2.6 (3.8)	4	18.4 (4.1)	3
F1.1 - Cu	----	-	3.5 (3.3)	3

Table 6.3 shows the amount of ethanol (µmol/g(fwt)) produced by incubated leaf tissue in a 24 hour period. The WT leaves do not show any significant difference in ethanol production whether sprayed with $CuSO_4$ or not. This further supports the evidence that the AP in WT plants is not affected by copper treatment. Wild-type plant tissue was able to utilise the AP when presented with the CP inhibitor AA. This is evident in the (+)AA results which do not vary significantly from the (-)AA results. Leaf tissue from the transformed plant line F1.1, in the absence of copper treatment, showed similar results to the WT tissue (data not shown). When incubated in the presence of AA, non-induced F1.1 leaf tissue was able to utilise the AP and did not undergo aerobic fermentation. However following treatment with a foliar spray of 5.0 µM $CuSO_4$ leaf tissue from F1.1 did not appear to

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utilise the AP with the same efficacy, resulting in the production of ethanol. This was not apparent in leaves that were incubated without AA and therefore were able to use the CP. Copper-induced F1.1 leaves produced up to 8.8 times more ethanol than WT tissue indicating that the native *aox1* gene expression was reduced by the antisense construct.

Figure 6.6 shows the production of ethanol over a 72 hour period. It was apparent that leaves taken from the copper-induced transgenic plant line F1.1, and incubated in the presence of AA (F1.1 +/+) showed an obvious increase in ethanol production well above that of the other treatments over the 72 hour sampling period. When non-induced leaves from the same plant line were incubated with AA (F1.1 -/+), the production of ethanol was not significantly different from the WT leaves. These results were encouraging because they indicated that the copper-inducible system was not being expressed in the absence of copper, as evident by the non-induced F1.1 leaves behaving as WT leaves. These results also supported the belief that copper treatment alone in WT tissue was not able to induce *aox*, as all of the WT samples were within the same range of ethanol production.

The 72 hour ethanol concentration results presented in figure 6.6 represented the mean of two samples. This was due to the high incidence of infection after 48 hours.

Ethanol production by leaf samples over a 72 hour period

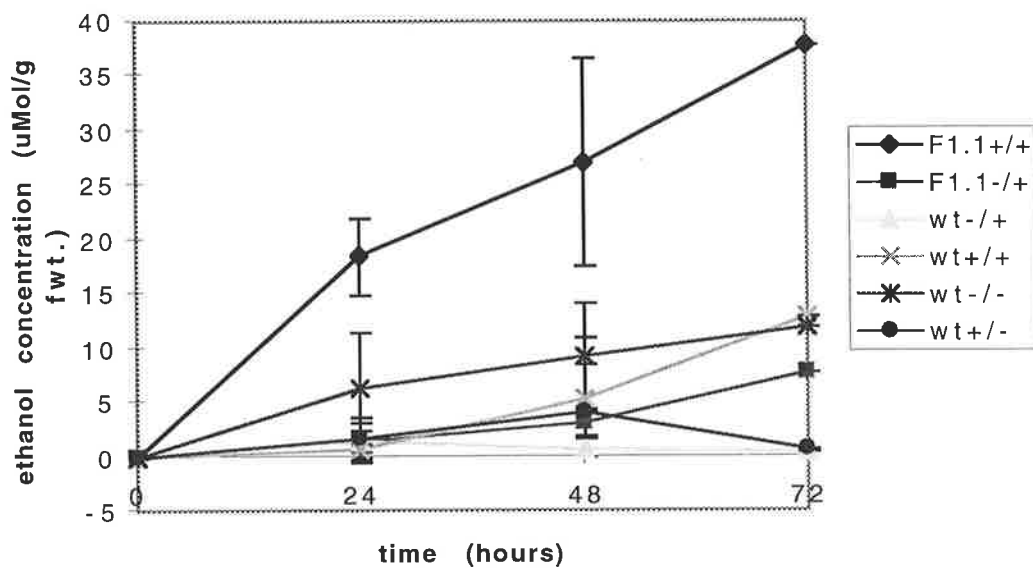**Figure 6.6 :** Ethanol production by leaf tissue over a 72 hour period. Measurements taken at 72 hours represent an average of two samples. For other values n ≥ 3. (Figure legend abbreviations ; +/+ copper induced leaves in the presence of AA, +/- induced leaves in the absence of AA, -/+ non-induced leaves in the presence of AA, -/- non-induced leaves in the absence of AA., and wild-type = wt.)

The 72 hour sample values (figure 6.6) show the continued increase in the production of ethanol in leaves from the induced plant line F1.1, however while leaves from the non-induced F1.1 were still within the WT values, some had increased to values approximately one third of that seen in F1.1(+/+). This only occurred in two WT samples and may have been due infection (potentially by yeast species) by day 3. The non-induced F1.1 leaf samples were also increasing their ethanol production by 72 hours, but were still within the WT values. This increase in ethanol production was expected by day three due to the presence of copper in the nutrient solution, which would induce F1.1 -/+ tissue to exhibit F1.1 +/+ characteristics.

The F1.1 plant line was made into a cell suspension culture which was tested for respiratory activity in the presence of inhibitors.

6.3.5. Oxygen electrode analysis of wild-type and transgenic cell cultures.

Table 6.4 shows the results of the oxygen electrode analysis of the induced and non-induced F1.1 cell cultures in comparison to copper-treated and untreated WT cell cultures. Wild-type cells showed no significant difference in the percentage SHAM and KCN-sensitivity, however overall control rates (in the absence of any inhibitor) of the cells without copper were twice that of cells with copper present. The reason for this was unclear. It was not due to substrate limitation because all control rates were measured in the presence of the uncoupler FCCP (0.02 µM).

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Table 6.4 : *Oxygen consumption results of wild-type and F1.1 cell suspension cultures expressed as a percentage of the control rate (cells respiring in the presence of 0.02 μM FCCP). Standard deviations are given in parentheses. (n=the number of separate experiments.)*

cell cultures	SHAMsens %rate	n	KCNsens %rate	n	Control rate nmolO$_2$/min/g(fwt)	n
F1.1 (- Cu)	7.7 (5.4)	7	57.4 (9.1)	7	1159.4 (329.1)	15
F1.1 (+Cu)	22.5 (6.6)	5	71.4 (5.6)	9	1716.2 (530.5)	17
WT (- Cu)	30.2 (4.9)	5	70.1 (14.7)	6	1264.5 (344.5)	8
WT (+Cu)	33.5 (10.3)	2	75.9 (4.2)	2	812.0 (93.5)	8

F1.1 cells cultured in the presence of 5 μM CuSO$_4$ showed no significant difference, in any of the rates measured, from the WT cells cultured without copper. This implied that the antisense construct was ineffective or not efficiently expressed in the cell cultured state. F1.1 cells cultured without copper behaved quite differently. Whilst they possessed similar control rates, they showed significantly less sensitivity to SHAM and showed a slight trend towards decreased KCN sensitivity. This indicated that non-induced F1.1 cells had a lower AP activity prior to SHAM addition and yet also showed a lower dependency on the KCN-sensitive pathway, when compared to WT cells. These results were not expected, and further experiments are required to clarify the behaviour of the cell cultures. This type of discrepancy between transgenic whole plants and cell culture was also observed by Vanlerberghe et. al. (1994). They found that the plant line S11, transformed with a sense *aox1* cDNA under the control of the 35S

promoter, showed no detectable leaf AOX protein however, once cultured, these cells became over-expressors with very high levels of AOX protein. This "antisense effect" of the sense construct in the tobacco plant line was explained by "reversible co-suppression", namely that the homologous, sense copy of the *aox1* gene was able to suppress expression of the native gene in whole plant tissues. The *A.thaliana* results presented here could also reflect the complex interference that occurs when plants contain a number of homologous genes. The inability of the induced antisense F1.1 culture to respond in the same manner as the leaf tissue (seen by the ethanol assay) could be due to the expression of different *aox* genes in different plant tissues. From the work by Saisho et. al. (1997) the four copies of *aox* in *A.thaliana* are expressed in different tissues. The gene *aox1a* is expressed in flowers, buds, stems, rosettes and roots of 8 week old plants. The other three homologous genes are expressed in some, but not all of these tissues. When plant cells are grown in suspension culture they are present as rapidly dividing, largely undifferentiated cells. The expression of *aox1a, b, c* and *aox2* has not been fully investigated in cell suspension cultures. Why the *aox1a* antisense construct did not function in these cultures may be answered by such studies. It is likely that in cell suspension cultures a different *aox* gene is expressed and the homology between this gene and *aox1a* is not sufficient to confer the antisense phenotype after copper induction. Further experiments, such as Northern blot analysis of the different

aox mRNA transcripts expressed in cell cultures, are required before these results can be fully understood.

6.4. Conclusions

Transgenic *A.thaliana* plants were successfully produced containing a copper-inducible system controlling the expression of an antisense *aox1a* cDNA insert. The presence of the construct within transformed plants was screened by growth on kanamycin and confirmed by PCR analysis. The copper-inducibility of these plants was tested indirectly via an assay, measuring ethanol production, in leaf tissue inhibited with AA. The copper-inducible system was found to be tightly controlled such that the non-induced plant tissue behaved like the WT controls. This project focussed on one transgenic line (F1.1) and found that, when cultured as a cell suspension, this cell line did not behave as expected. The mechanism of antisense construct interference in *aox1* gene expression is likely to be complex in a system involving a gene family with four homologous members, however these results show that the inducible antisense plant F1.1 will be a valuable tool for future research that investigates the role of the AP in the plant's stress response.

6.5 Future Studies

The lack of available tissue prevented the purification of mitochondria from transgenic plants within the time scope of this project. After the amplification of seeds and plant tissue, the

purification of mitochondria would be valuable in assessing the different AOX protein levels in wild-type, non-induced and induced transgenic plants. This will be a major focus for future studies.

Northern blot analysis of cell suspension cultures, induced and non-induced, would allow investigation of the complex processes occurring between the expression of the construct and native *aox* genes. This may explain the results in table 6.4 which showed that transgenic cell suspension cultures did not act in a way that was similar to the original transgenic leaf tissue.

The main goal of future studies, now that an inducible *aox1* antisense plant has been made, is to further investigate the stress responses of plants in an effort to comprehend the involvement of the AP, AOX protein or gene. This goal may be approached by two paths; one studying the bioenergetic and protein-protein interactions (such as the putative role of AOX in reducing damaging reactive oxygen species that proliferate during cellular stress) or the other, by attempting to elucidate the nuclear-mitochondrial intercommunication that occurs when the nuclear *aox1* gene is induced during mitochondrial stress. Either approach would be assisted by using an inducible antisense *aox1* plant and both approaches are necessary to fully investigate the role of alternative oxidase during plant stress.

CHAPTER SEVEN : GENERAL DISCUSSION

7.1 Discussion

A.thaliana was chosen for this study because of its short life-cycle, its abundant production of seeds, and the relative ease of *in planta* transformation (via the vacuum-infiltration technique) and production of cell culture. In addition to this, mitochondria purified from *A.thaliana* cell suspension culture had previously exhibited high AP activity of 49 ± 4.0 nmolO_2/min/mg protein (de Virville et. al. 1994), which was comparable to *G.max* root mitochondria (98 ± 20 nmolO_2/min/mg protein (Day et. al. 1995). The AOX protein has previously been shown by immunoblot analysis to be 36, 35, 38 kDa in size in *S.tuberosum* (Hiser and McIntosh 1990), *N.tabacum* (Vanlerberghe et. al. 1994) and *B.vulgaris* (Johnson 1993) respectively and as multiple bands in *S.guttatum* (35, 36 and 37 kDa), *G.max* (32 and 34 kDa) and *M.indica* (27, 33 and 36 kDa) (Elthon and McIntosh 1987b, Day et. al. 1993 and Cruz-Hernandez and Gomez-Lim 1995 respectively). The same antibodies, directed against the AOX of *S.guttatum*, were used in each analysis. The AOX in *A.thaliana* is slightly smaller in size (28 and 64 kDa for AOX monomer and dimer respectively) than that of other known species. This was detected via immunoblotting using antibodies specific for the *S.guttatum* AOX.

Application of stress to either plant tissue or cell culture changed the potential activity of the AP in mitochondrial respiration.

The work by previous investigators facilitated the choice of the four types of stress treatments used, namely (i). chilling (Vanlerberghe and McIntosh 1992), (ii). chilling plus dark treatment (Atkin et. al. 1993; Mawson 1994), (iii). SA treatment (Kalpulnik et. al. 1992; Johnson 1993; Rhoads and McIntosh 1993) and (iv). AA treatment (Vanlerberghe and McIntosh 1994). Previous investigation had shown an increase in either AP capacity, AP activity, AOX levels and/or *aox* expression with each of these treatments using other plant species. Measurements of leaf slice respiration showed that the cold plus dark treatment of plants increased the percentage SHAM sensitivity (chapter three). SA foliar spraying of plants also led to increased percentage SHAM sensitivity when measuring the respiration leaf slices. However, of all the different types of applied stress, the addition of AA gave the most dramatic response, and in *A.thaliana* cell culture, this response was far greater than that observed with SA. While AA application provides an excellent experimental method for continued stress studies, if one wished to ascertain the role of AOX, it may be more appropriate to study a stress similar to that experienced by plants *in vivo*. Further investigation of these and other types of stress is ideally required for future studies on the role of AOX during plant stress.

The copper-inducible system produced by Mett et. al. (1993) had been successfully transformed and used in tobacco and *Lotus corniculatum* (Mett et. al. 1993, P. H. Reynolds pers. comm.). In order to test the system in *A.thaliana*, the marker gene glucuronidase (*gus*),

under the control of the copper-inducible system, was transformed into plants. This gave rapid and easily identifiable results on the efficiency of the copper-inducible promoter system in *A.thaliana* as well as the day of maximal expression (Chapter five). The time of maximal GUS activity staining occurred on day 4 post-induction with a foliar spray of 5.0 μM $CuSO_4$ in four different transformant lines (Chapter five). These results both encouraged the use of this system with the antisense construct in *A.thaliana* and provided a temporal guide for testing antisense expression in this small plant (Chapter six).

Once the presence of the antisense construct in *A.thaliana* had been confirmed by PCR, leaf AP activity was indirectly tested using a method requiring only small amounts of harvested tissue, such that tested plants could be grown to seedset. The production of ethanol by copper-induced leaves, floated in a nutrient solution containing AA, was a reliable method of detecting an increased production of ethanol Leaf tissue with AA-inhibited CP activity displayed any reduction in AP activity via the production of ethanol. Of the successful transformants tested for a copper-inducible reduction in functional AOX, the plant line F1.1 showed the largest production of ethanol (up to 8.8 times that of the WT control) indicating the greatest reduction of the AP's capacity to absorb the electrons inhibited from flowing through the CP by the presence of AA. The results also showed that the application of copper to wild-type leaves had no apparent effect on the AP and, most importantly, that the copper-inducible system did not appear to be

expressed in the absence of copper. This ensured that the yeast promoter and transcription factor, used in producing the copper-inducible system, was working efficiently in *A.thaliana*.

This project has been successful in producing an inducible antisense *aox1* plant and in opening up a whole field of future research. Studies on induced and non-induced antisense plant tissue and cell suspensions have been presented in this project. The future work now planned is summarised in figure 7.1 and discussed in detail below, beginning with the initial stage (stage I) which follows on from the identification and investigation presented within this project.

7.2 Future Research

7.2.1. Stage I

The ethanol assays used in this project have identified that there is a decrease in AOX function when copper is applied to inducible antisense *aox* plant leaves. Whether this decrease in enzymic function relates to an absence of AOX protein is one of the initial questions to answer. Subsequent studies to accurately determine the time of maximal expression of the antisense cDNA in leaf tissue, are required for future expression studies. Maximum expression of the construct could be determined using Northern Blot analysis and a specific antisense *aox* probe.

STAGE I

- Accurate determination of the maximal day of antisense transcript expression, potential AOX reduction and AP inactivity
- Physiological studies of the growth of induced antisense plants compared to WT, under ideal conditions
- Determination of effects of *aox* silencing upon other mETC components under ideal conditions

STAGE II

- Studying the role of AOX during stress by repeating the physiological studies in STAGE I, comparing non-induced, WT and induced antisense plants during stress
- Determination of effects of *aox* silencing upon other mETC components under stressful conditions and comparing this with the results found in STAGE I
- Examination of the potential, ROS-protective role of AOX by studying the levels of ROS, other ROS-scavenging enzymes and cellular changes due to increased ROS activity

STAGE III

- Investigation of nucleo-mitochondrial interactions

Figure 7.1 : A flow diagram indicating the stages of future research using the inducible antisense *aox1* plants.

To determine when this expression had an effect upon the ability of the AP, ethanol production could be measured in samples (taken each day after induction) incubated in nutrient media plus AA. Similarly immunoblots using mitochondria purified from leaf tissue would show variations in AOX protein levels along a post-induction time course. These three approaches using leaf and cell suspension cultures would give information about the times at which the native gene expression, protein levels and enzymic function were "silenced". It would also discern whether cell suspension cultures were appropriate to use in the study of *aox1a* silencing and it would give more information on the turn-over of the AOX enzyme in *A.thaliana*.

Another area future of interest could be the study of how the antisense *aox* genotype affects plant growth and general physiology with and without stress. Initial observations have shown that phenotypic differences occur between the wild-type and the transgenic plants (see appendix figures A2 and A3). However these occur without induction and may be due more to the nuclear site of construct integration. Continuing this examination with growth measurements of transgenic plants grown with and without copper, and compared to wild-type controls, would allow observation of any effects on growth caused by a decrease in AOX function. Analysis of this kind could take place both in the absence and presence of environmental stress such as drought or chilling. The results from such a study may relate to how important AOX is to the overall growth of the plant in the field.

7.2.2. Stage II

Examination of the effects of stress on non-induced and induced plants could provide some answers to the role of the AOX in plant cells. For example, one could examine the potential role of AOX in diminishing the reactive oxygen species (ROS) which damage the cell during the plant's response to stress or disease. Signs of increased ROS in transformed plants upon induction with copper would suggest that AOX has an important, protective role. It is expected that as the antisense expression increased from the time of induction, so too would the levels of ROS. These could be detected in many ways, for example in the changes in plant physiology, the expression levels and activity of known ROS scavenging enzymes and levels of membrane lipid peroxidation and membrane integrity.

The expression of other mETC components before and during plant stress could be investigated in both induced and non-induced plants. The effect of diminishing the level of AOX protein on the expression of other mETC components could be studied. This would answer the question of whether there is any feedback or intercommunication between the component levels and their expression with respect to AOX.

7.2.3. Stage III

"Nucleo-mitochondrial interaction", or communication between the nucleus and mitochondrion, is an area of great interest when considering *aox* expression and induction. The *aox* nuclear gene

with its mitochondrial protein product is the ideal study for understanding how the cell co-ordinates mitochondrial demand and nuclear gene expression. Attention could be focussed on the communication with the nucleus when induction of *aox* expression is required in the plant cell during stress. Some sort of "signal" must be exchanged between the mitochondria and nucleus to turn on *aox* transcription during stress. The combination of differential display techniques and inducible antisense plants may help to focus this work. Differential display is a PCR approach whereby a suite of primers generate cDNA species representing those genes expressed in a plant (Welsh et. al. 1992). Comparison of a control plant with a treated plant allows detection of cDNA fragments appearing only in the treated plant. This allows identification of transcripts expressed in response to the treatment. With an inducible antisense plant, both induced and non-induced plants can be compared. Those plants that have been induced to down-regulate the *aox* gene expression are expected to have artifically high levels of the mitochondrial "signal" that tells the nucleus that *aox* expression is needed. Such "signals" could then be screened by the expected increase along a time course from the point of induction during stress. This would provide a more accurate assessment of potential "signals" than the use of constitutive antisense in comparison to wild-type plants. Constitutive plants may show a high background in this comparison for a number of reasons, including unrelated events such as the location of the antisense

construct within the genome. (The use of inducible antisense plants provides a comparison that would help eliminate any such background). In addition, constitutive antisense plants may have made long term changes in their mETC components due to the continual low level or absence of AOX protein. Inducible antisense plants are like wild-type plants until they are induced, which means that no long term adaptations are expected to have taken place.

Any studies involving the copper-inducible antisense plants would require detection of any other plant genes turned on by copper. The copper-inducible system was made from yeast elements, which respond to lower levels of copper than those deemed toxic by the plant. Nevertheless it is not unreasonable to assume that some plant genes may be activated by the low levels of copper used to induce the yeast promoter system and these could present as false positives when attempting to identify the nucleo-mitochondrial "signals". Elimination of these false positives could be achieved by either ; (i) comparing them to the wild-type transcripts expressed after copper spraying or (ii) identifying of the "false positives" sequences with the copper-inducible genes already present in the Arabidopsis genome project database.

In summary the inducible antisense plants provide a wealth of exciting future study that will assist our further understanding of the AOX role in plant cells and the mechanisms by which induction occurs.

APPENDIX

Figure A1 : The seed harvesting set-up for vacuum-infiltrated plants grown to seed-set. Each plant was confined within a wax paper cone. Seed pods were cut off and the entire paper cone removed. Seed pods were then tipped into tubes marked with the appropriate parent plant name and number.

Figure A2 : Photographs displaying the phenotype of the tease-ball mutants **(A)**. Plants had little or no root system and continued to regenerate small rosette-like structures at larger leaf axils **(B)**. These could be easily teased apart (see diagram C) from the parent plant. They would grow up to an adult size and then begin to form the tease-ball mutant morphology. In this manner F1.1 clones were produced and used throughout the project.
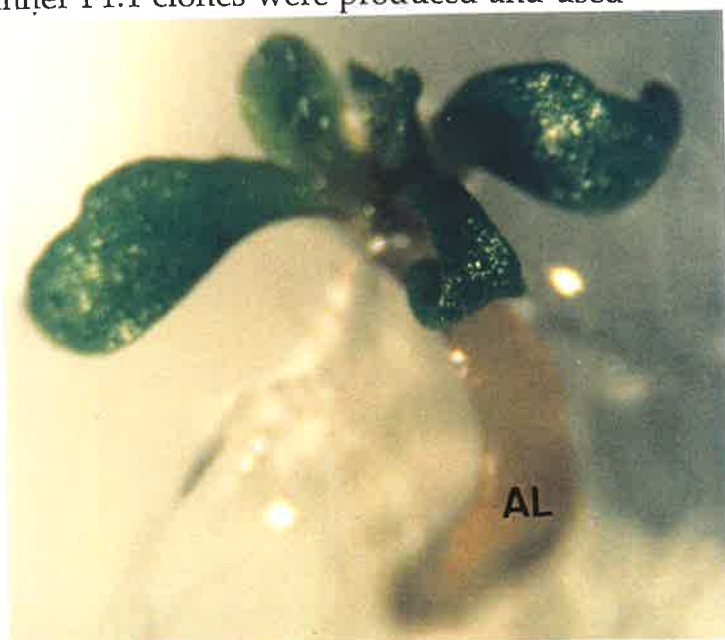
B. A dissecting microscope photograph of a detached, adult leaf (AL) with a tiny rosette growing from petiolar tissue.
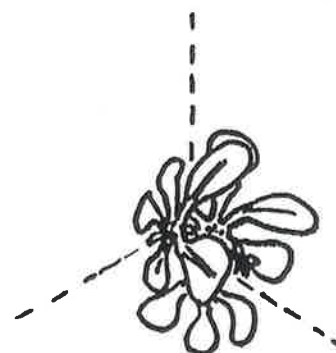
C. A hand-drawn diagram of how to divide a tease-ball mutant.

E3 F1.1 wild-type

Figure A3 : A photograph displaying the varying phenotypes of flowering transgenic (F1.1 compared with E3) and wild-type plants.

unsprayed

sprayed

Figure A4 : A photograph showing no apparent difference between unsprayed plants and those sprayed with copper and photographed after day 5.
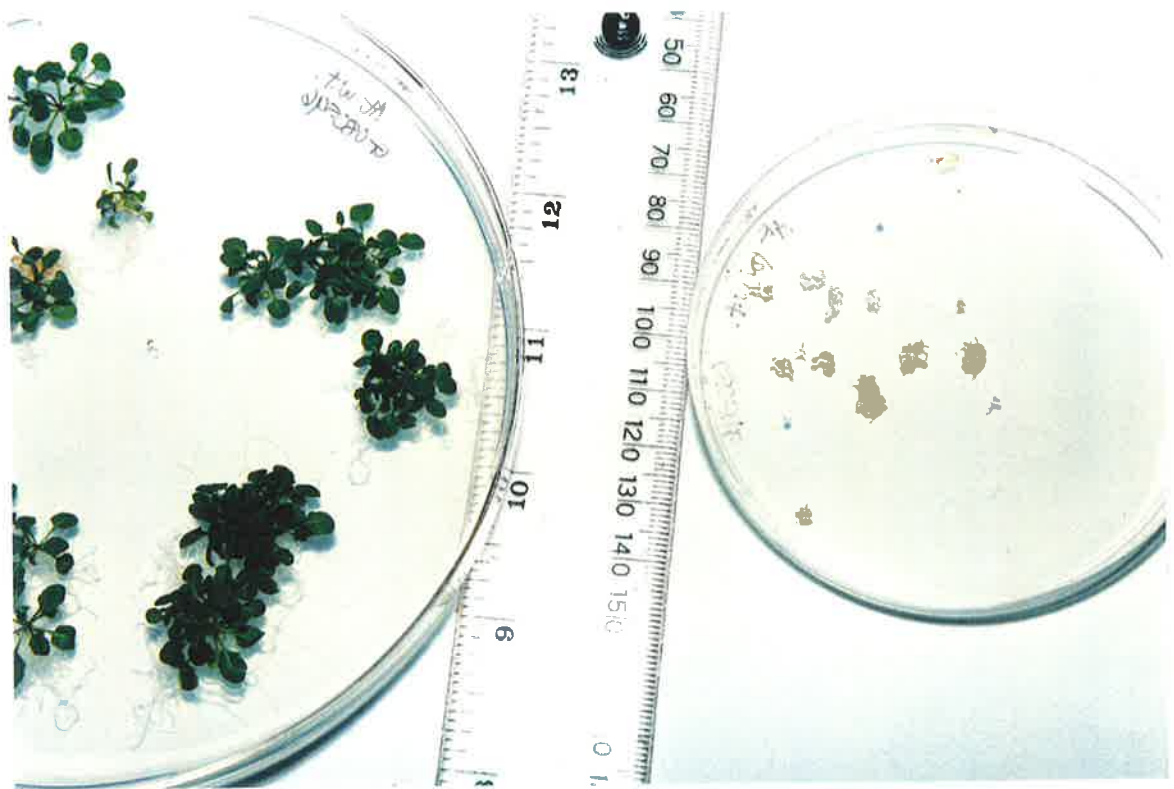
Figure A5 : A photograph showing WT *A.thaliana* germinants on media containing no kanamycin (left) and 30 mg/L kanamycin (right).

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