A TRANSGENIC APPROACH TO THE STUDY OF THE FUNCTION OF THE ADENINE NUCLEOTIDE TRANSLOCATOR.

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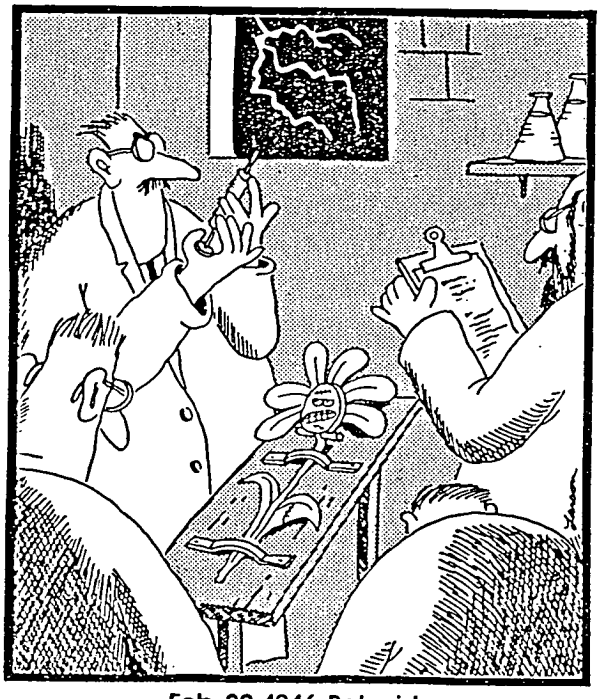

Feb. 22, 1946: Botanists
create the first artificial flower.

Declaration

I declare that all the work presented in this thesis is my own work, unless otherwise stated.

Index

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Abbreviations.

3'	3' carbon of a nucleotide or nucleoside, hydroxy terminus of a nucleic acid backbone, towards the 3' carbon.
5'	5' carbon of a nucleotide or nucleoside, hydroxy terminus of a nucleic acid backbone, towards the 5' carbon.
35S	promoter of the 35S cauliflower mosaic virus transcript.
ADP	adenosine-5'-diphosphate
AMP	adenosine-5'-monophosphate
ANT	adenine nucleotide translocator
ATP	adenosine-5'-triphosphate
BAP	6-benzylaminopurine
BSA	bovine serum albumin
bp	base pairs
c	flux control coefficient
chv	chromosonal virulence region of *Agrobacterium tumefaciens*
CIP	calf intestinal phosphatase
cm	centimetre
CMS	cytoplasmic male sterility
CaMV	cauliflower mosaic virus
CCC	(2-chloroethyl)-trimethylammonium chloride
°C	degrees centigrade
dCTP	2'-deoxycytidine-5'-triphosphate
DCCD	N,N'-dicyclohexylcarbodiimide
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
ds	double-stranded
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid (disodium salt)
FAD	flavine adenine dinucleotide
g	gram
GA_3	Gibberellic acid
kb	kilobase (pair(s))

l	litre
LB	Luria broth
Ltd.	limited
µg	microgram
µl	microlitre
µM	micromolar
µm	micrometre
M	molar
MES	2-[N-morpholino]ethanesulphonic acid
mg	milligram
ml	millilitre
mM	millimolar
mm	millimetre
mRNA	messenger ribonucleic acid
M&S	Murashige and Skoog
MU	methylumbelliferone
nm	nanometre
ng	nanogram
NAA	α-naphthaleneacetic acid
NaCl	Sodium chloride
NAD	nicotinamide adenine dinucleotide
NADH	β-dihydronicotinamide adenine dinucleotide
NCS	non-chromosomal stripe
NOS	nopaline synthase gene
NPT	neomycin phosophotransferase
OCS	octopine synthase gene
ORF	open reading frame
%	percent
pH	negative log of the hydrogen ion concentration
Pi	orthophosphate
poly(A)	polyadenylation site
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
S.C.R.I.	Scottish Crops Research Institute
SDS	sodium dodecyl sulphate
TAE	tris-acetate electrophoresis buffer

TBE	tris-borate electrophoresis buffer
TCA	tricarboxylic acid
T-DNA	DNA transferred from *A. tumefaciens* to plant cells
TEMED	N,N,N',N'-tetramethylethylene-diamine
Tris	tris(hydroxymethyl)aminomethane
USA	United States of America
U.V.	ultraviolet
V	volt
vir	*Agrobacterium tumefaciens* virulence genes encoded on the Ti plasmid
v / v	volume per volume (given as a percentage)
w / v	weight per volume (given as a percentage)

Abstract

The primary role of mitochondria in eukaryotic organisms is to generate ATP by oxidative phosphorylation to meet the organism's energy needs. The nuclear encoded adenine nucleotide translocator (ANT) is believed to play an important role in controlling mitochondrial function, by regulating the transport of ATP and ADP across the mitochondrial inner membrane. The aim of the work described in this thesis is to investigate the function of ANT by producing transgenic potato plants with introduced sense or antisense ANT genes, under the control of either constitutive or tissue specific promoters, to elevate or reduce the level of ANT protein.

The steady state levels of endogenous potato ANT expression appeared to vary between different tissues within the plant, in response to the energy requirements of the tissue, but not in response to a diurnal light dark cycle. The control coefficient of ANT in mitochondria isolated from tubers was found to be 0.45 to 0.51. This high control coefficient suggests that ANT can play an important role in controlling mitochondrial phosphorylation.

Binary vector constructs for *Agrobacterium tumefaciens* mediated transformation were prepared with both a heterologous ANT cDNA from maize and a homologous potato ANT cDNA. Both sense and antisense constructs were prepared with each cDNA under the control of the constitutive C_{α}MV 35S promoter, and two tissue specific promoters from potato. The expression of the patatin promoter is tuber specific, whilst the light induced ST-LS1 promoter is expressed in the leaves in association with chloroplasts.

A number of different protocols were used to attempt to regenerate transgenic potato plants. The overexpression of either heterologous or homologous ANT under the control of either the constitutive or tisue specific promoters did not affect the plants regenerated. The steady state level of ANT transcript was reduced in transgenic plants regenerated with both the heterologous and homologous ANT antisense genes under the control of the C_{α}MV 35S promoter, but the amount of ANT protein was not affected. No differences could be detected in transgenic plants with the tissue specific ANT constructs, apart from in one clone. Plants transformed with the homologous potato ANT antisense gene, under the control of the leaf specific ST-LS1 promoter, showed a reduction in both the level of ANT transcript and the amount of ANT protein in leaves. The expression of the antisense gene appeared to follow the expected leaf specific pattern as no change in ANT expression was found in root tissue. The steady state level of another nuclear encoded mitochondrial gene, NAD malic enzyme, was found to be increased in the leaves of these plants. The dark respiration rate of leaves from these plants was measured and was reduced by one third compared to that of wild types.

CHAPTER 1
INTRODUCTION

1.1. Introduction

Mitochondria are subcellular structures that provide energy and a variety of metabolic intermediates to eukaryotic organisms. The requirements of an organism for energy must vary during its growth, development and with changing environmental conditions. How mitochondrial functions are regulated to maintain a constant supply of energy is an area of much interest. The work described in this thesis was designed to investigate the role of the adenine nucleotide translocator (ANT) in the control of mitochondrial phosphorylation. The aim was to create transgenic plants that either over or under produce this specific protein so that its role and importance in the regulation of mitochondrial function could be determined.

1.2. Mitochondrial Function

The primary role of mitochondria in all eukaryotic organisms is the generation of ATP by oxidative phosphorylation. The mechanism by which this is achieved was first described by Mitchell (1966) and has become known as the chemiosmotic theory. The metabolic steps which lead to the generation of ATP are summarised in Fig.1.1. The oxidation of carbohydrates in the cytosol and the operation of the TCA cycle in mitochondria results in the production of NADH, which is oxidised by Complex 1 of the electron transfer chain. Electrons resulting from this reaction are transferred to oxygen by the components of the electron transfer chain, which in turn generates a proton gradient across the inner mitochondrial membrane from the matrix to the inner-membrane space. The proton gradient thus generated drives protons across the inner-membrane and this is coupled through the F_1-F_O ATP synthase to the synthesis of ATP from ADP and phosphate in the matrix. The ATP produced is transported from the matrix across the inner mitochondrial membrane by ANT in exchange for ADP. As a consequence of this coupling between oxidation and phosphorylation, if ATP synthesis is blocked by specific inhibitors (e.g. oligomycin) or lack of substrates (ADP, Pi), oxygen consumption is inhibited. Thus the control of this

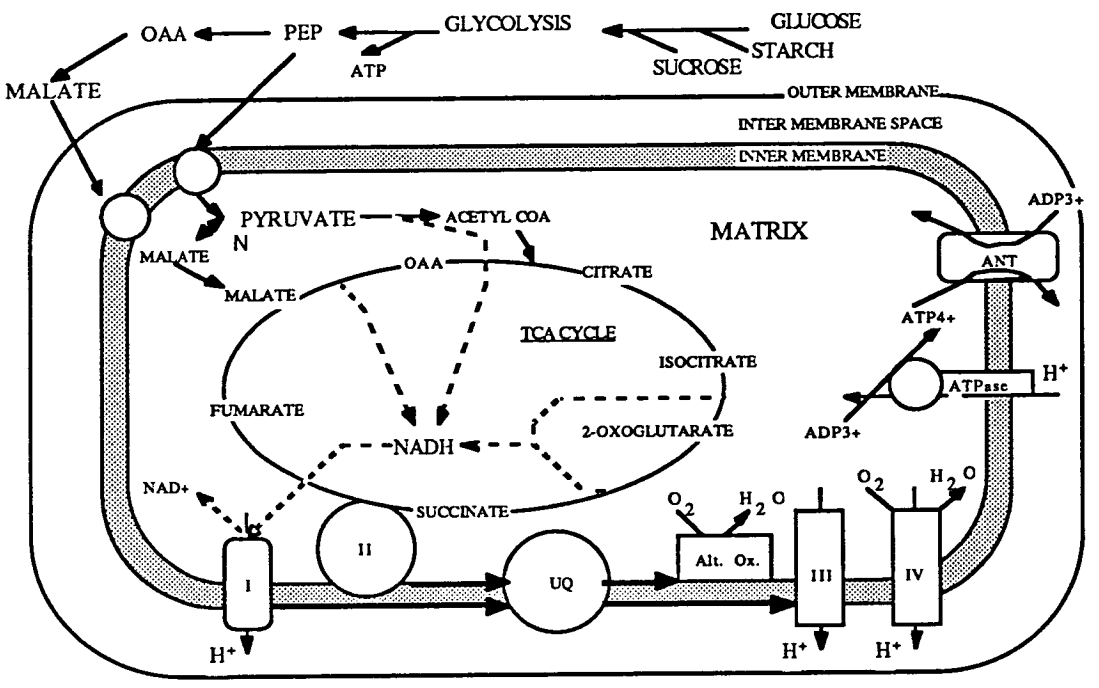


Fig.1.1. Metabolic Steps In Mitochondria Resulting in ATP Generation.

The production of ATP in mitochondria is the result of the TCA cycle in the mitochondrial matrix producing NADH which is oxidised by complex 1. The flow of electrons through the electron transport pathway in the inner mitochondrial membrane generates a proton gradient. ATP synthesis is coupled to the flow of electrons back into the matrix through ATP synthase. The ATP generated in the mitochondrial matrix is exchanged with ADP from the cytosol via ANT.

I, II, III and IV, the complexes of the electron transport chain.

UQ, ubiquinone pool.

Alt. Ox., alternative oxidase.

ATPase, ATP synthase.

ANT, adenine nucleotide translocator.

PEP, phosphoenolpyruvate.

OAA, oxaloacetate.

N, NAD malic enzyme

pathway in response to the energy requirements of a cell is of fundamental importance.

1.3. Features Unique to Plant Mitochondria

Although mitochondria from plants and other organisms share a common basic structure and many common functions plant mitochondria in addition possess a number of unique features (reviewed by Douce, 1985; Douce and Neuburger, 1989).

(1) Modified TCA cycle. The pathway of the TCA cycle in plant mitochondria includes an additional enzyme, NAD-malic enzyme in addition to the normal enzymes (Macrae, 1971). This enzyme catalyses the reductive decarboxylation of malate to pyruvate (Fig. 1.1.). High levels of activity of this enzyme have been found in all plant tissues examined so far except the nitrogen fixing nodules of soybean (Bryce and Day, 1990). The role of malic enzyme is unclear, but it may allow the operation of the TCA cycle without a 3 carbon substrate, or the removal of intermediates from the cycle (Wiskich and Dry, 1985).

(2) Modified electron transport chain. The electron transport chain possesses a number of complexes not coupled to proton translocation.

(a) Rotenone insensitive NADH dehydrogenase (Brunton and Palmer, 1973). This enzyme allows a by-pass of complex 1 and is able to oxidise matrix NADH without translocating protons across the inner mitochondrial membrane. The function of this enzyme is unclear, but the capacity of this bypass is high in mitochondria from soybean cotyledons and nodules where there are high rates of state 4 (ADP limited) respiration (Bryce *et al.*, 1990). This implies that this enzyme may be important in maintaining respiration when ATP/ADP ratios are high.

(b) External NADH dehydrogenase (Douce *et al.*, 1972). Cytosolic NADH is also oxidised by at least two externally facing complexes as well as complex I (Palmer and Ward, 1985). The first is located in the outer mitochondrial membrane and is not linked to the inner membrane electron transport chain (Douce *et al.*, 1973). The other is located in the inner mitochondrial membrane and can pass electrons to the ubiquinone pool (Douce *et al.*, 1973).

(c) **Cyanide insensitive alternative oxidase** (Bendall and Bonner, 1971). Cytochrome oxidase is sensitive to cyanide. However, plants exhibit a cyanide insensitive respiration (as reviewed by Lance *et al.*, 1985). This alternative pathway provides a second route for the transfer of electrons from ubiquinone to oxygen, bypassing both complexes III and IV of the electron transport chain. It can be inhibited by salicyl hydroxamic acid (SHAM) (Lance *et al.*, 1985), and although the structure of the complex is unclear two proteins have been implicated (Hiser and McIntosh, 1990). Once again the role of this pathway is unclear, but it has been suggested that it may allow mitochondrial substrate oxidations to take place while ATP levels are high, enabling the continued supply of carbon skeletons for biosynthetic activity (Bahr and Bonner, 1973).

(3) **Glycine oxidation.** The lack of specificity of ribulose bisphosphate carboxylase results in photorespiration in C3 plants. Oxygen rather than carbon dioxide is fixed resulting in the synthesis of the C2 organic acid glycolate which is indirectly converted to 3-phosphoglycerate. The conversion of two molecules of glycine to serine, a crucial reaction in this scheme, is catalysed by two enzymes, glycine decarboxylase and serine hydroxymethyl transferase (Neuburger *et al.*, 1986). In isolated mitochondria the NADH generated in this reaction is reoxidised by the respiratory chain generating ATP (Douce *et al.*, 1977). However it is argued that *in vivo* NADH is required for the reduction of hydroxypyruvate to glycerate and so is transferred to the peroxisome by means of a malate-aspartate shuttle (Dry *et al.*, 1987). When photorespiration was inhibited in barley protoplasts both the cytosolic and mitochondrial ATP/ADP ratios fell (Gardestrom and Wigge, 1989). Recent studies into the simultaneous oxidation of glycine and malate by isolated pea leaf mitochondria has further complicated the picture (Wiskich *et al.*, 1990). The addition of malate stimulates state 4 glycine oxidation, suggesting that NADH synthesised during glycine oxidation can be reoxidised by malate dehydrogenase operating in the oxaloactate to malate direction. Wiskich *et al.*, (1990) hypothesise that there are two pools of malate dehydrogenase within the mitochondrial matrix.

1.4. Changes in Respiratory Activity During Plant Development

There is evidence that the mitochondrial respiration rate in plants is coordinated with the energy requirements of the cell or tissue, and that these changes can be due to developmental or environmental factors.

During seed germination, as the seeds imbibe, there is an increase in the respiration rate. This increase may be due to the *de novo* synthesis of proteins and mitochondrial biogenesis (Morohashi *et al.*, 1981) or the activation of existing mitochondria (Morohashi, 1980; Ehrenshaft and Brambl, 1990). The respiration rate in root tissue is greatest in the root tip, where the cells are actively dividing and mitochondrial biogenesis is also occurring (Kuroiwa *et al.*, 1992). Azcón-Bieto *et al.* (1983) have found that the dark respiration in *Phaseolus vulgaris* is 3 fold less in mature leaves compared to young ones. Characteristic rises in respiration are observed during both fruit ripening, the 'respiration climateric', and during senescence (Noodén, 1988). Pathogen infection or wounding of a plant also results in an increase in respiration, presumably to supply energy for the production of secondary products such as phenylpropanoids (Uritani and Asahi, 1980). In some plants, such as the *Arum* species, mitochondria have a specialised thermogenic function producing heat to volatilize insect attractants during flowering (Meeuse, 1975). In *Arum maculatum* this may raise the temperature by up to 15 °C above ambient.

1.5. The Adenine Nucleotide Translocator

Whilst ATP is synthesized in mitochondria the primary site of ATP utilization is in the cytosol. The exchange between these two pools of adenine nucleotides is mediated by the adenine nucleotide translocator (ANT). Although this is a mitochondrial protein it is encoded in the nucleus and synthesised in the cytosol. It is composed of two identical subunits with a molecular mass around 30 kDa and spans the inner mitochondrial membrane. It catalyses a one-for-one sequential exchange of ATP and ADP across the inner mitochondrial membrane (Klingenberg *et al.*, 1976), which is

otherwise impermeable to adenine nucleotides (Klingenberg and Pfaff, 1966). This exchange is tightly coupled so that no net uptake or release of adenine nucleotides occurs (Meisner and Klingenberg, 1968). The carrier is highly specific; it excludes AMP and all other nucleotides with bases other than adenine except formycin di- and tri-phosphate which are close analogues of ADP and ATP (Pfaff and Klingenberg, 1968). Magnesium adenine nucleotide complexes are also excluded (Heldt *et al.*, 1972; Duszynski *et al.*, 1978). The rate of turnover of the adenine nucleotide translocator is relatively slow, but the high density of the carrier in the membrane compensates for this (Weidemann *et al.*, 1970) and it is in fact the most abundant nuclear-encoded mitochondrial protein involved in oxidative phosphorylation (Klingenberg, 1981). ADP-ATP exchange is not energy dependent and when the membrane is deenergised ATP and ADP are still transported in both directions. However energisation of the mitochondria makes the exchange directional and the uptake of ATP is strongly suppressed in favour of ADP (Pfaff and Klingenberg, 1968; Klingenberg *et al.*, 1969a, 1969b). When ADP and ATP are provided in equal amounts to the energised mitochondria the rate of ADP uptake exceeds that of ATP uptake by approximately ten fold (Klingenberg, 1972). A consequence of this is that the ratio of ATP/ADP can be up to 50 fold higher in the cytosol than in the mitochondria. It has been proposed that this directional exchange is driven by the transmembrane potential of the mitochondria combined with the charge difference between ADP^{3-} and ATP^{4-} (Pfaff and Klingenberg, 1968; Klingenberg *et al.*, 1969a). The adenine nucleotide translocator thus depends upon energy from the same source as ATP synthase and is thought to utilise approximately 25 % of the proton gradient to maintain the ATP/ADP ratios (Klingenberg, 1980). An ANT type protein that is able to mediate the uptake of ADP-glucose has also been identified in the inner membrane of amyloplasts from *Acer pseudoplatanus* (Ngernprasirtsiri *et al.*, 1989).

1.6. Adenine Nucleotide Translocator Genes

Initially immunological evidence, and more recently the isolation of multiple cDNA clones, have revealed that there are

multiple genes for this carrier in a number of different organisms, including yeast (O'Malley *et al.*, 1982; Lawson and Douglas, 1988; Kolarov *et al.*, 1990), humans (Battini *et al.*, 1987; Houldsworth *et al.*, 1988; Cozens *et al.*, 1989; Li *et al.*, 1989; Ku *et al.*, 1990), cattle (Schultheiss and Klingenberg, 1984; Rasmussen and Wohlrab, 1986; Powell *et al.*, 1988), maize (Baker and Leaver, 1985; Bathgate *et al.*, 1989; Winning *et al.*, 1991), *Arabidopsis thaliana* (Saint-Guily *et al.*, 1992; Schuster *et al.*, 1992) and potato (Emmerman *et al.*, 1992; Winning *et al.*, 1992). cDNA clones have been isolated from *Neurospora crassa* (Arends and Sebald, 1984), *Chlamydomonas reinhardtii* (Sharpe and Day, 1992) and *Chlorella kessleri* (Hilgarth *et al.*, 1991).

In yeast 3 genes have been identified, AAC1, AAC2 and AAC3. A single base change in one of these genes, AAC2, is enough to cause a 'petite' mutation, pet9 or op_1. However, a single copy of AAC2 will complement these mutations. AAC1, one of the other genes, can be disrupted without generating petites, but must be present in multiple copies to complement the pet9 and op_1 mutations (Lawson and Douglas, 1988). These gene disruption experiments suggest that the genes are differentially expressed with AAC2 encoding the majority of the translocator protein. The third gene, AAC3, was found to be expressed almost exclusively under anaerobic conditions, in contrast to the other two genes which are expressed in derepressed (aerobic) conditions (Kolarov *et al.*,1990; Sabová *et al.*, 1993). The deletion of AAC2 but not AAC1 reduced mitochondrial cytochrome content and respiratory activity (Gawaz *et al.*, 1990). Further, the rate of nucleotide transport by the ANT protein encoded by AAC1 is 40 % of that of AAC2. When both AAC2 and AAC3 were disrupted anaerobic growth was completely inhibited. The triple mutation proved to be lethal under anaerobic conditions (Drgoň *et al.*, 1991). However, if all three genes were disrupted the mutant could still grow on a fermentable carbon source under aerobic conditions, but very slowly. Drgoň *et al.* suggest that ATP inside mitochondria is essential for growth, and in the presence of oxygen there is a small leak of nucleotides that may support oxidative phosphorylation and growth, which does not occur under anaerobic conditions.

The two bovine translocator genes T1 and T2 share 78.8 % sequence identity in their coding regions and are differentially expressed, T1 mRNA predominating in heart and to a lesser extent kidneys, whereas T2 mRNA was found in intestine and kidney and also in other tissues (Powell *et al.*, 1989).

Three adenine nucleotide translocator genes (77 to 79 % homologous) have been identified in humans. ANT1, isolated from a muscle cDNA library (Neckelmann *et al.*, 1987), is also called T1 (Cozens *et al.*, 1989) or pHAT14 (Houldsworth and Attardi, 1988); ANT2 was isolated from a fibroblast library and has also been named pHAT3 (Houldsworth and Attardi, 1988), T3 (Lunardi and Attardi, 1991) or hp2F1 (Battini *et al.*, 1987) and ANT3, isolated from a liver cDNA library, has been named T2 (Cozens *et al.*, 1989) or pHAT8 (Houldsworth and Attardi, 1988). In mammalian cell lines these genes are differentially expressed. ANT3 is highly expressed in proliferating human cells, but is absent or low in nonprofilerating cells (Battini *et al.*, 1987; Hirshcorn *et al.*, 1984; Rittling *et al.*, 1986; Lunardi and Attardi, 1991), whereas the steady state mRNA levels of ANT2 decreased progressively during prolonged growth of HeLa cells (Lunardi and Attardi, 1991) and also in Balb/c/3T3 cells, a mouse line (Battini *et al.*, 1987). Expression of the muscle specific ANT1 gene could not be detected in cell lines (Lunardi and Attardi, 1991). Stepien *et al.* (1992) have investigated the expression of the three ANT genes in human, bovine, and mouse tissues. The expression of all three genes is tissue specific in these mammals; ANT1 transcripts are most highly expressed in striated muscle (as found by Li *et al.*, 1989) whilst ANT3 transcripts are abundant in all the tissues investigated. ANT2 transcripts were absent or minimal in all the tissues. ANT expression (as well as other nuclear encoded mitochondrial genes) has also been shown to be responsive to thyroid hormone levels in mammalian cells (Luciakova *et al.*, 1992). These results have led to the suggestion that the isoforms identified have different kinetic properties related to the organ or specific energy requirements (Klingenberg, 1989; Lunardi and Attardi, 1991; Stepien *et al.*, 1992).

Two nuclear genes for ANT have been identified in maize (Bathgate *et al.*, 1989), and the corresponding cDNAs have been sequenced (Baker and Leaver, 1985; Winning *et al.*, 1991). The expression of one of these, pANT-1 was found to be greatest in the basal, non-photosynthetic and meristematic sections of leaves and to decrease further up the leaf as it becomes photosynthetically active (Bathgate *et al.*, 1989). Similar results to these have been observed in wheat (Topping, 1987). cDNAs corresponding to two genes have been isolated from *Solanum tuberosum* (Emmerman *et al.*, 1991; Winnning *et al*, 1992), and *Arabidopsis thaliana* (Saint-Gulley *et al.*, 1992; Schuster *et al.*, 1992). The expression of ANT mRNA in *Chlorella*, a unicellular green alga, is activated by sugar, increasing by approximately 10 fold (Hilgarth *et al.*, 1991).

The data presented in this section suggest that in a wide variety of organisms ANT genes are differentially regulated in different tissues, possibly in response to the demand for ATP in the cytosol.

1.7. Control of Oxidative Phosphorylation

Oxidative phosphorylation requires a supply of NADH, O_2, ADP and Pi. Whilst much work has been dedicated to the elucidation of the pathways of oxidative phosphorylation, in recent years attention has been directed towards the understanding of the control of these pathways (Dry *et al.*, 1987). Whilst there is no simple answer to the question, 'What controls respiration?', a basic mechanism has been established. Isolated mitochondria demonstrate 'respiratory control' whereby their rate of oxygen uptake is governed by the availability of phosphate and ADP (Wiskich and Dry, 1985). The basis for this mechanism was determined following observations made by Chance and Williams (1955) on isolated rat mitochondria. The following section will describe the development of this hypothesis and the evidence for ANT as the rate limiting step, and the consequent application of control theory to the control of oxidative phosphorylation.

1.7.1. Regulation By Adenylates

Chance and Williams (1955) defined two states in which rat mitochondria could exist, the 'resting state', 4 (lack of ADP limits respiration), and the 'active state', 3 (ADP is present in excess). They described a hyperbolic relationship between the rate of respiration and the concentration of ADP in the extra mitochondrial space and concluded that the extramitochondrial ADP concentration is the primary factor controlling the rate of oxidative phosphorylation. Subsequent work by Klingenberg (1969) indicated that ATP and Pi were also involved in the control of respiration and that the rate of respiration was a function of the cytosolic phosphate potential [ATP]/[ADP].[PI] implying a thermodynamic control of oxidative phosphorylation. Support for this work was provided by Wilson and coworkers (Wilson *et al.*, 1973; Owen and Wilson, 1974; Holian *et al.*, 1977; Erecinska and Wilson, 1982). Wilson further postulated that the regulation of oxidative phosphorylation occurred at cytochrome *c* oxidase, and that the first two sites of the respiratory chain are at near equilibrium with the cytosolic phosphate potential. This would mean that the transport of adenine nucleotides is also near equilibrium and that the adenine nucleotide translocator does not limit the rate of respiration.

However the results obtained by other groups have led to the conclusion that the ATP/ADP ratio of the cytosol is responsible for regulating the rate of oxidative phosphorylation, and that the kinetic control would be mediated by the adenine nucleotide translocator (ANT), which is far from equilibrium (Slater *et al.*, 1973; Davis *et al.*, 1974; Davis and Lumeng, 1975; Davis and Davis-van Thienen, 1978; Kuster *et al.*, 1976; Bohnensack and Kunz, 1978; reviewed by Klingenberg, 1980). Jacobus *et al.* (1982) supports the conclusion of Chance and Williams (1955) that control of oxidative phosphorylation is affected by ADP concentration. They point out that the free ADP concentration in some rat tissues maybe lower than previously thought (as low as 50 μM) (Ackerman *et al.*, 1980; Veech *et al.*, 1979). Their work indicates that respiration is directly controlled by the extramitochondrial ADP concentration with little or no absolute correlation with the the ratios of ATP/ADP or

ATP/ADP.PI. Dry and Wiskich (1982) also found that for isolated pea mitochondria at low external ADP levels the absolute levels of ADP were of importance in the control of respiration, having more effect than the ATP/ADP ratio, which only affects the flux at ratios greater than 20. Hence the development of a controversy over the exact mechanism of adenylate control and which step is rate limiting (has the lowest activity in the pathway and therefore is likely to be regulatory) for respiration, as at this stage it was assumed that only a single step per pathway would be rate limiting.

1.7.2. The Role of the Adenine Nucleotide Translocator in the Control of Respiration

A number of groups have proposed that the exchange catalysed by ANT is the rate-limiting step of oxidative phosphorylation in rat liver mitochondria and have produced data that supports this hypothesis. Wanders *et al.* (1981) have shown that ANT is displaced from equilibrium by -8.7kJ at 30 % of state 3 respiration and Letko *et al.* (1980) claim that ANT is already out of equilibrium at the resting state (4), with the disequilibrium becoming greater as the respiration rate is increased. Wanders *et al.* (1981) observed no direct unequivocal control of the rate of respiration by the extramitochondrial ATP/ADP ratio, but did observe that the rate of respiration was directly related to the intramitochondrial ATP/ADP ratio, regardless of the site of ATP utilization. The results of Davis *et al.* (1974) showed no direct relationship between the rate of oxidative phosphorylation and the phosphate potential, which they took as evidence for the nonequilibrium character of ANT. However there has always been some controversy over determining rate limiting steps. The groups of Davis and Kunz have proposed that the extramitochondrial ATP/ADP ratio determines the rate of ADP uptake by the mitochondria and therefore the rate of respiration. Hence when the ATP/ADP ratio is high, low rates of respiration are observed due to inhibition of ADP uptake by ATP. As extramitochondrial rates of ATP utilization increase, the ATP/ADP ratio falls, inhibition of ADP transport declines and the rate of oxidative phosphorylation

increases. They interpret this to mean that the importance of ANT in controlling oxidative phosphorylation decreases in the upper range of mitochondrial activity with the greatest influence being exerted in the submaximal range. ANT is therefore claimed to be rate limiting so long as the concentration of Pi is not limiting. However Stubbs *et al.* (1978) point out that this effect could be due to rate limitation by availability of substrate rather than enzyme capacity, which would be the most common definition of a rate limiting step. From this point of view the work of Davis *et al.* (1976 : 1978) and Bohnensack *et al.* does not indicate that the adenine nucleotide translocator is a rate limiting step.

1.7.3. Metabolic Control Theory

The idea of a pacemaker reaction, a single step that limits the flux through a metabolic pathway was first proposed by Krebs (1957), following from what has become known as the 'Law of Limiting Factors'. It is generally agreed that rate limiting enzymes operate out of equilibrium in a pathway. The invalidity of this approach is revealed by comparing the maximum activities of enzymes and the flux through pathways *in vivo*: most enzymes operate well below their maximum activities. Enzymes that are near equilibrium could play a role in the control of flux through a pathway. Instead of the concept of a rate limiting reaction Kacser and Burns, (1973) and Heinrich and Rapoport (1974) considered metabolism as an integrated system rather than a series of unrelated steps, an approach called 'the metabolic control theory' (Kacser, 1987). A fundamental concept of control analysis is that the control of a metabolic pathway may be spread over all the enzymes of pathway rather than in a single step as previously thought (Kunz *et al.*, 1981; Vignais, 1976; Stubbs *et al.*, 1978; Wilson, 1980). The flux control coefficient (sometimes called the control strength) is the percentage change in the steady state rate of the pathway divided by the percentage change in the enzyme level causing the flux change. This can be represented by the equation:

$$C_j = \frac{dJ/J}{dE/E}$$

where Cj is the flux control coefficient, J is the pathway flux and $\dot{E}$ is the concentration or activity of an enzyme in the pathway. The sum of the control coefficients of all the enzymes of a pathway is equal to unity. Thus if the control coefficient of an enzyme was zero, the enzyme exerts no control over the flux at all. However, if the control coefficient of the enzyme was one, then all the control of the pathway is with that enzyme. So, if control of a pathway is shared by several enzymes in that pathway, the control coefficient of each enzyme must be less than one. Furthermore Kacser and Burns (1973, 1979) developed a connectivity theorem that relates the flux control coefficients of two adjacent enzymes to their elasticity coefficients. The elasticity coefficient takes into account the internal interactions within a system; that is metabolites involved in one step can act as substrates or modulators of another step. The theorem states that there is a reciprocal relationship between the flux control coefficients of adjacent enzymes in a metabolic pathway and the elasticity coefficients of the enzymes towards their common intermediates.

1.7.4. The Application of Control Theory

A number of groups have applied the control theory in studying the control of oxidative phosphorylation in isolated mitochondria from animals (Groen *et al.*, 1982), yeast (Mazat *et al.*, 1986) and plants (Padovan *et al.*, 1989; Hill, 1990).

Groen *et al.* (1982) used a number of inhibitors of specific components of the respiratory chain to titrate the respiratory flux. The inhibitors included carboxyatractyloside (inhibits adenine nucleotide translocator), oligomycin (inhibits ATP synthase), azide (inhibits cytochrome c_1 oxidase) and phenylsuccinate (inhibits succinate dehydrogenase), and the control strengths of these enzymes were calculated from the initial slopes of the inhibitor titrations. These control strengths were quantified at a variety of respiration rates, as it is thought that the rate of respiration *in vivo* lies between state 3 and state 4, as defined by Chance and Williams (1955). The distribution of control was found to vary considerably according to the rate of respiration of the rat liver mitochondria.

At low levels of respiration almost all control is exerted by the passive permeability of the mitochondrial membrane to protons. However, at intermediate and high levels of respiration the control is distributed so as to make it impossible to identify one rate controlling step. The control coefficient of ANT rose to a maximum of approximately 0.3 at 80 % of maximum respiration; the control coefficient of the dicarboxylate carrier increased in an almost linear fashion to a maximum of 0.4 in state 3. Other groups have obtained slightly different results. Moreno-Sanchez (1985) measured flux control coefficients in rat liver mitochondria for ANT of 0.2 to 0.48 and of 0.05 to 0.57 for ATP synthase in state 3 respiration. The control strengths for these two enzymes were found to vary in a reciprocal manner with Ca^{2+} and Pi concentrations, which together with other data suggests that Ca^{2+} may have an important role in controlling ANT activity. The *b*-c_1 complex (c=0.30) and cytochrome *c* oxidase (c=0.23) were found to be other important sites of control. Moreno-Sanchez concluded that at two different rates of respiration ANT is an important point of control in rat liver mitochondria.

Mazat *et al.* (1986) investigated the control of various steps in the flux of ATP synthesis and the oxygen consumption flux of yeast mitochondria, because although these processes are closely coupled this does not imply that the fluxes are, or that their control is identical. Their results show that the adenine nucleotide translocator has no control on either flux except possibly on ATP synthesis at Pi concentrations greater than 7.4 mM. However the control coefficient of cytochrome *c* oxidase is always high (c=0.3 to 0.6) and the proton leak is only controlling when the respiration rate is low (as shown by Groen *et al.*, 1982).

It has been shown that the control of oxidative phosphorylation by ANT depends on the mode of ATP utilization and the complexity of the metabolic framework (Lemasters and Sowers, 1979; Gellerich *et al.*, 1983; Doussiere, 1982; Kunz *et al.*, 1981). Lemasters and Sowers (1979) observed that atractyloside inhibited respiration far more effectively at any one rate if this rate was set by the external phosphate concentration rather than if a glucose-hexokinase system was used to deplete external ATP.

Hexokinase effectively lowers the control coefficient of ANT due to the relative dependence of both reactions on the ATP and ADP concentrations. The presence of pyruvate kinase and phosphenolpyruvate in the system reversed the effect of hexokinase due to the synthesis of ATP catalysed by this system (Gellerich *et al.*, 1983; Kunz *et al.*, 1981).

Padovan *et al.* (1989) measured the control coefficients of cytochrome *b*-c_1, cytochrome *c* oxidase, ATPase and ANT in isolated turnip mitochondria under both state 3 and state 4 conditions. Under state 3 conditions the control of respiration is primarily through the electron transport chain, (cytochrome *c* oxidase and cytochrome *b*-c_1) and the control contributed by ATP synthase and ANT is negligible. The importance of these control steps was found to vary with different respiratory substrates and with the respiratory flux as modified by ADP supply, so that the control strengths of cytochrome *c* oxidase and cytochrome *b*-c_1 were virtually zero in state 4. In contrast, Hill (1990) has shown that in cucumber cotyledons during early seedling development ANT can exhibit significant control of respiration at specific stages of development. During the initial phase of lipid metabolism in cucumber cotyledons the control coefficient of ANT was measured at approximately 0.3.

The variation in the importance of the control coefficients of several enzymes in the pathway of oxidative phosphorylation reported above illustrate the point that, by its nature, the accuracy with which the control coefficient can be determined is limited (Westerhoff *et al.*, 1987). However apart from the results reported by Mazat *et al.* (1986) in yeast and Padovan *et al.* (1989) in turnip, most data support the hypothesis that ANT does have a role in the control of oxidative phosphorylation under conditions that simulate the *in vivo* situation. It is generally agreed that the control strength of ANT increases from zero at state 4 to a maximum of around 0.3 at 75 to 80 % of state 3 respiration and then decreases slightly as 100 % state 3 respiration approaches.

1.7.5. Regulation of Oxidative Phosphorylation *in vivo* by Adenylate Levels

Whether the control coefficients measured *in vitro* represent the *in vivo* situation is not clear. The states 3 and 4 defined by Chance and Williams (1955) are artificial and *in vivo* mitochondria are probably at some stage between these two extremes. Biochemical methods have been used to determine ATP/ADP ratios *in vivo* (Gröller *et al.*, 1982; Stitt *et al.*, 1982). These indicate that the extramitochondrial ATP/ADP ratios are between 1 and 10, below the ratio of 20 required to have any effect on respiration found by Dry and Wiskich (1982). However the methods used may be subject to large errors, as perchloric acid (used to stop biochemical reactions) may hydrolyse ATP, and the short half life of ATP means that it may be turned over before metabolism is stopped (Dry *et al.*, 1987). Further, if ATP and ADP are bound to proteins or membranes then the actual ratios of 'free' ATP/ADP may be significantly different and high enough to exert an influence on regulation.

Nuclear magnetic resonance spectroscopy can distinguish between the protein bound and 'free' adenine nucleotides in living tissue. Data from Ackerman (1980) suggest that 'free' ADP is much lower than the total measured and the availability of ADP may be important in the control of respiration (Jacobus *et al.*, 1982). Roberts *et al.* (1985) measured ATP/ADP ratios in maize roots of greater than 25, in the range that may have significant control on respiration.

1.8. Use of Mutants to Analyse Regulation

Metabolic mutants have been used to determine flux control coefficients in a number of systems, including the control of photosynthate partitioning in leaves. Measurement of metabolite levels in a number of mutants in enzymes of the sucrose and starch synthesis pathways has revealed that the control of sucrose synthesis is shared between fructose-1,6-bisphosphatase and sucrose phosphate synthase, whilst control of starch is shared by a number of steps, with most of the control residing at ADP-glucose pyrophosphorylase (Kruckeberg *et al.*,1989; Neuhaus *et al.*, 1989;

Stitt, 1989; Neuhaus *et al.*, 1990; Neuhaus and Stitt, 1990; Smith *et al.*, 1990). This work has been achieved only by using several different plant species (*Arabidopsis thaliana*, *Pisum sativum*, *Spinacia oleracea* and *Clarkia xantiana*) in which mutations in the gene under investigation have been identified. This illustrates the problem of this approach as a mutant for the required gene must be available or readily identified. Also, unlike yeast where mutants in proteins associated with respiration result in the generation of the petite series, such mutations in plants may be lethal. A number of respiratory deficient mutants, affecting the cyanide sensitive cytochrome pathway, have been isolated in *Chlamydomonas reinhardtii* (Dorthu *et al.*, 1992), but only one of these was due to a mutation in a nuclear encoded mitochondrial gene (encoding part of complex IV).

1.9. Transformation and Transgenics

Molecular biological techniques enable the transfer into cells of a defined DNA sequence, of any origin, so that it may be transiently expressed or create a new and stably inherited genetic locus. This powerful tool is called transformation. The coding sequence of a gene can be manipulated so that it is disrupted, or expressed in a novel temporal or spatial pattern either in a whole organism or in specific cells, allowing the investigation of its role. In plant science transformation has stimulated research into gene expression and function.

1.9.1. Plant Transformation

The ability to introduce specific foreign genes into plants has become routine over the last decade. Several different methods have been developed, using a variety of techniques to express the genes of interest in plant cells either transiently or stably. These have been extensively reviewed by Potrykus (1990; 1992), but only a few techniques have been applied successfully to many plant species.

Purified DNA was originally introduced into protoplasts by incubating them with DNA in the presence of polyethylene glycol and poly-L-ornithine (Draper *et al.*, 1982), or using calcium

phosphate to produce a coprecipitate (Krens *et al.*, 1982). The efficiency of these processes is usually quite low, and the difficulty of regeneration of plants from protoplasts limits its application. As an alternative to the use of chemicals to introduce DNA into protoplasts electroporation has proved successful although the same problems as found with chemical transformation apply (Fromm *et al.*, 1985; 1986). Tobacco and alfalfa protoplasts have been successfully microinjected (Crossway *et al.*, 1986; Reich *et al.*, 1986), but once again these methods are dependent on systems for regenerating transformed plants from protoplasts.

Alternative methods for plant transformation avoiding the preparation of protoplasts have been developed. De la Peña *et al.*, (1987) reported that DNA injected into juvenile inflorescences of rye would become incorporated into the genome of some of the progeny. Imbibition of wheat, barley and rye embryos with solutions containing a bacterial gene led to the expression of the gene transiently in developing plants (Töpfer *et al.*, 1989). 'Biolistics', the bombardment of tissue under a slight vaccum with DNA coated tungsten particles of 1 to 2 μm is a method that may well prove of great value (Klein *et al.*, 1987).

1.9.2. *Agrobacterium* Mediated Transformation

The most widely used plant transformation technique exploits the capacity of *Agrobacterium tumefaciens* and *A. rhizogenes* to transfer DNA into plant cells. Transformation using *A. rhizogenes* has been reviewed recently by Tepfer (1990). However techniques using *A. tumefaciens* are most frequently employed in plant transformation. The transfer of a novel foreign DNA sequence (the transposable element Tn7 of *E. coli*) into plants using *A. tumefaciens* was first achieved by Hernalsteens *et al.* (1980). The successful transfer and phenotypic expression of a synthetic chimaeric gene in plants (the bacterial neomycin phosphotransferase conferring resistance to the antibiotic kanamycin) was reported by Herrera-Estrella *et al.* (1983a, b). Reviews by Klee *et al.* (1987), Weising *et al.* (1988) and van Wordragen and Dons (1992) report that gene transfer has been claimed for more than 60 species. However, in many cases the

claim is from a single laboratory and the production of regenerated transgenic plants, confirmed by molecular evidence has been reported for only 27 species (van Wordragen and Dons, 1992).

Much has been discovered about the processes that occur during the transfer of DNA between the bacterium and the plant. These events will not be considered in depth as there are many reviews on this area (e.g. Zambryski, 1988; Zambryski *et al.*, 1989; Zambryski, 1992; Howard and Citovsky, 1990): most of our knowledge is about events that occur in the bacterium and the other aspects of the interaction with the plant are less well understood.

The Crown Gall disease caused by *A. tumefaciens* is the result of genes carried on the transferred T-DNA region of a large (200 kb) extra-chromosomal tumour inducing (Ti) plasmid. These genes encode enzymes involved in the production of the plant growth regulators auxin and cytokinin, responsible for the tumourous gall growth, and the synthesis of opines which are catabolised by the infecting bacteria. The Ti plasmid also carries virulence genes (the *vir* region) that are essential for tumour formation, along with virulence genes on the bacterial chromosome (the *chv* locus). The *chv* locus is involved in the attachment of the bacterium to the plant cell wall (Douglas *et al.*, 1985). Phenolic signal molecules such as acetosyringone released by the wounded plant cell result in the transcription of the *vir* region of the Ti plasmid (Stachel *et al.*, 1986). These gene products are involved in the molecular events leading to T-DNA transfer. The T-DNA region is flanked by two 25 bp directly repeated border sequences. Foreign DNA sequences inserted within these borders will be transferred with the T-DNA. Two observations led to the development of the more convenient 'disarmed' binary vector systems; first, none of the sequence normally within the T-DNA borders is required for T-DNA transfer, and second, T-DNA could be transferred from one plasmid even if the *vir* region was encoded on a separate plasmid (Hoekema *et al.*, 1983).

One of the most commonly used binary vectors is pBIN19 (Bevan, 1985). This vector is based on the broad host range replicon pRK252 and can replicate in both *E. coli* and

Agrobacterium. It carries the T-DNA border sequences flanking a multiple cloning site and a gene conferring resistance to kanamycin when expressed in plant cells, which acts as a selectable marker for transformed plant cells. There is also a kanamycin gene outside the T-DNA allowing the selection in the bacteria.

DNA manipulations are carried out in *E. coli* and the pBIN 19 vector transferred to *A. tumefaciens* by conjugation using the helper plasmid pRK2013. The *Agrobacterium* host used with pBIN19 is LBA4404, which carries a Ti plasmid (pAL4404) from which the T-DNA has been deleted. This plasmid contains an intact *vir* region which mediates the transfer of the T-DNA from pBIN19 to the plant cell in *trans*.

As recovery of transgenic plants is often desirable *A. tumefaciens* mediated transformation is combined with tissue culture systems to regenerate plants from single cells, often directly from cut tissue pieces or by the formation of callus and plant regeneration. Sterile explants are infected with *Agrobacterium* harbouring the required T-DNA sequence. After a few days the bacteria are killed with an antibiotic such as carbenicillin that does not interfere with plant growth. The application of an antibiotic which the T-DNA encodes resistance for, such as kanamycin, selects for plant cells in which the T-DNA has integrated, from which transgenic plants can be regenerated.

Using transformation it is possible to affect the expression of genes so that novel genes are expressed in an organism, or the expression of the endogenous genes is altered. The gene expression pattern can be changed so that the expression of the gene is altered either temporally or spatially, increased or reduced.

1.9.3. Over-Expression of Gene Products

Walsh and Koshland, (1985) transformed *E. coli* to produce a range of citrate synthase levels, allowing the direct determination of flux control coefficients. A synthetic and adjustable promoter was used to vary the expression of citrate synthase from 10 to 5000 % of the wild type level. An increase in the amount of a rate controlling enzyme should increase the flux through the pathway, whereas an increase in a non-rate controlling enzyme should have no effect on the flux. Walsh and Koshland (1985) showed that in *E.*

coli that citrate synthase is a rate controlling enzyme only under certain conditions.

The control of carbohydrate metabolism in potato is an area where molecular biology has been used in plants to understand the regulation of a pathway. For example, the over expression of *E. coli* phosphofructokinase to 60 fold higher than in controls altered the relative concentration of intermediates in glycolysis but did not alter the flux through the pathway (Burrell, 1991). The expression of the *E. coli* pyrophosphatase altered photoassimilate partitioning, increasing the ratio between soluble sugars and starch by 3 to 4 fold (Sonnewald, 1992). The constitutive over expression of yeast invertase in tobacco and *Arabidopsis* resulted in dramatic changes in plant phenotype, the accumulation of carbohydrates and the inhibition of photosynthesis (von Schaewen *et al.*, 1990). Gene over expression is being used to investigate the role of enzymes involved in nitrogen assimilation in plants. The constitutive expression of a glutamine synthetase gene from soybean in tobacco resulted in the expression of the endogenous tobacco gene being induced in tissues such as the leaves where it is normally not expressed (Hirel *et al.*, 1992).

Molecular approaches have been applied to the study of plant development, for example to confirm a model of floral development proposed following the characterisation of various mutant phenotypes. The constitutive expression of a gene involved in the fate of floral organ primordia, AGAMOUS, resulted in the transformation of sepals into carpels, and petals into stamens (Mizukami and Ma, 1992; Mandel *et al.*, 1992). These results were consistent with the genetic model that had been proposed.

1.9.4. Down-Regulation of Gene Expression

The transformation of plants has been combined with another recently discovered technique in molecular biology, the use of antisense RNA molecules, to create new and novel plant mutants. These plants have proved very useful in the study of a target gene in biochemical and/or developmental pathways where conventional mutants are unavailable. The principle of the antisense approach is to inhibit the expression of a specific gene by the synthesis of complementary RNA or DNA sequences that may potentially

hybridise to sense mRNA transcripts of the gene (Colman, 1990). There are many reviews about antisense technology (e.g. van der Krol *et al.*, 1988b, c; 1990; Colman, 1990), but recently Takayama and Inouye (1990) have comprehensively reviewed the accumulated knowledge on both natural and artificial antisense regulation. The possible mechanisms of action of antisense RNA molecules will be considered in Chapter 3.

1.10. Naturally Occurring Antisense Gene Regulation

1.10.1. In Prokaryotes

Control by antisense transcripts was initially identified in prokaryotes as a method of regulating the replication of the plasmid ColE1 (Itoh and Tomizawa, 1980). In prokaryotes regulation by natural antisense transcripts has been found to occur at three levels: DNA replication, translation and transcription (as reviewed by Green *et al.*, 1986; Simons and Kleckner, 1988; Takayama and Inouye, 1990). These regulatory RNAs have been classified into three groups: class I, consisting of antisense RNAs that are complementary to the Shine-Delgrano sequence and/or coding sequences of the target mRNAs; class II, antisense RNAs that hybridise to noncoding regions of the target mRNA or to untranslated RNAs; and class III, antisense RNAs regulating transcription of the target mRNA by a mechanism similar to transcriptional attenuation, whereby a homologous RNA binds to the 5' end of the target mRNA, causing RNA polymerase to stop and transcription to be prematurely terminated.

1.10.2. In Eukaryotes

In eukaryotes naturally occurring antisense transcripts have been found in several systems including *Drosophila* (Spencer *et al.*, 1982), *Xenopus* (Kimelman and Kirshner, 1989), mouse (Farnham *et al.*, 1985; Nepveu and Marcu, 1986), rat (Sbisà *et al.*, 1992) and barley (Rogers, 1988). However their role, if any, in regulating gene expression remains obscure.

In *Drosophila* an overlapping transcription unit has been found in the Dopa carboxylase region (*Ddc*) of the genome. An 88 bp overlap exists between the genomic region encoding the 3' terminus of Ddc mRNA and the 3' terminus of an adjacent gene of

unknown function on the opposite strand. This transcript occurs maximally in testes tissues whereas the Ddc transcript is at low levels. This raises the possibility of regulatory interactions between the two RNAs, or regulation by transcriptional interference (Spencer *et al.*, 1982).

Small nuclear poly(A)$^-$ RNAs transcribed from the antisense strand that encodes the mouse DHFR mRNA were discovered during the course of studying DHFR transcription (Farnham *et al.*, 1985). These RNAs ranged from 180 to 240 nucleotides in size with heterogenous 5' ends that map near the DHFR promoter region and are complementary to the first 10 nucleotides of the major DHFR transcript and to a short region immediately following the DHFR mRNA translational stop codon.

The detection in rat mitochondria of a variety of antisense transcripts complementary to the region of the genome containing the origin of replication of the L DNA strand implied that they may play a regulatory role in the replication and expression of mitochondrial DNA (Sbisà *et al.*, 1991).

In *Xenopus* oocytes an antisense RNA to a 900 nucleotide region of the basic fibroplast growth factor (bFGF) mRNA has been found (Kimelman and Kirshner, 1989). This antisense RNA has an open reading frame encoding a 2.5×10^4 Mr protein. It appears that all the bFGF mRNA is in hybrid form (duplexed with the antisense RNA), since on maturation of the oocyte all the bFGF mRNA molecules were shown to become modified by RNA:RNA duplex unwinding activity that is released into the cytoplasm by the events of maturation.

In barley seeds antisense RNAs complementary to both type A and B isozymes of α-amylase mRNAs has been detected (Rodgers, 1988). S1 nuclease mapping of these has shown that the complementarity is not perfect, indicating that the antisense RNA is not transcribed from the type A and type B coding regions. The antisense transcripts are developmentally regulated, like the α-amylase RNAs. They are present in the developing endosperm tissue and in mature aleurone tissue, but are absent in shoot and root tissue. A second type of antisense α–amylase RNA was detected by a less stringent assay with Northern blot analysis.

Three distinct hybridizing species of 1.6, 1.4 and 1.0 kb were identified in RNA from mature aleurone and/or shoot tissues. It is possible that either type of the antisense RNAs may play a role in regulating the stability and/or translation of α-amylase mRNA.

Another example of naturally occurring antisense transcripts in plants has been found in *Antirhinum majus*. The semi dominant allele *niv-525* of the nivea locus (which encodes the enzyme chalcone synthase) has been found to have the potential to be transcribed to give an antisense RNA (Coen and Carpenter, 1988). The *niv-525* allele is the result of a transposon induced rearrangement, in which the transposon *Tam3* excised leaving an inverted duplication consisting of 207 bp of the chalcone synthase promoter and the 5' end of the mRNA coding sequence. Although the chalcone synthase gene (which is required for anthocyanin pigment formation) is still intact, the enzyme activity is reduced. As a result an altered flower pigmentation is obtained, with a semi dominant inheritance pattern. This may be explained by the synthesis of a 40 nucleotide antisense RNA complementary to the 5' end of the chalcone synthase transcript resulting in a decrease in expression of the gene.

1.11. Regulation by Artificial Antisense RNA:

1.11.1. Down-Regulation of Exogenous Genes

It was demonstrated *in vitro* that short antisense oligodeoxynucleotides would inhibit translation of mRNA (Hastie and Held, 1978), and then shown that Rous sarcoma virus development could be inhibited in cultured cells by oligomers complementary to 5' and 3' reiterated sequences of the virus (Zamecnik and Stephenson, 1978). Since these experiments oligodeoxynucleotides and antisense RNAs synthesised *in vitro* have been used extensively to inhibit gene expression in cell cultures and when injected into ooyctes, eggs or embryos (examples include Melton, 1985 and Rosenberg *et al.*, 1985). However the limited stability of oligomer or antisense RNA and the difficulty of introducing it into the cell precludes their use in many situations.

Transient inhibition of a specific gene by an artificial antisense RNA was demonstrated when a plasmid containing an

antisense herpes simplex virus thymidine kinase gene was coinjected with a plasmid containing the wild type gene into mouse LTK$^-$ cells (Izant and Weintraub, 1984). Ecker and Davies (1986) were the first to demonstrate that the approach could be applied in plants. The expression of chloroamphenicol acetyl transferase (CAT) was transiently inhibited when antisense CAT plasmids were coelectroporated with sense CAT plasmids at a ratio of 100:1 respectively.

Following this the expression of introduced marker genes (CAT, Phosphinotricin acetyl transferase (the bialaphos resistance (*bar*) gene), β-glucuronidase (GUS) and nopaline synthase (NOS)) was inhibited in transgenic plants when the plants were further transformed using *Agrobacterium* containing an antisense construct complementary to the marker gene (Delauney *et al.*, 1988; Rothstein *et al.*, 1987; Sandler *et al.*, 1988; Cornelissen, 1989; Cornelissen and Vandewiele, 1989; Robert *et al.*, 1989; Cannon *et al.*, 1990). These experiments set the precedent for the application of the technique to investigating the effects of antisense transcripts on the expression of endogenous plant genes. The ease of transforming and regenerating transgenic plants has resulted in this technique being applied more sucessfully in plants than other organisms (although for example, transgenic mice containing antisense genes have been created (Katsuki *et al.*, 1988)).

1.11.2. Down-Regulation of Endogenous Genes

Van der Krol *et al.*, (1988) were the first group to report the down-regulation of an endogenous plant gene. Chalcone synthase (CHS) is a key enzyme of the flavonoid biosynthesis pathway, encoded by multigene family. Antisense constructs with a full length cDNA complementary to chalcone synthase from petunia were constitutively expressed in transgenic petunia, tobacco and potato plants, resulting in decreased levels of steady state mRNA and protein. These changes correlated with phenotypic changes in the floral pigmentation patterns. The different types of pigmentation observed were not correlated with the alteration in the steady state mRNA level. The levels of antisense mRNA were found to be independent of the number of antisense genes integrated into the plant genome. This suggests that the DNA

sequences adjacent to the inserted gene may affect both the quantity and the quality of transgene expression. Sub-genomic fragments from the 3' end of the mRNA also showed an effect, although the 5' end was not effective (Van der Krol *et al.*, 1990). Interestingly Van der Krol *et al.* (1990) and Napoli *et al.*, (1990) have observed that when additional 'sense' copies of the chs gene were introduced some plants exhibited a reduced floral pigmentation, associated with a reduced level of chs mRNA. They have termed this effect 'co-suppression'. Van der Krol *et al.* have summarised their results in a review (1991).

Antisense technology has been applied to the genes involved in the ripening of tomato with considerable success. Polygalacturonase (PG) is synthesized *de novo* from the translation of accumulated mRNA when the fruit ripens, leading to partial solubilizing of pectin in the cell wall. Smith *et al.* (1988) and Sheehy *et al.* (1988) transformed tomato plants with antisense constructs containing either a fragment from, or the complete cDNA for, PG. In both cases plants expressing the antisense gene had lower steady state levels of PG mRNA and PG protein in ripe fruit. PG enzyme activity was inhibited by up to 90% in the transgenic plants. In further experiments Smith *et al.* (1990) investigated the effect of reducing PG activity to 1% of the wild type level. Several ripening characteristics were measured and found to be unchanged, but the depolymerisation of soluble pectin was inhibited. However there was no change in the compressibility of these fruit (Smith *et al.*, 1990) When Smith *et al.* (1990) expressed the truncated cDNA used in the antisense experiments, but in the sense orientation the endogenous PG expression was reduced. This result supports the conclusion that sense genes can down regulate the expression of endogenous genes at the level of RNA. The enzyme pectin methylesterase (PME) may also play a role in fruit ripening. Fruits from transgenic plants expressing high levels of PME antisense mRNA showed less than 10% of wild type PME enzyme activity and undetectable amounts of both PME protein and mRNA (Tieman *et al.*, 1992). This reduced PME activity was associated with an increased molecular mass and methylesterification of pectins and

decreased levels of total and chelator-soluble polyuronides in the cell walls. The ripening process was not affected.

Hamilton *et al.* (1990) investigated the function of the gene pTOM13, the expression of which is correlated with ethylene production in ripening fruit and senescing leaves. In plants expressing an antisense pTOM13 reduced ethylene synthesis in a gene dosage-dependent manner was observed. It was suggested that pTOM13 encodes the enzyme 1-aminocyclopropane-1-carboxylate acid (ACC) oxidase, one of the specific enzymes of the ethylene biosynthesis pathway. The other specific enzyme in this pathway is ACC synthase. Oeller *et al.* (1991) used the antisense approach to down-regulate this enzyme and inhibited ethylene production. The antisense fruits never ripened unless they were treated with exogenous ethylene or propylene, an ethylene analogue. E8 is another enzyme of the ethylene biosynthesis pathway related to ACC oxidase, although its function is not yet clear. However when it is down-regulated by transformation with an antisense gene the production of ethylene is increased (Penarrubia et al., 1992).

Even the most abundant enzyme in plants, ribulose bisphosphate carboxylase (RUBISCO), can be reduced using the antisense technique. The multimeric protein is composed of small (SS) and large (LS) subunits, encoded by a nuclear encoded multigene family (*rbc*S) and a single chloroplast gene respectively. Rodermel *et al.* (1988) investigated the effect of an antisense *rbc*S gene on the levels of LS mRNA and protein production and accumulation. The *rbc*S antisense gene reduced the levels of *rbc*S mRNA and SS protein, with an accompanying effect on the levels of LS protein, but not the levels of LS mRNA. The accumulation of the LS protein appeared to be regulated by translational and posttranslational events. Plant growth rate and antisense gene dosage appeared to be related; the greater number of antisense genes the lower the growth rate. From this initial work several elegant studies have investigated the effect of decreasing RUBISCO levels in transgenic plants (Quick *et al.*, 1991a, b; 1992; Stitt *et al.*, 1991; Fichtner *et al.*, 1993). A series of plants with progressively less RUBISCO was generated by using different transformant lines and crossing them to alter the number of copies of antisense genes.

This decrease in RUBISCO is selective and other photosynthetic enzymes were not affected until RUBISCO was decreased three to four fold (Quick *et al.*, 1991a). The flux-control coefficient of RUBISCO was determined in these plants under a variety of conditions. It varied from 0.8 under high irradiance and ambient CO_2 to marginal under low irradiance or high CO_2 concentration. The flux-control coefficient also varied depending on the fraction of the available RUBISCO capacity that was used (Stitt *et al.*, 1991). By applying the connectivity theory of Kacser and Porteous (1987) the control coefficients of stomata were estimated to vary between zero and 0.25. In plants with decreased photosynthetic capability the leaf area ratio (leaf area per g plant dry weight) increased 3 to 4 fold and although the level of free starch in the leaf fell, the volume of the diurnal starch turnover remained nearly constant (Quick *et al.*, 1991b).

Stockhaus *et al.* (1990) used the antisense approach to reduce the levels of the 10 kd protein (part of the water splitting apparatus of photosystem II) to 1 to 3% of wild type levels. This did not affect the accumulation of mRNAs or proteins associated with photosystem II. They were not able to detect any phenotypic differences between the transgenic and wild type plants. However the functional integrity of photosystem II did appear to be altered.

Antisense techniques have been used to investigate a number of other biochemical pathways. The formation of starch in potato tubers has been a target for a number of groups. Visser *et al.* (1990; 1991) have generated transgenic potato plants with reduced levels of granule-bound starch synthase (GBSS). GBSS determines the presence of amylose in starch reserves. Both heterologous (maize) and homologous (potato) genes have been used as source of the antisense gene and gene expression could be totally inhibited, producing amylose free starch. The inhibition of ADP-glucose pyrophosphorylase (AGPase) which forms ADP-glucose (essential to the formation of starch) created potato tubers without starch. Instead these tubers contained up to 30% sucrose and 8% glucose (Müller-Röber *et al.*, 1992). The other starch biosynthesis enzymes were not affected, but the mRNA levels of the major sucrose

forming enzyme, sucrose phosphate synthase, were increased. The expression of the major tuber proteins was greatly reduced.

The fatty acid composition of *Brassica rapa* and *B. napus* was modified by an antisense gene to stearoyl-acyl carrier protein (stearoyl-ACP) desaturase. This enzymes catalyses the first desaturation step in seed oil biosynthesis. The concentration and activity of stearoyl-ACP desaturase in the resulting transgenic plants was reduced and the levels of stearate in the seeds dramatically increased (Knutzon *et al.*, 1992).

The antisense method has been used to inhibit the expression of other plant genes including nitrite reductase (Vaucheret *et al.*, 1992) and the tonoplast H^+ ATPase (Gogarten *et al.*, 1992).

These are examples of the successful application of the antisense technique in plants. However failure to achieve inhibition is often not reported. Complementary viral RNAs have been used in an attempt to inhibit viral infection (Cuozzo *et al.*, 1988; Hemmenway, 1988). The experiments were successful when a low inoculum concentration was used, but protection was overcome at high inoculate levels. The antisense experiments were less successful than experiments expressing the sense coat protein gene in plants.

1.12. The Aims of This Project

The aim of the research described in this thesis is an investigation of the role of the adenine nucleotide translocator in controlling oxidative phosphorylation. There is evidence that this gene has a pivotal role in the regulation of mitochondrial phosphorylation. At present there are no known natural mutants in any plant species with altered levels of ANT expression (unlike in yeast). Using genetic manipulation artifical ANT genes will be constructed and introduced into plants using *Agrobacterium* mediated transformation. These transgenic plants will either over- or under-express the gene, with the gene under both constitutive and tissue specific promoters. Molecular and biochemical analysis of these plants will hopefully provide insights into the role of the gene in regulating respiration in plants.

The model system chosen for the investigation of ANT function is the potato, *Solanum tuberosum* L. There are a number of reasons for using the potato as the model system:

The ease of genetic transformation of potato, especially by *Agrobacterium* mediated transformation has been well documented (Vayda and Belknap, 1992). Although ANT genes have previously been isolated from maize in this laboratory, maize transformation is not a regular event (although it has been reported e.g. Gordon-Kamm *et al.*,1990). The potato also has a number of other advantages for this work. It can be easily propagated by clonal methods and there is extensive tissue culture experience. A number of tissue specific promoters have been isolated and characterised that can be used to direct the expression of an introduced gene. The respiration of potato tubers has been extensively studied at the biochemical and physiological level (as reviewed by Dizengremel, 1985) and the tuber is an excellent source of mitochondria for biochemical experiments. The potato goes through a number of distinct developmental stages in which respiratory activity dramatically changes. However, commercial potato cultivars are tetraploid and exhibit a high degree of genetic complexity and heterozygosity.

CHAPTER 2
MATERIALS AND METHODS

2.1. Biological Materials

2.1.1. Plant material

Sterile micropropagated plantlets and pathogen free tubers of potato, *Solanum tuberosum* L. cv. Desiree were obtained from the Department of Agriculture and Fisheries for Scotland, East Craigs, Edinburgh.

2.1.2. Bacterial strains

Escherichia coli	JM101	Δ(lac proAB), thi, SupE, (F', traD 36,proAB, lacI qZ ΔM15) (Yanisch-Perron *et al.*, 1985)
	HB101	F^-,racA 13, ara-14, proA 2, lacY 1, galK 2, rpsL 2, xyl-5, mtl-1, supE 44, hsdS 20 (r_B^-,m_B^-), λ^- (Manniatis *et al.*, 1982)
Agrobacterium tumefaciens	LBA4404	Genotype not available. This strain carries a cryptic Ti plasmid and a 'disarmed' Ti plasmid lacking the entire T-DNA, but with an intact vir region. Streptomycin resistance is carried on the bacterial chromosome and rifampicin resistance on the disarmed Ti plasmid (Hoekema *et al.*, 1983).

2.1.3. Bacterial plasmids

pGEM-1	Promega Corporation
pUC 19	Yanisch-Perron *et al.*, (1985)
pIC 19H	Marsh *et al.*, (1984)
pBIN 19	Bevan (1984)
pMOGEN	Gift from Dr. B. Dekker, MOGEN Int B.V., based on pBIN 19.
pBluescript II KS	Short *et al.*, (1988)

2.1.4. Adenine Nucleotide Translocator cDNA Clones

The cDNA of the adenine nucleotide translocator from *Zea mays* L., MANT-A was isolated in this laboratory as described previously (Winning *et al.*, 1991). The potato adenine nucleotide translocator cDNA, ANT1, was also isolated in this laboratory (Winning *et al.*, 1992).

2.2. Miscellaneous

2.2.1. Chemicals

All chemicals were purchased from Sigma Chemical Co. Ltd., Boehringer Mannheim, or British Drug Houses (BDH) Chemicals Ltd., unless otherwise stated.

2.2.2. Enzymes

Restriction enzymes were purchased from GIBCO BRL Life Technologies Ltd, Stratagene, Northumbria Biologicals Ltd (NBL), and Amersham International Plc.

2.2.3. Radiochemicals

$\alpha(^{32}P)$ dCTP (3000 Ci/mmol) was purchased from Amersham International Plc.

2.2.4. Autoradiography film

Cronex ®4 or Kodak X-OMAT AR X-ray film was used for autoradiography and developed in an Agfa-Gaevert Gevamatic 60 automatic developer.

2.2.5. Bacteriological media

All strains of *E. coli* and *A. tumefaciens* were grown in Luria broth (LB) or LB agar

Luria broth	10 g/l Bacto tryptone (Difco Laboratories, Detroit), 5 g/l Bacto yeast extract (Difco), 10 g/l sodium chloride, pH 7.2.
LB agar	As above, but solidified with 1 % (w/v) Bacto agar (Difco, Detroit).

2.2.6. Tissue Culture Media

All media were based on the basic medium as described by Murashige and Skoog (1962). Murashige and Skoog (M&S) basal salt mixture or M&S basal medium (Flow Laboratories, Irving, or Sigma Chemical Co. Ltd.) was used depending on the vitamins required and the media solidifed with 10 g/l Bacto agar (Difco, Detroit) if required. In all the media the pH was adjusted to 5.8 using 0.1 M potassium hydroxide.

HS medium	4.4 g/l M&S medium (Sigma Chemical Co.), 30 g/l sucrose, 2 mg/l 6-benzylaminopurine (BAP). After autoclaving 1 g/l (2-chloroethyl)-trimethylammonium chloride (CCC).
NM medium	2.2 g/l M&S medium, 15 g/l sucrose, 0.1 mg/l kinetin, 0.2 mg/l GA_3
MS30	4.6 g/l M&S salts (Flow Labs.), 30g/l sucrose.
MS30-R3	4.6 g/l M&S salts, 30 g/l sucrose, 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine HCl, 0.1mg/l thiamine HCl.
L/SR1	4.4 g/l M&S medium, 30 g/l sucrose, 2.00 mg/l zeatin riboside, 0.2 mg/l naphthaleneacetic acid (NAA), 0.02 mg/l GA_3.
L/SR2	4.4 g/l M&S medium, 30 g/l sucrose, 2.00 mg/l zeatin riboside, 0.02 mg/l naphthaleneacetic acid, 0.02 mg/l GA_3.
Rooting medium	4.6 g/l M&S salts (Flow Labs.), 15 g/l sucrose.

2.2.7. Centrifugation

Beckman JA-14, JA-20 and JS-13.1 rotors were used in a Beckman J2-21 centrifuge. 250ml bottles were used with the JA-14. Disposable 15 and 30ml polypropylene tubes (Sarstedt Ltd.) or Corex® tubes (Fisons Scientific Equipment) were used in the JA-20 and JS-13.1 rotors.

Two Beckman ultracentrifuges, a TL-100 and a L-70, were used with TLA 100.3 and 70.1Ti rotors respectively for the isolation of DNA. Beckman polyallomer bell-top Quick-Seal™ centrifuge tubes were used with these rotors for caesium chloride gradients. Eppendorf™ 1.5 ml microcentrifuge tubes were used with an Anderman 5414 microcentrifuge for subsequent reactions and manipulations with isolated DNA.

2.2.8. Plant growth conditions

Sterile plants were grown in Gallenkamp Fi-Totron T.C. growth rooms at 23 to 25 °C with 16 hours of light, 8 hours of dark, at 1000 to 3000 Lux per day unless otherwise stated. Transgenic plants were potted out into greenhouses built to genetic containment standards. Supplementary mercury vapour lighting was used as required to maintain a 16 hour daylength during winter months and the temperature regulated to 25 °C using the greenhouse climate control system.

2.2.9. Genetic Manipulation Code of Practice

All molecular biological and transgenic plant manipulations were carried out according to the local genetic manipulation committee code of practice.

2.3. Plant tissue culture techniques

2.3.1. Potato propagation

Sterile plants were grown by axial bud propagation. Five to six internodal sections of potato were embedded with the basal end in 1/2 or 1 litre glass preserve jars containing 100 ml of NM medium. These were incubated under the conditions described (2.2.8) and a new shoot developed from the axilary bud of each section. After four weeks these shoots would typically be up to 15 cm long with 6-7 nodes on each shoot. If smaller plants were required up to 12 internodal sections could be propagated on 25 ml of NM medium in a petri dish.

2.3.2. *In vitro* tuberisation

Tubers were induced following the protocol of Hussey and Stacey (1984). The basal end of internodal sections were embedded in HS medium and incubated at 18 to 19 °C with 8 hours illumination, 16 hours darkness in a VSL illuminated growth cabinet. Microtubers were harvested after 8-12 weeks.

2.4. Molecular biology techniques

2.4.1. General conditions

All solutions, pipette tips and microfuge tubes used in the isolation and manipulation of nucleic acids were autoclaved at 15 p.s.i. and 121 °C for 15 minutes before use. All solutions were prepared with water obtained from a Millipore Mill-RO® system. All manipulations were carried out on ice unless otherwise stated. The purity of nucleic acids was determined by measuring the optical density (OD) between 200 and 300 nm using a Beckman DU® series 70 spectrophotometer, and the concentration determined from the OD at 260 nm. For DNA 1 OD unit is equivalent to 50 ug/ml, and for RNA, 1 OD unit is equivalent to 40 ug/ml.

2.4.2. Isolation of nucleic acids

2.4.2.1. Isolation of plasmid DNA

Plasmid DNA was prepared from *E. coli* following standard methods (Maniatis *et al.*, 1982). Plasmid DNA was isolated from *A. tumefaciens* using a method based on the alkaline lysis procedure used with *E. coli*. However each step in the lysis stage was extended to 30 minutes. Otherwise the protocol was identical.

2.4.2.2. Isolation of total RNA from plant tissue

Total RNA was isolated from various potato tissues following the protocol of Logemann *et al.*, (1987).

2.4.2.3. Isolation of total DNA from plant tissue

1g of potato tissue was frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. The powder was transferred to a 30 ml centrifuge tube, and 6 ml of extraction buffer (42 % (w/v)

urea, 0.32 M NaCl, 50 mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0, 0.4 % (v/v) sarkosyl), 3 ml phenol and 3 ml of a 24:1 mixture of chloroform and isoamyl alchohol were added. These were mixed well with a vortex machine and then centrifuged at 1000 rpm for 10 minutes. The resulting upper aqueous layer was recovered and mixed with 3 ml 7.5 M ammonium acetate and 3.6 ml isopropanol. This was centrifuged at 1000 rpm for 5 minutes. The supernatant was poured off and the pellet air dried, then redissolved in 400 µl of T.E. buffer. 5 µl of 10 mg/ml RNase was added and the sample incubated at 37 °C for 30 minutes. The sample was then phenol/chloroform extracted again and the nucleic acids precipitated with ethanol at -80 °C for 30 minutes. The pellet was resuspended in 200 µl of T.E. buffer.

2.4.3. Isolation of DNA from Agarose Gels

The desired band in an electrophoresis gel stained with ethidium bromide and visualised under U.V. light was isolated from the gel by the insertion of a piece of NA 45 nitrocellulose paper (Schleicher and Schuell) in a cut in the gel in front of the band. The band was transferred to the membrane by further electrophoresis and the membrane removed. The membrane was then incubated with 200 µl of elution buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 1.5 M sodium chloride) at 65 °C for 45 minutes. The DNA recovered was diluted with 200 µl of water, and precipitated with 1 ml of ethanol at -70°C. The pellet was resuspended in 10-50 µl T.E. buffer.

Bands from gels were also isolated using 'Geneclean' or 'Geneclean II' (Bio101 Inc, La Jolla, Ca., USA) following the manufacturers protocol.

2.4.4. Ligation of DNA Molecules

DNA used for ligation was resuspended in T.E. buffer pH 7.1. Vector and insert were included at an estimated molar ratio of between 1:2 and 1:4. The ligation was performed in BRL ligation buffer (50 mM Tris-HCl pH 7.6, 10 mM magnesium chloride, 1 mM DTT, 1 mM ATP, 5 % (w/v) polyethylene glycol-8000) with one unit of DNA ligase. The reaction was performed at room temperature for four hours, or overnight at 4 °C.

2.4.5. Preparation and Transformation of Competent Bacterial Cells

The most consistent and efficient method for transformation of *E. coli*, especially when cloning into binary vectors, was found to be the method detailed here. An overnight culture of *E. coli* was started from a glycerol stock frozen at -20 °C, and grown at 37 °C. This stock was diluted 100 fold into 100 ml of LB, and grown at 37 °C with shaking at 300 rpm until the optical density at 595 nm was 0.3. The cells were pelleted in sterile 30 ml glass tubes at 3000 rpm, and resuspended in 50 ml of ice cold sterile 0.1 M calcium chloride. The cells were left on ice for at least 20 minutes, then pelleted again at 4 °C and 3000 rpm and resuspended in 2 ml of 0.1 M calcium chloride.

Transformations were performed with 100 µl of competent cells, to which 1/10th of the ligation mix was added. The cells were left on ice for 30 minutes and then heat shocked at 42 °C for 2 minutes. The cells were then incubated with 1 ml of LB for 1 hour at 37 °C, concentrated by centrifugation, and plated on LB agar plates which contained 50 µg/ml of the appropriate antibiotic to select for plasmid bearing transformants.

2.4.6. Radiolabelling of nucleic acids for hybridisation studies

2.4.6.1. Random Primer Labelling

Hybridisation probes were labelled by incorporation of $\alpha[^{32}P]$dCTP by the random primer extension method (Feinberg and Vogelstein, 1984), using the Amersham Multiprime DNA labelling kit. Unincorporated nucleotides were separated on a Pharmacica NICK column containing Sephadex G-50.

2.4.6.2. RNA Probes

Plasmid DNA was linearised with the appropriate restriction enzyme. RNA probes were synthesised with $\alpha[^{32}P]$UTP using the Promega® Riboprobe Gemini II system as described in the 'Promega Protocols and Applications Guide'.

2.4.7. Nucleic acid hybridisation

2.4.7.1. Southern blot analysis

Southern blots (Southern, 1975) were made by cleaving 10 µg of genomic DNA with 40 units of restriction endonuclease and 20 mM spermidine added to the restriction enzyme buffer for five hours at 37 °C. Digested DNA was fractionated by electrophoresis through a 1 % (w/v) agarose gel and transferred to Hybond™-N (Amersham International plc.) following the manufacturers recommended protocols. The air dried nylon membranes were UV crosslinked using a Stratalinker™ UV crosslinker1800 (Stratagene) set at 120 mJoules/cm^2. Prehybridisation of the filters was carried out at 65 °C in 3x SSC, 1 % (w/v) SDS, 0.1 % (w/v) sperm DNA for at least five hours. The filter was probed at 65 °C overnight in the same mix, but with the inclusion of 10% (w/v) dextran sulphate. After hybridisation the filter was washed in 3x SSC, 1.0 % (w/v) SDS, 0.1 % (w/v) sodium pyrophosphate for 30 minutes at 65 °C. The filter was then washed in 1x SSC, 1.0 % (w/v) SDS, 0.1 % (w/v) sodium pyrophosphate for 15 minutes at 65 °C. Washed filters were exposed to X-ray film at -70 °C with intensifying screens (Bonner and Laskey, 1974).

2.4.7.2. Northern Blot analysis

5 µg or 10 µg of total RNA was fractionated by electrophoresis through a 1 % agarose gel in an agarose/formaldehyde denaturing gel system (as described in the manufacturers protocol, (Amersham International plc, 1985)) and transferred to Hybond™-N as described in the manufacturers protocol. The air dried filters were UV crosslinked using a Stratalinker™ UV crosslinker1800 (Stratagene) set at 120 mJoules/cm^2.

Filters probed with a random primed DNA probe were prehybridised at 42 °C in a buffer of 50 % (v/v) formamide, 5x SSPE, 5x Denhardts solution, 0.1 % (w/v) SDS and 100mg/ml single stranded salmon sperm DNA for at least 1 hour. Hybridisation was carried out overnight using the conditions described above with the relevant probe.

The filter was washed twice at 42 °C in 5x SSPE for 15 minutes, then in 1x SSPE, 0.1 % (w/v) SDS for 30 minutes. The filter was then exposed to preflashed X-ray film, with intensifying screens at -70 °C.

Filters probed with a riboprobe were prehybidised in 50 % (v/v) formamide, 0.25 M sodium phosphate buffer pH 7.2, 0.25 M NaCl, 7 % (w/v) SDS, 1 mM EDTA and 200 mg/ml single stranded salmon sperm DNA at 55 °C for four hours. Hybridisation was carried out under the same conditions, with the addition of the relevant probe. The filter was washed in 0.25 M sodium phosphate buffer pH 7.2, 2 % (w/v) SDS at 55 °C for 20 minutes. This wash was repeated and then the filter was washed in 0.05 M sodium phosphate buffer pH 7.2, 1 % (w/v) SDS at 55 °C for 10 minutes. Washed filters were treated with RNase A (1mg/ml RNase A in 2x SSC) at room temperature for 15 minutes . The filter was washed for a further 30 minutes at 50 °C in 0.1x SSC, 0.1 % (w/v) SDS, dried and exposed to preflashed X-ray film, with intensifying screens at -70 °C.

2.5. Protein techniques

2.5.1. Isolation of proteins

Approximately 0.5 g of potato leaf or tuber tissue was ground in an eppendorf microfuge tube with a teflon homogenizer, and then further ground with one volume of extraction buffer:

100 mM Tris-HCl buffer, pH 8.0
10 mM $MgCl_2$
18 % (w/v) sucrose
4 % (w/v) SDS
40 mM βmercaptoethanol

This was vortexed and centrifuged for 10 minutes at 14,000 rpm in an eppendorf microfuge and the supernatant was removed for further analysis.

2.5.2. SDS-polyacrylamide gel electrophoresis

Proteins were separated by SDS-polyacrylamide gel electrophoresis in 15 % (w/v) polyacrylamide gels using the

discontinuous buffer system of Laemmli (1970). Gels 1.0 mm thick were made from the following stock solutions:

20 ml 30 % (w/v) acrylamide (Kodak)
0.2 % (w/v) N, N'-methylenebisacrylamide
8 ml 1.875 M Tris-HCl, pH 8.85
20 µl TEMED
made to 40 ml with distilled water.
200 µl 10 % (w/v) ammonium persulphate.

This mixture was poured into a previously assembled gel cassette and allowed to polymerise at room temperature for an hour under a layer of gel overlay solution (0.375 M Tris-HCl, pH 8.85, 0.1 % (w/v) SDS, 80 % (w/v) isopropanol). The gel overlay solution was removed before pouring a stacking gel made from the following:

2 ml 30 % (w/v) acrylamide (Kodak)
0.2 % (w/v) N, N'- methylenebisacrylamide
1.2 ml 0.6 M Tris-HCl, pH 6.8
12 µl TEMED
made to 12 ml with distilled water.
60 µl 10 % (w/v) ammonium persulphate

A well forming comb was inserted and the stacking gel allowed to polymerise for 30 minutes at room temperature before use.

Samples of total protein were mixed with 1/3 volume of 4X sample loading buffer (0.24 M Tris-HCl pH 6.8, 30 % (v/v) glycerol, 0.4 % (w/v) SDS, 0.02 % (w/v) bromophenol blue) to give a final protein concentration of 5-10 mg/ml. The samples were boiled for 90 seconds, cooled on ice, then allowed to warm to room temperature. Samples were loaded onto gels using a Gilson pipette.

Gels were run in Laemmli electrode buffer (50 mM Tris, 192 mM glycine, pH 8.2) with the upper tank containing 0.1 % (w/v) SDS in addition. Gels were run at 100 mA for 1.5 mm gels until the bromophenol blue reached the bottom, then were stained for protein (see 2.5.4) or electroblotted onto PVDF membrane (Biorad) for the immunodetection of specific polypeptides (see 2.5.5).

2.5.4. Staining of gels for proteins

Following electrophoresis gels were stained for 1 hour in 45 % (v/v) methanol, 8 % (v/v) acetic acid, 0.2 % (w/v) Coomasie brilliant blue R250 and were destained over a period of 4 hours with several changes of 45 % (v/v) methanol, 8 % (v/v) acetic acid. The gels were rinsed in distilled water before being dried under vacuum onto Whatman 3MM paper.

2.5.5. Immunoblotting

Proteins were transferred electrophoretically using a Hoeffer electrophoresis system from 1.0 mm thick polyacrylamide gels onto PVDF membranes (Biorad) following the manufactuers protocol in a buffer containing 25 mM Tris-192 mM glycine pH 8.2, 20 % (v/v) methanol and 0.1 % (w/v) SDS (Towdin *et al.*, 1979) for one hour at 500 mA and 4 °C. The membranes were then washed for at least 1 hour in 5 % (w/v) milk protein (Marvel) in TBST buffer (0.9 % (w/v) sodium chloride, 10 mM Tris-HCl pH 8.0, 0.05 % (v/v) Tween-20). The filters were incubated overnight at room temperature in 5 % (w/v) milk protein (Marvel) in TBST buffer containing the appropriate dilution of antisera (1:500) and then washed in five changes of 5 % (w/v) milk protein TBST buffer (5 minute washes) to remove excess antisera. The filters were then incubated for a further 2 hours at room temperature in 5 % (w/v) milk protein (Marvel) in TBST buffer containing a 3000-fold dilution of alkaline phosphatase conjugated anti-rabbit immunoglobulin antisera (Sigma Chemical Company) and then washed once more in five changes of TBST buffer (5 minute washes). Antibody binding was detected by an alkaline phosphate specific stain. The filter was incubated in 100 mM Tris-HCl pH 9.5 containing 4 mM magnesium chloride, 0.1 mg/ml nitro-blue tetrazolium and 0.06 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (toluidine salt) until intense purple bands appeared.

The filters were probed with ANT antibodies raised in rabbits against purified ANT protein from *Zea mays* (as described in Winning *et al.*, 1992).

2.6. Generation of transgenic plants

2.6.1. Construction of plant transformation vectors and their conjugation into *A. tumefaciens*

Recombinant plasmids derived from binary vectors as described in chapter 3 were constructed in *E. coli* strain JM101 using standard procedures (Maniatis *et al.*, 1982), apart from the isolation of DNA fragments following gel electrophoresis as described in section 2.4.3., and the transformation of competent *E. coli* cells which was according to section 2.4.5. Conjugation of these plasmids into *A. tumefaciens* involved a triparental mating of the donor and host strains along with *E. coli* strain HB101 carrying the conjugation plasmid, pRK2013, as described in Draper *et al.*, (1988). The presence of the recombinant plasmid was confirmed by plasmid DNA preparation from *A. tumefaciens* as described in section 2.4.2.1. The DNA extracted was retransformed into *E. coli* and plasmid DNA isolated from these analysed by restriction endonuclease digestion and gel electrophoresis.

2.6.2. Potato transformation techniques

2.6.2.1. General conditions

All tissue culture proce dures were performed under sterile conditions in a laminar flow cabinet using standard aseptic techniques. All sterile plant material was propogated as in section 2.3.1. Potato tubers used for transformation were either stored in the dark at 4 °C for up to 6 months, or harvested from potato plants the same day.

In both methods *A. tumefaciens* strain LBA 4404, carrying the appropriate plant transformation vector, was steaked on a LB agar plate containing the appropriate antibiotics, 100 µg/ml rifampcin, 300 µg/ml streptomycin and 100 µg/ml kanamycin, the resistance to these antibiotics being carried on the bacterial chromosome, the virulence helper plasmid, and the binary vector respectively. The plates were incubated at 28 °C for 48 hours and a single colony was used to innoculate 5 ml of LB containing the same antibiotics. When acetosyringone (Aldrich Chemical Co. Ltd.) was used it was added to

the overnight culture at a concentration of 200 μM (from a stock solution of 10 mM in MES buffer, pH 5.5). The overnight culture was incubated at 28 °C for 24 hours at 300 rpm in a New Brunswick shaker.

2.6.2.2. Tuber transformation

This protocol was developed by MOGEN Int N.V., Lieden, Holland and was learnt whilst on a visit to the company, and from H. Haagsman, who worked in Prof. Leaver's laboratory in Edinburgh for six months. The protocol is a modification of that described by Hoekema *et al.* (1989). Whole potato tubers were peeled and surface sterilised in 0.7 % (v/v) sodium hypochlorite with 20 drops Tween 20 per litre for 20 minutes. The tubers were washed in three to five changes of sterile water for 30 minutes. Tubers sections were taken using a sterile cork borer in a laminar flow hood, and 0.5 cm from each end of the section discarded. The segments were then sliced into discs, approximately 1 to 2 mm thick and kept in liquid MS30 medium (as described in Sec.2.2.6) until required. The *Agrobacterium* overnight culture was pelleted at 3000 rpm for 15 minutes and washed in MS30-R3 (described in Sec.2.2.6), before being diluted 1:100 in MS30-R3. Between 25 and 50 ml of *Agrobacterium* suspension was added to 25 to 100 tuber discs in a sterile petri dish. After 20 minutes the discs were transfered individually with tweezers onto sterile filter paper and blotted dry. 20 discs per plate were then placed on MS30-R3 medium containing 5 μM zeatin riboside and 0.3 μM indole acetic acid. These petri dishes were then sealed with Parafilm™ and left for 2 days in a growth chamber at 23 to 25 °C and a 16 hour light, 8 hours dark cycle. The discs were then transferred to identical MS30-R3 plates, with the addition of the antibiotics carbenicillin (500 mg/l) and kanamycin (100 mg/l) and plates were incubated in a growth chamber under standard tissue culture conditions and sub-cultured every 3 weeks. After 3 weeks the concentration of carbenicillin was reduced to 50 mg/l. The shoots that developed were excised and transferred to

rooting medium containing 50 mg/l carbenicillin and 100 mg/l kanamycin.

2.6.2.3. Leaf and Stem transformation

The method described is the one used to successfully transform leaf and/or stem explants. Many other methods were attempted without success, and are not described here in detail but are mentioned in the results section.

2.6.2.3.1. Scottish Crop Research Institute Potato Transformation Protocol

This protocol is essentially that described by Knapp *et al.* (1989). Micropropagated potato plants were grown for 3 to 5 weeks under usual tissue culture conditions. 20 to 30 leaf sections, cut at the base of the leaf, and internodal stem sections from the micropropagated plants were cut with a sterile scalpel and transferred immediately to 20 ml MS3O liquid medium (as described in Sec.2.2.6.) in a petri dish. 100 µl of an overnight culture of *Agrobacterium* was added to the petri dish which was then was incubated with gentle shaking at room temperature in the dark for 10 minutes. The infected explants were blotted dry on sterile filter paper and placed upside down on solid LS/R1 medium with hormones (as described in Sec.2.2.6.) and incubated for 48 hours in the dark. Subsequently the explants were transferred to fresh LS/R1 medium containing hormones and 500 mg/l carbenicillin and 100 mg/l kanamycin, and incubated under standard tissue culture conditions. These explants were subcultured onto fresh plates every two weeks, until calli started to appear on the cut surfaces. The explants were transferred to L/SR-2 medium supplemented with 50 mg/l carbenicillin and 100 mg/l kanamycin (as descibed in Sec.2.2.6.) to regenerate shoots. Shoots that appeared were transferred to rooting medium (as described in Sec.2.2.6.) with 100 mg/l kanamycin selection. If the shoot was transgenic root growth should have been observed within 14 days, and a full root system would develop.

2.6.2.3.2. Alternative Transformation Protocols

The protocol described by Twell and Ooms (1987) was used without modification, except that carbenicillin (500 mg/l) was used as the bacteriostatic antibiotic instead of cefotaxime.

2.6.3. Propagation of transgenic plants

Rooted transgenic plants were propagated following the method described in section 2.3.1, with the exception that kanamycin at 50 mg/l was added to the NM medium.

2.7. Aging of Potato Tuber Slices

Potato tuber slices were aged for up to 18 hours in a phosophate solution in the dark at 28 °C as described by Logemann *et al.* (1988), in the presence of 50 µg/ml chloramphenicol.

2.8. Isolation of Mitochondria From Potato Tubers

Mitchondria were isolated from from fresh and aged potato tubers as described in Winning *et al.* (1992).

2.9. Measurement of Isolated Mitochondrial Respiration

Mitochondrial respiration was assayed by making polarographic measurements of oxygen concentration using a Clark Type oxygen electrode (Walker, 1987).

Reactions were carried out in two chambers containing oxygen electrodes (Hanastech, U.K.) linked to two CB1 controlboxes (Hanasatech, U.K.). These control boxes provide the necessary polarising voltage to allow the operation of the electrodes and amplify the signal produced by it. The output was displayed on a dual pen chart recorder (Rikadenki, Japan). The temperature of the reaction chambers was maintained at 25 °C using water circulating through surrounding jackets from a water bath maintained at this temperature.

2.9.1. Calibration of Electrodes

Two points were used for calibration:

1. Oxygen saturated water, which was taken to correspond to 253 µM oxygen at 25 °C.

2. Oxygen free water produced by the addition of a few crystals of sodium dithionite to the water in the reaction chamber.

$Na_2S_2O_4 + O_2 + H_2O \rightarrow NaHSO_4 + NaHSO_3$

The calibration procedure was initiated with distilled water in the reaction chamber. The gain control on the control box was adjusted so that a full scale deflection was obtained on the chart recorder. A few crystals of sodium dithionite were added to the reaction chamber. The oxygen concentration declines rapidly at first, then more slowly until it reaches a steady state. This point corresponds to an oxygen concentration of zero and should be at or near zero on the chart recorder. The oxygen free distilled water was removed and the reaction chamber washed with distilled water until the full scale deflection was achieved again.

2.9.2. Oxidation of Respiratory Substrates

Reactions were carried out in 1 to 2 ml of standard reaction medium (0.3 M sucrose, 10 mM TES buffer, 10 mM potassium dihydrogen orthophosphate, 5 mM magnesium chloride, 0.1 % (w/v) bovine serum albumin, pH 6.8 containing mitochondria (0.2-1.0 mg protein). Respiration was initiated by the addition of 10 mM succinate and 0.25 mM ATP, to activate succinate dehydrogenase, and 1 µmol ADP per ml of reaction mixture for prolonged state three respiration.

2.9.3. Estimation of Mitochondrial Outer Membrane Intactness

Outer mitochondrial membrane intactness was assayed by measuring cyanide sensitive cytochrome c dependent ascorbate oxidation (Neuburger, 1985). The rate obtained with the intact mitochondria was compared to that obtained by lysis of the mitochondria in pure water for 10 seconds. The reaction was carried out in standard reaction medium (Sec.2.7.3.) at pH 7.2 in the presence of 8 mM isoascorbate and 30 µM cytochrome c. The cyanide sensitive

component was measured by subtraction after the addition of 0.25 mM potassium cyanide.

2.9.4. Inhibitor Titrations

Inhibitor titration curves for the adenine nucleotide translocator were obtained by the addition of carboxyatractyloside to the reaction chamber containing mitochondria as described in Sec.2.7.3. Following the establishment of a constant rate, another addition of the inhibitor was made and a new rate established. Inhibitor titrations were compared to a control carried out under identical conditions, but in the absence of the inhibitor. The inhibitor titration curve was constructed by plotting the relative rate of respiration against the inhibitor concentration.

2.10. Measurement of Respiration *in vivo*

Oxygen uptake due to dark respiration of potato leaves was measured polarographically using a leaf disc oxygen electrode (Hansatech, UK) and the output displayed on a Compaq Portable III™ personal computer running Flashcard software, according to Walker (1987).

2.11. Measurement of Membrane Potential

Potential sensitive dyes were used to investigate mitochondrial function as described in Liu *et al.*, (1987), except that the tissue was incubated with the dye for 15 to 30 minutes, and the fluorescence was observed immediately after the dye solution was replaced by distilled water. The sections were observed with a Zeiss Axiophot microscope and photographs were taken on Fuji RHD film.

CHAPTER 3
THE PREPARATION OF ANT CONSTRUCTS IN BINARY VECTORS

3.1. Introduction

The introduction of either sense or antisense transgenes is an established technique to investigate gene function by creating functional mutants. Antisense technology has been applied to many organisms, but has proved especially powerful in plant science, where it is possible to regenerate transgenic plants. Despite these applications the mechanisms by which antisense transcripts cause inhibition in eukaryotes remains unclear. This chapter aims to review the information available on the mode of action of antisense RNA in eukaryotes, and the antisense transgenes that have proved successful, especially in plants. The construction of sense and antisense constructs with the ANT cDNAs will be described, and their ligation into binary vectors used for plant transformation will be described.

3.2. Mechanisms of Action of Antisense Transcripts in Eukaryotic Cells

Possible mechanisms for the mode of action of antisense RNA in eukaryotes are illustrated in Fig.3.1. The regulatory effect is believed to be due to base-pairing between sense and antisense RNA strands, by which the expression of mRNA is blocked. In eukaryotes, such interactions could occur in the nucleoplasm as well as at other locations in the cell. The mechanisms proposed suggest that double stranded RNA hybrids generated in the nucleus are rapidly degraded, whereas double stranded cytoplasmic RNA interacts less efficiently with the translational apparatus, or has an increased turnover.

Melton (1985) and Harland and Weintraub (1985) found that antisense RNAs injected in *Xenopus* oocytes appeared to exert its effect by interfering with mRNA translation of co-injected non-*Xenopus* transcripts. Wormington (1986) showed that expression of endogenous oocyte mRNA was also susceptible to inhibition. These studies showed that stable duplexes were formed between the sense and antisense RNAs, but required a large ratio of antisense to sense RNA to be effective.

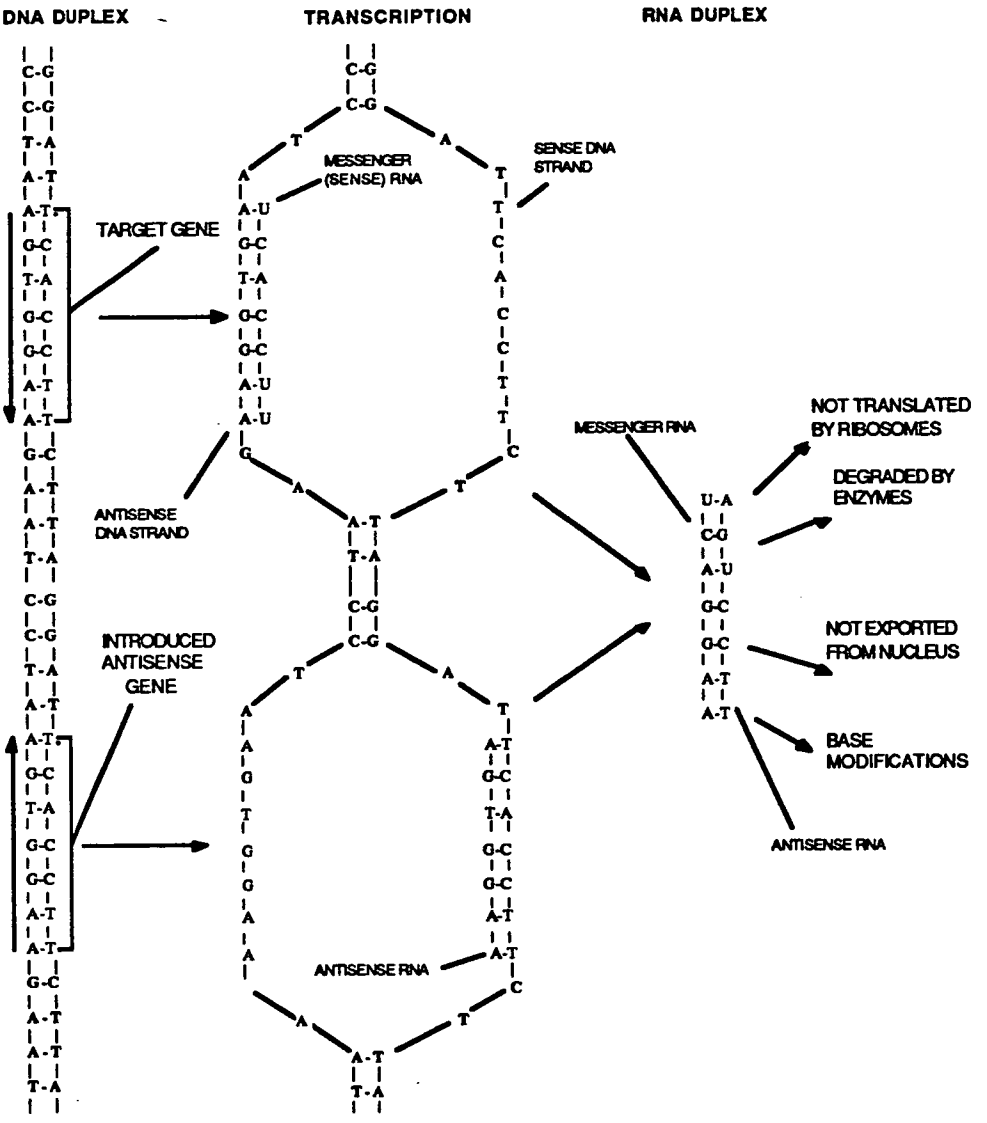


Fig.3.1.Base pair specificity is the key to antisense RNA inhibition of genes.

The matched sense and antisense strands of a gene complement each other. During transcription of DNA into RNA the antisense DNA strand acts as a template for assembling a complementary (sense) messenger RNA. A single stranded messenger RNA is translated into protein by ribosomes. The expression of the target gene can be reduced if another copy of the gene, in the reverse orientation, is introduced into the genome. An antisense RNA will be made from what was the sense DNA strand of the original gene. The antisense RNA and the messenger RNA can bind with each other. A variety of factors may then prevent the translation of the protein: the RNA duplex may never leave the nucleus; it may be degraded by enzymes; it may be rejected by the ribosomes; the adenine (A) bases may be chemically modified to become inosine, thus scrambling the genetic code on the messenger RNA.

Translational interference was demonstrated when retroviral mediated gene transfer was used to introduce antisense sequences directed against creatine kinase B (CK-B) into human U937 lymphona cells (Ch'ng *et al.*, 1989).

The failure of injected antisense RNAs to inhibit the expression of specific genes in *Xenopus* oocytes surprisingly led to the discovery of another possible mechanism for the inhibition of translation, an 'unwindase' activity (Rebagliati and Melton, 1987; Bass and Weintraub, 1987). The 'unwindase' activity is present in the cytoplasm of embryonic cells, modifing as many as 50 % of adenine residues to inosine, thus destabilising the duplex (Bass and Weintraub, 1988). An inevitable consequence of this modification would be the corruption of translational reading frames in any coding region involved in the duplex structure. However, this does not explain the failure of this injected antisense RNA to inhibit expression.

Such results are most easily accomodated into a model where duplex formation between antisense and sense RNAs interferes with either the binding or the translocation of ribosomes on the mRNA. However, there is evidence that antisense transcripts can interfere with transcription or post transcriptional processing, although the mode of inhibtion remains obscure. Duplexes between sense and antisense RNAs have been reported (Kim and Wold, 1985; de Benedetti *et al.*, 1987; Yokoyama and Imamoto, 1987) and Kim and Wold observed the duplexes only in the nucleus. This led to speculation that the reduction in activity obtained was primarily due to duplexes formed in the nucleus trapping the sense transcripts. Unusally in this case they found a 300-fold excess of antisense to sense transcripts. It may be that duplexes are rapidly degraded (Crowley *et al.*, 1985) or that unstable interactions greatly retard the transport of mRNA to the cytoplasm, leading to reduced steady state levels of mRNA.

In transgenic tobacco it was observed that the inhibition of the bialaphos (*bar*) gene expression by an antisense-*bar* gene occured at two levels; firstly the *bar* mRNA pool was decreased; secondly

protein synthesis per *bar* mRNA was strongly reduced, to about 30 % of control levels (Cornelissen and Vandewiele, 1989; Cornelissen, 1989). Duplex RNA could not be detected in the nucleus or the cytoplasm, but approximately equal levels of *bar* and antisense-*bar* RNA was detected in the cytoplasm. These results suggested that the antisense effect was exerted at two levels, both nuclear and cytoplasmic. In the nucleus RNA duplexes formed between *bar* and antisense-*bar* RNA may be largely degraded, with only small amounts of *bar* and antisense-*bar* RNA being transported to the cytoplasm. In the cytoplasm the translational efficiency of the *bar* mRNA might be reduced by unstable association with anti-*bar* RNA.

3.3. The Design of Artificial Antisense Genes

The uncertainity in the mechanism by which antisense RNA exerts its effect in eukaryotes obviously undermines any attempt to rationally design antisense constructs. This is compounded by our lack of knowledge of secondary structures of both sense and antisense RNAs and regions which may be coated in protein. Such concerns about how the technique works would be less important if the technique worked equally well in all cases. In the 17 cases surveyed by Van der Krol *et al.* (1988) a range of inhibition from 0 to 99 % was observed. Different regions of genes have been used to suppress both introduced model transgenes (such as *bar*) and endogenous gene expression in animals and plants. Often the full length cDNA has proved to be a successful antisense agent, but sections of cDNAs spanning either the 5' or 3' regions have proved effective in inhibiting expression of the target gene.

3.3.1. Antisense Genes to Inhibit Model System Genes

Expression of herpes simplex virus (HSV) thymidine kinase (TK) was the first specific gene to be successfully inhibited in eukaryotes. An antisense construct of the full structural TK gene (from +51 to +1415 bp) inhibited wild type TK expression when coinjected on plasmids (Izant and Weintraub, 1984). A similar inhibitory effect was observed with a 5' region of the HSV TK gene

(from -80 to +343 bp). This contruct also inhibited a coinjected hybrid HSV-Chicken TK fusion gene with only 52 complementary bp. However the expresson of the chicken TK, which shares no homology with the HSV TK, was not inhibited demonstrating the specifity of the inhibition. Transient inhibition of bacterial chloramphenicol acetyltransferase (CAT) was observed in a line of mouse LTK- cells when a plasmid containing the full coding region was co-injected into the cells (Izant and Weintraub, 1985). Kim and Wold (1985) demonstrated that the 3' end of the TK gene could also act as an antisense agent, inhibiting TK activity by up to 90 % when transcriptionally fused to dihydrofolate reductase (DHFR) to form a chimeric antisense gene using DHFR as a selectable marker.

The first antisense inhibition in a plant system was demonstrated by Ecker and Davis (1986). A full length antisense *cat* gene electroporated into carrot protoplasts inhibited the expression of a transiently introduced sense *cat* gene. A selection of these model transgenes that have been used in plants are summarized in Table 3.1.

Rothstein *et al.* (1987) inhibited nopaline synthase (NOS) activity in transgenic tobacco plants previously transformed with the *nos* gene by transformation with an antisense construct containing the 5' region of the gene. Sandler *et al.* (1988) also demonstrated the inhibition of NOS activity in tobacco plants transformed with a sense copy of the nos gene, but using the 3' end of the gene. However, the full length gene or a shorter 5' construct than that used by Rothstein *et al.* were not effective in inhibiting gene expression. These results were explained by differences in RNA stability and/or three dimensional structure for each antisense RNA, affecting its hybridisation with the sense mRNA. An antisense construct generated with 172 nucleotides of the 5' terminal CAT sequence was found to be less effective in suppressing CAT activity than a full length CAT antisense transcript (Delauney *et al.*, 1988). β-glucuronidase (GUS) activity in previously transformed plants expressing GUS has been inhibited by antisense constructs, with either a full length gene (Robert *et al.*, 1989), or as little as 41

TARGET GENE	HOST CELL	PROMOTER	COMPLEMENTARY TO TARGET	RATIO	INHIBITION	REFERENCE
Chloroamphenicol acetyltransferase	Carrot protoplasts	NOS CaMV 35S PAL	Full	100:1	>95%	Ecker and Davies, 1985.
Chloroamphenicol acetyltransferase	Tobacco plants	CaMV 35S rbcS	Full cDNA 5' end cDNA Full	1:2-20 1:10	100% 60% None	Delauney *et al.*, 1988.
Nopaline synthase	Tobacco plants	CaMV 35S	5' two thirds	10:1	10-50X	Rothstein *et al.*, 1985.
Nopaline synthase	Tobacco plants	Cab22R	Full cDNA 3' end	-	Up to 85%	Sandler *et al.*, 1988.
β-glucuronidase	Tobacco plants	CaMV 35S	Full	1-5X	>90%	Robert *et al.*, 1989.
β-glucuronidase	Tobacco plants	ca/b	41 base pairs 5' end	-	0-100%	Cannon *et al.*, 1990.
Phosphinotricin acetyl transferase	Tobacco protoplasts and plants	CaMV 35S	Full	1-2:1	97% 3-78%	Cornelissen and Vandeweile, 1989; Cornelisson, 1989.

Table 3.1. (Above) Regulation by antisense inhibition of model target genes in plant systems

Table 3.2. (Below) Regulation by antisense inhibition of endogenous genes in plant systems.

Target gene: exogenous or endogenous gene target

Host cell: Transient or stably transformed plant species

Promoter: promoter in antisense gene construct

Complementarity: degree of sequence homology of antisense gene with target gene

Ratio: ratio of antisense to sense gene plasmids introduced into cells, or the ratio of antisense transgenes to endogenous plant genes, if determined.

Inhibition: reduction in protein levels, target gene activity, or change in phenotype.

TARGET GENE	HOST CELL	PROMOTER	COMPLEMENTARY TO TARGET	RATIO	INHIBITION	REFERENCE
Chalcone synthase A	Petunia and tobacco plants	CaMV 35S chs CaMV 35S + anther box	Full	-	Phenotypic changes in floral pigmentation	van der Krol *et al.*, 1988; 1990. van der Meer *et al.*, 1992.
Polygalacturonase	Tomato plants	CaMV 35S	5' end	-	90%	Smith *et al.*, 1988.
Polygalacturonase	Tomato plants	CaMV 35S	Full	-	69-93%	Sheehy *et al.*, 1988.
Ribulose bisphosphate carboxylase small subunit	Tobacco plants	CaMV 35S	5' end cDNA	1-4:3	67-88%	Rodermel *et al.*, 1988.
Ribulose bisphosphate carboxylase small subunit	Tobacco plants	CaMV 35S	Full coding region + 3' end cDNA	1+:3	82%	Hudson *et al.*, 1992.
Granule-bound starch synthase	Potato plants	CaMV 35S	Full (potato) 78% (maize)	1-4:1	70-100%	Visser *et al.*, 1991.
Coat protein of CAMV	Tobacco plants	CaMV 35S	Full	-	at low levels	Cuozzo *et al.*, 1988.
Coat protein of potato X virus	Tobacco plants	CaMV 35S	Full	-	at low levels	Hemmenway *et al.*, 1988.
pTOM13	Tomato plants	CaMV 35S	1.1 kb	1-2:2	66-93%	Hamilton *et al.*, 1990.
ADP-glucose pyrophosphylase	Potato plants	CaMV 35S	85%	-	100%	Müller-Röber *et al.*, 1992.
Nitrite reductase	Tobacco plants	2X CaMV 35S	Full	-	5 fold	Vaucheret *et al.*, 1992
ACC Synthase	Tomato plants	CaMV 35S	Full	10:1	99.5%	Oeller *et al.*, 1991
Pectin methylesterase	Tomato plants	CaMV 35S	95%	-	90%	Tieman *et al.*, 1992
E8	Tomato plants	CaMV 35S	Full	-	90%	Peñarrubia *et al.*, 1992
Tonoplast H+ ATPase	Carrot plants	CaMV 35S	Full coding/5' non coding	-	30-40%	Gogarten *et al.*, 1992
Systemin	Tomato plants	CaMV 35S	Full	-	>60%	McGurl *et al.*, 1992.

nucleotides spanning the translation start site (Cannon *et al.*, 1990). The full phosphinotricin acetyl transferase (PAT) antisense gene reduced PAT synthesis to only 8 % of that observed in the original tobacco plant, previously transformed with a sense copy of the gene (Cornelissen and Vandeweile, 1989).

3.3.2. Antisense Constructs to Inhibit Wild-type Target Genes

The expression of endogenous genes in a number of eukaryotic systems has been inhibited by antisense RNA. Mammalian cells, *Drosophila, Dictyostelium,* and *Xenopus* are all systems where the principle has been shown to work, but the ease of regenerating transformants makes the technique especially powerful in plants. Endogenous genes that have been down regulated in plants are listed in Table 3.2.

The first endogenous plant gene which was shown to be down-regulated by antisense RNA was chalcone synthase (CHS) in petunia and tobacco (van der Krol *et al.*, 1988a). Transformation with a full length antisense cDNA construct regenerated plants which showed a high frequency of altered pigmentation in petals. Antisense constructs using either the 3' half or the last quarter of the CHS cDNA were also effective antisense agents (van der Krol *et al.*, 1990). However, a construct expressing the 5' half of the CHS gene was not effective. The RNA encoded by this construct had a much lower steady state level in leaf tissue than RNAs encoded by the other constructs. The full length CHS cDNA has been used in antisense constructs to inhibit gene expression in the anthers of transgenic petunia plants (van der Meer *et al.*, 1992).

Fruit ripening in tomato is a process that has been extensively investigated with antisense RNA. Smith *et al.* (1988) demonstrated inhibition of polygalacturonase (PG) in tomato using an antisense construct containing 730 nucleotides from the 5' end of the PG cDNA, including 50 nucleotides of untranslated region. A full length antisense cDNA construct has also been used to inhibit PG activity in tomato (Sheehy *et al.*, 1988). Hamilton *et al.* (1990) inhibited

expression of the gene for the mRNA pTOM13 using a 1.1 kb fragment of the cDNA to the gene. One copy of the 1-amino-cyclopropane-1-carboxylic acid-oxidase (ACC) gene reduced activity by 66 %, but two copies reduced activity by 93 %. Oeller *et al.* (1991) inhibited the expression of ACC synthase with an antisense construct containing a full length cDNA to ACC synthase, whilst Peñarrubia *et al* (1992) also used a full length cDNA in an antisense construct to E8, another enzyme involved in fruit ripening, to inhibit protein accumulation. Another gene involved in fruit ripening, pectin methylesterase, has been down regulated by an antisense construct with a 1.6 kb genomic fragment, which includes 185 bp of the untranslated 5' region (Tieman *et al.*,1992). Even the expression of one of the most abundant leaf mRNA species, the small subunit of ribulose bisphosphate carboxylase (*rbc*S), could be greatly reduced by the expression of an antisense construct (Rodermel *et al.*, 1988). This construct contained 22 nucleotides upstream and 300 nucleotides downstream of the initation codon from an *rbc*S cDNA. These plants have been subject to extensive physiological and biochemical investigation (described in Sec.1.11.2.) Recently Hudson *et al.* (1992) have inhibited the expression of rbcS in tobacco using a 600 nucleotide rbcS cDNA. This cDNA spans the complete coding region and the 3' untranslated region.

A 2.3 kb full length granule-bound starch synthase (GBSS) cDNA antisense construct inhibited potato GBSS activity by 70 to 100% (Visser *et al.*, 1990). Potato ADP- glucose pyrophosphorylase has been successfully inhibited by an antisense construct containing a 1.6 kb DNA fragment spanning the full coding region of the cDNA (approximately 85 % of the full size) of the B subunit (Müller-Röber *et al.*, 1992). A 1.8 kb antisense cDNA for nitrite reductase successfully reduced the expression of nitrite reductase, despite lacking approximately 80bp from the 5' region (Vaucheret *et al.*,1992). A full length cDNA was also used by Knutzon *et al.* (1992) to inhibit expression of stearoyl-acyl carrier protein desaturase in *Brassica* oil seeds.

Antisense technology has also being applied to protecting plants from viral infection. The entire coat protein sequence of the cauliflower mosaic virus (CaMV) has been introduced into tobacco plants. Transgenic plants expressing this CaMV CP antisense RNA were resistant to CaMV infection, but only at low levels of innoculum (Cuozzo *et al.*,1988). Plants expressing an antisense transcript to the potato virus X coat protein were protected on innoculated and systemic leaves, but once again only at the lowest levels of innoculum (Hemenway *et al.*, 1988). The inhibition of infection was not as effective as that observed in plants expressing the coat protein itself. The expression of a 5' region of the coat protein gene of 135 nucleotides failed to confer any protection.

3.3.3. Heterologous Genes Used for Antisense Constructs

In some of the cases described in Sec.3.3.2. a heterologous gene from different species was used to inhibit gene expression. A multigene family encodes CHS in petunia and two genes, *chsA* and *chsJ*, are expressed in the flower. *chsA* accounts for 90 % of the mRNA level in wild type flowers whilst *chsJ* accounts for the remaining 10 %. The introduction of an antisense gene to *chsA* in petunia reduced mRNA levels of both *chsA* and *chsJ* to 1% of the wild type. Similarly the introduction of this construct into tobacco down regulated the level of the endogenous tobacco *chs* mRNA. The down regulation of petunia *chsJ* and *chs* in tobacco demonstrates that heterologous genes may act as antisense templates. The Petunia *chsJ* gene is 86 % homologous to the *chsA* gene, while the sequence divergence between the petunia and tobacco genes is estimated to be around 20 % (Van der Krol *et al.*, 1988).

The successful, but partial inactivation of GBSS in potato by a heterologous antisense gene has also been reported (Visser *et al.*, 1990). A 2.1 kb antisense maize genomic *gbss* clone, covering both introns and exons, partially inactivated the endogenous gene. This gene contains about 34 complementary regions of at least 10 nucleotides, showing a homology of greater than 75 % to the potato gene.

Rodermel *et al.* (1988) also used a heterologous cDNA, from *Nicotiana sylvestris,* to successfully inhibit expression of the endogenous *Nicotiana tabacum rbcS* genes which account for the bulk of the mRNA production in the leaves of this species. However, the sequence homology of these cDNAs is 99.7 %. The stearoyl-acyl carrier protein desatur ase from *Brassica napus* was used by Knutzon *et al.* (1992) to inhibit gene expression in *Brassica rapa,* but the homology was not reported.

3.3.4. Choice of Promoters For Antisense Gene Expression

The injection of antisense RNAs into animal cells suggested that a high ratio of antisense to sense RNA was required for effective action (Melton, 1985; Harland and Weintraub, 1985; Wormington, 1986). Although ratios of antisense to sense RNA as low as 4:1 (Stickland *et al.*, 1988) and 10:1 (Harland and Weintraub, 1985) were successful, in most reports the ratio of antisense to sense RNA injected was much higher (e.g. 50:1 reported by Melton, 1985). Similarly, the ratio of plasmids expressing antisense genes to those with sense genes required to obtain an effect when coinjected into mammalian cells was 100:1 (Izant and Weintraub, 1984, 1985; Kim and Wold, 1985).

Ecker and Davies (1985) compared the inhibitory effect of antisense CAT constructs driven by three different promoters when electroporated into carrot protoplasts. Promoters from three genes were compared, NOS, cauliflower mosaic virus 35S (CaMV 35S) and phenylalanine ammonia-lyase (PAL). There was a correlation between promoter strength and the degree of inhibition of the target gene, chloroamphenicol acetyltransferase (CAT). They found that the level of expression of the sense CAT gene was similar with the NOS and CaMV 35S promoters, but using the PAL promoter expression was a factor of 4 or 5 lower. The NOS and CaMV 35S antisense constructs were equally effective in inhibiting expression of the target gene when the sense gene was under the control of the same promoter. When the PAL antisense plasmid was used to inhibit expression of

the sense gene the level of inhibition observed was lower by a factor of 4.

Van der Krol *et al.* (1990) used both the CaMV 35S promoter and the endogenous CHS promoter in antisense CHS constructs. The endogenous CHS promoter did not produce an excess of antisense RNA compared to endogenous CHS mRNA, but it was sufficent to reduce CHS steady state mRNA levels and inhibit flower pigmentation. The pigmentation of anthers in these flowers was not affected by either construct. The CaMV 35S promoter is known not to be expressed in tapetum and sporogenous layers of the anther whereas the CHS promoter is active in these tissues. The failure to achieve inhibition may be due to low levels of expression in the transgenic plants (van der Meer *et al.*, 1990). The insertion of an 'anther box', a homologous sequence found in other genes expressed in anthers, resulted in increased expression in the anthers, and an inhibition of pigment synthesis and male sterility (van der Meer *et al.*, 1992).

Cannon *et al.*, (1990) demonstrated leaf specific inhibition of GUS gene expression (which was driven by the CaMV 35S promoter) using the light regulated (*cab*) promoter to drive the antisense gene. This promoter does not produce a large excess of antisense transcripts, but is expressed at similar levels to the CaMV 35S promoter. This variation was thought to be due to position effects. Knutzon *et al.* (1992) used stearoyl-ACP desaturase antisense constructs driven by two promoters, the stearoyl-ACP desaturase promoter and the napin promoter to limit the expression of the antisense genes to developing oil seeed rape seeds.

The most common promoter used to express antisense genes is the CaMV 35S promoter. This is a strong, constitutive promoter, highly active in most plant organs and during most stages of development when integrated into the genome of transgenic plants (Nagy *et al.*, 1985; Odell *et al.*, 1985; Jefferson *et al.*, 1987; Kay *et al.*, 1987). It may be that this constitutive expression provides a pool of antisense RNA already present when a developmentally regulated target gene is switched on. This creates a large excess of antisense to

sense template, which has been suggested as being important, but not an absolute requirement in causing antisense inhibition.

3.3.5. Polyadenylation Signals For Antisense Constructs

Ecker and Davies (1986) compared the effect of adding an effective polyadenylation signal (from the NOS gene) on the efficiency of antisense inhibition. When the polyadenylation signal was included the amount of antisense plasmid required to inhibit CAT activity to the same extent as a control without such a sequence was reduced by a factor of at least 2. The NOS polyadenylation signal has become the most commonly used terminator in plant antisense genes, being used in most of the constructs described in the previous section (Sec.3.4.4.). Other terminators that have been used in sucessful antisense constructs include the transcript 7 3' terminator from *Agrobacterium* (Sheehy *et al.*, 1988; Cannon *et al.*, 1990) and the octopine synthase polyadenylation signal (Sandler *et al.*, 1988).

3.4. Design of Antisense Expression Cassettes

The antisense constructs described in the previous section illustrate the approaches to the design of antisense genes. However, it appears that the factors likely to lead to a successful antisense construct are; 1, the antisense gene should span either the majority or all the coding region of the gene, and that the fragments of genes that have been used and which work vary with each gene and 2, the promoter used to drive the gene should be capable of high expression levels in the target tissue.

3.4.1. Constitutive CaMV 35S Promoter

As described in Sec.3.3.4., the CaMV 35S promoter is the most commonly used promoter in antisense constructs. However, the use of a constitutive promoter may prevent the regeneration of transgenic plants if the gene inhibited is essential for cell function. Further, the use of a tissue specific promoter should result in the levels of antisense transcripts synthesised in the target tissue being sufficient to cause the desired effect.

3.4.2. Tissue Specific Promoters

An effective antisense construct driven by a tissue specific promoter would appear to require a promoter of equal strength (at least) to the CaMV 35S promoter. Using potato as the model system allows the use of two tissue specific promoters that have been isolated from potato, the ST-LS1 promoter and the patatin promoter.

3.4.2.1. The Patatin Promoter

The morphological changes during tuberization are accompanied by a variety of biochemical changes. The most dramatic of these are the deposition of starch and the appearance of new proteins, such as patatin. Patatin represents around 40 % of the total soluble protein of mature potato tubers (Racusen and Foote,1980). The patatin gene family can be divided into two classes based on their expression pattern: class I genes are mainly expressed in the tubers, whereas expression of class II genes is found in both roots and tubers, although at a much lower level when compared to class I genes (Pikaard *et al.*, 1987).

Rocha-Sosa *et al.* (1989) have isolated a 1.5 kb genomic DNA fragment of upstream sequence from a class I promoter which is able to direct a high level of tuber specific gene activity. When a class I promoter was linked to the β-glucuronidase (GUS) reporter gene and transformed into potato GUS activity was on average 100 to 1000 fold higher in tubers when compared to leaf, stem and roots. High levels of sucrose, which mimic the conditions that stimulate the accumulation of starch in storage organs activated the promoter in leaves. The level of GUS activity observed in tubers (2000 to 15 000 pmol MU/mg protein/min) was within the same range as that observed when the GUS gene was linked to the CaMV 35S promoter (2000 to 15 000 pmol MU/mg protein/min).

Another class I patatin promoter has been isolated on a 3.5 kb genomic DNA fragment by Bevan *et al.* (1986). This promoter also induces tuber specific gene expression and high levels of activity on high concentrations of sucrose, but not at low concentrations of sucrose or high concentrations of sorbitol or glucose. Comparison of GUS activity in plants transformed with either the CaMV 35S or the

class I patatin promoter linked to the GUS reporter gene showed that both gave similar high levels of expression in the tubers (3875 pmol/mg protein/min and 6000 pmol/mg protein/min respectively). These patatin promoters are thus probably strong enough to be useful in antisense experiments directed at modifying target gene expression in a specific tissue, the tuber.

In tubers the mitochondria are the principle source of ATP for cell development and differentiation. This is not only true during tuber formation, but also when sprouting starts and as shoot growth and development occurs. Thus the role of ANT, linking the sites of ATP production (the mitochondria) and the sites of ATP consumption (in the cytosol), in these tissues may be crucial. Thus by modifying expression of genes encoding mitochondrial proteins and thus mitochondrial function in this tissue an insight into the role of mitochondria during tuber development may be obtained.

3.4.2.2. The ST-LS1 Promoter

In many plant tissues, such as leaves, mitochondria share a close metabolic interaction with other organelles including chloroplasts. The ability to perturb ANT expression in such tissues could provide information on the effects of modifying mitochondrial function in these tissues where photophosphorylation may partially satisfy the cells' energy requirements. A light-inducible, organ-specific promoter, ST-LS1, has been isolated on a 1.5 kb fragment of genomic DNA from potato (Eckes *et al.*,1985, 1986). The ST-LS1 gene encodes the 10 kd protein of photosystem II. This protein is part of a complex in the thylakoid lumen that is closely associated with the water splitting function, although its role has not been fully characterised (Stockhaus *et al.*, 1990). It has been shown that ST-LS1 gene expression is qualitatively and quantitatively very similar in transgenic potato and tobacco plants (Stockhaus *et al.*, 1987a). The expression of this promoter appears to be closely associated with the presence of chloroplasts (Stockhaus *et al.*, 1987b; Stockhaus *et al.*,1989). The levels of expression of a β-glucuronidase reporter

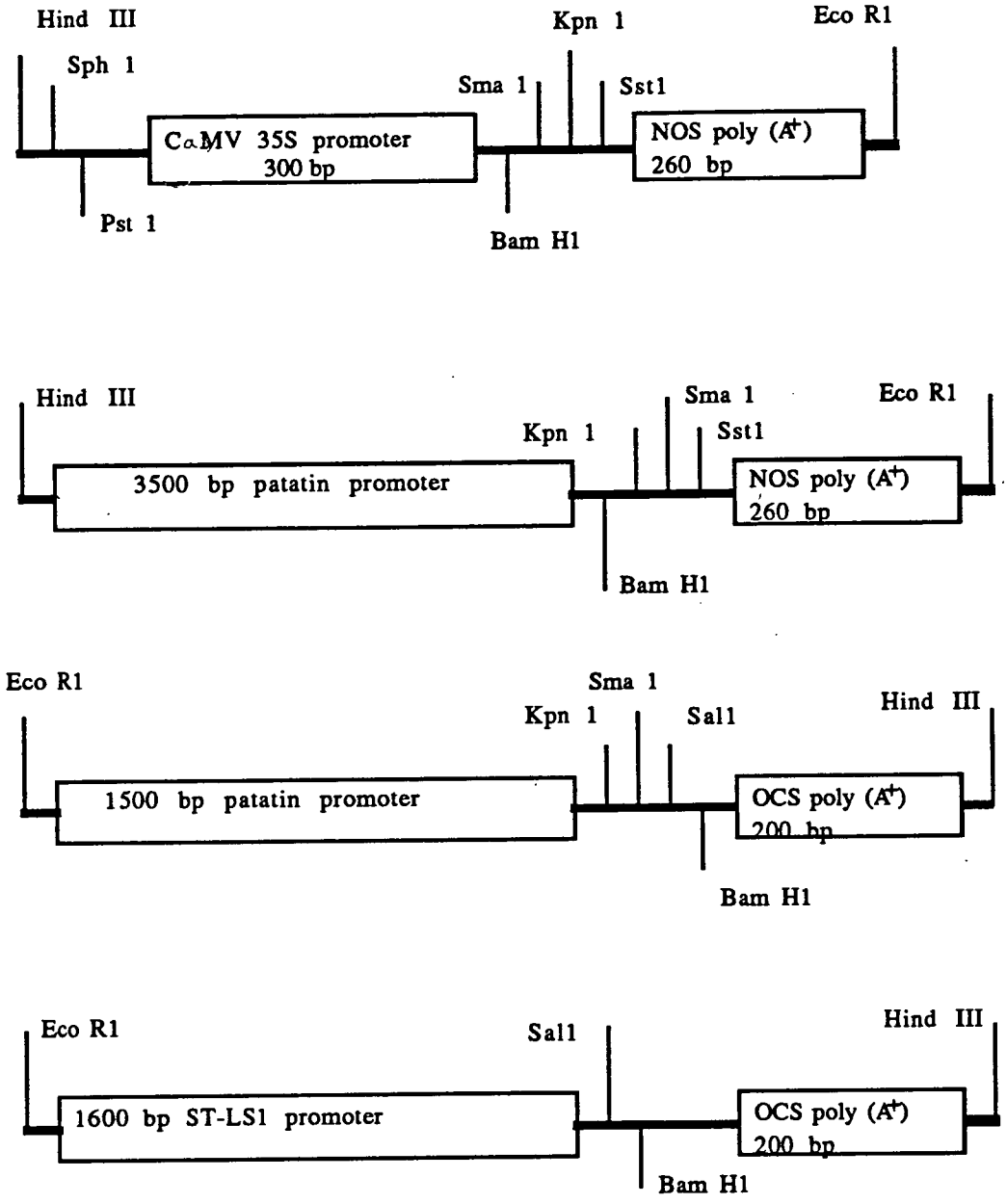


Fig.3.2. Structure of the gene expression cassettes uesd for constitutive or tissue specific gene expression.

The unique restriction enzyme sites in the multiple cloning site of each expression cassette are shown, with the *Bam* H1 site in each cassette which was used to subclone the ANT cDNA marked below. The borders of each cassette are delimited by *Eco* R1 and *Hind* III restriction enzyme sites.

gene driven by this promoter in leaf tissue are also in the same range as those observed for the CaMV 35S promoter.

3.5. Promoter Expression Cassettes

All the promoters described above were available in or were subcloned into "expression cassettes", with the promoter upstream of a short multiple cloning site linked to a NOS polyadenylation sequence (Fig.3.2.). The borders of each cassette were defined by an *Eco* RI restriction enzyme site at the 5' end and a *Hind* III restriction enzyme site at the 3' end. This complete cassette could be excised as an *Eco* RI-*Hind* III fragment and cloned into the *Agrobacterium* binary vector, replacing the existing multiple cloning site.

In each of the cassettes the number of restriction enzyme sites in the polylinker was limited. Furthermore, due to the restriction enzyme sites present in the promoter fragment, ANT cDNA or the binary vector, there were only two single restriction enzyme sites, *Bam* HI and *Sac* I, in the multiple cloning site that were common to all the cassettes. This lack of restriction enzyme sites dictated the cloning strategy for creating the sense and antisense constructs.

3.6. Adenine nucleotide translocator cDNA clones

Previous work in this laboratory has led to the isolation of a 1579 bp ANT cDNA from maize, MANT-A (Winning *et al.*, 1991). This was contained on an *Eco* RI fragment and encoded a 987 bp open reading frame. The sequence for this cDNA was found to correspond with one of two nuclear genes found in maize (Bathgate *et al.*, 1989). As only one short cDNA corresponding to the other maize nuclear gene had been isolated MANT-A was the only putative full length clone for the adenine nucleotide translocator available at the start of the project. Although Visser *et al.* (1991) have partially inhibited expression of a potato gene with an antisense construct using a maize genomic clone, most antisense transcripts have been generated from a homologous cDNA. Some genes that have been transferred between monocots and dicots are not faithfully processed, as the introns are inefficiently spliced, e.g. *rbcS* from wheat expressed in

tobacco is not correctly processed, whilst the pea *rbcS* is correctly processed (Keith and Chua, 1986). In this work the cDNA from maize was used in both sense and antisense constructs.

Comparison of MANT-A with the corresponding maize genomic clone shows that MANT-A extends 153 nucleotides beyond the 3' terminus of the genomic clone. In addition, the 5' end of the cDNA contained 64 nucleotides of apparently unknown origin, as they showed no similarity with 442 bp of pANT-1 upstream region (Fig.3.3.). The constructs created with this cDNA where designed so that this non homolgous region was not present. However experiments performed in this laboratory during this work have shown that this 64 bp region is an exon located upstream of the genomic ANT DNA fragment isolated previously (Day, 1992).

In the course of this work several cDNA clones were isolated from a potato tuber cDNA library, of which one 1453 bp cDNA, pPANT-1, was fully sequenced (Winning *et al.*, 1991). Translation of the sequence gives a predicted polypeptide sequence that is one amino acid shorter than the predicted maize ANT proteins (Fig.3.3). Transcript analysis of potato tuber and leaf RNA suggests that the transcript is approximately 1600 nucleotides in length. The cDNAs from maize and potato were found to be 67 % homologous in the coding region at the nucleotide level.

3.7. Subcloning of the Maize MANT-A cDNA

As described in Sec.3.5. the *Bam* HI restriction enzyme site in the polylinker is common to all the expression cassettes. As the maize ANT cDNA is encoded on an *Eco* RI fragment it was necessary to modify it before cloning into the expression cassettes. MANT-A was subcloned into the plasmid pGEM 1 at the *Eco* RI site in the multiple cloning site. Clones were picked in which the orientation of the cDNA resulted in the *Bam* HI site of pGEM 1 being at the 5' end of the cDNA. There is a *Bgl* II restriction enzyme site at 1396 bp in

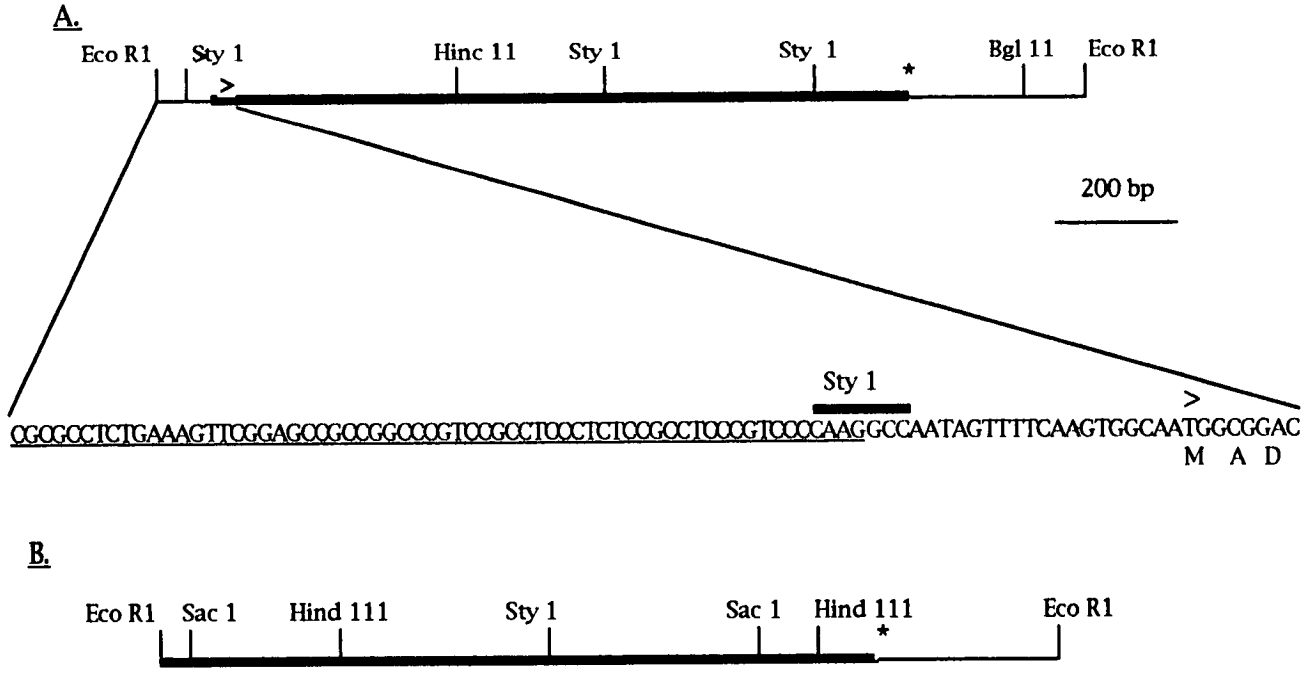


Fig.3.3. Organization and restriction maps of ANT cDNA clones from (A) maize, MANT-A, and (B) potato, pANT-1.

The open reading frame of each cDNA is indicated by the thick line. In (A) the position of the first ATG codon is marked by > and the stop codon is marked with an asterisk. The first 64 bp of MANT-A that were not thought to be homologous to the genomic clone are shown below the diagram of MANT-A, and are underlined. The Sty I restriction enzyme site at the end of the 64 bp region is shown, with an overline.

MANT-A which is compatible on ligation with the *Bam* HI restriction enzyme site in the multiple cloning sites of all the expression cassettes. This ligation destroys both sites, but generating a *Xho* II restriction enzyme site. A double digestion of this plasmid with the restriction enzymes *Bam* HI and *Bgl* II would result in the isolation of a fragment of MANT-A spanning the complete protein coding region of the cDNA, in a form that could be easily subcloned into the expression cassettes. The constructs would lack the 3' non coding 179 bp of the cDNA MANT-A.

3.7.1. Removal of the non-homologous MANT-A 5' region

As described in Section 3.6. MANT-A used in this work appeared to have a region of 64 bp at the 5' end which was not homologous to the genomic clone that had been isolated. This sequence was presumed to be a cloning artefact. To eliminate the possiblity of this non homologous sequence affecting the antisense efficiency of the constructs it was decided to remove this region from the cDNA used in the constructs. Other researchers have removed such cloning artefacts from antisense constructs they have made (e.g. Hamilton *et al.* (1990) deleted a cloning artefact of 200 bp at the 3' untranslated region from pTOM13).

Since the isolation of this region, described in the following sections was completed, further research on the upstream regions of maize ANT genes has revealed a more complicated structure than previously believed. cDNA clones to the other maize ANT gene also had a short region of 57 bp similar to the 64 bp region. When these regions were used as probes to the upstream regions of both genomic clones, they were found to be exons (Day, 1992).

3.7.2. Isolation of the 5' end of MANT-A and Insertion of a Linker

The cloning strategy designed to create the cDNA used in the expression cassettes is summarised in Fig.3.4. Sequence analysis of MANT-A revealed that there was a *Sty* I restriction enzyme site exactly at the point of divergence between the cDNA and genomic clone. This would be convenient for subcloning of the 64 bp region.

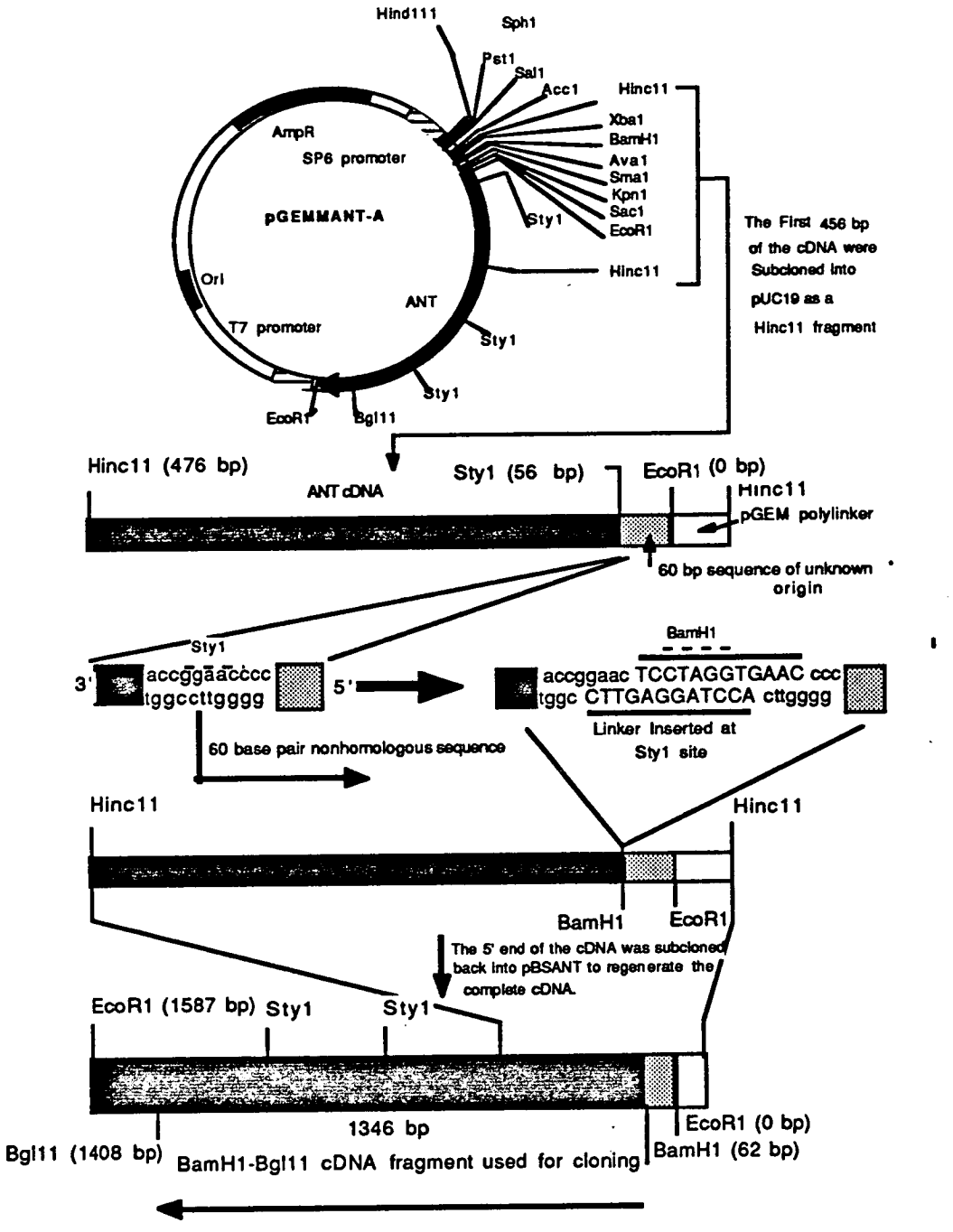


Fig.3.4. Strategy used to modify the MANT-A cDNA. The cDNA was modified so it could be cloned without the non homologous 64 bp region by isolating the first 456 bp of the cDNA as a *Hinc* II fragment in pUC 19. A linker with a *Bam* HI restriction enzyme site was inserted at the *Sty* I site that marks the point of divergence of the 64 bp. The *Hinc* II fragment was cloned back into the original plasmid to regenerate MANT-A.

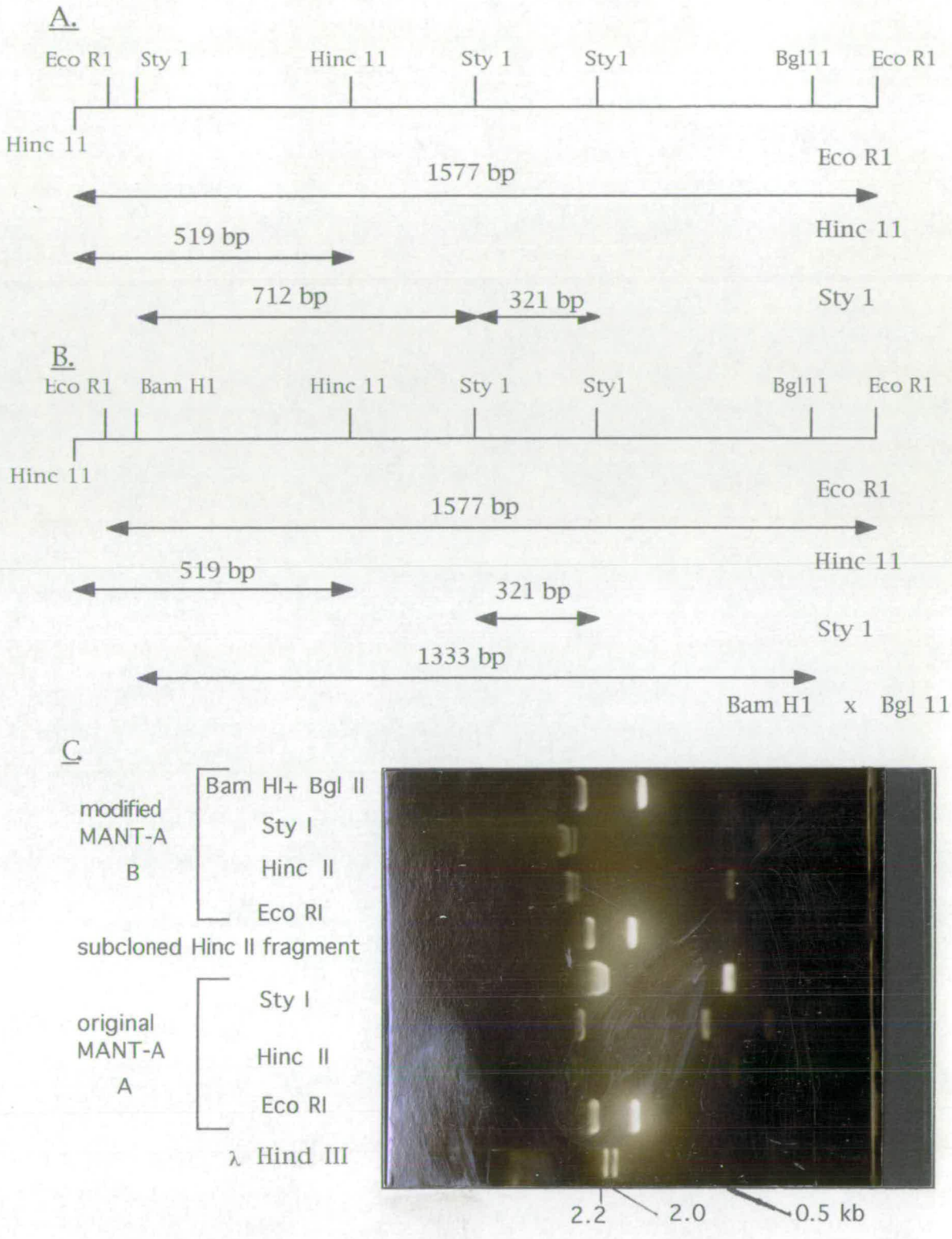


FIG.3.5. Restriction enzyme maps of (A), original MANT-A cDNA clone, showing the restriction enzyme sites and expected digestion fragments.
(B) Modified MANT-A cDNA clone, showing restriction enzyme sites, including the introduced Bam HI site in place of the Sty I site and expected digestion fragments.
(C) Restriction enzyme digests showing original pUCMANT-A and regenerated modified pUCMANT-A, with introduced Bam HI site. Markers are λ DNA cut with Hind III.

However, the presence of two other *Sty* I restriction enzyme sites in MANT-A (at 761 and 1082 bp) prevented a simple restriction digest of the complete cDNA to remove the 64 bp sequence. By isolating the 5' region of the MANT-A cDNA the 64 bp region could be separated from these two *Sty* I restriction enzyme sites. A linker designed with a *Bam* HI restriction enzyme site was ligated into the *Sty* I site at the end of the 64 bp region, and the cDNA regenerated by religating the 5' cDNA fragment into the original plasmid. Using this newly created *Bam* HI restriction enzyme site the coding region of the cDNA could be subcloned into the expression cassettes without the non homologous region.

The 5' region of the cDNA was isolated as a *Hinc* II fragment, using the *Hinc* II site in the multiple cloning site of pGEM-1 and a *Hinc* II site at 486 bp in the cDNA. This fragment, containing only the first *Sty* 1 restriction enzyme site of the cDNA, was subcloned into another pGEM-1 plasmid at the *Hinc* II restriction enzyme site in the multiple cloning site.

The cloning strategy for the insertion of the cDNA into the expression cassettes requires a *Bam* HI site at the 5' end of the cDNA. To achieve this aim a strategy was designed so that a linker containing a *Bam* HI restriction enzyme site was inserted at the *Sty* I site. Thus, when the whole cDNA was regenerated, a *Bam* HI-*Bgl* II fragment without the 64 bp, could still be isolated. This linker was designed so that it contained a *Bam* HI restriction enzyme site and also so the *Sty* I site was disrupted by the insertion of another base, without the creation of another restriction enzyme site. The resulting disruption of the *Sty* I site provided a convenient marker for the insertion of the linker. The linker was created from two seperate oligonucleotides, annealed together. This linker was ligated into the *Sty* I restriction enzyme site of an alkaline phosphatase treated plasmid and plasmids that had religated without the linker being inserted were cut by digesting the ligation mixture with *Sty* I before using the ligation mixture to transform into *E.coli*. Clones containing the linker were identified by restriction enzyme digestion and anaylsis on agarose gels. Plasmids that released a 60 bp fragment on

restriction enzyme digestion with *Bam* HI and failed to digest with *Sty* I were those where the linker had been inserted.

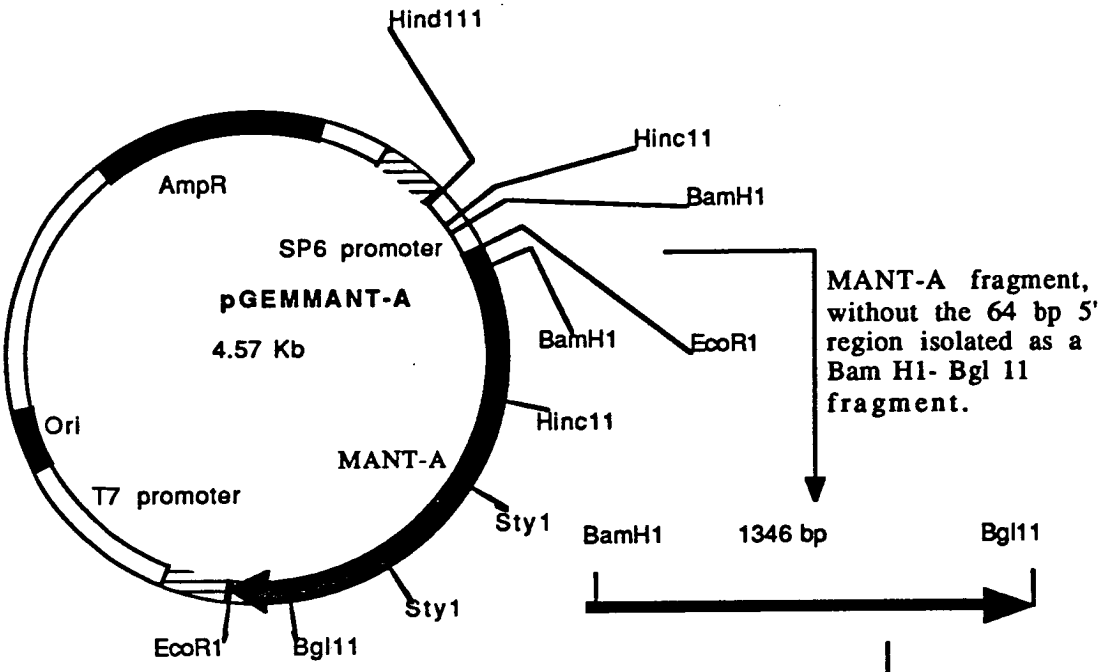
3.7.3. Regeneration and Verification of the MANT-A cDNA

Once a clone had been identified in which the linker had been inserted the *Hinc* II fragment containing the 5' region of the MANT-A cDNA was excised and isolated from an agarose gel. This *Hinc* II fragment was then religated back into the original *Hinc* II digested pUCMANT-A plasmid from which it had been isolated to regenerate the complete MANT-A cDNA. Restriction enzyme analysis was used to confirm the orientation of the fragment and that the cDNA had been regenerated. The regenerated clone compared to the original cDNA is shown in Fig.3.5.

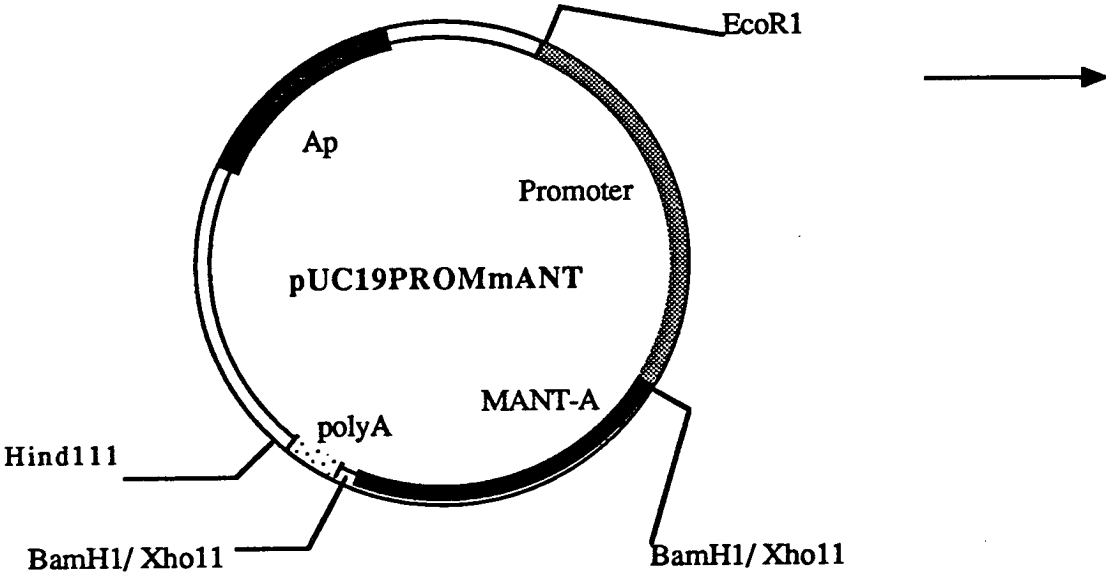
The work described in these sections was undertaken at MOGEN Int. B.V., Leiden, Holland. I am grateful to Dr. P. van der Elsen and Dr. B. Dekker for their help and advice whilst working in their laboratory.

3.8. Construction of Plant Expression Cassettes Containing MANT-A

Potato transformation was acheived by the *Agrobacterium* mediated system, so it was necessary to subclone the expression cassettes into suitable vectors. The *Agrobacterium* binary vector pMOGEN (a derivative of BIN19) was obtained from MOGEN B.V. and is a large plasmid (approximately 10kb in size) maintained in a single copy in *E.coli* cells. This makes cloning exercises difficult to perform due to the size of the plasmid and its relatively low abundance. As the expression cassettes were already available in the plasmid pUC 19 it was easier to assemble each complete expression cassette, with the maize ANT cDNA MANT-A in the sense or antisense orientation, in the pUC 19 plasmids and then subclone the completed cassette into the binary vector. This cloning strategy is summarised in Fig.3.6.



The Bam H1- Bgl 11 fragment is subcloned into a pUC 19 based plasmid containing the appropriate expression cassette at the unique Bam H1 site in the polylinker of the expression cassette.
The orientation of the plasmid was determined by restriction enzyme digestion.



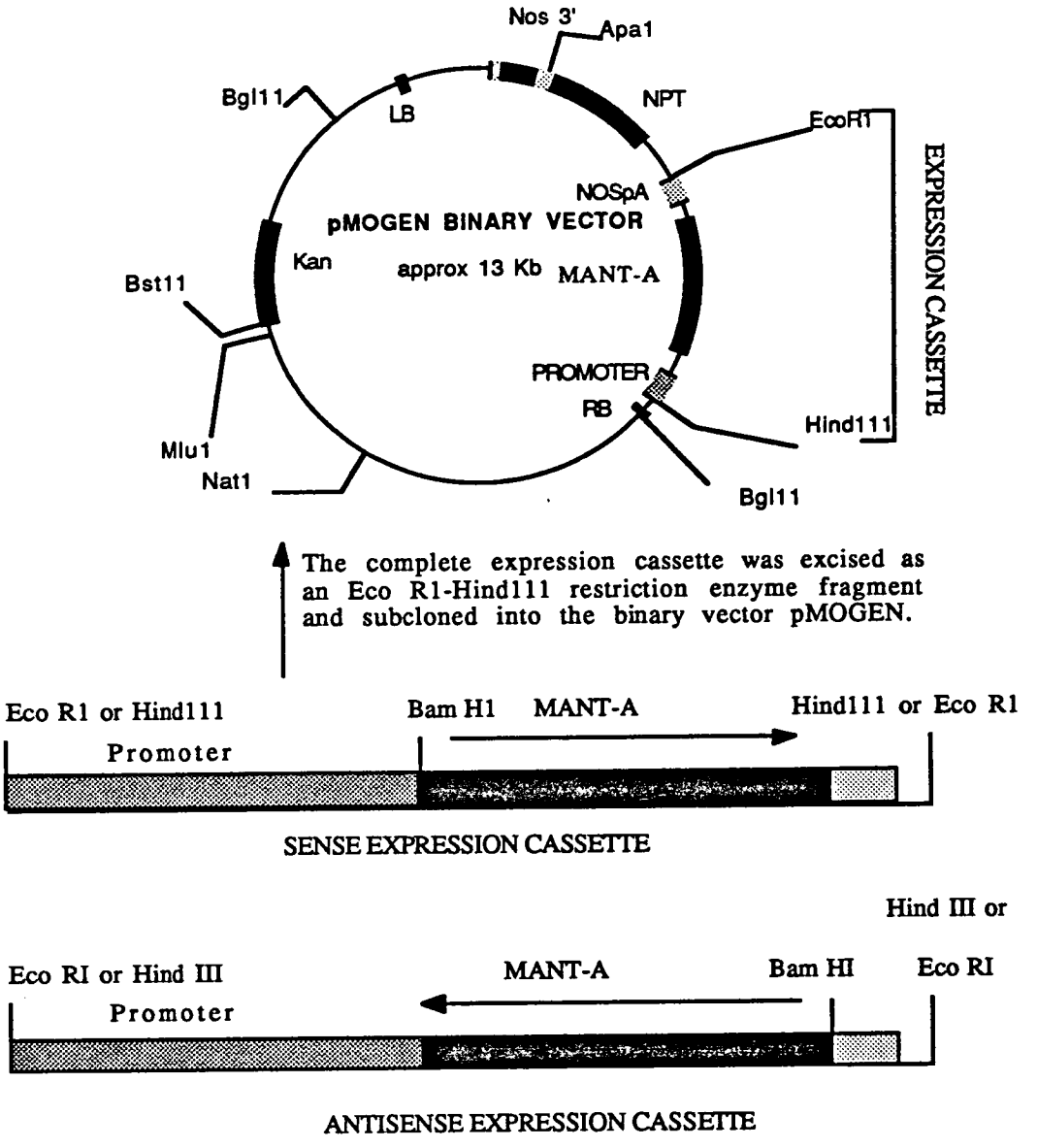


Fig.3.6. Cloning strategy to clone either sense or antisense expression cassettes with the MANT-A cDNA into the binary vector pMOGEN.

A 1346 bp *Bam* HI- *Bgl* II fragment of MANT-A was isolated and subcloned into the multiple cloning site of an expression cassette in pUC 19 at the *Bam* HI restriction enzyme site. The orientation of the fragment was determined by restriction enzyme digestion. The complete expression cassette was excised as an *Eco* RI-*Hind* III fragment and cloned into the binary vector pMOGEN in place of the original *Eco* RI-*Hind* III multiple cloning site.

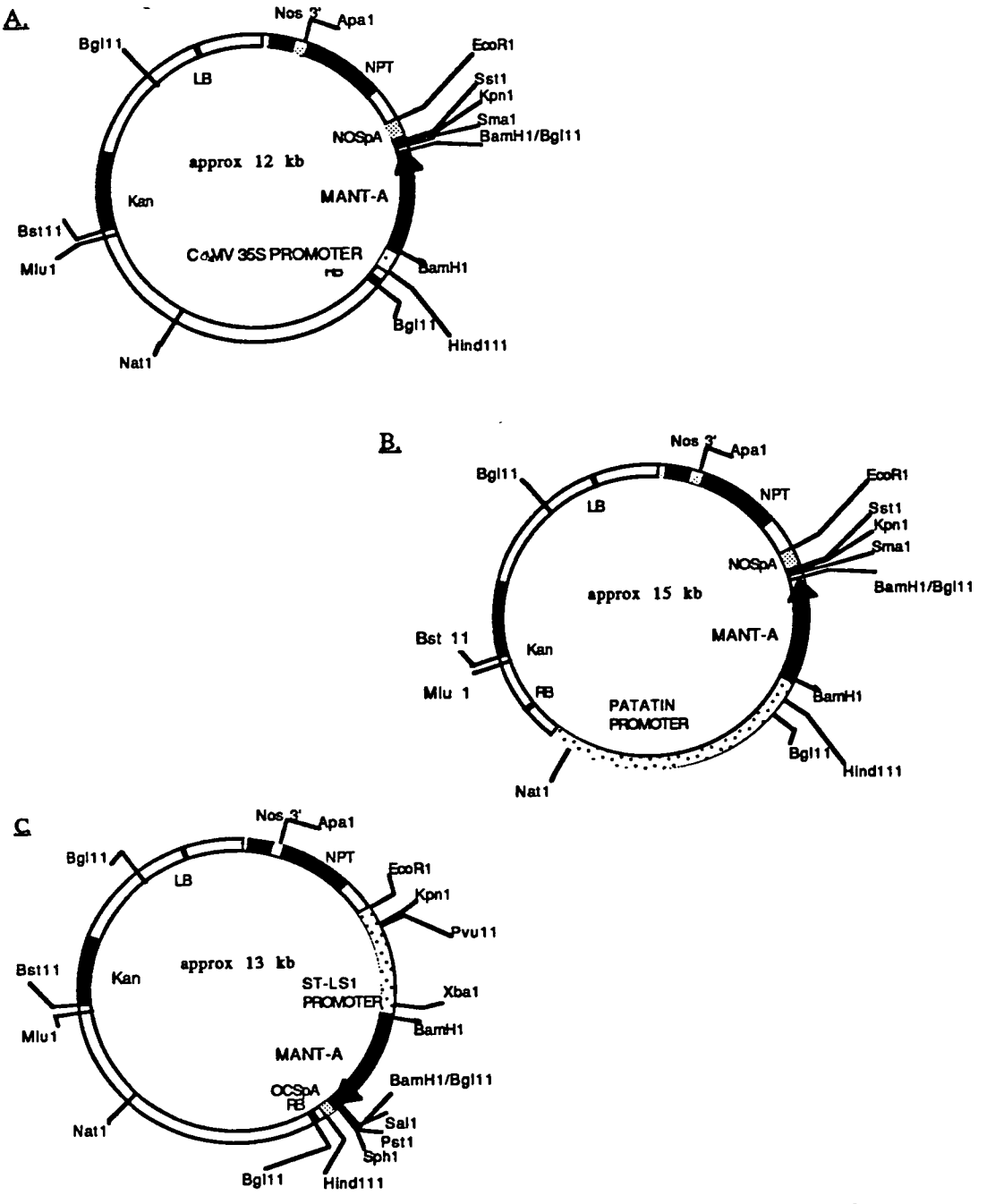


FIG.3.7. pMOGEN binary vector constructs designed to express the maize MANT-A cDNA in the sense orientation.

A. CAMV 35S MANTF, constitutive expression construct.

B. PATMANTF, tuber specific expression construct.

C. ST-LS1MANTF, leaf specific expression construct.

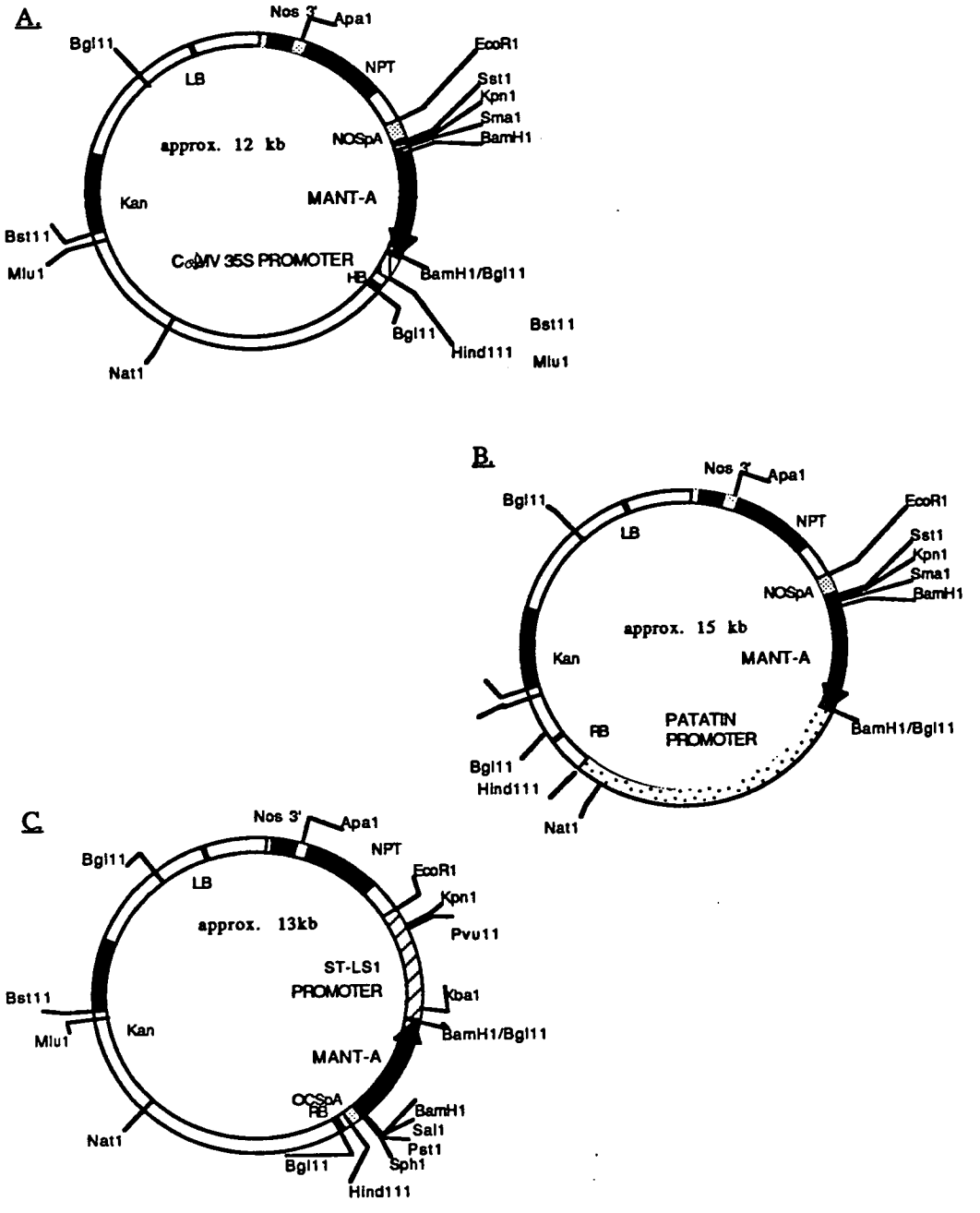


Fig. 3.8. pMOGEN binary vector constructs designed to express maize MANT-1 cDNA in the antisense orientation.

A. CAMV35SMANTR, constitutive expression construct.

B. PATMANTR, tuber specific expression construct.

C. ST-LS1MANTR, leaf specific expression construct.

3.8.1. Cloning MANT cDNA into the Expression Cassettes

The sequence modifications described performed with MANT-A enabled it to be isolated as a *Bam* HI-*Bgl* II fragment, without the 'non homologous' 5' region of the cDNA but spanning the complete coding region of the cDNA. This was ligated into the expression cassettes at the *Bam* HI site present in all of the expression cassettes. The orientation of the fragment was determined by restriction enzyme mapping using the *Bam* HI site at the 5' end of the cDNA and the *Eco* RI and *Hind* III sites at either end of the expression cassettes as reference points. The results of these digests were used to identify clones that contained the MANT-A cDNA in both the forward and the reverse orientations.

3.8.2. Cloning of Complete Expression Cassette into the Binary Vector

Once a construct containing MANT-A in the appropriate orientation was identified it was subcloned into the binary vector pMOGEN. The completed expression cassette was excised from a pUC plasmid as an *Eco* RI-*Hind* III fragment and ligated into the binary vector, which had been digested at the *Eco* RI and *Hind* III restriction enzyme sites found in the original multiple cloning site of the binary vector. The completed vector was characterised by restriction enzyme analysis to confirm the presence of the expression cassette. The completed binary vectors are illustrated in Figs.3.7. and 3.8.

3.9. Preparation of Constructs with ANT-1

The potato ANT cDNA ANT-1 was used to make a replica set of clones corresponding to the maize ANT constructs. This cDNA is also contained on an *Eco* RI fragment, but sequence analysis showed that there were no convenient restriction enzyme sites which would have allowed the use of the same strategy as used with MANT-A. The presence of two *Hind* III restriction enzyme sites in the potato ANT-1 cDNA meant that the expression cassette could not be created in a pUC intermediate vector and then be transferred into the binary vector. Thus a different cloning strategy was used to create

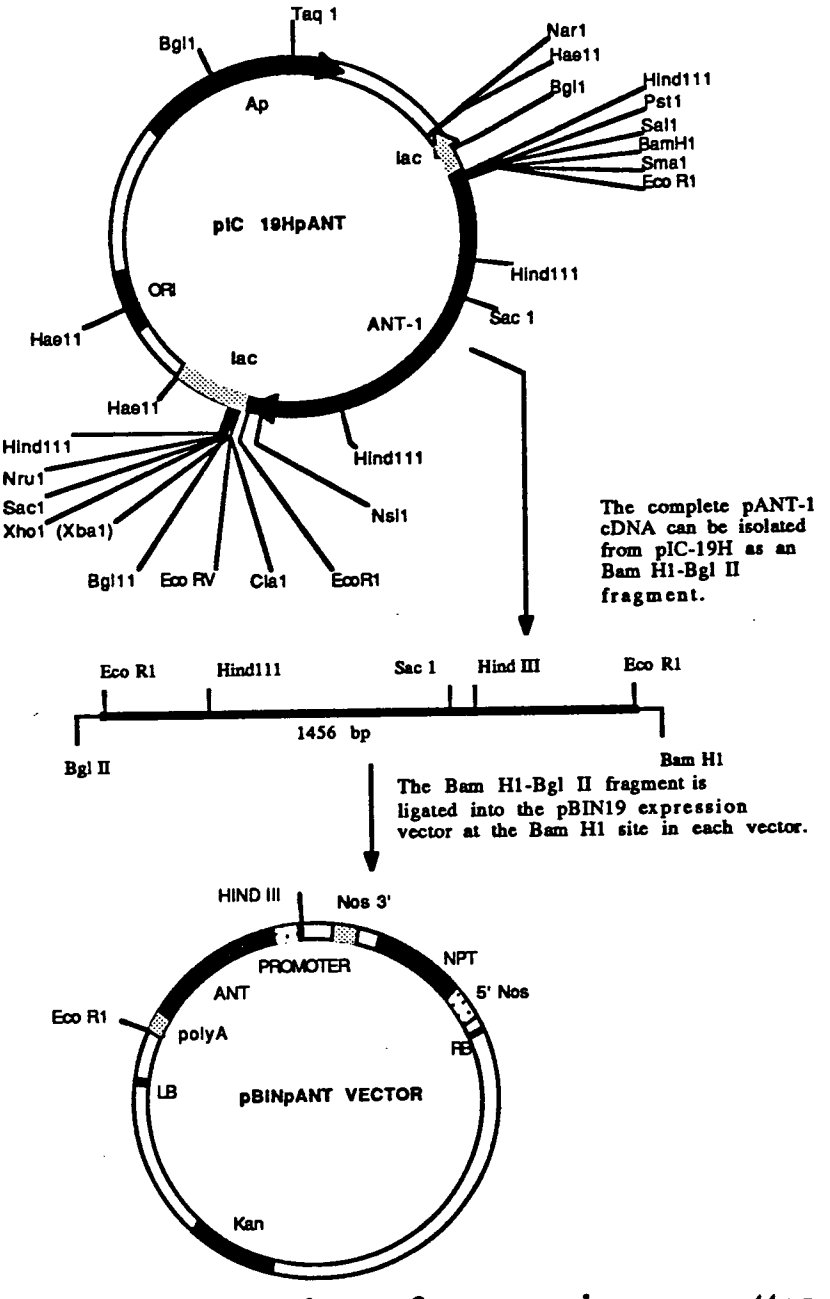


Fig.3.9. Construction of expression cassettes with the potato ANT-1 cDNA.

Restriction map of plasmid pIC19HpANT, showing the insertion of the potato ANT-1 cDNA fragment as an *Eco* RI fragment, and its excision as a *Bam* HI-*Bgl* II fragment and insertion into a binary plasmid based expression vector at the *Bam* HI restriction enzyme site in the multiple cloning site of the vector.

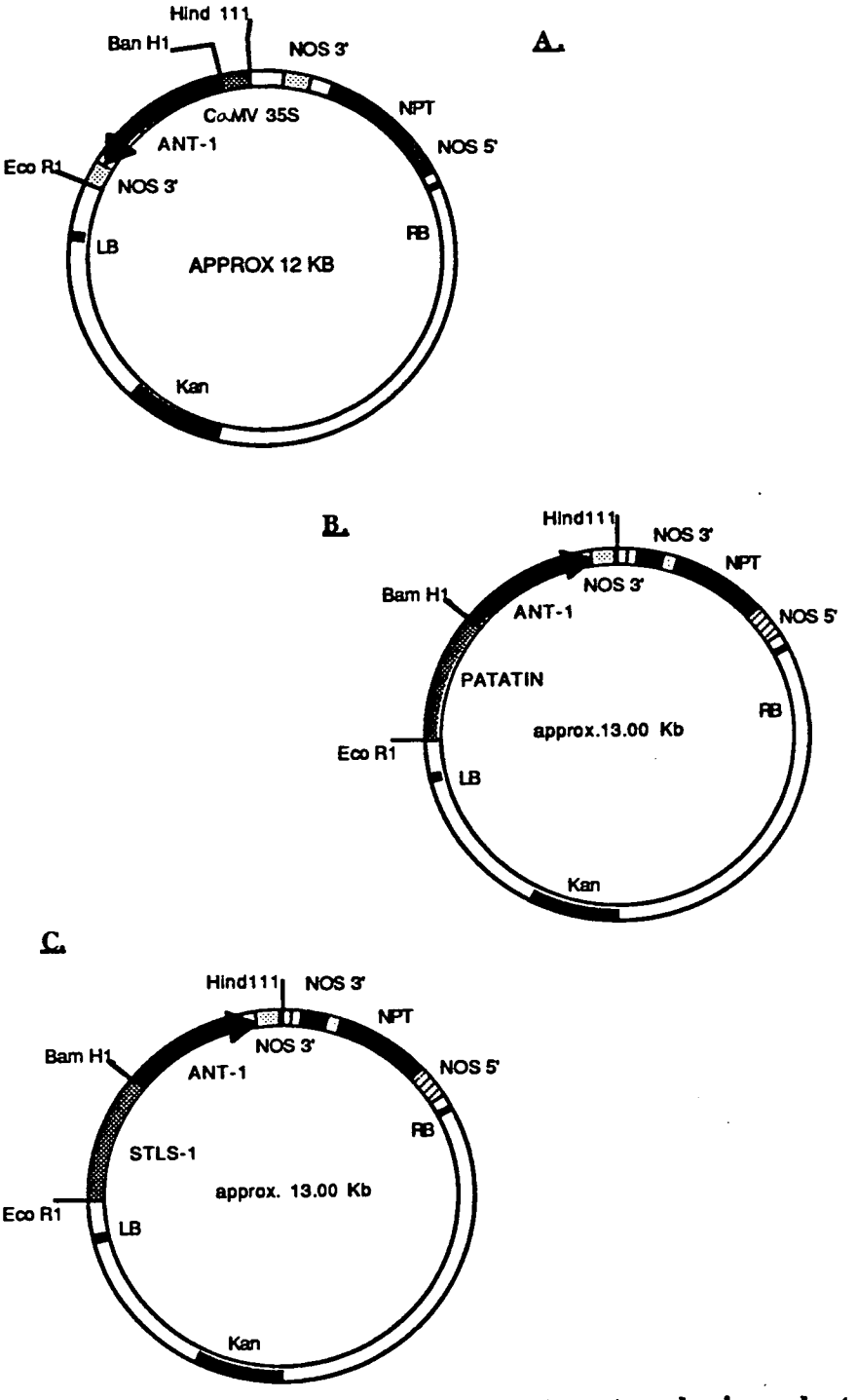


Fig. 3.10. pBIN19 binary vector constructs designed to express the potato ANT-1 cDNA in the sense orientation.

A. CAMV 35SpANTF, constitutive expression construct.

B. PATpANTF, tuber specific expression construct.

C. ST-LS1pANTF, leaf specific expression construct.

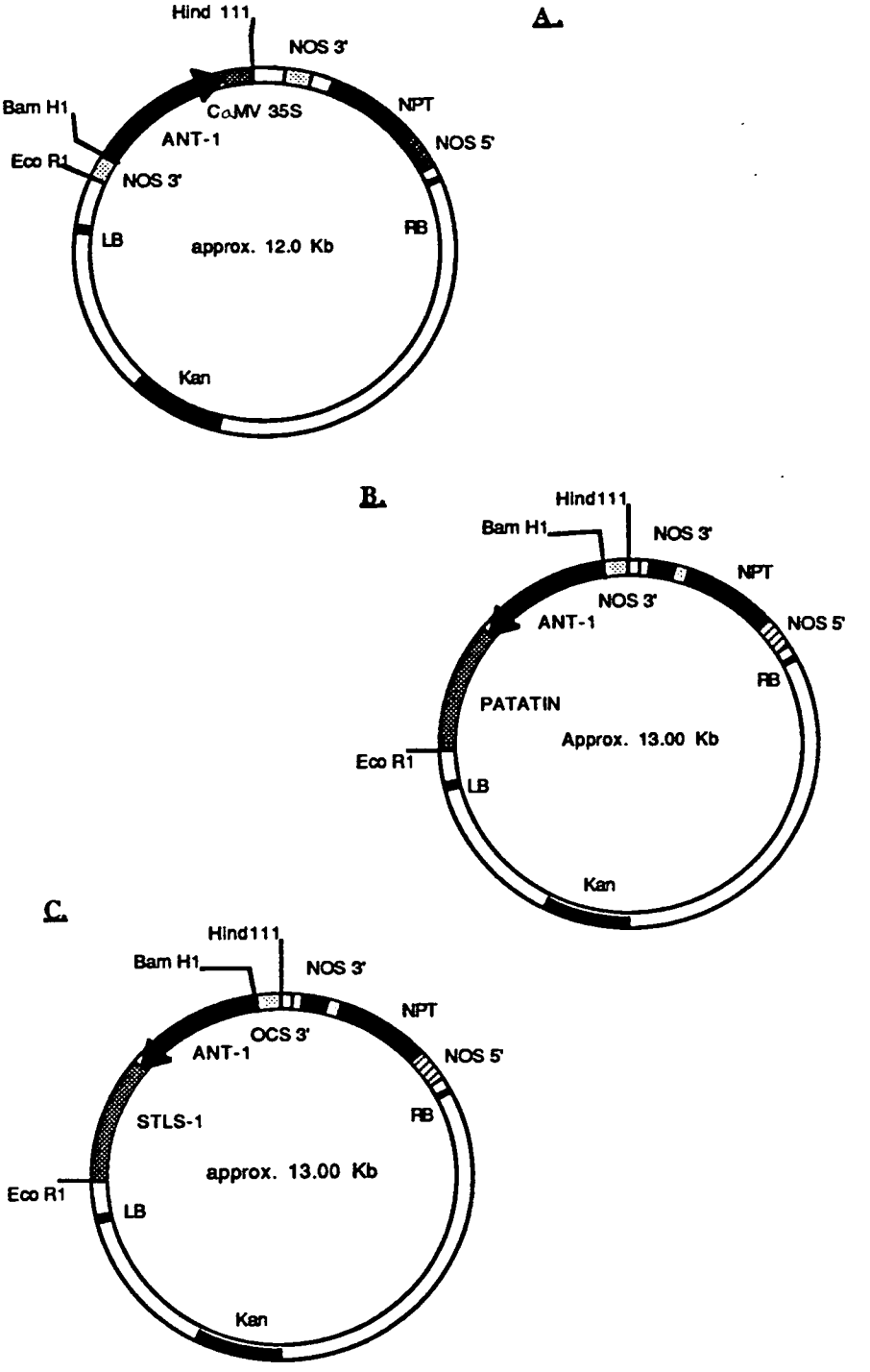


Fig. 3.11. pBIN 19 binary vector constructs designed to express the antisense potato ANT-1 cDNA.

A. CAMV 35SpANTR, constitutive expression construct.

B. PATpANTR, tuber specific expression construct.

C. ST-LS1pANTR, leaf specific expression construct.

expression cassettes with ANT-1, which involved cloning the ANT-1 cDNA directly into binary vectors with the appropriate promoter. The strategy is described in Fig.3.9.

The *Eco* RI fragment containing the ANT-1 cDNA was subcloned into the plasmid pIC19H (Marsh *et al.*, 1984). The *Eco* RI restriction enzyme site is in the middle of the multiple cloning site of this plasmid. It is flanked by a *Bam* HI restriction enzyme site on one side and a *Bgl* II restriction enzyme site on the other side of the *Eco* RI site. The orientation of the fragment was determined by restriction enzyme analysis. The plasmid is described in Fig.3.9. Using these sites the complete ANT-1 cDNA could be isolated on a *Bam* HI-*Bgl* II fragment and subcloned into the polylinker of each expression cassette at the *Bam* HI restriction enzyme site.

3.10. Creation of Binary Vector Constructs

The strategy described required that the promoters were subcloned into the binary vector before potato ANT-1 cDNA was cloned into the vector. A pBIN19 vector containing the short 1.5 kb patatin promoter described by Rocha-Sosa *et al.* (1989) was obtained (the gift of Prof. L. Willmitzer), and the CaMV 35S and ST-LS1 promoter expression cassettes were cloned into pBIN19 as *Eco* RI-*Hind* III fragments replacing the existing polylinker in the binary vector. ANT-1 was excised from pIC 19H as a *Bam* HI-*Bgl* II fragment and ligated into each binary vector at the *Bam* HI restriction enzyme site. The orientation of the insert in each cassette was determined by restriction enzyme mapping so that clones containing ANT-1 in both sense and antisense orientations were identified. The vectors are shown Figs.3.10 and 3.11.

3.11. Mobilization of Binary Vectors into *Agrobacterium tumefaciens*

The binary vectors were mobilized into *A. tumefaciens* as described in Sec.2.6.1. In order to determine that the strains isolated.harboured the binary vector and that no rearrangements had occured during the conjugation event plasmid DNA was isolated

and retransformed into *E. coli.* as described in Sec.2.6.1. Restriction enzyme digestion of plasmid DNA isolated from these cells demonstrated the same restriction enzyme pattern as the original plasmid conjugated into the *A. tumefaciens* strain.

3.12. Summary

This chapter has considered the nature of the antisense inhibition mechanism and the antisense constructs that have been used to successfully inhibit gene expression in plants. In the light of this information the choice of promoters to direct expression of the genes, both sense and antisense, used in the constructs has been described. The CαMV 35S promoter was chosen as one of the promoters as it has been extensively used in other systems and is constitutively expressed in most tissues. Two other tissue specific promoters, the patatin and the ST-LS1 promoter were also chosen to try and perturb gene expression only in specific tissues where the mitochondria are functioning in different cellular environments. The preparation of constructs to overexpress and inhibit gene expression with a heterologous gene, the maize ANT cDNA MANT-A, and with a homologous gene, the potato ANT cDNA ANT-1 has been described.

CHAPTER 4

THE ORGANISATION AND EXPRESSION OF ADENINE NUCLEOTIDE TRANSLOCATOR GENES IN POTATO

4.1. Introduction

At the outset of this project nothing was known about the organization and expression of ANT genes in potato. In maize two cDNAs had been identified and Southern blot analysis of maize nuclear DNA showed only two hybridising bands (Bathgate *et al.*, 1989). ANT genes have been studied in many other organisms, including cattle and humans, in which multiple nuclear encoded genes that are differentially expressed have been found (described in Sec.1.6.). These changes in gene expression are presumably in response to varying requirements for energy in different tissues in an organism.

This chapter will describe the genomic organisation of the genes encoding ANT in potato and the expression pattern in various tissues. The effect of environmental changes, the aging of tissue slices from potato tubers and the diurnal light/dark cycle on the expression of ANT was investigated. Metabolic control theory was used to determine the importance of ANT in controlling mitochondrial oxidative phosphorylation in isolated mitochondria from fresh and 'aged' tuber slices.

4.1.1. Potato ANT cDNA Clones

Two different classes of ANT cDNA clones have been isolated in this laboratory, corresponding to two genes, ANT-1 and ANT-2 (Winning *et al.*, 1991). One of these cDNA classes, ANT-2, corresponds to a potato ANT cDNA isolated by another laboratory (Emmermann *et al.*, 1991). Both cDNAs were isolated from mRNA libraries made from potato tubers. The coding regions of the two cDNAs are strongly conserved, but the 3' non coding regions show little sequence similarity. The complete ANT-1 cDNA was used as a non-specific probe in Southern and northern blot analysis. The 3' region of the ANT-2 cDNA was used as an ANT-2 specific probe.

4.2. ANT Genomic Organisation

4.2.1. Southern Blot Analysis of ANT in Tetraploid Potato

Ten micrograms of total potato DNA was digested with each of the restriction enzymes *Ava* II, *Bgl* II, *Eco* RI, *Eco* RV, *Kpn* I, *Pst* I and *Xba* I which were chosen as they did not cut within either of

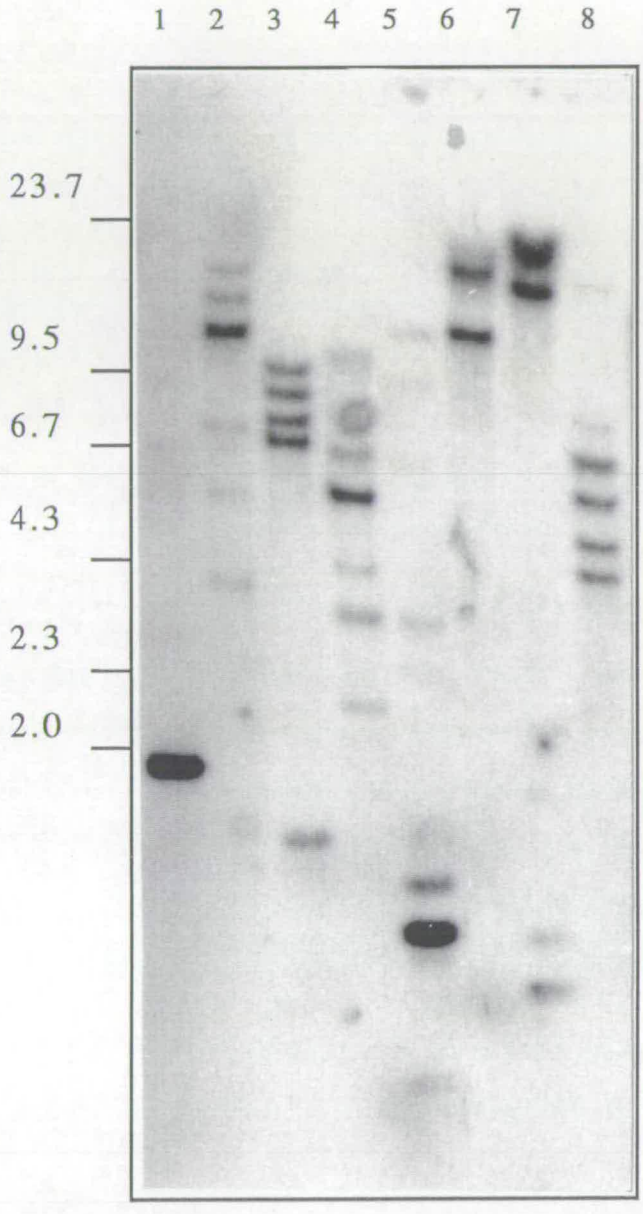


Fig.4.1. Southern blot analysis of total DNA isolated from potato.

10 μg of DNA was used per lane. The DNA was digested with the restriction enzymes (1) *Ava* II, (2) *Bgl* II, (3) *Eco* RI, (4) *Eco* RV, (5) *Hind* III, (6) *Kpn* I, (7) *Pst* I and (8) *Xba* I. The ANT cDNA ANT-1 was used as a probe. Markers were λ DNA cut with *Hind* III.

the cDNA clones that had been isolated, and *Hind* III which cuts twice in ANT-1 and ANT-2.

The Southern blot was hybridized with the ^{32}P radiolabelled cDNA probe ANT-1 (Fig.4.1.). The hybridisation pattern generated with this probe varied with different restriction enzymes between a single strongly hybridising band with a number of much less intense larger bands, as in the *Ava* II digest, and several bands of similar intensity, as in the *Eco* RI and *Xba* I digestions. In two of the samples, with *Kpn* 1 and *Pst* 1, the DNA was only partially digested.

The number of cDNAs that have been isolated from potato indicate that there are at least two genes encoding ANT (Emmerman *et al.*, 1991; Winning *et al.*, 1991), as found in maize. The information from Southern blot analysis suggests that these genes are present in multiple copies in the potato genome, as in most digestions several large bands were found to hybidize to the probe. This is in contrast to results from maize, where only two strongly hybridising bands (and a very faintly hybridizing band) have been identified by Southern blot analysis of genomic DNA (Bathgate *et al.*, 1989). These two bands appear to correspond to the two cDNAs that have been identified.

4.2.2. Southern Blot Analysis of ANT in a Dihaploid Potato

The cultivated potato is an autotetraploid: 2n=4x=48 chromosomes. The result described in the previous section illustrates the complexity of performing genetic analysis of potato genome. It is possible to produce dihaploid potato plants (2n=2x=24) and these have proved useful in the study of the complex patatin gene family (Twell and Ooms, 1988). To simplify the hybridization patterns observed on Southern blot analysis of total potato DNA, and to gain some insight into the chromosomal segregation of ANT in potato, a dihaploid potato line derived from the cultivar Desiree, DDH5, was obtained from Dr. G. Ooms, Rothamstead Experimental Station (Twell and Ooms, 1988). Genomic DNA from an autotetraploid Desiree plant and the dihaploid was digested with either *Eco* RI or *Hind* III restriction enzymes and analysed by Southern hybridization (Fig.4.2.). While

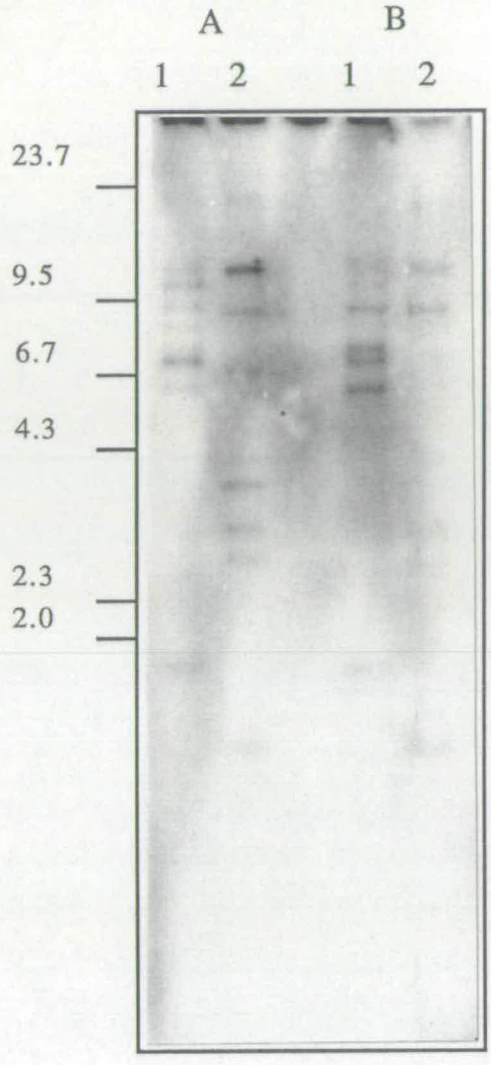


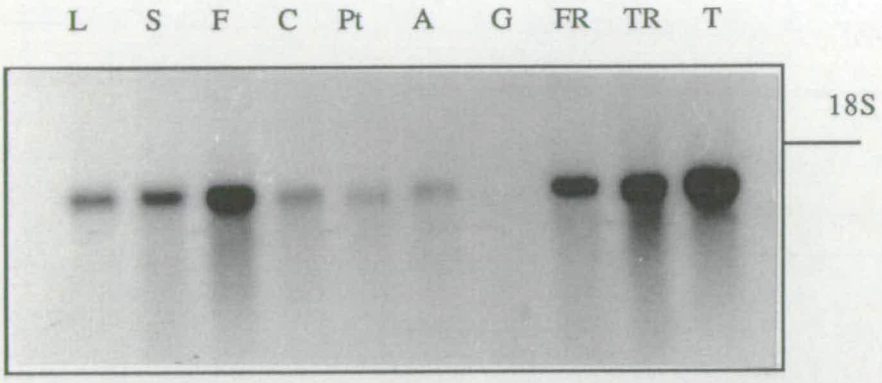
Fig.4.2. Southern blot analysis of genomic DNA isolated from (A) tetraploid Desiree potato and (B) dihaploid DDH5 potato line. 10 μg of DNA was used per lane. The DNA was digested with (1) *Eco* RI and (2) *Hind* III. The ANT cDNA ANT-1 was used as a probe. Markers were λ DNA cut with *Hind* III.

the hybridisation pattern found in the dihaploid was very similar to that in the autotetraploid Desiree, a number of hybridising bands found in the autotetraploid were missing in the dihaploid. This suggests that the ANT gene complements of each haploid genome are different. As potato is known to maintain a high level of heterozygosity such a variation may be expected. However, this is in contrast to the patatin complement which shows very little variation between the dihaploid and autotetraploid (Twell and Ooms, 1988).

4.3. Expression of ANT in Different Potato Tissues

As reviewed in Sec.1.6. the multiple ANT genes in other organisms, such as yeast and mammals, are differentially expressed in a number of tissues or in response to changing environmental conditions. Previous work in this laboratory on maize has shown that the expression of ANT mRNA varies along the length of the developing maize leaf. The transcripts were most abundant in the basal meristem, whilst being undetectable in green leaves (Bathgate *et al*, 1989). Topping (1987) also observed that the levels of an ANT mRNA decreased from the base to the apex of wheat leaves. However, another smaller ANT mRNA was observed to increase from the base to the apex of the leaf. Day (1992) found that ANT transcript steady state levels varied in a tissue specific manner in maize. Lower transcript levels were found in tissues with low metabolic activity or that were photosynthetically active, such as leaves, whilst high steady state ANT transcript levels were found in tissues which exhibited high metabolic activity and were non-photosynthetic, such as the root tip.

The steady state levels of expression of ANT mRNA were determined by northern blot analysis of total RNA isolated from various potato tissues (Fig.4.3.a.). 10 μg of total RNA from each tissue was loaded on the gel. When the complete pANT-1 cDNA was used as a probe a single transcript of approximately 1.6 kb was detected in each case. The levels of this transcript varied between the tissues, with the highest level being found in tuber and root tissues, whilst no signal was detected in the gynoecium.



L	S	F	C	Pt	A	G	FR	TR	T
1	1.5	3.4	1.0	0.6	0.7	0	2.1	3.1	5.1

Fig.4.3a. Northern blot analysis of different tissues from potato, probed with the complete ANT-1 cDNA.

10 µg of total RNA was loaded in each lane. (L) leaf, (S) stem, (F) flower bud pre anthesis, (C) calyx, (Pt) petal, (A) androecium, (G) gynoceium, (FR) fine root, (TR) tap root and (T) tuber. The position of the 18S ribosomal is marked. The ratios of the transcript levels determined by densitometry relative to the signal in the leaf track are shown below the blot.

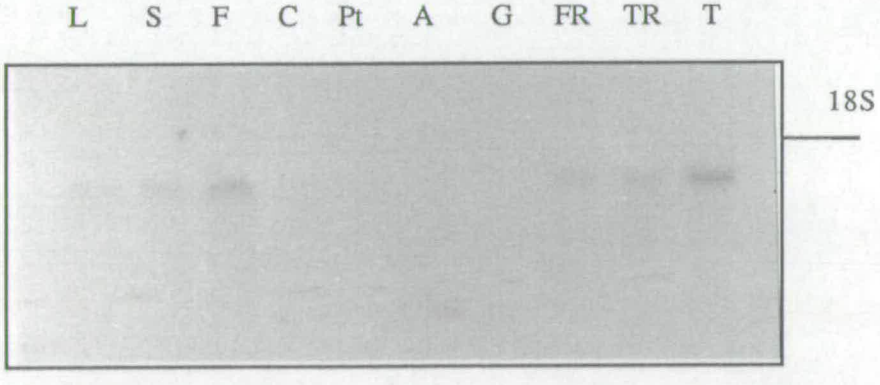


Fig.4.3b. Northern blot analysis of different tissues from potato, probed with the 3' region of pANT-2.

10 µg of total RNA was loaded in each lane. (L) leaf, (S) stem, (F) flower bud pre anthesis, (C) calyx, (Pt) petal, (A) androecium, (G) gynoceium, (FR) fine root, (TR) tap root and (T) tuber. The position of the 18S ribosomal is marked.

ANT steady state levels were highest in non-photosynthetic vegetative tissues. In tubers it was up to 5 times the level found in leaves, whilst in the tap and fine roots the level of ANT was 2 to 3 times that of the leaf. High levels of expression (3.5 times the level found in leaves) were found in flower buds sampled pre-anthesis, but not in the tissues of mature flowers where expression had decreased. Whilst the level of expression in the calyx was very similar to that found in leaves, it declined in the petals and androecium, and no signal was detected in the tissues forming the gynoecium.

These results suggest that there may be a relationship between the level of ANT expression and the photosynthetic competence or metabolic activity of a tissue, with lower levels of ANT expression reflecting a lesser reliance on mitochondrial function. In tissues such as leaves and the calyx, where chloroplasts are active, the mitochondria are not the sole suppliers of energy to the cell and so ANT expression is reduced. The high level of expression seen in flower buds harvested pre-anthesis may be due to high levels of metabolic activity in this tissue whilst cell division and development are occurring, whilst in root and tuber tissue mitochondria are the sole source of energy. These results for the steady state levels of ANT expression are similar to those observed in maize tissues by Day (1992). However, northern blot analysis by Emmerman *et al.* (1991) showed that, although they didn't quantify the levels of ANT expression found in a number of potato tissues, the expression in tuber tissue was lower than that in leaf tissue. The transcript they detected was approximately 1.7 kb, except in root tissue where a much larger transcript was found. In repeated northern blot analysis of potato tissue in this laboratory this larger transcript size was never detected.

There are multiple copies of ANT genes in many organisms, and the expression of these can vary under different conditions or tissues (Sec.1.6.). As it was possible to prepare a probe from the 3' region of one of the potato ANT cDNAs (a 344 bp HindIII–EcoRI fragment of pANT-2), this probe was used to investigate if there was any difference in the expression pattern of this gene (or genes) compared to the expression

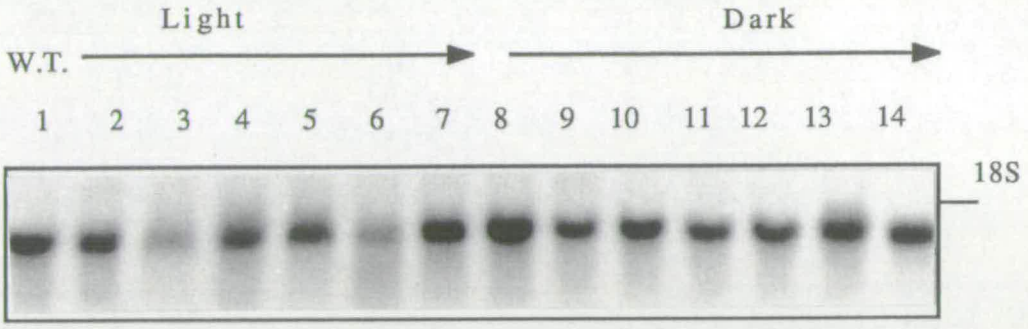
of total ANT transcripts. A single transcript of approximately 1.6 kb was detected using the 3' pANT-2 specific probe in the various tissues investigated (Fig.4.3.b.). It appeared that pANT-2 was not the major component of the signal detected with the full length non specific probe, as the signal obtained was weak. However, the pattern of expression of pANT-2 followed that detected with the non specific ANT probe. The expression of pANT-2 was greatest in tuber tissue, whilst only low levels of expression were detected in tissue from mature flower tissues.

4.4. Changes in ANT Expression in Response to Light

The northern blot analysis described above showed that ANT gene expression varied between different tissues. However, the importance of mitochondrial oxidative phosphorylation in meeting a cell's energy requirements presumably varies with environmental changes. One of the most important environmental changes that a plant faces is the regular diurnal change between light and dark. There has been much debate about the role of mitochondrial oxidative phosphorylation, whether it contributes to metabolism in tissue with chloroplasts or if the chloroplasts supply ATP via the triose phosphate shuttle. However there is evidence (e.g. Kromer *et al.*, 1988) that mitochondria do play an important role in illuminated tissue. In view of the variation in ANT expression in various plant tissues, it seemed relevant to investigate whether the expression of ANT varies during the diurnal light/dark cycle.

The steady state level of ANT expression in the leaves of a potato plant was measured by northern blot analysis of total leaf RNA, using the full length pANT-1 clone as a probe (Fig.4.4.a.). The plant was grown in a 16 hour light, 8 hour dark cycle in a temperature controlled growth chamber. Single leaves were removed at time points indicated in Fig.4.4., spanning the complete 24 hour cycle, and total leaf RNA was extracted from these samples.

A single transcript of approximately 1.6 kb was identified in all the RNA samples, but no significant change in the steady state



C	2	3	4	5	6	7	8	9	10	11	12	13	14
1	1.1	0.9	0.9	0.9	0.9	1.3	1	0.9	1	1.1	0.9	1.0	1.1

Fig.4.4a. Northern blot analysis of ANT expression in leaf tissue during a 24 hour light/dark cycle.

10 µg of total leaf RNA was loaded in each sample and the filter was probed with the complete pANT-1 cDNA. (1) RNA isolated during the day from leaf tissue of greenhouse grown potato, (2-7) Light period, (2) 10 minutes, (3) 20 minutes, (4) 40 minutes, (5) 1 hour, (6) 2 hours, (7) 16 hours, (8-14) dark period, (8) 10 minutes, (9) 20 minutes, (10) 40 minutes, (11) 1 hour, (12) 2 hours, (13) 3 hours and (14) 8 hours. The position of the 18S ribosomal RNA is indicated. The ratios of transcript level relative to the greenhouse grown plant, as measured by densiotometry, shown below the blot.

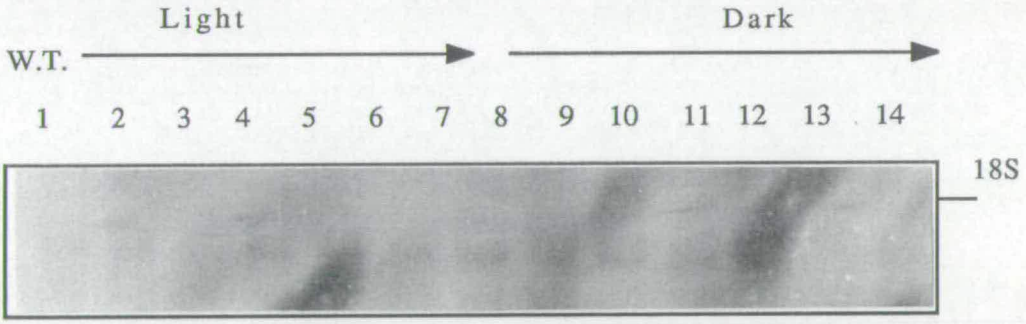


Fig.4.4b. Northern blot analysis of ANT expression in leaf tissue during a 24 hour light dark cycle.

10 µg of total leaf RNA was loaded in each sample and the filter was probed with the 3' region of pANT-2. (1) RNA isolated during the day from leaf tissue of greenhouse grown potato, (2-7) Light period, (2) 10 minutes, (3) 20 minutes, (4) 40 minutes, (5) 1 hour, (6) 2 hours, (7) 16 hours, (8-14) dark period, (8) 10 minutes, (9) 20 minutes, (10) 40 minutes, (11) 1 hour, (12) 2 hours, (13) 3 hours and (14) 8 hours. The position of the 18S ribosomal RNA is indicated.

level of ANT transcripts was found. There appeared to be no diurnal regulation of ANT expression in response to light.

When the 3' probe from pANT-2 was used to probe an identical filter, no change in the expression of pANT-2 in response to light was observed (Fig.4.4.b.). The size of the transcript detected was again approximately 1.6 kb, as found when the full length probe was used. The expression of genes recognised by pANT-2 remained at a very low level at all the time points measured, as found when RNA samples from different tissues were probed.

4.5. ANT Expression in Fresh and 'Aged' Potato Tuber Slices

In freshly isolated plant tuber mitochondria the normal cytochrome pathway is active. However mitochondria isolated from potato tuber slices that have been incubated in an aerated aqueous environment exhibit another pathway, the alternative oxidase. This pathway, which is cyanide resistant, bypasses two sites of ADP-phosphorylation (Theologies and Laties, 1978), and is associated with a partial uncoupling of electron transport from ATP synthesis. These changes in mitochondria from aged tissue are accompanied by an increase in respiration, the 'wound induced respiration rate' (Theologis and Laties, 1974). The rise in alternative oxidase capacity is correlated with changes in mitochondrial membrane composition and an increase in cytoplasmic protein synthesis (Hiser and McIntosh, 1989).

To investigate if these changes in mitochondrial activity are accompanied by changes in the expression of ANT or the accumulation of ANT protein, potato tuber slices were aged for 18 hours as described in Logemann *et al.* (1988). Mitochondria were isolated from the slices as described in Winning *et al.* (1992) and the respiration rate of the mitochondria was measured in an oxygen electrode (as described in Sec.2.9.). The respiration rate was higher in aged mitochondria compared to fresh mitochondria (Table 4.1).

Total RNA was isolated from the aged tuber slices and from freshly prepared tuber slices. Northern blot analysis of this RNA with a full length probe to ANT-1 showed that there was no

Fig.4.5. Northern blot analysis of total RNA from (A) aged tuber slices and (F) fresh tuber slices. 18 µg of RNA was loaded in the aged tuber sample and 16 µg in the fresh tuber sample. The filter was probed with the complete pANT-1 cDNA. This blot was provided by Dr. B. Winning.

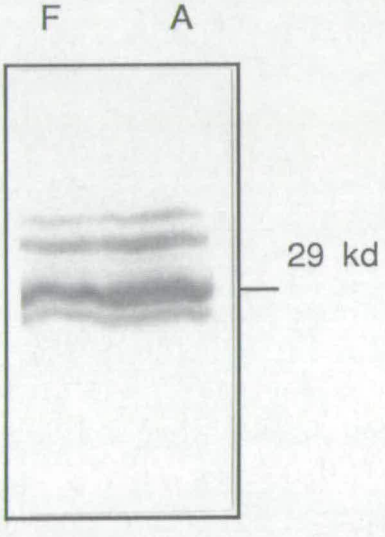


Fig.4.6. Western blot analysis of total protein samples of mitochondria. The mitochondria were isolated from (A) aged tuber slices and (B) fresh tuber slices, probed with an ANT antibody.

change in the steady state levels of ANT transcripts between the mitochondria isolated from fresh and aged tissue slices (Fig.4.5.). Immunblotting of equal amounts of mitochondria isolated from fresh and aged tissue showed no major difference in the amount of ANT protein (Fig.4.6.). These results show that the increase in respiration rate and the increase in the alternative oxidase is not associated with changes in the expression of the ANT genes or accumulation of ANT protein.

4.6. Control Coefficients of ANT

The flux control coefficient of a step in a metabolic pathway is defined as the fractional change in flux through the pathway induced by a fractional change in the enzyme under consideration (Kacser and Burns, 1973). The flux control coefficient of ANT was determined in isolated mitochondria from fresh and aged tuber slices oxidising succinate in the presence of excess ADP (state 3), using the inhibitor titration approach of Groen *et al.* (1982), as described in Sec.2.9. The specific inhibitor of ANT activity, carboxyatractyloside (CATR), was used to titrate the ANT protein (Vignais *et al.*, 1976). As CATR is an irreversible inhibitor the flux control coefficient was calculated using the following equation:

$$C_j = \frac{dJ/J}{dI/I_{max}} = \frac{dJ}{dI} \times \frac{I_{max}}{J}$$

where Cj is the flux control coefficient, J is the pathway flux and I_{max} is the minimum amount of inhibitor required to give maximum inhibition. The term dI/dJ can be determined by measuring the initial slope of the inhibitor titration curve and I_{max} can be determined by titrating the enzyme with inhibitor. Typical titration curves are shown in Fig. 4. 7.

The flux control coefficients for ANT from fresh and aged tissues are shown in Table 4.1. The control coefficients measured demonstrate that significant amounts of control (more than 40%) are located at ANT in mitochondria from both fresh and aged tuber slices under these conditions. No significant change in the level of control exerted by ANT was measured between the mitochondria isolated from fresh and aged tissue. These control coefficients are

Table 4.1. Respiration rates and control coefficients of mitochondria from fresh and aged potato tuber slices.

Uninhibited (state 3) respiration rates and the flux control coefficient of the adenine nucleotide translocator of isolated mitochondria from fresh and aged potato tuber slices as measured in the oxygen electrode in the presence of 10 mM succinate, 0.25 mM ATP and 0.1 mM ADP. The respiration rates are measured in nmol O_2 min^{-1} mg^{-1} mitochondrial protein. The flux control coefficient was determined by titration with carboxyatractyloside. The values are the mean (± SEM) of four readings of mitochondria from fresh tissue and six readings of mitochondria from aged tissue and are an average of two independent experiments.

Mitochondrial preparation	Fresh	Aged
Respiration Rate	735±126	1074±97
Control coefficient	0.45±0.08	0.51±0.10

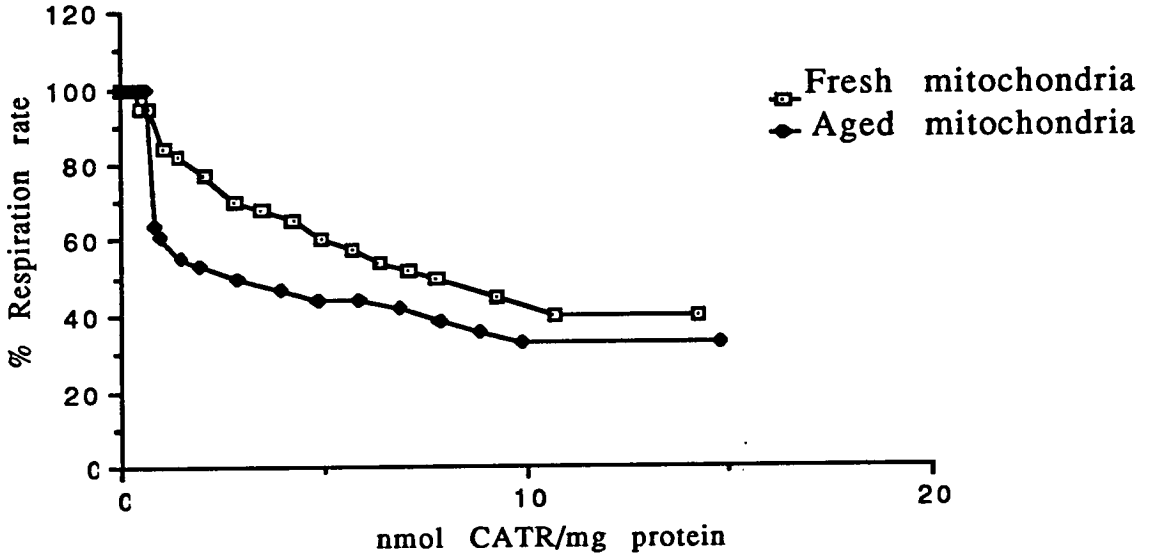


Fig.4.7. Effect of carboxyatractyloside on the state 3 rate of succinate oxidation by isolated potato mitochondria from fresh and aged tuber slices. Oxygen uptake was measured in the presence of 10 mM succinate, 0.25 mM ATP, 0.1 mM ADP and increasing amounts of carboxyatractyloside. The initial uninhibited respiration rates were 897 nmol O_2 min^{-1} mg^{-1} mitochondrial protein for mitochondria from fresh tuber slices and 1117 nmol O_2 min^{-1} mg^{-1} mitochondrial protein for mitochondria from aged tuber slices.

in the same range (0.1-0.6) as those previously determined with potato mitochondria in this laboratory (Haagsman, pers. comm.). Similar values have been obtained by workers with animal mitochondria, where ANT has a flux control coefficient of the order of 0.25 to 0.48 (e.g. Groen *et al.*, 1982; Moreno-Sanchez, 1985). Hill (1990) found that the flux control coefficient of ANT in mitochondria from cucmber cotyledons was up to 0.30 during lipid metabolism. The data presented in this section suggest that ANT does play a major role in the control of mitochondrial oxidative phosphorylation. This is in contrast to the results found by Padovan *et al.* (1989) that ANT exerted no influence on respiratory flux in mitochondria from turnip root under state 3 conditions.

4.7. Summary

The results presented in this chapter show that the genomic organisation of ANT in potato is more complicated than that found in maize, with multiple copies of ANT genes being present in the potato genome. The organization of ANT in a dihaploid potato was not significantly simpler than that found in the autotetraploid Desiree. The steady state level of ANT mRNA appears to vary between various tissues and organs of the potato plant. This variation suggests that the levels of expression reflect the energy requirements of the tissue and its photosynthetic state. The steady state level of expression of one of the ANT genes, pANT-2 appeared to be much lower than the overall ANT expression. No change in ANT mRNA was found during a diurnal light/dark cycle. When tuber slices were aged no change in the expression of ANT or the level of ANT protein was detected. ANT exhibited a significant control coefficient in mitochondria isolated from fresh tubers, indicating that it can play a role in controlling mitochondrial oxidative phosphorylation. This control coefficient did not alter in mitochondria from tuber tissue that was aged for 18 hours.

CHAPTER 5
POTATO TRANSFORMATION

5.1. Introduction

The potato has proved to be amenable to *Agrobacterium* mediated genetic transformation. Efficient and reproducible transformation has been claimed by a number of different groups since the first report of potato transformation with *Agrobacterium* (Ooms *et al.*, 1983). *A tumefaciens* has been the mostly widely used bacterium to mediate potato transformation, although *A. rhizogenes* has also proved to be a successful transformation agent. In this chapter the success of the different protocols used to transform potato will be considered and the effects of using alternative growth conditions and tissues on the efficiency of potato transformation will be described.

5.2. The Development of Potato Transformation

There are several reviews of plant transformation in general but potato transformation, reflecting the importance of potato as a crop, has been specifically reviewed recently (Mitten *et al.*, 1990; Vayda *et al.*, 1992). There have been more than fifteen separate reports of either stable or transient potato transformation. Table 5.1. lists many of the recently published protocols. Whilst potato has proved amenable to a variety of transformation techniques, such as transient gene expression in electroporated protoplasts (Jones *et al.*, 1989) and *A.rhizogenes* mediated transformation (e.g. Ooms *et al.*, 1986; Hanisch ten Cate *et al.*, 1987), the majority of reports concern *A. tumefaciens* mediated transformation.

In all *Agrobacterium*-mediated transformation systems there are two common steps; a co-cultivation stage during which *Agrobacterium* can infect the plant tissue and transfer the plasmid carrying the transgene to the plant cell, followed by a tissue culture step during which transformed shoots are regenerated and selected. Whilst these are common stages, the conditions used at each step can vary greatly between different protocols, especially at the plant regeneration step. In potato these conditions have often proved to be specific to particular cultivars.

Potato		Agrobacterium		Plant regeneration	Reference
Cultivar	Tissue	Strain	Plasmid		
Maris Piper	shoots	T37, LBA4060, LBA1501	?	yes	Ooms *et al.*, 1983
Russet Burbank, ADX262-9	leaf/stem	?	pGA472	yes	An *et al.*,1986
NDD-277-2	leaf callus	LBA404	pARC 8	yes	Shahin and Simpson, 1986
not stated	tuber	C58C1	pMPK110	yes	Stockhaus *et al.*, 1986
Maris Piper, Desiree	shoots	LBA1834/LBA4404	pBIN6	yes	Ooms *et al.*, 1987
Desiree	stem	LBA4404	pPOT100	yes	Twell and Ooms, 1987
Berolina, Bintje, Desiree, Russet Burbank	leaf	C58C1	pGSRFR760A/780A	yes	DeBlock, 1988
H81.1506/60	leaf	GV3850/GGV3850HPT	pGV3850::pKU2/pGV3850::pKU3/pGV3850HPT::pKU3	yes	Knapp *et al.*, 1988
Desiree, Pentland Dell, Golden Wonder, Maris Piper, Maris Bard	tuber	LBA4404	pBIN6	yes	Sheerman and Bevan, 1988
Desiree, Bentje	tuber	LBA4404	pBI121	yes	Stiekema *et al.*, 1988
Desiree	leaf	LBA4404	pGA492	yes	Tavassa *et al.*, 1988
Bintje, Desiree, Escort	tuber	GV2260	pPVX102	yes	Hoekema *et al*, 1989
Lemhi Russet/Russet Burbank	tuber	PC2760	pBI121	yes	Ishida *et al.*, 1989
MQ1	callus	A281	pTOK119	no	Komari, 1989
Desiree	leaf	LBA4404/GV2260	pBI101	yes	Rocha-Sosa *et al.*, 1989
Mn 79.7322	leaf/stem	LBA4404	pVU1011	yes	Visser *et al.*, 1989
FL1607, Desiree, Russet Burbank, Superior	leaf	LBA4404	pBI121/pPS20A-G	yes	Wenzler *et al.*, 1989

Table 5.1. Published transformation protocols for potato using *Agrobacterium tumefaciens*. Cultivar, the potato cultivars used in the transformation; Explant, the tissue types used for transformation; Plasmid is either the binary T-DNA plasmid or the cointegrative plasmid identified as pXX::pYY; Regeneration, regeneration of transformed plants; ?, unknown.

5.2.1. Potato Transformation Systems

The first reported potato transformation was by Ooms *et al.* (1983), infecting potato shoots with an oncogenic strain of *A. tumefaciens*. Infected shoots developed galls, from which shoots could be excised. Shoots that did not root were found to produce opines, suggesting that they contained the T-DNA sequence encoding the genes for opine synthesis.

An *et al.* (1986) were the first to use a binary vector system with *A. tumefaciens* to transform potato (the tetraploid cultivar Russet Burbank, and a diploid line, ADX262-9). This protocol was also used to transform tobacco, tomato and *Arabidopsis thaliana*. Evidence of transformation was provided by assaying for neomycin phosphotransferase activity encoded by a neomycin phosphotransferase II (NPT II) gene introduced in the T-DNA transferred from *A. tumefaciens* to the plant genome. Leaf or stem segments were co-cultivated with *A. tumefaciens* for two days. These explants were washed and placed on selective medium to promote callus formation, although the stem sections proved less amenable to regeneration than leaf pieces. It was possible to regenerate transgenic plants from such callus, but this took up to five months from the initial co-cultivation and at a low efficiency. Stockhaus *et al.* (1986) reported the transformation of potato tuber discs, but the details of the protocol were not published. Shahin and Simpson (1986) were able to transform callus derived from potato leaf tissue and recover transgenic plants. Ooms *et al.* (1987) developed a protocol that required the use of mixed disarmed and shoot inducing strains of *Agrobacterium* to obtain a small number of transgenic plants after 8 to 16 weeks.

5.2.2. Potato Tuber Transformation

The requirement for a more efficient and rapid method of potato transformation lead to the adaptation of the protocol developed for tobacco leaf discs by Horsch *et al.* (1985). Sheerman and Bevan (1988) and Stiekema *et al.* (1988) were able to successfully transform potato tuber discs with such a protocol.

In both these protocols, after the initial co-cultivation step with *A. tumefaciens*, tuber discs were placed on media containing a feeder layer of cells, before being transferred to the selection media. Stiekema *et al.* were able to transform both Bintje and Desiree potato cultivars, whereas Sheerman and Bevan attempted to transform four different cultivars. They found cultivar specific differences in the ability to regenerate transgenic plants. This method proved rapid, with shoots being obtained within four to six weeks, without having to go through an extensive callus stage. Sheerman and Bevan found that tuber discs were consistently superior to either leaf discs or stem segments in their transformation ability. However, they also found that the age of the tubers used and the conditions in which they had been stored affected the transformation efficiency: young tubers, less than 6 months old and stored at 4 °C were most easily transformed. Ishida *et al* (1989) have demonstrated that it is possible to transform minitubers grown *in vitro*. Untransformed shoots emerged from the 'eyes' of these tubers within one week. If these shoots were removed secondary shoots emerged from the eyes. Approximately 5 % of these secondary shoots were found to be transgenic when removed after four months. Hoekema *et al.* (1989) further optimised the protocol developed by Sheerman and Bevan (1988) for three potato cultivars, Bintje, Desiree and Escort.

5.2.3. Transformation of Other Potato Tissues

Other potato tissues, including leaf discs and stem sections, have been successfully transformed. These protocols are reported to be very efficient and reproducible. Twell and Ooms (1987) were able to regenerate transgenic plants from Desiree stem sections infected at the apical end with *A. tumefaciens* from a bacterial loop.

The tobacco leaf disc transformation protocol developed by Horsch *et al.* (1985) to infect tobacco leaves has been successfully used on potato leaf explants. De Block (1988) reported the transformation of potato leaves cut at the base and floated in liquid medium for 2 days before infection. After infection with *A.*

tumefaciens in this liquid medium the explants were transferred to a solid glucose based medium. The explants were incubated on this medium for one week before being transferred to fresh medium for two weeks and then onto a medium with antibiotic selection. Calli that developed were transferred to a shoot induction medium. The transformation efficiency of this protocol was independent of the four cultivars used, but including the cytokinin zeatin in the regeneration medium improved the efficiency. The addition of silver to the medium dramatically increased the ability of the cultivar Russet Burbank to produce calli. Knapp *et al.* (1988) were able to use a leaf disc transformation protocol with success to regenerate transgenic potato plants. Tavazza *et al.* (1988) transformed leaf discs from the potato cultivar Desiree using feeder plates to accelerate shoot formation, obtaining transgenic shoots within four weeks.

Rocha-Sosa *et al.* (1989) transformed leaf discs of Desiree, without using feeder plates. In this protocol the co-cultivation was performed in darkness, whilst callus and shoot formation was promoted on medium containing glucose, not sucrose as the carbon source.

The transformation protocol for leaf pieces developed by Wenzler *et al.* (1989) required the pretreatment of the tissue before co-cultivation, by floating the explants in liquid medium for four days. A pretreatment stage was also used by Visser *et al.* (1989) for the transformation of both stem and leaf pieces. In this method the tissue pieces were pretreated overnight by floating in liquid medium before the *A. tumefaciens* infection stage.

5.3. Transformation Experiments With Potato Tubers

There have been several reports that the efficiency of potato transformation varies between potato cultivars (e.g. Sheerman and Bevan, 1986). As a consequence one cultivar, Desiree, was chosen for these experiments. This cultivar had been used with success in many of the transformation protocols described in the previous section. Pathogen tested, micropropagated Desiree

plantlets and field grown tubers were obtained from the Department of Agriculture and Fisheries for Scotland, East Craigs.

Tuber disc transformations were performed using a protocol developed at MOGEN Int N.V. This protocol, described in Sec.2.6.2., is a modification of that described by Hoekema *et al.* (1989). After cocultivation with the *A. tumefaciens* strain harbouring the construct to be introduced, the tuber discs are transferred to MS30-R3 regeneration medium with a selective antibiotic (100 mg/l kanamycin) and a bacteriostatic antibiotic (500 mg/l carbenicillin).

30 to 40 tuber discs were cocultivated with each *A. tumefaciens* strain harbouring the vector, initially with only the heterologous sense and antisense maize ANT constructs described in Chap. 3, and then also with the homologous potato sense and antisense ANT constructs. Six independent transformation experiments were attempted using this protocol, but they were not successful in regenerating transgenic plants. In parallel control experiments, uninoculated tuber discs treated identically to inoculated discs produced multiple shoots per disc on MS30-R3 regeneration medium without kanamycin selection. These shoots started to develop three weeks after the discs were transferred onto the MS30-R3 regeneration medium. Shoots excised from each tuber disc failed to root on rooting medium with kanamycin selection, but did when placed on rooting medium without antibiotic selection. Uninoculated tuber discs placed on regeneration medium with kanamycin selection failed to produce shoots. Transformations with *A. tumefaciens* harbouring a control binary vector plasmid, pBIN19, without an expression cassette introduced into the multiple cloning site were also unsuccessful. As observed with the uninoculated discs, multiple shoots per tuber disc inoculated with pBIN19 were formed on MS30-R3 regeneration medium without the antibiotic selection, but none were observed on regeneration medium with kanamycin selection. Those shoots that were regenerated failed to produce roots when transferred to rooting medium with kanamycin selection. Similar results were obtained when tuber discs were incubated with *A. tumefaciens* harbouring both the binary vector sense and

antisense ANT constructs. When placed on MS30-R3 regeneration medium without selection these discs produced multiple shoots within three to four weeks of the initial incubation. However shoots excised from these discs failed to root when tranferred on rooting medium with kanamycin selection.

Whilst no shoots were regenerated with the control plasmid pBIN19, shoots did emerge on some of the tuber discs innoculated with *A. tumefaciens* harbouring constructs with ANT constructs. These shoots were observed on discs innoculated with ANT constructs in either the sense or antisense orientation. The number of shoots produced on the inoculated tuber discs was very low, despite being maintained for more than two months on MS30-R3 shoot regeneration medium, with subculturing onto fresh medium every three weeks. Most tuber discs did not produce shoots, and the number of shoots produced varied between each transformation experiment. However, all the shoots produced in these transformation experiments failed to root on selective rooting medium with 100 mg/l kanamycin. The response of the tuber discs on MS30-R3 regeneration medium was not consistent (as seen in Fig.5.1.). In most of the experiments the tuber discs either showed no visible changes when placed on the regeneration medium, or they developed numerous small bumps over their surface, but did not produce shoots. Eventually these discs discoloured and died. In some transformation experiments the response of the tuber discs on the regeneration medium was very different, as these tuber discs produced a friable white callus on their surface. This callus did not grow and eventually senesced, without producing shoots.

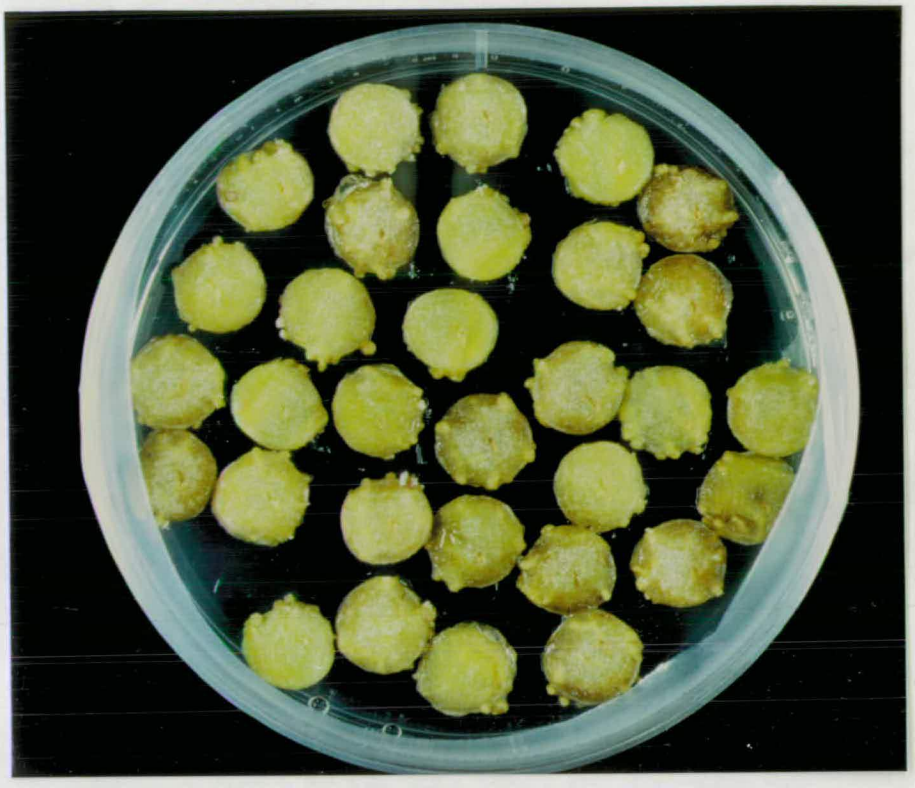
Untransformed shoots ('escapes') either did not root on selective rooting medium, or produced short, stubby, morphologically distinct roots that remained at the surface of the rooting medium (as illustrated in Fig. 5.2.). These shoots were able to survive for up to two months on medium with kanamycin, but eventually bleached and died (as reported by Stiekema *et al.* (1988)).

Fig. 5.1. Different responses of tuber discs on MS30-R3 regeneration medium with 100 mg/l kanamycin after being in tissue culture for eight weeks.

A. The 'typical' response observed of tuber discs. The tuber discs retain their shape, changing colour to a faint green. When maintained on the medium for longer the discs still retain their shape, but change colour becoming black. As can be seen most of the discs have formed 'bumps' on their surface, but these have not developed any further.

B. The 'aberrant' response observed with some tuber dics in which a callus is seen to form on the tuber discs. This callus growth did not continue beyond the stage seen in this photograph.

A.



B.

Fig.5.2. Rooting response of (A.) a non-transgenic potato shoot and (B.) a transgenic potato shoot on rooting medium with 50 mg/l kanamycin antibiotic. These shoots were regenerated from leaf or stem explants as described in Sec.5.6. The untransformed 'escape' has produced only short, thick roots, which do not branch extensively, in the surface of the rooting medium. In contrast the roots of the transgenic shoot are more extensive, growing throughout the medium.

A.

B.

5.4. Transformations With Tubers of Different Ages

The initial tuber transformation experiments described in the previous section had not regenerated any transgenic plants. Sheerman and Bevan (1988) reported that the age of the tubers was a, "Critical parameter," on the efficiency of transformation . They found that the most consistent successful results were obtained with tubers less than six months old which had been stored at 4 to 6 °C. The transformations described in the previous section were performed with tubers that were 4 to 6 months old and had been stored at 4 °C. However, to determine if tuber age or condition was affecting transformation efficiency fresh tubers were used in the next transformation experiments. These tubers were harvested on the same day as they were required from either glasshouse or field grown plants. The same transformation protocol was followed, with 20 to 40 tuber discs per ANT construct being inoculated with *A. tumefaciens*.

Both uninnoculated and innoculated tuber discs placed on MS30-R3 regeneration medium without antibiotic selection produced numerous shoots, as observed when old tubers were used. These multiple shoots started to appear on the discs within three weeks. When shoots were transferred to rooting medium with kanamycin selection they failed to root. Tuber discs that were innoculated with *A. tumefaciens* harbouring the ANT constructs and incubated on regeneration medium with antibiotic selection showed no improvement in transformation efficiency. Whilst shoots did emerge from the discs the number that was produced was not improved and those that did develop failed to root when transferred to rooting medium with selection. The use of fresh tubers in place of older stored tubers had no effect on the transformation efficiency achieved with this protocol.

5.5. Increasing the Efficiency of Transformation

Methods have been developed to enhance transformation efficiency with *A. tumefaciens* that rely on the effects of phenolic compounds secreted by wounded plant cells on *A. tumefaciens*. The transfer of T-DNA from the bacterium to the plant is mediated by virulence genes, which form the *vir* region of the Ti

plasmid and *chv* genes on the bacterial chromosome. Transcription of the *vir* region is induced by various phenolic compounds, such as acetosyringone and alpha-hydroxy-syringone, which are released for example, by wounded tobacco cells (Stachel *et al.*, 1985). Whilst species such as tobacco produce enough quantities of these signal molecules to ensure that the *vir* genes are induced and T-DNA transfer occurs, other species are not so amenable as they do not produce such high quantities of phenolic compounds. One method that has been used to provide these phenolic compounds is to use a feeder layer of cells (Sheerman and Bevan, 1988). However, the addition of acetosyringone to the bacterial culture or co-cultivation medium has been reported to beneficially affect transformation efficiency (Sheikholeslam and Weeks, 1987).

200 μM acetosyringone was added at inoculation to the *A. tumefaciens* overnight cultures used in the cocultivation. Otherwise the same tuber transformation protocol as described previously was followed. However, the transformation efficiency achieved with these *A. tumefaciens* cultures was not increased compared to the experiments in which acetosyringone was not used. No difference was observed in the number of shoots produced on the tuber discs, and as found in the previous experiments those shoots that were produced failed to root on rooting medium with selection.

5.6. Transformation of Alternative Potato Tissues

As tuber transformation had proved unreliable an alternative transformation protocol, using *in vitro* grown potato plantlets, was used to generate transgenic plants. This protocol, in use at S.C.R.I., is derived from the leaf disc method described by Knapp *et al.* (1985), using both leaf and stem explants. It is described in detail in Sec. 2.6.3.1. and has proved successful with the cultivar Desiree. The use of *in vitro* grown plant material avoids the initial tissue sterilization stage of the tuber transformation protocol. The explants were prepared from micropropagated Desiree plants grown for three to five weeks as described in Sec. 2.3.1. The leaves were wounded by slicing just

above the base as this region is reported to regenerate most easily, and extensive wounding lowers the transformation efficiency (De Block, 1988).

Initial transformations with this protocol, using *A. tumefaciens* strains harbouring ANT binary vector constructs with both the maize and potato ANT cDNAs, were not successful in regenerating transgenic plants. Uninoculated control explants, placed on callus inducing LS-R1 medium without antibiotic selection, rapidly regenerated callus on their cut surfaces and multiple shoots were produced within three weeks of infection when transferred to LS-R2 regeneration medium without antibiotic selection. Uninoculated explants placed on medium with antibiotic selection (100 mg/l kanamycin) produced much less vigorous callus growth and shoots were not observed. These calli rapidly changed colour, from green to brown and growth was inhibited. When these explants were transferred to shoot regeneration medium without antibiotic selection no shoots were produced. Explants inoculated with *A. tumefaciens*, harbouring the binary vectors, but regenerated on medium without antibiotic selection behaved as the uninoculated controls, regenerating multiple shoots on the LS-R2 shoot regeneration medium, having first produced callus. Shoots excised from these explants failed to root on rooting medium with selection. Those explants placed on regeneration medium with kanamycin antibiotic selection also produced calli. However, the callus response of these explants was slower than that of the control explants not under antibiotic selection, being delayed by up to two weeks. The extent of callus growth was reduced and most of the callus appeared to stop growing, discoloured and died after several rounds of tissue culture. Whilst shoots did emerge on some explants when they were transferred to LS-R2 shoot regeneration medium, these shoots were mostly from callus produced around the veins of the leaf explants and not from callus associated with stem explants. Such shoots failed to root when placed on rooting medium with 100 mg/l kanamycin antibiotic selection. If the incubation time with the *A. tumefaciens* suspension was prolonged to 30 minutes, the explants senesced more rapidly, without producing callus.

This protocol had proved more promising than the tuber disc protocol as, although transgenic plants were not regenerated, the tissue culture steps appeared more successful. In the transformation experiments described above stringent kanamycin selection (a concentration of 100 mg/l) was used in both the regeneration media. Twell and Ooms (1987), Rocha-Sosa *et al.* (1989) and Visser *et al.* (1989) have reported that a less stringent level of selection (a concentration of 50 mg/l kanamycin) was sufficient to select for transgenic potato plants. A series of transformations were then attempted with this less stringent selection pressure of a lower kanamycin concentration.

Uninoculated control explants did not regenerate shoots on the media with reduced levels of kanamycin selection, although callus was produced. However, this callus growth was not sustained, and they senesced after being subcultured onto fresh medium. More extensive callus growth was observed on the explants inoculated with *A. tumefaciens* harbouring the binary vector constructs. After four weeks on the selection medium this callus tissue started to turn brown and no further growth could be observed. When this occurred new callus growth could be distinguished on some of the old callus (as seen in Fig. 5.3.). This new callus was green, but the growth would be variable; some calli continued to grow, but others stopped growing and senesced. When the explants were transferred to LS-R2 shoot regeneration medium shoots did start to emerge from the proliferating callus. This response was not observed on all the transformation plates in an experiment or with all of the binary vector constructs. Indeed transformations performed with the control plasmid pBIN19 did not produce these calli. When the shoots were excised and transferred to rooting medium, a number of shoots did root successfully in the presence of 100 mg/l kanamycin. However the transformation efficiency was very low and many of the shoots transferred to rooting medium did not root sucessfully.

Despite the low transformation achieved with this protocol transgenic potato plants, that rooted on kanamycin selection, were

Fig. 5.3. Leaf and stem explants on LS-R2 shoot regeneration medium with 50 mg/l kanamycin antibiotic selection.

The initial callus growth on the expants has ceased, but areas of fresh, vigourous green callus growth have appeared. A shoot has emerged from one of the explants.

Table 5.2. The number of the shoots regenerated with the leaf and stem explant transformation protocol that rooted in the presence of 50 mg/l kanamycin.

Maize ANT binary vector constructs	Number of rooted plants regenerated
CAMmANTF (sense construct)	5
CAMmANTR (antisense construct)	6
PATmANTF (sense construct)	0
PATmANTR (antisense construct)	0
STLSImANTF (sense construct)	3
STLS1mANTR (antisense construct)	0
Potato ANT binary vector constructs	
CAMpANTF (sense consrtruct)	6
CAMpANTR (antisense constrcut)	7
PATpANTF (sense construct)	6
PATpANTR (antisense construct)	9
STLS1pANTF (sense construct)	0
STLSIpANTR (antisense construct)	2
control binary vector	
pBIN19 with no expression cassette inserted	0

generated. Unfortunately it was not possible to regenerate plants with all the constructs that had been prepared. Also transformations with the control pBIN19 plasmid, without an ANT expression cassette, did not regenerate any transgenic plants. The results of these transformations are summarised in Table 5.2.

5.7. Other Potato Transformation Protocols Used

The transformation efficiency of the protocol described above, whilst successful in regenerating transgenic plants, was lower than expected and transgenic plants had not been generated with all the ANT constructs used. To achieve a higher transformation efficiency a number of other protocols using stem and leaf explants were attempted to increase the transformation efficiency. These varied in the treatment of the tissue (Visser *et al.*, 1989), the method of cocultivation with *A. tumefaciens* (Twell and Ooms, 1987) or the conditions used for plant regeneration (Rocha-Sosa *et al.*, 1989).

5.7.1. Transformation of Pretreated Explants

One of the protocols used was described by Visser *et al.* (1989). Stem explants are used as the tissue for inoculation, and the steps in this protocol are similar to those in the previous protocols, with a cocultivation step and then tissue regeneration. However, the potato explants are pretreated before cocultivation by floating them in liquid medium overnight. The protocol also differs from the pevious protocols used as the explants are not subject to kanamycin selection immediately after cocultivation with the *A. tumefaciens* culture, but after a period of 7 days whilst they are on medium without selection. During this time the explants are subject only to a bacteriostatic antibiotic. The explants are then transferred to fresh medium with 50 mg/l kanamycin antibiotic selection in addition to the bacteriostatic antibiotic.

However, the pretreatment process appeared to accelerate tissue senescence of the explants. Both uninnoculated and innoculated explants grown on medium with and without kanamycin selction applied after seven days failed to produce

callus. There was no difference in the response observed between leaf and stem explants as both types rapidly bleached and died without callus being produced.

5.7.2. Stem Explant Transformation

At Rothamsted Experimental Station potato transformation has been regularly achieved with a protocol developed by Dr. G. Ooms. This protocol is described in Twell and Ooms (1987), and was used without modification. Selection for transgenic shoots was achieved with 50 mg/l kanamycin in the regeneration media. This protocol differs from the S.C.R.I. protocol used before as the explants are not infected by incubation with *A. tumefaciens* from an overnight culture, but with *A. tumefaciens* colonies taken from a freshly grown plate with a sterile flamed loop. The loop is brushed over the upper cut surface of stem explants embedded into the regeneration medium. Transformations with this protocol were performed with the potato line used in the other transformation experiments and with a line of micropropagated Desiree obtained from Rothamsted Experimental Station.

The response of the explants to this protocol was very similar to that observed of explants subjected to the S.C.R.I. protocol. Uninoculated control explants, on medium without antibiotic selection, readily and rapidly regenerated calli from which multiple shoots were produced. Explants transformed with either the control plasmid pBIN19 or the ANT constructs did not form callus as readily, although all the stem explants did eventually form callus. However, these calli failed to continue growing, turning brown and apparently dying. On a small proportion of these apparently dead calli areas of more vigorous green callus growth appeared. This callus would survive for two or three rounds of tissue culture. Some of those calli that did survive produced shoots, either individual ones or small numbers of multiple shoots. Whilst these shoots were produced under kanamycin selection when they were transferred on the rooting medium they failed to root.

5.7.3. Transformation With The Protocol of Rocha-Sosa

Rocha-Sosa *et al.* (1989) describe a potato leaf disc transformation protocol that has been extensively used in their laboratory. An overnight culture of *A. tumefaciens* is used to inoculate the leaf explants in liquid medium and they are incubated in the dark for two days. The explants are transferred to callus induction medium, which unlike the medium used in the other protocols utilizes glucose, not sucrose, as the carbon source. When this protocol was used callus growth was observed on the cut surfaces of those leaf explants infected with *A. tumefaciens*, but the rate of callus development was slower than that of control explants on regeneration medium without selection. Shoots were regenerated from some of these calli, but these shoots failed to root when transferred to rooting medium with kanamycin selection. Many of these shoots originated from callus close to the mid rib or veins in the leaf explant.

5.8. Discussion

The work discussed in this chapter illustrates the difficulty of achieving efficient potato transformation in our laboratory. A wide number of different transformation protocols were used to regenerate transgenic potato plants. Although the protocols used were reported to be successful, none of these proved to be reliable in regenerating transgenic plants. The efficiency of even the most successful protocol tested, as used at S.C.R.I., was much lower than expected. The inefficiency of these protocols is illustrated as it was not possible to regenerate plants when transformed with a control binary vector, pBIN19 without an expression cassette inserted into its multiple cloning site. Whilst transgenic plants were regenerated with some of the ANT constructs prepared, it was not possible to regenerate plants with all of the constructs used. Constructs prepared with the homologous potato ANT cDNA seemed more effective in the transformation experiments than those prepared with the heterologous maize ANT cDNA. Transgenic plants were regenerated with all but one of the potato ANT constructs, including those with the CaMV 35S and patatin promoters, but not with the ANT cDNA, in the sense orientation,

under the control of the ST-LS1 light induced promoter. In contrast, transgenic plants were regenerated with the maize ANT cDNA in the sense orientation under the ST-LS1 promoter, but not when the cDNA was in the antisense orientation. Transgenic plants were produced with both of the CaMV 35S promoter maize ANT constructs, but not with either of the patatin maize ANT constructs.

Many factors have been implicated as parameters that may influence the transformation efficiency of a protocol. The variation between different cultivars in the ease with which transformed plants can be regenerated is well documented. This cultivar dependence has been observed in both pea (Hobbs *et al.*, 1989) and tomato (Davies *et al.*, 1991), as well as potato (e.g. Higgins *et al.*, 1992; Ooms *et al.*, 1987; Sheerman and Bevan, 1986; Wenzler *et al.*, 1988). Higgins *et al.* (1992) have reported an approximate correlation between the overall transformation efficiency and regeneration of transgenic plants. Cultivars that were poor for regeneration had the fewest transformed areas (determined by GUS staining), while the best cultivars for transformation, such as Desiree, had more stained areas. The reasons for these varietal differences are not clear, but most protocols are optimised for a particular potato cultivar (e.g. Hoekema *et al.*, 1989), most commonly Desiree. However other potato transformation protocols have been developed in which the transformation efficiency is cultivar independent (De Block, 1988; Stiekema *et al.*, 1988). The optimization often involves altering the balance and concentration of the phytohormones used to regenerate shoots from the explants. In most protocols shoot regeneration is promoted by a high ratio of cytokinins to auxins. Sheerman and Bevan (1988) reported that the shoot induction medium used in their protocol (containing 5 μM zeatin riboside and 0.3 μM indole acetic acid (IAA)) was markedly superior to others that were tried. Hoekema *et al.* (1989) similarly reported that this medium was most effective for the regeneration of shoots from Desiree. Desiree was consistently superior to the other cultivars tested in its regenerative properties, even with the different balances and concentrations of phytohormones that were

tested. However, for the other cultivars investigated a medium containing 10 μM zeatin riboside and 1 μM indole acetic acid was more effective for regenerating shoots. De Block (1988) compared media containing different hormones, finding that medium containing the cytokinin trans-zeatin was more effective for shoot regeneration than medium with benzylaminopurine. He reported that the auxin naphalene acetic acid was more effective than IAA for regenerating plants, as the calli formed on medium containing IAA were smaller and more difficult to regenerate into plants. All the protocols used in this study, except that of Visser *et al.* (1989), were able to regenerate shoots when uninoculated explants were placed on medium without antibiotic selection. These controls produced multiple shoots from each explant. However, when kanamycin antibiotic selection was applied the controls did not regnerate shoots. Yet it was not possible to regenerate shoots from explants innoculated with *A. tumefaciens*when subjected to stringent selection pressure. Explants innoculated with *A. tumefaciens* but not under kanamycin selection behaved as the control explants, producing large numbers of shoots which did not root in the presence of kanamycin.

A possible factor in the failure of the inital tuber transformation experiments was the condition of the tubers. Sheerman and Bevan (1988) reported that tubers under 6 months old gave the best transformation results. The tubers used in the transformations were stored in similar conditions (less than 6 months old and stored at 4 °C) as described by Sheerman and Bevan, but transgenic plants were not regenerated. It has been found with other plant species (e.g. *Lotus corniculata* (Armstead and Webb, 1987)) that the age and type of tissue is an important factor in determining the efficiency of transformation. Visser *et al.* (1989) have reported a seasonal influence on the efficiency of potato transformation. Although uninoculated explants on medium without antibiotic selection always produced numerous shoots transformation experiments were more successful in the spring compared to the autumn. They suggest that this was due to the physiological state of the tissue. However, when fresh tubers instead of stored tubers were used in the transformation

experiments, no increase in transformation efficiency was found. The variability in the response of tuber discs on the regeneration medium is seen in Fig 5.1. Whilst the tuber discs often did not produce callus on the regeneration medium, showing no response apart from forming an number of 'bumps', occasionally some tuber discs did form a very friable callus. Shoots did not emerge from such callus, and there seemed to be no apparent reason for this response.

As tubers had not proved susceptible to transformation, alternative potato tissues were used. The transformation of leaf and stem explants is well established and a number of protocols for these are available. These protocols were reported to work at reasonable efficiency, but in the present work none was totally reliable. All the methods used, except one, successfully regenerated control plants when no antibiotic selection was applied, but were not very efficient in regenerating transgenic plants. These tissues appeared to be more susceptible to infection by *A. tumefaciens* as they formed callus very rapidly. However this callus was mostly untransformed as its initial growth was not maintained, although smaller areas of callus were able to continue to grow for some time. This pattern is reflected in some investigations into the early processes of *A. tumefaciens* infection. The early stages of infection have been monitored using the β-glucuronidase (GUS) gene (Janssen and Gardner, 1989; Higgins *et al.*, 1992). Janssen and Gardner reported that transient GUS activity was detected after 2 to 4 days over a large area on cut surfaces of petunia and potato explants. These large areas were replaced by much smaller areas that persisted after the initial areas had peaked and correlated with stable integration as measured by kanamycin resistance. In contrast Higgins *et al.* (1992) only observed the much smaller areas of staining. Many, but not all, of the transgenic shoots that were produced in these experiments arose from these young calli that appeared on the initial callus.

The transfer of the T-DNA from *A. tumefaciens* to a plant cell depends on the function of the virulence (vir) genes. These genes are induced by certain phenolic compounds, which are

secreted by wounded plants. One of these compounds, acetosyringone, has been used to promote transformation efficiency by a number of groups working with a number of plant species (Sheikoleslam and Weeks, 1987; Godwin *et al.*, 1991; Higgins *et al.*, 1992). Higgins *et al.* concluded that the effect of acetosyringone on transformation efficiency was relatively minor with some potato cultivars such as Maris Piper, but with other genotypes there was a pronounced effect (in association with other factors, such as using a different *A. tumefaciens* strain and the addition of silver thiosulphate to the *in vitro* stock cultures). When acetsyringone was added to the overnight cultures of *A. tumefaciens* used to infect the potato tuber explants the efficiency of transformation was not enhanced, and no transgenic plants were recovered. Despite this acetosyringone was added to the *A. tumefaciens* overnight cultures used in the S.C.R.I. leaf and stem explant transformation protocol. Whilst this protocol was successful in regenerating plants the low frequency at which this occured implied that the effect of the acetosyringione was realitively minor.

The most effective alteration to the protocols used, which resulted in the generation of transgenic plants, was changing the selection regime used to select for transgenic plants. In the initial tuber transformation and leaf disc transformation experiments a stringent level of selection, of 100 mg/l kanamycin, was used. At this stringency it was not possible to regenerate shoots that then rooted on rooting medium with the same selection stringency. Reducing the selection pressure to 50 mg/l resulted in the production of many more shoots on the explants. Not all of these were transgenic and they failed to root on rooting medium with kanamycin selection. This lower level of kanamycin selection has been used with a number of the protocols described, including those of Twell and Ooms (1987), Rocha-Sosa *et al.* (1989), Visser *et al.* (1989) and Wenzler *et al.* (1989). Stiekema *et al.* (1989) used 100 mg/l kanamycin to select for transformants with Desiree, but used only 50 mg/l for selecting transformants with the cultivar Bentje. Higgins *et al* (1992) observed that when 100 mg/l kanamycin was used for the selection fewer transformation

foci (determined by GUS reporter gene) were detected compared to selection at 50 mg/l. However if no selection pressure was applied to the explants fewer transformed areas were observed than when selection was applied. They suggest that selection is a compromise between providing a competitive advantage for transformed cells and killing those transformants with a relatively low expression of the NPTII gene.

The *A. tumefaciens* strain used in all the experiments described in this chapter was LBA4404. This strain has been used successfully in many reports to transform potato. It is an octopine producing strain, but some researchers report that virulence plasmids from nopaline strains are more effective in plant transformation. These nopaline virulence plasmids have a trans zeatin synthesis gene (*tzs*) which may account for their increased effectiveness, but the addition of zeatin riboside to the medium in most of the potato transformation protocols used in this thesis should reduce this effect.

In summary, none of the potato transformation protocols tried in the course of this work proved reliable and efficient. Whilst transgenic plants were generated, they were not produced in large numbers. Reducing the stringency of the antibiotic selection pressure did result in the production of a number of transgenic plants, but the number of 'escapes' (non-transformed shoots produced) increased. Transformations with a control binary vector that carried no transgene apart from the selective NPTII marker gene did not regenerate any transgenic plants. This was also found with some of the transgene constructs which did not regenerate any transgenic plants. However those transgenic plants that rooted on rooting medium with kanmycin antibiotic selection.were taken on for fur ther investigation.

CHAPTER 6
ANALYSIS OF TRANSGENIC PLANTS

6.1. Analysis of Transgenic Potato Plants

The transgenic plants that had been regenerated following *Agrobacterium* mediated transformation (described in Chap. 5) were transferred to soil and grown in a containment greenhouse. In this chapter the analysis of these plants will be described. None of the plants showed any phenotypical difference compared to control plants regenerated and grown under the same conditions. For each set of plants transformed with a specific construct, the organization of the integrated T-DNA was investigated by Southern blot analysis. Northern blot analysis was used to investigate the effect of the transgene on the expression of the introduced and endogenous ANT genes. The accumulation of ANT protein was estimated by immunoblotting to determine if it had been altered.

6.2. Analysis of Potato Plants Transformed with the CAMmANTF Construct

Five shoots that rooted on kanamycin selection after transformation with the maize ANT cDNA, in the sense orientation under the control of the CaMV 35S promoter, were transferred from the growth chamber to soil and grown in the greenhouse. When these plants were analysed by Southern blotting (Fig.6.1.) two pairs of plants (A7 and A13, and A14 and A15) produced the same banding pattern, indicating that each pair arose from the same transformation event. These plants had a single hybridizing band integrated into their genome. The banding pattern of the other plant, A8, indicated that two hybridizing bands had integrated into its genome.

6.2.1. Expression of ANT Genes

Northern blot analysis of total leaf RNA from these plants showed that the steady state level of expression of endogenous ANT mRNA was not significantly different between any of the transgenic plants and the wild type control (Fig.6.2).

As the expression of the endogenous ANT transcript appeared unaltered, northern blot analysis, using a double stranded mANT specific probe, was used to investigate the expression of the introduced mANT gene in leaf tissue (Fig.6.3.).

FIG.6.1. Southern blot analysis of *Eco* R1 digested DNA from transgenic plants transformed with a CAMmANTF construct,(A). 10 μg DNA was loaded in each lane. A 1.2kb *Pst*1 fragment from pBIN19 spanning the NPTII gene was used as the probe. Markers are λ DNA (kbp) digested with *Hind* III.

FIG.6.2. Northern blot analysis of total RNA isolated from leaves of transgenic plants transformed with a CAMmANTF construct. W.T. is RNA from an untransformed control plant. 5 μg of total RNA was loaded in each lane. The probe used was the homologous pANT cDNA. The position of the smaller ribosomal RNA from potato is marked.
The numbers below indicate the relative amount of ANT transcript in the transgenic plants (determined using densitometry) compared with the level observed in a wild type plant .

A7	A12	A13	A14	A15	W.T.
0.84	1.16	1.00	1.20	0.84	1.00

FIG.6.3. Northern blot analysis of total RNA isolated from leaves of transgenic plants transformed with a CAMmANTF construct. W.T. is RNA from an untransformed control plant. 5 μg of total RNA was loaded in each lane. The probe used was the heterologous mANT cDNA. The position of the smaller ribosomal RNA from potato is marked.

FIG.6.4. Western blot analysis of total leaf proteins isolated from leaves of transgenic plants transformed with a CAMmANTF construct, probed with an antibody specific for ANT. W.T. is protein from an untransformed control plant. $1/20^{th}$ of the total protein sample, equivalent to the proteins extracted from 25 mg of leaf tissue by fresh weight, was loaded in each lane. A separate gel was run and stained with Coomasie brilliant blue to check that the protein loadings in each lane were similar. The position of the 29 kDa molecular weight marker is indicated on the right.

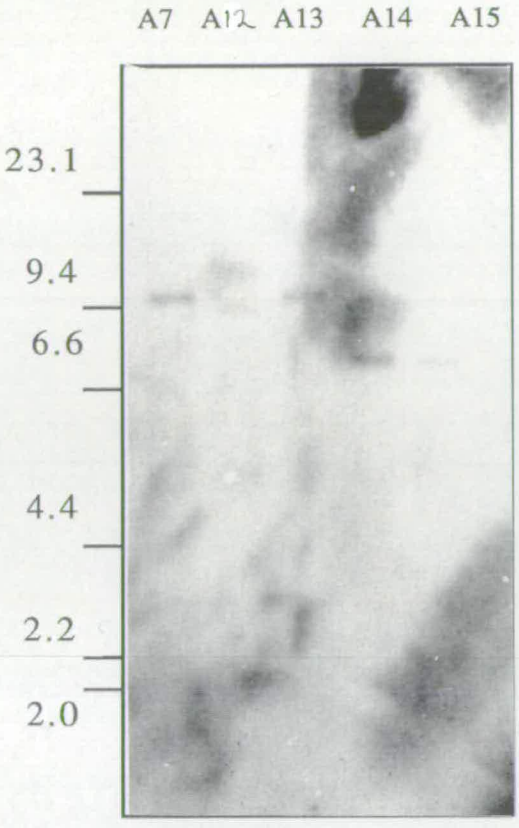


FIG.6.1.

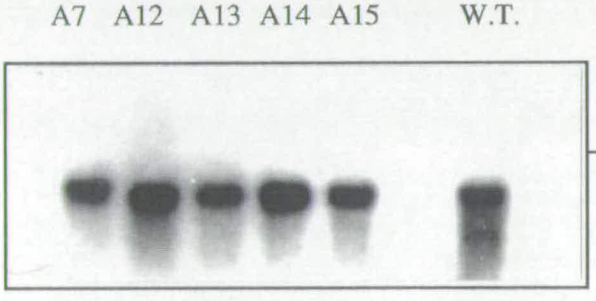


FIG.6.2

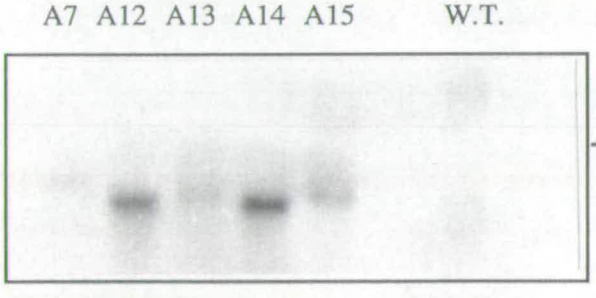


FIG.6.3.

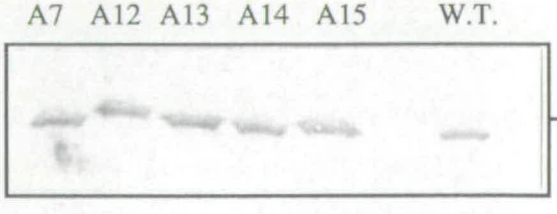


FIG.6.4.

Using this probe a signal was detected in all the transgenic plants, but not in the control plant RNA. The signal was very weak in three of the plants, but in two plants a much stronger signal was detected.

6.2.2. Abundance of ANT Protein

The effect of expressing a heterologous maize ANT gene on the accumulation of ANT protein was estimated by immunoblotting of total leaf proteins (Fig.6.4.). There was no observable difference in the amount of ANT protein in the leaves of the transgenic plants and the wild type plant, even in those transgenic plants with high levels of expression of the heterologous gene.

6.3. Analysis of Potato Plants Transformed with the CAMmANTR Construct

Six plants that rooted on kanamycin selection medium after transformation with the maize ANT cDNA, in the antisense orientation under the control of the CaMV 35S promoter, were transferred to soil and grown in the greenhouse. Southern blot analysis of these plants showed that two plants (B5 & B6) had identical hybridisation patterns and arose from the same transformation event (Fig.6.5.). In these two plants the hybridisation pattern suggested that four hybridizing bands had integrated into the plant genome. All the other plants were the result of different transformation events. Two of the other plants, B2 and B8, generated two bands on the blot, whilst in the other plants a single band was found.

6.3.1. Expression of ANT Genes

Northern blot analysis of total leaf RNA was performed to determine the steady state levels of endogenous ANT mRNA in the transgenic plants (Fig.6.6.). There was a decrease in the level of endogenous ANT mRNA in all of the transgenic plants except one, B3, in which expression was the same as the wild type. The reduction in ANT expression varied between the transformants, from 1/3 to 2/3 of the level of the control.

FIG.6.5. Southern blot analysis of *Eco* R1 digested DNA from transgenic plants transformed with a CAMmANTR construct,(B). 10 μg DNA was used in each lane. A 1.2kb *Pst*1 fragment from pBIN19 spanning the NPTII gene was used as the probe. Markers are λ DNA (kbp) digested with *Hind* III.

FIG.6.6. Northern blot analysis of total RNA isolated from leaves of transgenic plants transformed with a CAMmANTR construct. W.T. is RNA from an untransformed control plant. 5 μg of total RNA was loaded in each lane. The probe used was the homologous pANT cDNA. The position of the smaller ribosomal RNA from potato is marked.
The numbers below indicate the relative amount of ANT transcript in the transgenic plants (determined using densitometry) compared with the level observed in a wild type plant .

B2	B3	B5	B6	B7	B8	W.T.
0.66	1.00	0.48	0.46	0.34	0.44	1.00

FIG.6.7. Northern blot analysis of total RNA isolated from leaves of transgenic plants transformed with a CAMmANTR construct. W.T. is RNA from an untransformed control plant. 5 μg of total RNA was loaded in each lane. The probe used was the heterologous mANT cDNA. The position of the smaller ribosomal RNA from potato is marked.

FIG.6.8. Western blot analysis of total leaf proteins isolated from leaves of transgenic plants transformed with a CAMmANTR construct, probed with an antibody specific for ANT. W.T. is protein from an untransformed control plant. $1/20^{th}$ of the total protein sample, equivalent to the proteins extracted from 25 mg of leaf tissue by fresh weight, was loaded in each lane. A separate gel was run and stained with Coomasie brilliant blue to check that the protein loadings in each lane were similar. The position of the 29 kDa molecular weight marker is indicated on the right.

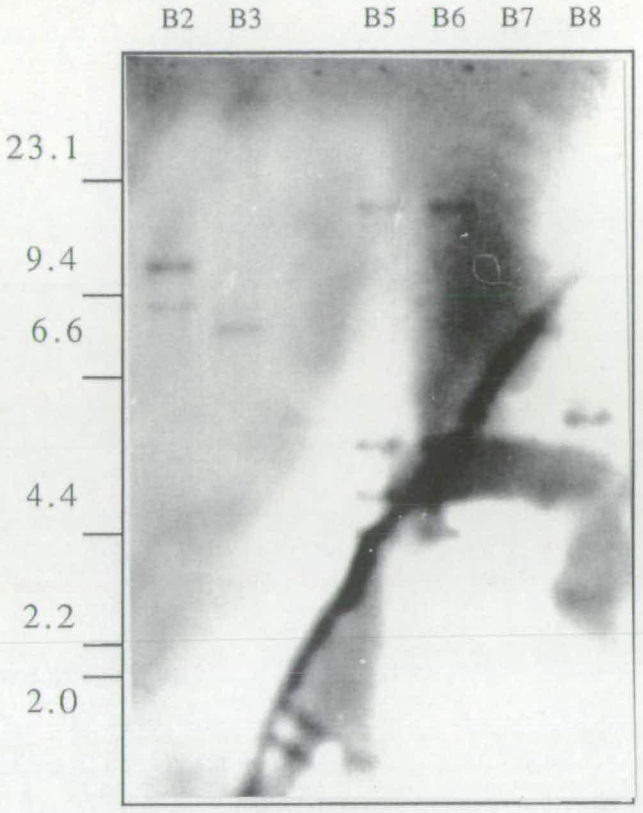


FIG.6.5.

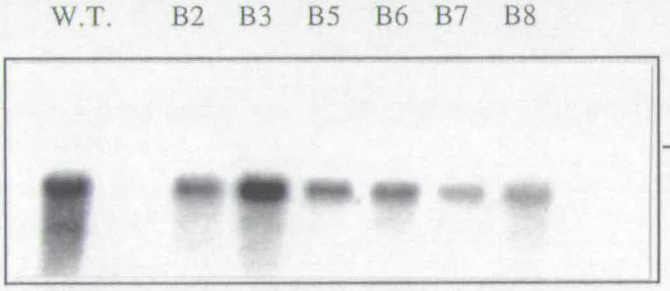


FIG.6.6.

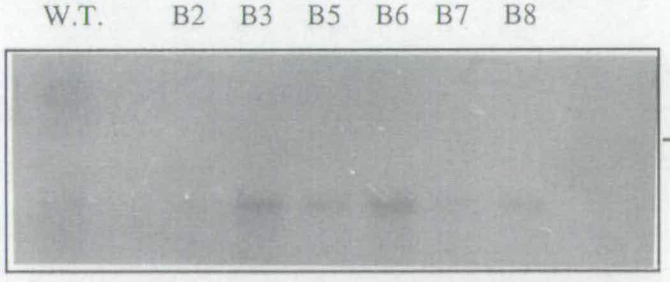


FIG.6.7.

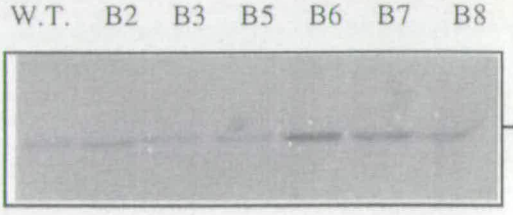


FIG.6.8.

When the expression of the antisense gene was investigated using a double stranded mANT specific probe, antisense mANT expression was detected in most of the transgenic plants, but only at very low levels (Fig.6.7.). One of the plants in which expression of the antisense mANT was greatest was B3 (in which expression of the endogenous gene was not reduced). The other two plants with relatively high levels of antisense gene expression were B5 and B6 which had multiple copies of the introduced transgene. These levels of antisense gene expression did not correlate with the reductions in the level of endogenous gene expression observed in the plants.

6.3.2. Abundance of ANT Protein

When total leaf proteins were probed with an ANT antibody by immunoblotting there was no detectable reduction in the amount of ANT protein between the transgenic plants, including those with reduced levels of endogenous ANT mRNA, and the control sample (Fig.6.8.).

6.4. Analysis of Plants transformed with the ST-LS1mANTF Construct

Three shoots that rooted on kanamycin selection medium after transformation with the maize ANT cDNA, in the sense orientation under the control of the ST-LS1 promoter, were transferred to soil to grow in the greenhouse. Southern blot analysis showed that two plants (E10 & E11) were the result of the same transformation event, with a single hybridizing band integrated into the potato genome, whilst the other plant (E4) contained two hybridizing bands (Fig.6.9.).

6.4.1. Expression of ANT Genes

Northern blot analysis of total RNA from the leaves of the transgenic plants showed that all three plants had elevated levels of endogenous pANT expression (Fig.6.10.), to almost twice that of the wild type control .

When total leaf RNA was probed with a mANT specific double stranded probe very little signal was detected in the wild type control, but a signal was detected in all the transgenic plants (Fig.6.11.). The expression of the mANT transcript was greatest in

FIG.6.9. Southern blot analysis of *Eco* R1 digested DNA from transgenic plants transformed with a ST-LS1mANTF construct,(E). 10 µg DNA was used per lane. A 1.2kb *Pst* 1 fragment from pBIN19 spanning the NPTII gene was used as the probe. Markers are λ DNA (kbp) digested with *Hind* III.

FIG.6.10. Northern blot analysis of total RNA isolated from leaves of transgenic plants transformed with a ST-LS1mANTF construct. W.T. is RNA from an untransformed control plant. 5 µg of total RNA was loaded in each lane. The probe used was the homologous pANT cDNA. The position of the smaller ribosomal RNA from potato is marked.
The numbers below indicate the relative amount of ANT transcript in the transgenic plants (determined using densitometry) compared with the level observed in a wild type plant .

E4	E10	E11	W.T.
1.86	1.62	1.33	1.00

FIG.6.11. Northern blot analysis of total RNA isolated from leaves of transgenic plants transformed with a ST-LS1mANTF construct. W.T. is RNA from an untransformed control plant. 5 µg of total RNA was loaded in each lane. The probe used was the heterologous mANT cDNA. The position of the smaller ribosomal RNA from potato is marked.

FIG.6.12. Western blot analysis of total leaf proteins isolated from leaves of transgenic plants transformed with a ST-LS1mANTF construct, probed with an antibody specific for ANT. W.T. is protein from an untransformed control plant. $1/20^{th}$ of the total protein sample, equivalent to the proteins extracted from 25 mg of leaf tissue by fresh weight, was loaded in each lane. A separate gel was run and stained with Coomasie brilliant blue to check that the protein loadings in each lane were similar. The position of the 29 kDa molecular weight marker is indicated on the right.

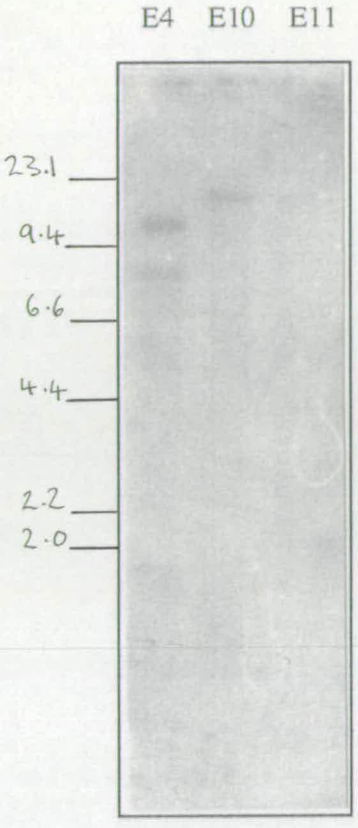


FIG.6.9.

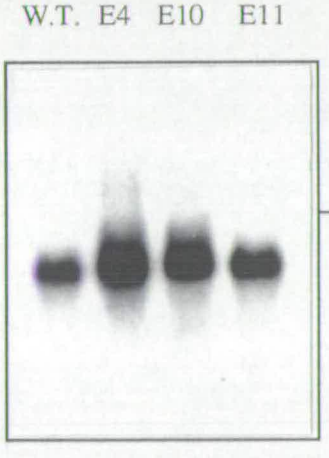


FIG.6.10.

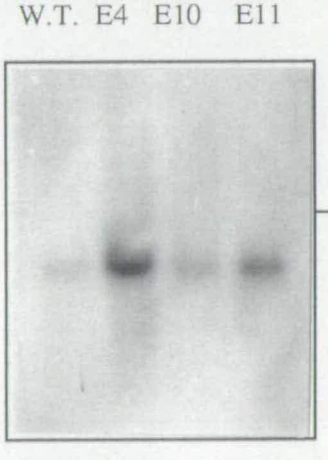


FIG.6.11.

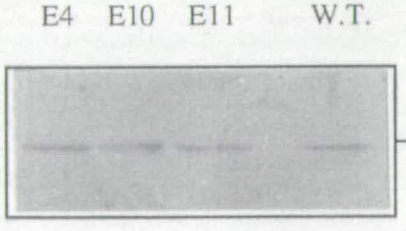


FIG.6.12.

plant E4, which had two copies of the gene integrated into its genome.

6.4.2. Abundance of ANT Protein

The increase in expression of the endogenous ANT and the expression of the introduced mANT did not affect the level of ANT protein as revealed by probing an immunoblot of total leaf proteins with an ANT antibody (Fig.6.12.).

6.5. Analysis of Potato Plants Transformed with CAMpANTF Construct

Six plants which after transformation with the potato ANT cDNA, in the sense orientation under the control of the CaMV 35S promoter, were rooted on kanamycin selection and transferred to soil in the greenhouse. Southern blot analysis of these plants showed that they were all the result of separate transformation events (Fig.6.13.). In one plant (G2) the hybridisation pattern suggested that it was transformed with two hybridizing bands, whilst in all the others a single transformation event had occurred.

6.5.1. Expression of ANT Genes

The steady state level of endogenous pANT expression in the transgenic plants was investigated by northern blot analysis (Fig.6.14.). The steady state level of expression in three of the transgenic plants, G1, G2 and G3, was increased by up to twice the level of the control. No difference in the level of ANT expression compared to the control was found in the other three transgenic plants.

6.5.2. Abundance of ANT Protein

The amount of ANT protein in the leaves of the transgenic plants was estimated by immunoblotting (Fig.6.15.). There were only small detectable differences in the level of ANT protein between the transgenic and wild type plants, even in those plants over expressing ANT mRNA.

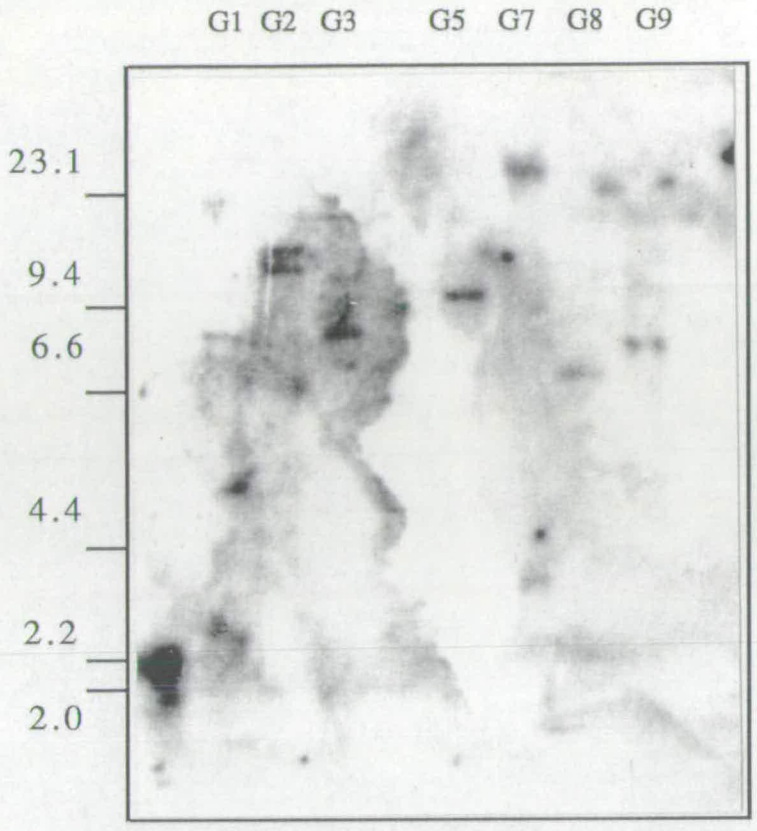


FIG.6.13.

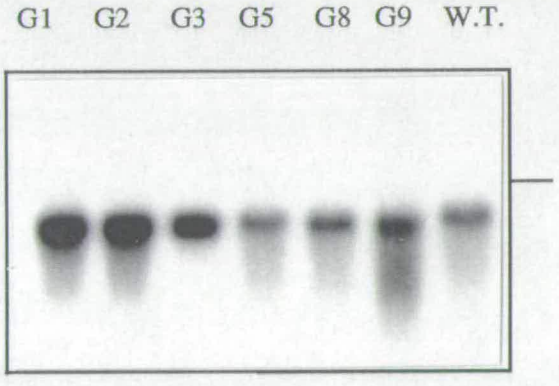


FIG.6.14.

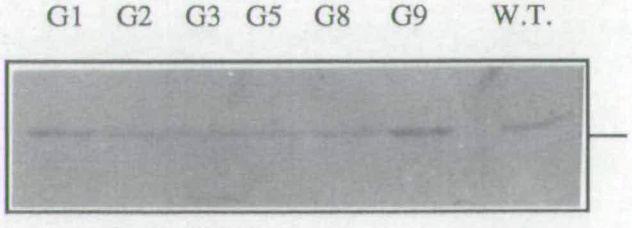


FIG.6.15.

6.6 Analysis of Potato Plants Transformed with CAMpANTR Construct

Seven shoots were rooted on selective kanamycin rooting medium after transformation with the potato pANT cDNA in the antisense orientation, under the control of the CaMV 35S promoter and transferred to the greenhouse. When T-DNA integration was investigated by Southern blot analysis (Fig.6.16.) two plants were found to originate from the same transformation event (H1 & H4), generating the same hybridisation pattern. Both these plants contained two hybridizing bands. The other plants were all independent transformants, with one plant (H8) having four hybridizing bands integrated into its genome. H7 also had multiple sites of T-DNA integration (three bands were identified), but in the others only a single band was detected.

6.6.1. Expression of ANT Genes

Northern blot analysis of total leaf RNA from the transgenic plants and a wild type control revealed that there were differences in the steady state levels of ANT mRNA between the plants (Fig.6.17.). Expression of the endogenous ANT was reduced in most of the transgenic plants to approximately 1/2 of the wild type. However, endogenous gene expression was reduced to less than a 1/3 of the wild type in H12, the plant showing the greatest inhibition. In one plant, H4, the expression of the endogenous gene was not affected.

Using a strand specific riboprobe for the pANT antisense transcript to probe these northern blots it was not possible to detect a signal in either the wild type control or any of the transgenic plants (data not shown).

6.6.2. Abundance of ANT Protein

Immunoblotting of total leaf protein from the transgenic and control plants (Fig.6.18.) revealed no major difference in the amount of ANT protein detected between the transgenic plants and the control.

FIG.6.16. Southern blot analysis of *Eco* R1 digested DNA from transgenic plants transformed with a CAMpANTR construct,(H). 10 μg DNA was used per lane. A 1.2kb *Pst* 1 fragment from pBIN19 spanning the NPTII gene was used as the probe. Markers are λ DNA (kbp) digested with *Hind* III.

FIG.6.17. Northern blot analysis of total RNA isolated from leaves of transgenic plants transformed with a CAMpANTR construct. W.T. is RNA from an untransformed control plant. 5 μg of total RNA was loaded in each lane. The probe used was the homologous pANT cDNA. The position of the smaller ribosomal RNA from potato is marked.
The numbers below indicate the relative amount of ANT transcript in the transgenic plants (determined using densitometry) compared with the level observed in a wild type plant .

H1	H4	H5	H6	H7	H8	H12	W.T.
0.64	0.80	0.52	0.53	0.4	0.44	0.34	1.00

FIG.6.18. Western blot analysis of total leaf proteins isolated from leaves of transgenic plants transformed with a CAMpANTR construct, probed with an antibody specific for ANT. W.T. is protein from an untransformed control plant. $1/20^{th}$ of the total protein sample, equivalent to the proteins extracted from 25 mg of leaf tissue by fresh weight, was loaded in each lane. A separate gel was run and stained with Coomasie brilliant blue to check that the protein loadings in each lane were similar. The position of the 29 kDa molecular weight marker is indicated on the right.

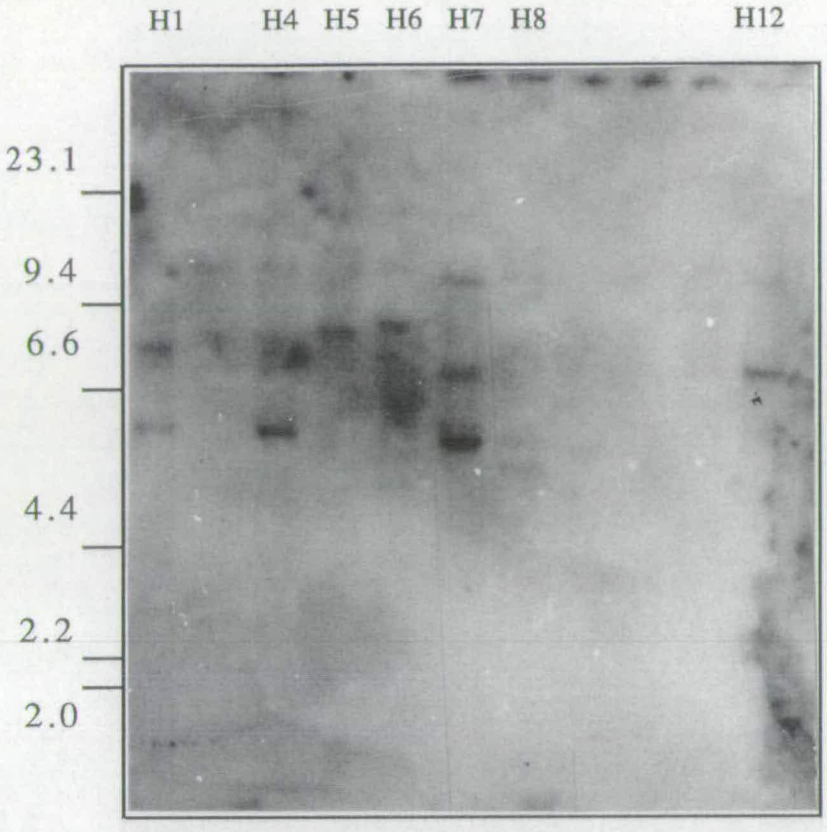


FIG.6.16.

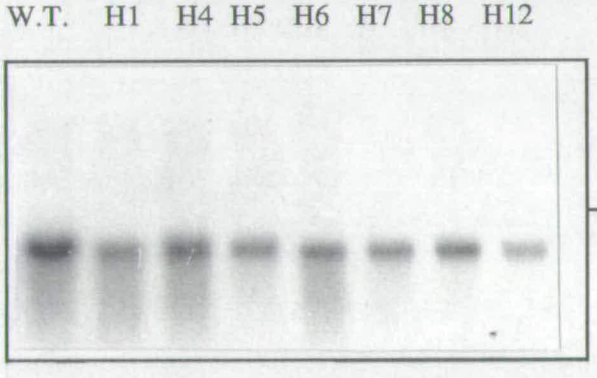


FIG.6.17.

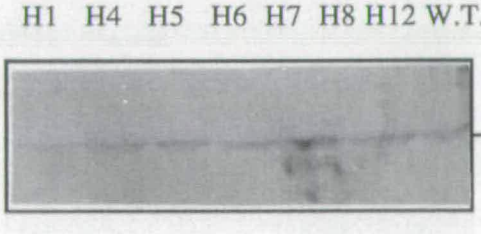


FIG.6.18.

6.7. Analysis of Potato Plants Transformed with PATpANTF Construct

Six plants that rooted on kanamycin selection medium after transformation with the potato pANT cDNA in the sense orientation, under the control of the patatin promoter, were transferred to soil and grown in the greenhouse. The organisation of the inserted T-DNA was investigated by Southern blot analysis (Fig.6.19). This showed that two of the plants, I5 and I6, originated from the same transformation event as they had the same banding pattern, generating at least four bands. Two other plants, I1 and I3, also originated from another transformation event, although only a single band was detected, whilst a single band was also seen in plant I2. The other plant, I7 had three copies of the T-DNA into the genome. The position of two of these bands suggested that the T-DNA had integrated at similar sites in this plant to two of the T-DNA copies in the I5 and I6.

6.7.1. Expression of ANT Genes:

6.7.1.1. In Leaf Tissue

Northern blot analysis of total leaf RNA from the transgenic plants showed no difference between the steady state levels of ANT mRNA in these plants and the wild type control (Fig.6.20.). As the transgene is under the control of a tuber specific patatin promoter the introduced gene is not expected to affect ANT expression in the leaves.

6.7.1.2. In Tuber Tissue

The expression of ANT genes in minitubers (shown in Fig.6.22.) from transgenic and control plants was investigated by northern blot analysis. As illustrated in Fig.6.23. the expression of ANT did vary between the transgenic and control plants, with the level of ANT transcript being slightly increased.

6.7.2. Abundance of ANT Protein:

6.7.2.1. In Leaf Tissue

Immunoblot analysis detected no difference in the level of ANT protein in total proteins isolated from the leaves of the transgenic plants compared to the wild type (Fig.6.21.).

FIG.6.19. Southern blot analysis of *Eco* R1 digested DNA from transgenic plants transformed with a PATpANTF construct,(I). 10 μg DNA was used per lane. A 1.2kb *Pst* 1 fragment from pBIN19 spanning the NPTII gene was used as the probe. Markers are λ DNA (kbp) digested with *Hind* III.

FIG.6.20. Northern blot analysis of total RNA isolated from leaves of transgenic plants transformed with a PATpANTF construct. W.T. is RNA from an untransformed control plant. 5 μg of total RNA was loaded in each lane. The probe used was the homologous pANT cDNA. The position of the smaller ribosomal RNA from potato is marked.
The numbers below indicate the relative amount of ANT transcript in the transgenic plants (determined using densitometry) compared with the level observed in a wild type plant.

I1	I2	I3	I5	I7	W.T.
1.00	1.00	0.77	0.77	0.91	1.00

FIG.6.21. Western blot analysis of total leaf proteins isolated from leaves of transgenic plants transformed with a PATpANTF construct, probed with an antibody specific for ANT. W.T. is protein from an untransformed control plant. $1/20^{th}$ of the total protein sample, equivalent to the proteins extracted from 25 mg of leaf tissue by fresh weight, was loaded in each lane. A separate gel was run and stained with Coomasie brilliant blue to check that the protein loadings in each lane were similar. The position of the 29 kDa molecular weight marker is indicated on the right.

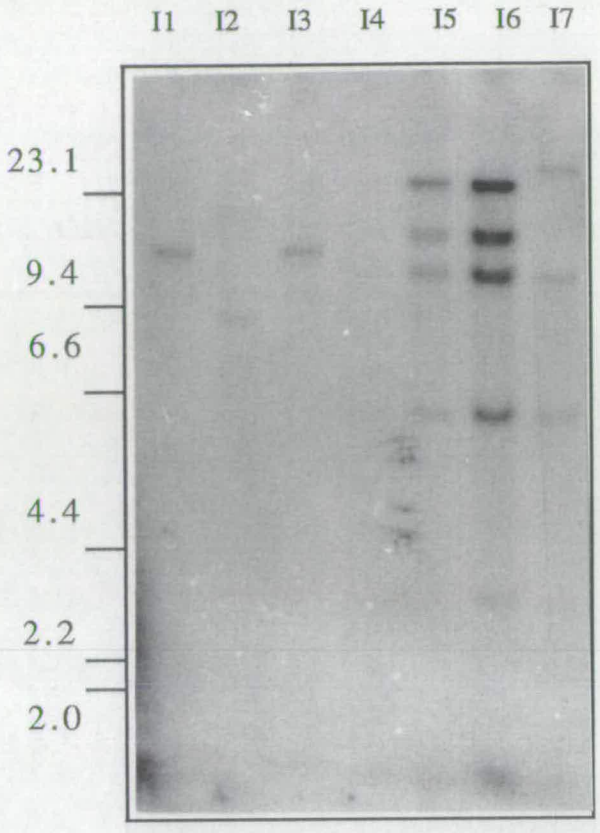


FIG.6.19.

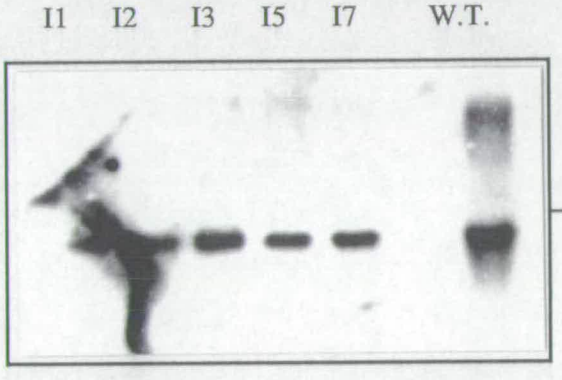


FIG.6.20.

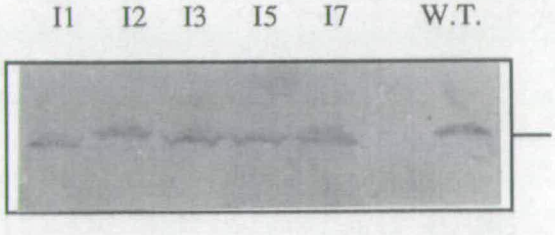


FIG.6.21.

FIG.6.22. Minitubers of transgenic potato plant transformed with a PATpANTAF construct. The tubers were grown for 12 weeks as described in Sec.2.3.2.

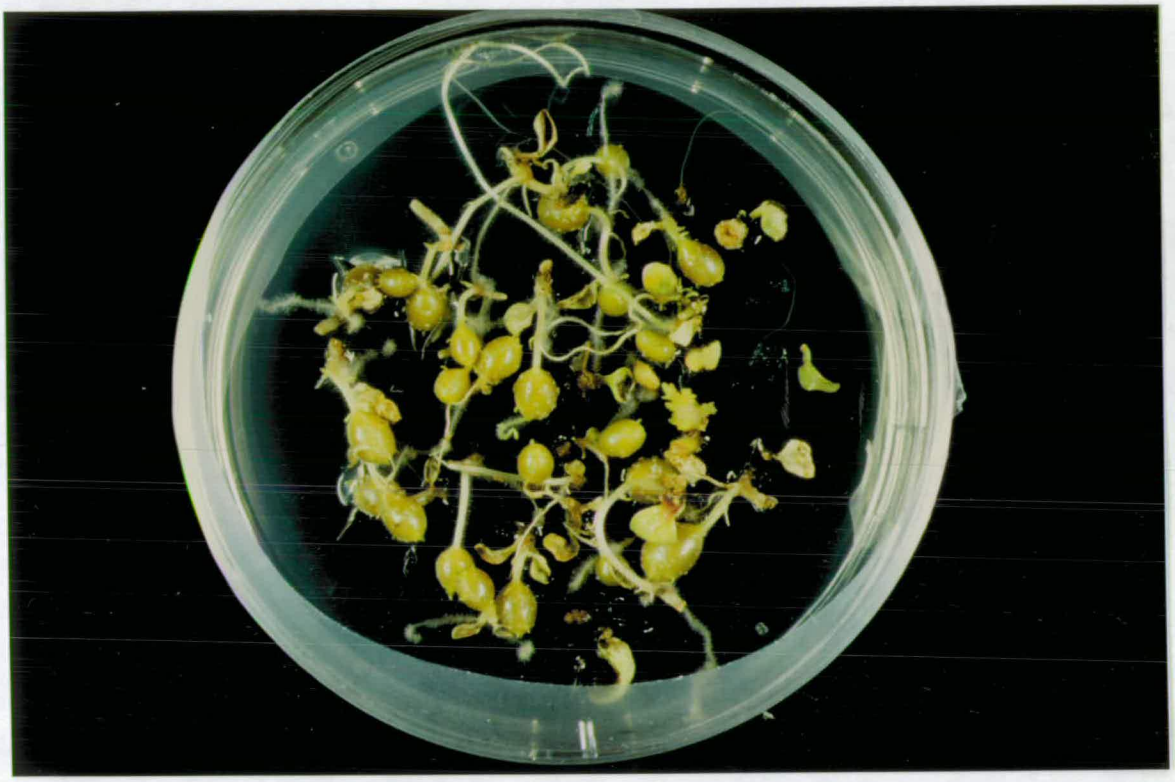


FIG.6.23. Northern blot analysis of total RNA isolated from tubers of transgenic plants transformed with a PATpANTF construct. W.T. is RNA from an untransformed control plant. 5 µg of total RNA was loaded in each lane. The probe used was the pANT cDNA. The position of the smaller ribosomal RNA from potato is marked.
The numbers below indicate the relative amount of ANT transcript in the transgenic plants (determined using densitometry) compared with the level observed in a wild type plant.

I1	I2	I3	I5	I7	W.T.
2.00	1.50	1.25	1.60	1.00	1.00

FIG.6.24. Western blot analysis of total tuber proteins isolated from tubers of transgenic plants transformed with a PATpANTF construct, probed with an antibody specific for ANT. W.T. is protein from an untransformed control plant. 1/20th of the total protein sample, equivalent to the proteins extracted from 25 mg of leaf tissue by fresh weight, was loaded in each lane. A separate gel was run and stained with Coomasie brilliant blue to check that the protein loadings in each lane were similar. The position of the 29 kDa molecular weight marker is indicated on the right.

FIG.6.25. Northern blot analysis of total RNA isolated from tubers of transgenic plants transformed with a PATpANTR construct. W.T. is RNA from an untransformed control plant. 5 µg of total RNA was loaded in each lane. The probe used was the homologous pANT cDNA. The position of the smaller ribosomal RNA from potato is marked.
The numbers below indicate the relative amount of ANT transcript in the transgenic plants (determined using densitometry) compared with the level observed in a wild type plant.

J2	J3	J5	J6	J7	J10	W.T.
0.63	0.61	0.72	0.90	0.76	0.66	1.00

FIG.6.26 Western blot analysis of total tuber proteins isolated from tubers of transgenic plants transformed with a PATpANTR construct, probed with an antibody specific for ANT. W.T. is protein from an untransformed control plant. 1/20th of the total protein sample, equivalent to the proteins extracted from 25 mg of leaf tissue by fresh weight, was loaded in each lane. A separate gel was run and stained with Coomasie brilliant blue to check that the protein loadings in each lane were similar. The position of the 29 kDa molecular weight marker is indicated on the right.

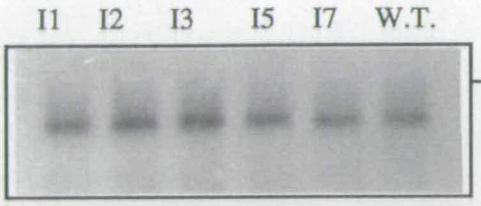


FIG.6.23.

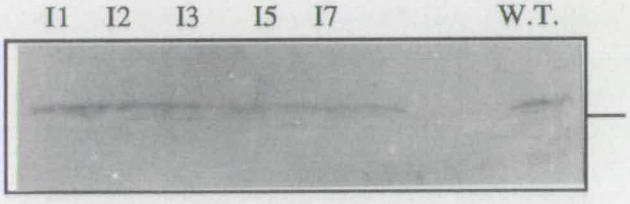


FIG.6.24.

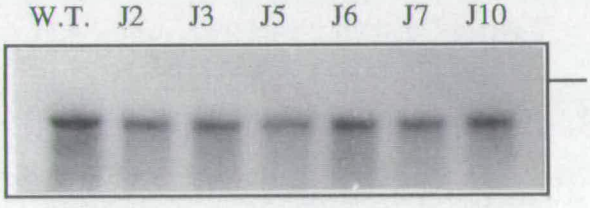


FIG.6.25.

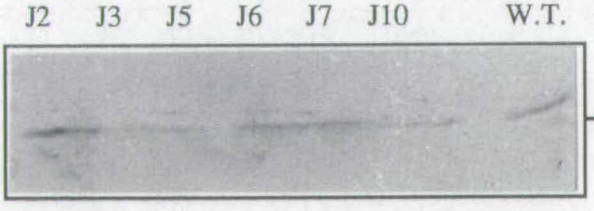


FIG.6.26.

6.7.2.2. In Tuber Tissue

Immunoblot analysis of total tuber proteins with the ANT antibody showed no detectable difference in the level of ANT protein in the transgenic and the wild type plants, despite the increased steady state level of ANT expression observed in some of the tubers (Fig.6.24.).

6.8. Analysis of Potato Plants Transformed with PATpANTR Construct

Nine plants that rooted on selective rooting medium after transformation with the pANT cDNA, in the antisense orientation under the control of the patatin promoter, were transferred to the greenhouse. Southern blot analysis of these plants showed that four of these plants (J1-J4) were the result of the same transformation event, with a single hybridizing band introduced into the genome (Fig.6.27.). Two plants (J10 & J11) were the result of another single transformation event. One of the other plants (J5) was the product of a double transformation event, but the other two plants were the result of a single integration event.

6.8.1. Expression of ANT Genes:

6.8.1.1. In Leaf Tissue

The expression of the endogenous gene in leaves was investigated by northern blot analysis of total RNA. No difference was observed in the steady state level of expression of pANT in the transgenic plants compared to the wild type, except for plant J3, in which the sample was degraded and the signal reduced (Fig.6.28.). The result expected from this tissue is no change in the steady levels of pANT expression as the antisense gene is under the control of the tuber specific patatin promoter.

6.8.1.2. In Tuber Tissue

The level of steady state ANT expression in minitubers of these plants was also determined by northern blot analysis (Fig.6.25.). The steady state level of ANT transcripts was inhibited by up to a maximum of 1/3, compared to wild type levels. The

FIG.6.27. Southern blot analysis of *Eco* R1 digested DNA from transgenic plants transformed with a PATpANTR construct,(J). 10 μg DNA was used per lane. A 1.2kb *Pst* 1 fragment from pBIN19 spanning the NPTII gene was used as the probe. Markers are λ DNA (kbp) digested with *Hind* III.

FIG.6.28. Northern blot analysis of total RNA isolated from leaves of transgenic plants transformed with a PATpANTR construct. W.T. is RNA from an untransformed control plant. 5 μg of total RNA was loaded in each lane. The probe used was the homologous pANT cDNA. The position of the smaller ribosomal RNA from potato is marked.
The numbers below indicate the relative amount of ANT transcript in the transgenic plants (determined using densitometry) compared with the level observed in a wild type plant.

J2	J3	J5	J6	J7	J10	W.T.
1.00		1.00	1.00	0.98	0.90	1.00

FIG.6.29. Western blot analysis of total leaf proteins isolated from leaves of transgenic plants transformed with a PATpANTR construct, probed with an antibody specific for ANT. W.T. is protein from an untransformed control plant.

1/20th of the total protein sample, equivalent to the proteins extracted from 25 mg of leaf tissue by fresh weight, was loaded in each lane. A separate gel was run and stained with Coomasie brilliant blue to check that the protein loadings in each lane were similar. The position of the 29 kDa molecular weight marker is indicated on the right.

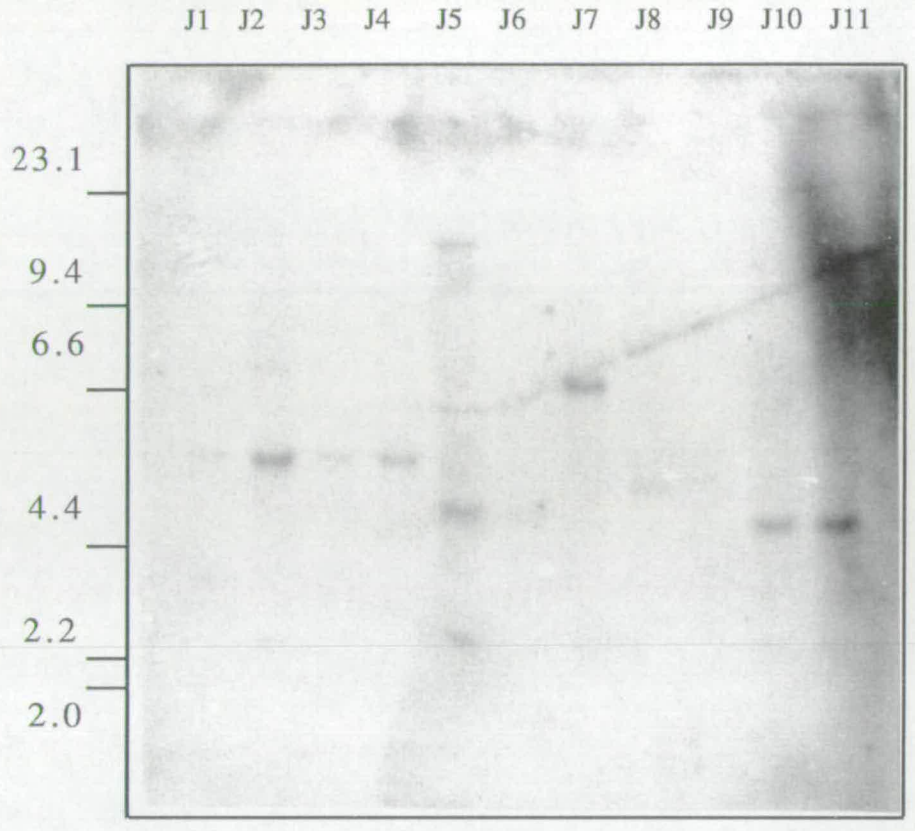


FIG.6.27.

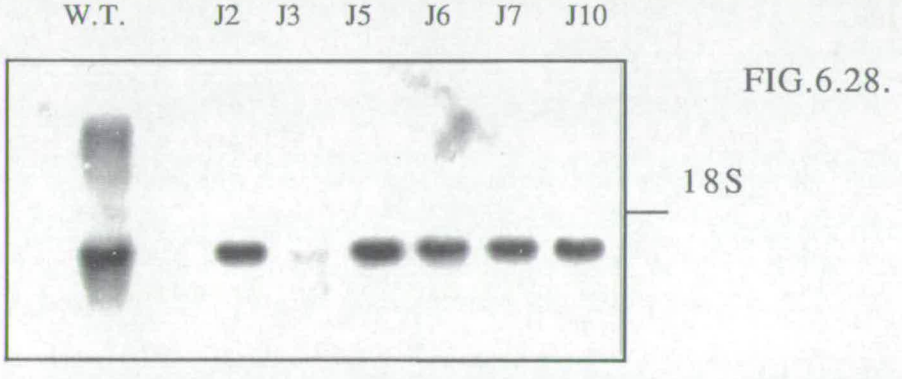


FIG.6.28.

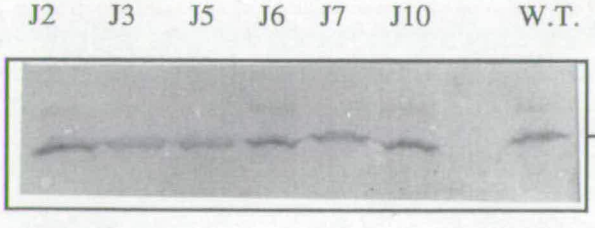


FIG.6.29.

antisense transcript could not be detected when a strand specific sense probe was used.

6.8.2. Abundance of ANT Protein:

6.8.2.1. In Leaf Tissue

The level of ANT protein in leaves of the transgenic plants was estimated by immunoblotting. No measurable difference was detected between the wild type and control plants (Fig.6.29.).

6.8.2.2. In Tuber Tissue

Small differences in the level of ANT protein were detected in minitubers from the transgenic and control plants (Fig.6.26).

6.9. Analysis of Potato Plants Transformed with ST-LS1pANTR Construct

Two shoots isolated from transformations with the ST-LS1 pANT antisense construct rooted on kanamycin selection. When these plants were transferred to soil and grown in the greenhouse they were visually similar from non-transformed control plants that had been through the same micropropagation procedure (Fig.6.30.). Southern blot analysis of these transgenic plants showed that both plants originated from the same transformation event (Fig. 6.31.). It appears that multiple copies (more than three) of the T-DNA had integrated into the potato genome.

6.9.1. Expression of ANT Genes in Leaves

Northern blot analysis of total leaf RNA isolated from these plants and a control plant revealed that the steady state level of ANT transcript was greatly reduced in the transgenic plants compared to the control plant (Fig. 6.32.). The level of expression in both the transgenic plants was reduced to less than 20% of that in the wild type. This result suggested that the antisense gene was affecting the accumulation of endogenous ANT mRNA.

As the levels of ANT mRNA had decreased, the expression of the antisense gene in leaves was investigated. However, when an antisense strand specific pANT riboprobe was used to probe total leaf RNA on a northern blot, it was not possible to detect ANT

FIG.6.30. Phenotype of transgenic plants transformed with the ST-LS1pANTR construct. A L3 plant is shown on the right and a L4 plant on the left of a wild type Desiree potato plant, grown in the containment greenhouse.

FIG.6.31. Southern blot analysis of *Eco* R1 digested DNA from transgenic plants transformed with a ST-LS1pANTR construct,(L). 10 μg DNA was used per lane. A 1.2kb *Pst* 1 fragment from pBIN19 spanning the NPTII gene was used as the probe. Markers are λ DNA (kbp) digested with *Hind* III.

FIG.6.32. Northern blot analysis of total RNA isolated from leaves of transgenic plants transformed with a ST-LS1pANTR construct. W.T. is RNA from an untransformed control plant. 5 μg of total RNA was loaded in each lane. The probe used was the homologous pANT cDNA. The position of the smaller ribosomal RNA from potato is marked.
The numbers below indicate the relative amount of ANT transcript in the transgenic plants (determined using densitometry) compared with the level observed in a wild type plant.

L3	L4	W.T.
0.28	0.16	1.00

FIG.6.33. Western blot analysis of total leaf proteins isolated from leaves of transgenic plants transformed with a ST-LSIpANTR construct, probed with an antibody specific for ANT. W.T. is protein from an untransformed control plant. $1/20^{th}$ of the total protein sample, equivalent to the proteins extracted from 25 mg of leaf tissue by fresh weight, was loaded in each lane. A separate gel was run and stained with Coomasie brilliant blue to check that the protein loadings in each lane were similar. The position of the 29 kDa molecular weight marker is indicated on the right.

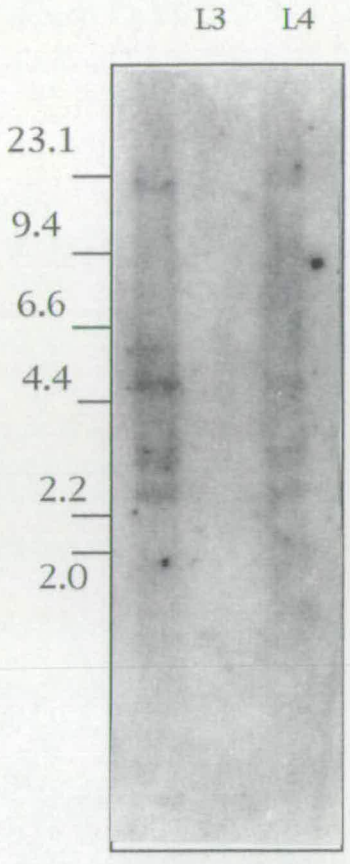


FIG.6.31.

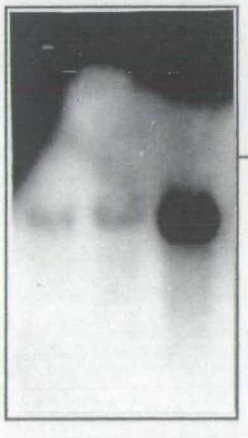


FIG.6.32.

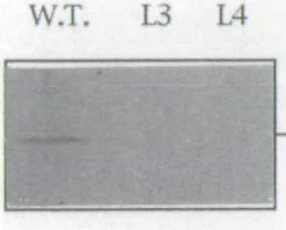


FIG.6.33.

antisense transcripts in either of these transgenic plants or the control.

6.9.2. Abundance of ANT Protein

The reduction in the level of ANT transcript detected was reflected in the amount of ANT protein detected by immunoblot analysis (Fig.6.33). In both the transgenic plants it was not possible to detect any ANT protein on a western blot of total leaf proteins with an ANT antibody, although it was detected in the untransformed control.

6.9.3. ANT Expression in Other Plant Tissues

The expression of the transgene in these potato plants was driven by the ST-LS1 promoter which is a tissue specific promoter and is restricted to photosynthetically active cells (Stockhaus *et al.*, 1989). If the pattern of expression of the transgene is faithful to the control of this promoter, ANT expression in non-photosynthetic tissues of the transgenic plants should not be altered. Northern blot analysis of total RNA isolated from root tissue of the transgenic plants and a control plant showed that no difference in ANT transcript levels was detected between the transgenic and control plants (Fig.6.34.). This suggested that the transgene was faithfully following the expression of its promoter.

6.9.4. Expression of Other Nuclear Encoded Mitochondrial Genes

ANT is one of a large number of nuclear encoded mitochondrial genes. The steady state level of ANT expression had been reduced in the leaves of these transgenic plants which raised the question of whether the expression of other nuclear encoded mitochondrial genes was affected by this change? Recent work in this laboratory has led to the isolation of cDNA clones from potato to NAD malic enzyme. Using α probe to NAD malic enzyme B subunit provided by Dr. B. Winning the steady state level of malic enzyme mRNA was determined by northern blot analysis of total leaf RNA (Fig.6.35.). The expression of NAD malic enzyme was elevated in leaves of the transgenic plants to nearly 5 times that found in leaves of a wild type control plant.

The expression of the 'A' subunit gene of NAD malic enzyme was also similarly increased (data not shown).

FIG.6.34. Northern blot analysis of total RNA isolated from roots of transgenic plants transformed with a ST-LS1pANTR construct. W.T. is RNA from an untransformed control plant. 5 µg of total RNA was loaded in each lane. The probe used was the pANT cDNA. The position of the smaller ribosomal RNA from potato is marked.
The numbers below indicate the relative amount of ANT transcript in the transgenic plants (determined using densitometry) compared with the level observed in a wild type plant .

L3	L4	W.T.
0.91	0.85	1.00

FIG.6.35. Northern blot analysis of total RNA isolated from leaves of transgenic plants transformed with a ST-LS1pANTR construct. W.T. is RNA from an untransformed control plant. 5 µg of total RNA was loaded in each lane. The probe used was the B subunit NAD malic enzyme cDNA. Markers are the cytosolic ribosomal RNAs from potato.
The numbers below indicate the relative amount of ANT transcript in the transgenic plants (determined using densitometry) compared with the level observed in a wild type plant.

L3	L4	W.T.
5.00	4.20	1.00

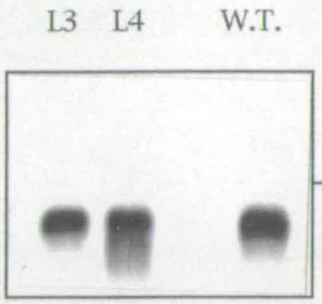


FIG.6.34.

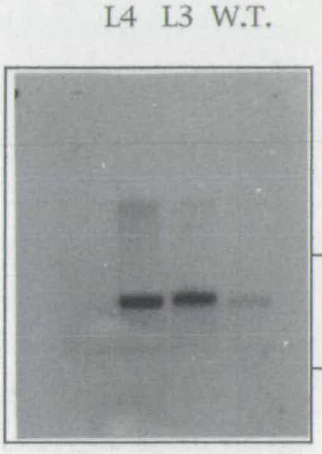


FIG.6.35.

6.9.5. Oxygen Uptake by Leaf Discs From Wild Type and Transgenic Plants

The transgenic plants containing the STLSIpANTR antisense construct regenerated did not show any phenotypic differences compared to wild type control plants. To investigate whether mitochondrial activity was affected in these transgenic plants leaf sections were placed in a leaf disc oxygen electrode to measure the rate of oxygen uptake in the dark. This oxygen uptake is due to mitochondrial respiration and provides a measure of mitochondrial activity. As shown in Table 6.1. the level of oxygen uptake in the transgenic plants was reduced to approximately two thirds of that measured in wild type control potato plants.

Plant	Control	L3	L4
Oxygen Uptake	0.145 0.151	0.109 0.106	0.107 0.097

TABLE 6.1. Oxygen uptake in the dark of leaves from wild type control plants and plants transformed with a ST-LS1pANT antisense construct. The oxygen uptake is measured in nmol O_2 $min^{-1}cm^{-2}$ leaf tissue. Values are two readings of separate leaves from each plant.

6.9.6. Visualisation of Mitochondrial Activity *in vivo*

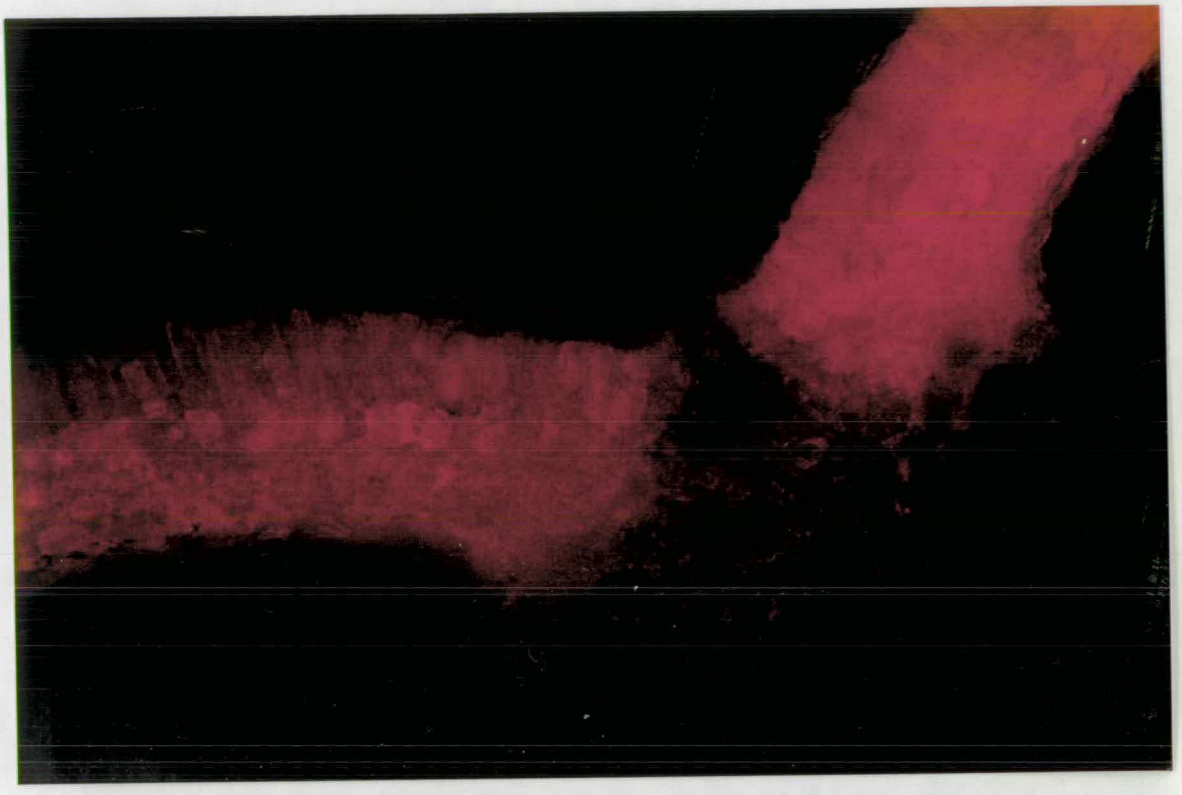
The results obtained with the leaf disc oxygen electrode suggested that mitochondrial function had been altered in the transgenic plants. Fluorescent dyes that are sensitive to membrane potentials have been used as a method of visualising mitochondrial activity *in vivo* (Matzke and Matzke, 1986; Liu *et al.*, 1987). These cationic dyes accumulate in organelles that have a membrane potential, such as mitochondria in which oxidative phosphorylation is occuring. As the level of ANT protein had decreased and mitochondrial respiration was reduced in the transgenic plants potential sensitive dyes were used to visualise mitochondrial function *in vivo*. A variety of different dyes have been reported to stain mitochondria, including rhodamine 123, 3-3'-diheptyloxacarbocyanine iodide ($DiOC_7(3)$) and 3-3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$). These three dyes were used to stain both leaf tissue sections and protoplasts isolated

FIG.6.36A, B. Transverse leaf section from transgenic potato plant L4, transformed with construct ST-LS1pANTR, stained with $DiOC_6(3)$ to visualise mitochondrial activity. (Magnification x100).

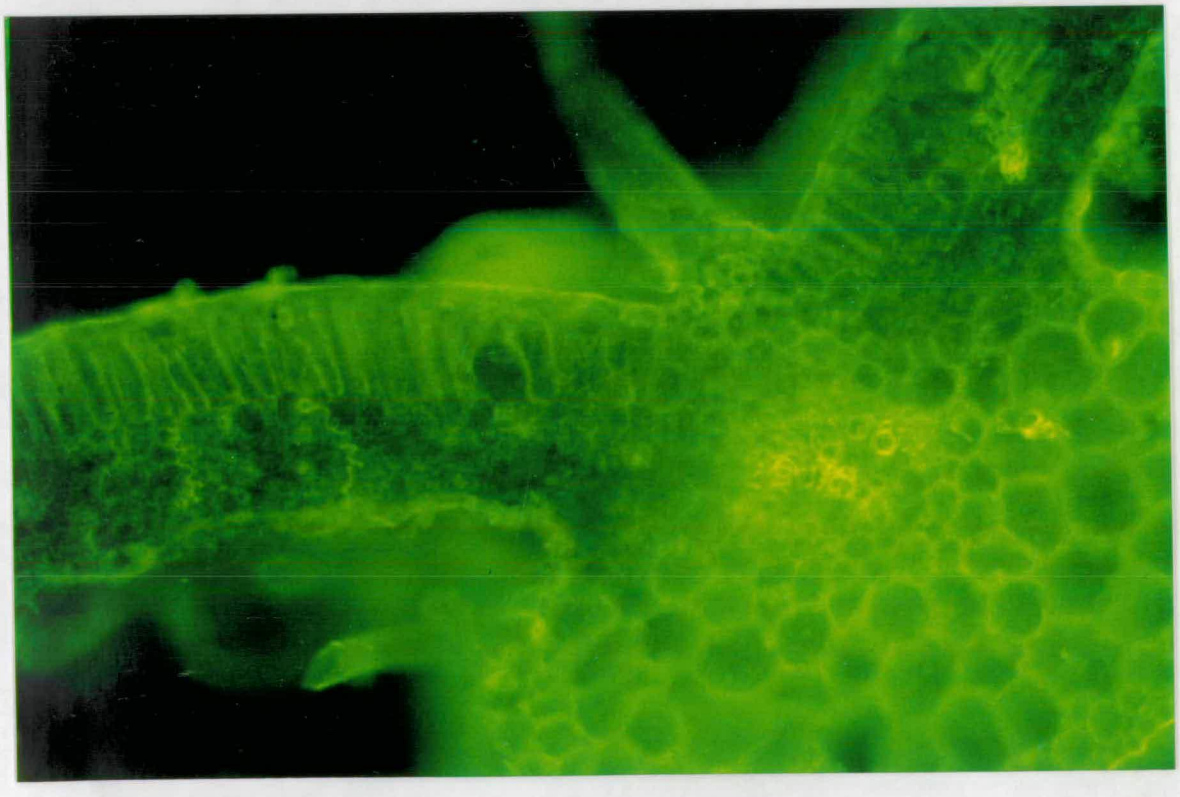
A. Autofluorescence of chlorophyll in the leaf section is confined to the mesophyll tissue, resulting in the intense red staining.

B. Variation in the flourescence of the leaf section veiwed at the excitation wavelength of the dye. Low fluorescence is observed in the mesophyll tissue of the leaf compared to the greater fluorescence in the midrib of the leaf section. The dye also appears to be taken up in tissue such as the vascular bundle.

a.



b.



from leaves of potato plants. It was not possible to successfully stain any preparation of protoplasts. When the dyes were applied to transverse sections of leaf tissue from the transgenic plants no staining was obtained with rhodamine 123 or $DiOC_7(3)$, but fluorescence was observed with $DiOC_6(3)$, (Fig6.36a+b). There is a large difference in the observed fluorescence between different tissues within the leaf section (Fig.6.36b.). The spongy mesophyll and palisade mesophyll cells did not stain very intensely with the dye (in the same cells where chlorophyll, visualised by its autofluorescence in Fig.6.36a., was very abundant), whereas the main vein and midrib showed a high level of fluorescence. Intense fluorescence was also observed in the epidermis and the vascular bundle.

6.10. Discussion

In most of the regenerated plants examined the introduction of either sense or antisense ANT transgenes either did not result in a detectable change in the level of ANT transcripts or those changes in the level of ANT expression that were detected did not result in a measurable difference in the accumulation of ANT protein. However, in transgenic plants resulting from the introduction of an antisense transgene linked to the ST-LS1 promoter, which is active in green tissues, reduced expression of the endogenous ANT and a reduction in the accumulation of ANT protein was observed in these tissues. ANT gene expression was not altered in tissues where the antisense gene was not likely to be expressed. The rate of mitochondrial respiration and the expression of another nuclear encoded mitochondrial gene was altered in these plants. The preliminary evidence of Fig.6.36 suggests that there maybe a difference in mitochondrial activity in these plants. However control experiments with a wild type plant are required.

6.10.1. Effects of ANT Over Expression

When plants transformed with a transgene designed to constitutively express a heterologous maize ANT gene (construct CAMmANTF) were analysed, no change in the expression of the endogenous ANT was detected. Although expression of the introduced transgene was detected, it varied between different

transformants and did not result in a detectable change in the amount of ANT protein. Variation in the level of transgene expression between individual transformants has been observed in other experiments (Willmitzer, 1988), and is normally explained by neighbouring sequences of the random chromosomal insertion site exerting a dominant 'position effect' on the transgene.

Some researchers have found that over expressing plant genes can have no effect e.g. van der Krol *et al.* (1990) increased the expression of genes for floral pigmentation, but this did not alter flower colour. However, the over expression of heterologous genes in plants can result in the expresion of the endogenous gene being induced, e.g. the soybean glutamine synthetase gene induces the expression of the homologous tobacco gene in leaves, presumably in response to changing metabolic activity in the plant (Hirel *et al.*, 1992).

The phenomenon of 'co-suppression', whereby the introduction of sense transgenes into a plant reduced gene expression (observed by van der Krol *et al.* (1990), Napoli *et al.* (1990), Smith *et al.* (1990), Tieman *et al.* (1992) and Gottlob-McHugh *et al.* (1992)) was not observed in the transgenic plants generated in this study.

Similarly, when plants were regenerated with a construct designed to constitutively overexpress the potato ANT gene (construct CAMpANTF) a range in the steady state levels of ANT transcripts was observed. In some plants ANT expression was elevated to almost twice that found in the control, although in other plants there was no measurable difference in expression. Those changes in the level of gene expression that were observed did not alter the amount of ANT protein detected in these plants.

Expression of the maize ANT sense transgene under the control of the light induced promoter (construct ST-LS1mANTF) was detected in leaf tissue of the transgenic plants. The expression of the endogenous ANT gene was elevated in these plants, unlike plants transformed with the CAMmANTF transgene. Why this transgene should have this effect on the expression of the endogenous ANT gene is unclear. The

expression of the transgene in these plants using the tissue specific ST-LS1 promoter could be greater than that achieved with the constitutive CaMV 35S promoter, and this results in increased endogenous gene expression. These increased levels of expression were not reflected in the accumulation of ANT protein in the plants, as no detectable increase or decrease in the level of protein was observed.

As the accumulation of ANT protein was not affected, despite the increase in the steady state level of ANT mRNA observed, factors acting at the translational or post translational level, in addition to transcription, may play a role in regulating the synthesis of ANT protein.

6.10.2. Down Regulation of ANT

The introduction of a heterologous antisense maize ANT transgene (construct CAMmANTR) resulted in the reduction of ANT mRNA levels in most of the plants examined to a level of 40% of that observed in the wild type control plants. The inhibition did not appear to correlate with the number of transgenes in the plant genome, as expression of the endogenous gene was reduced by similar amounts in plants with one or four T-DNA insertions. Despite reductions in ANT mRNA levels of more than 50%, these did not result in a detectable reduction in the amount of ANT protein in these plants. The antisense transcript was detected in leaves of some of these transgenic plants, but only at very low levels. The expression of this transcript also appeared to vary between transformants. The level of antisense gene expression did not correlate with the reduction of ANT expression observed, as the greatest antisense expression was seen in plant B3, but there was no change in ANT expression in this plant.

The homologous ANT antisense transgene, under the control of the constitutive promoter (construct CAMpANTR), produced similar effects to those obtained with the heterologous maize antisense gene. The level of inhibition of the endogenous transcript level was found to vary. ANT expression was unchanged in one plant, whilst in plants with the greatest inhibition ANT expression was reduced to approximately 34% of the control level. As in plants transformed with the maize

antisense transgene these differences in gene expression were not reflected in reduced levels of ANT protein.

The constitutive expression of the homologous or heterologous ANT antisense genes down regulated the endogenous ANT gene (by up to 66 % compared to the wild type), but did not alter the accumulation of ANT protein. The variation in the degree of inhibition observed was presumably due to the position effect of T-DNA integration into the plant genome. Müller-Röber *et al.* (1992) have regenerated a range of transformed potato plants with an antisense ADP-glucose pyrophosphorylase (AGPase) transgene. In some of these plants the expression of AGPase was significantly reduced (and no antisense transcript was identified), but this did not correlate with a reduction in AGPase protein. Tieman *et al.* (1992) classified the tobacco transformants they generated with an antisense construct to pectin methylesterase (PME) into different groups, including classes where the protein was still present, but no target transcript or antisense transcript could be detected, and in which the accumulation of mRNA and protein was unaffected, but no antisense transcript could be detected. Despite these variations all these plants had been selected as they showed reduced PME activity.

6.10.3. Down Regulation of ANT by a Light Inducible Promoter

The most dramatic result of altering endogenous ANT expression was seen in the plants transformed with the ST-LS1 potato ANT antisense construct. The two plants transferred to the greenhouse were the only shoots obtained with this construct that rooted on kanamycin selection, but Southern blot analysis showed that both plants were the result of the same transformation event. Steady state ANT transcript levels were dramatically reduced in the leaves of these plants, although they were not completely eliminated. As with the other plants transformed with potato ANT antisense constructs it was not possible to detect the antisense transcript in these plants. The reduction in ANT expression was greater in these plants than in any of the other

antisense plants regenerated. It was not possible to detect the ANT protein in the leaves of these plants by immunoblotting.

Whilst the low transformation efficiency prevents any definite conclusions being drawn, a possible reason for the amount of ANT protein in most of the transgenic plants being unaffected is that altering ANT expression is lethal in most cases. The presumption is that only plants in which the reduction in ANT expression is not severe enough to alter mitochondrial function are regenerated. The transformants regenerated with the constitutively expressed transgenes are those in which the changes in ANT expression do not affect ANT protein levels in mitochondria. This could explain why the only transgenic plants regenerated with patatin transgenes show no change in ANT expression or protein levels. The patatin promoter is induced by high (7%) levels of sucrose (Rocha-Sosa *et al.*, 1989). Sucrose is present in all the tissue culture and plant regeneration media, although only at 3%. This may induce enough transgene expression to prevent plant regeneration in most cases.

Most successful antisense experiments use the CaMV 35S promoter to regulate the antisense gene as this is thought necessary to produce an excess of antisense to sense transcripts for successful inhibition. This excess of antisense transcripts should permit their detection in many experiments, but has not been reported. Where the interaction between the sense and antisense transcripts occurs and how it is mediated is unclear. No experiments were performed to detect RNA duplexes but as no antisense transcripts could be detected in these plants (or any transformants with the homologous antisense gene) the site of action may be in the nucleus, preventing either the export or promoting degradation of the ANT mRNA. Alternatively it has been suggested that antisense transcripts are less stable than endogenous transcripts and they are degraded to mediate their effects through many small antisense molecules that individually interact with the mRNA, causing its breakdown or preventing translation (van der Krol *et al.*, 1991). It is conceivable that there is a 'threshold' level of mRNA required for protein synthesis. If the level of

mRNA is below this threshold then efficient translation of mRNA and accumulation of protein does not occur.

The effective antisense transgene was introduced under the control of a light inducible promoter which is active in photosynthetically active cells (Stockhaus *et al.*, 1989). Although it was not possible to detect the antisense transcript in the leaves of these plants, if the transgene followed its predicted expression pattern then ANT expression should not be affected in non photosynthetic tissues. This was confirmed by northern blot analysis of total RNA extracted from the roots of the transgenic plants. No difference in the steady state levels of ANT expression was observed in these plants compared to a wild type control, suggesting that the transgene was not being expressed in root tissue.

6.10.4. Consequences of Reducing the Levels of ANT Protein

The amount of ANT protein in leaves from these transgenic plants was dramatically reduced, to below the limits of detection by immunoblotting. Whilst the plants were phenotypically similar to control plants grown under the same conditions, the rate of oxygen uptake in the dark by leaves from these plants, when measured in a leaf disc oxygen electrode, was substantially reduced (by one third) compared to a wild type control. As this oxygen uptake is due to mitochondrial respiration, it would appear that reducing the level of ANT protein has altered mitochondrial activity.

The adenine nucleotide translocator mediates the export of ATP generated in mitochondia to the cytosol and the import of ADP in exchange. Electrons do not usually flow through the electron transport chain to oxygen and generate a proton gradient unless the F_1-F_0 ATP synthase is phosphorylating ADP to ATP. However, if the amount of ANT protein in the mitochondrial inner membrane is greatly reduced in these transgenic plants, the activity of the ATP synthase may be inhibited. In such a scenario, where electron transport is reduced and a potential gradient is not maintained, the rate of oxygen uptake by the mitochondria would be reduced.

ANT is the major transporter of adenine nucleotides across the inner mitochondrial membrane and the only transporter that allows a one for one exchange of ADP and ATP. Another mechanism for ADP or ATP uptake, insensitive to CATR, has been reported (Abou-Khalil and Hanson, 1979a; b). This mechanism enables the one way transport of adenine nucleotides, as opposed to the one for one exchange mediated by ANT and could play a role in maintaining mitochondrial function in the absence of a functional ANT. This transport process requires exogenous phosphate and Mg^{2+} for ADP uptake, although there is no consistent stoichiometry between phosphate and ADP uptake.

A lower mitochondrial respiration could result in a reduced mitochondrial membrane potential. It is possible to visualise this membrane potential *in vivo* using fluorescent dyes. Preliminary experiments were carried out with the dye $DiOC_6(3)$ to examine the effect of antisense transgene expression on mitochondrial membrane potential. Low levels of fluorescence were observed in the mesophyll tissue of leaf sections from the ST-LS1pANTR plants, indicating that the mitochondria in this tissue had a lowered membrane potential. In contrast the levels of fluoresence observed in leaf areas which did not contain many chloroplasts (as seen by the chlororphyll autofluoresence) were much higher. This may reflect the expression pattern of the introduced transgene, as ST-LS1 promoter expression correlates with the presence of chloroplasts. When leaf sections of potato plants transformed with a ST-LS1 GUS construct were examined by Stockhaus *et al.* (1989) the GUS staining was confined to the mesophyll section of the leaf (containing many chloroplasts), but not the midrib of the leaf (with few chloroplasts). Thus the antisense effect of mitochondria with reduced levels of ANT protein may be limited to the leaf mesophyll tissue of the transgenic plants, whilst the mitochondria in the midrib are unaffected. However, if the mitochondria in the main vein contain unaltered levels of ANT protein, this was still not enough to detect by immunoblotting of total leaf protein. The dye DiOc6(3) stains mitochondria (Matzke and Matzke, 1986), but as can be seen in Fig.6.36b. the dye appears to have stained other structures in the leaf, such as the

although control experiments with leaves from wild type plants are required to confirm these results.

lignified regions of the vascular bundle and other organs in the leaf such as the epidermis. Thus the intense staining seen in the main vein is not only due to mitochondrial activity, but also due to other structures in the tissue. However this technique offers further possibilities to visualise mitochondrial activity *in vivo* and investigate the consequences of introducing an antisense ANT transgene.

Although the ANT protein is localised in the mitochondrial inner membrane, the gene is encoded in the nucleus. Rodermel *et al.* (1988) found that down regulating the small subunit of RUBISCO did not affect the expression of the chloroplast encoded large subunit of RUBISCO. Sonnewald *et al.* (1992) inhibited expression of ADP-glucose pyrophosphorylase, but found that expression of most of the other enzymes in the starch biosynthetic pathway (except sucrose phosphate synthase) was not altered. The increase in expression of sucrose phosphate synthase was correlated with a rise in the amount of sucrose and other soluble sugars in tubers from these plants. Mitochondrial activity, as measured by mitochondrial oxygen uptake and membrane potential, appears to have been affected by the reduction in ANT protein, so the expression of other genes encoding mitochondrial proteins could also be altered in response to this change. NAD malic enzyme is another nuclear encoded mitochondrial protein, and plays a role in maintaining the TCA cycle. It has been linked with the functioning of the alternative electron transport pathways (the rotenone insensitive by-pass and the alternative oxidase) in the inner mitochondrial membrane (Rustin *et al.*, 1980). This protein is encoded by two mRNAs, and expression of both these mRNAs was found to be substantially elevated in the transgenic plants, by almost five fold in each case, compared to the wild type. (Data not shown) The possible significance of this increase in expression of NAD malic enzyme will be discussed in further detail in Chapter. 7.

CHAPTER 7
DISCUSSION AND FUTURE DIRECTIONS

7.1. Introduction

The results presented in this thesis indicate some of the problems encountered in applying molecular biology to the study of plant biochemistry. The ability to modify the expression of a specific gene and analyse the effects of such changes on gene function can potentially reveal much about the role and importance of the protein in an organism. The production of energy, as ATP, in eukaryotes is performed in specialised organelles, mitochondria (as well as by chloroplasts in plants). Metabolism in the cytosol depends upon the exchange of ADP for ATP from the mitochondrion and many metabolic intermediates produced in mitochondria.

The adenine nucleotide translocator has been intensively characterised at the biochemical level (reviewed by Klingenberg, 1989), and more recently gene structure and expression have been characterised in a number of diverse organisms (as described in Sec.1.5 and 1.6.). In many organisms it is encoded by multiple genes, which are differentially expressed (for example, in yeast and humans), presumably in response to functional and developmental factors. In yeast the disruption of ANT genes results in respiratory deficient mutants. When the control coefficient of ANT is determined (as a measure of the control ANT exerts over mitochondrial oxidative phosphorylation), in most cases it is found to exert considerable control over mitochondrial oxidative phosphorylation. This suggests that ANT plays a pivotal role in controlling mitochondrial function. The intention of the work described in this thesis was to use molecular biological techniques to create transgenic plants with altered expression patterns of ANT mRNA. It was hoped that these changes would result in plants with enhanced or reduced levels of functional protein and enable the investigation of the effects of these changes. By using tissue specific promoters to control the expression of the transgene, as well as a constitutive promoter, it was hoped to investigate the role of ANT during specific developmental changes or in response to environmental conditions.

7.2. Summary of Results

7.2.1. The Organisation and Expression of the ANT Gene in Potato

The genomic organisation of ANT in potato has proved to be more complex than that found in maize (Bathgate *et al.*, 1989). As in maize, two genes encoding ANT have been identified by cDNA cloning (Emmermann *et al.*, 1991; Winning *et al.*, 1992), but the genomic organization of these genes appears to be more complex and multiple bands hybridise in Southern blot analysis of the tetraploid potato DNA. This complexity is still apparent in the genome of a dihaploid progeny of Desiree, as most of the bands identified in the tetraploid are conserved in the dihaploid.

The steady state level of ANT transcripts in a range of potato tissues was found to vary, suggesting that expression is regulated during plant development. When a probe specific to one of the two cDNAs was used no evidence to indicate any difference in tissue specific expression between the two cDNAs was obtained. However the intensity of hybridisation of the specific probe suggests that only a minor constituent of the total ANT expression. A similar tissue specific pattern of ANT expression has been observed in maize (Day, 1992) where the variation in expression was found to be almost 10 fold, whereas in potato the difference was only 5 fold. The pattern of expression is similar in both plants, with expression being elevated in tissues where mitochondrial oxidative phosphorylation is the primary source of energy for the cells, or the tissue is metabolically active, and reduced in tissues that are photosynthetically competent. These results appear to contradict another study of ANT expression in potato, where it proved possible to distinguish another larger transcript in root tissue and ANT expression in leaf tissue was much greater than measured in this study (Emmermann *et al.*, 1991). The expression of ANT in leaf tissue did not appear to be affected by changes in light during a diurnal cycle.

Measurement of the control coefficient of ANT in mitochondria from potato tubers suggests that it plays a significant role in the control of mitochondrial oxidative

phosphorylation. - The control coefficient of ANT does not change when the tuber tissue is aged and the non phosphorylating mitochondrial alternative oxidase pathway is induced. These results suggest that ANT is a suitable target gene to investigate the regulation of mitochondrial function.

7.2.2. Design of Transgenes to Over-express or Inhibit the Expression of ANT

Transgene constructs were created to increase or reduce the expression of ANT by introducing either sense or antisense copies of a heterologous (maize) or homologous (potato) ANT cDNA into transgenic potato plants. The genes were under the control of either a constititutive or tissue specific promoters so that gene expression could be modified in either the whole plant or specific tissues. The transgenes were introduced into a binary vector for introduction into potato by *Agrobacterium* mediated transformation.

7.2.3. Potato Transformation

The transformation of potato using *Agrobacterium tumefaciens* has not proved a reliable and efficient process in this laboratory. Although a number of transformation protocols were attempted, using a variety of tissues as the source of explants, under a range of conditions, it was not possible to generate a large number of transgenic plants with all the constructs prepared. The reasons for this low transformation efficiency are not clear, but have been discussed in Chapter 5.

7.2.4. Analysis of Transgenic Plants

The overexpression of either the heterologous or homologous ANT transgene did not result in any detectable change in the level of ANT protein. The introduction of either homologous or heterologous antisense ANT genes, under the control of the constitutive CaMV 35S promoter, reduced the expression of the endogenous gene, but did not alter the accumulation of protein. The introduction of sense or antisense transgenes with the homologous ANT gene under the control of the patatin tuber specific promoter caused no change in the

expression of ANT or the levels of protein detected in either leaf or tuber tissue.

However, plants transformed with an homologous potato ANT antisense construct under the control of the light induced ST-LS1 promoter showed greatly reduced levels of ANT mRNA in their leaves. The level of ANT protein in these leaves was below the detection limits of immunoblotting. ANT expression in roots of these plants, a tissue not exposed to light, was not affected. The rate of oxygen uptake in the dark (due to mitochondrial activity) was reduced in these plants compared to that of a wild type control. The expression of NAD malic enzyme was elevated in leaf tissue of these plants. Mitochondrial activity, as determined by dyes sensitive to membrane potentials, appeared to be inhibited in the mesophyll tissue of leaf sections from these plants.

7.3. The Consequences of Introducing ANT Transgenes

7.3.1. Potato Transformation

Agrobacterium mediated transformation is, by its nature, a difficult and not well understood process even in the small number of plant species which can be regularly transformed. It involves the interaction of two different organisms, the infecting *Agrobacterium* and the plant host, and the resulting transfer of genetic information between them. Many factors can affect the success with which this process occurs and the ease with which transgenic plants are regenerated. The efficiency of transformation can vary between different cultivars of a plant species, as well as with different *Agrobacterium* genotypes (Hobbs *et al.*, 1989; Davies *et al.*, 1991). The potato was chosen as the model system for this investigation as amongst other factors it was reported to be amenable to *Agrobacterium* mediated transformation and protocols have been optimised for the cultivar Desiree, in contrast to many other potato cultivars which have proved recalcitrant (Sheerman and Bevan, 1988).

It was possible to regenerate untransformed plants from control tissue explants, not under antibiotic selection, with all the protocols attempted, except that of Visser *et al.* (1989). The low transformation efficiency obtained with all the protocols used is

illustrated by the inability to regenerate transgenic plants even when a binary vector without a transgene inserted into the polylinker was used in the transformations as a control. The transformation of tuber discs proved especially recalcitrant, as although it was possible to generate shoots at low frequency from discs on selection, these shoots did not root on selective medium and were not transgenic. Altering one of the factors reported to affect efficiency of transformation, by using tubers of different ages, did not improve the transformation rate.

Changing the source of the explants for transformation, from tuber discs to leaf and stem pieces led to an increase in the efficiency of regeneration of transgenic shoots. Untransformed escapes, which failed to root on selective rooting medium, still appeared. Such escapes were apparent when high levels of selection (100 mg/l kanamycin) were applied, but this appeared to be too stringent to allow regeneration of transgenic plants. The most effective method of actually regenerating transformants was to reduce the selection stringency to 50 mg/l kanamycin. Using this lower level of selection it was possible to regenerate transgenic plants, although a larger number of escapes were also regenerated. However untransformed shoots failed to root on rooting medium with 50 mg/l kanamycin, but transgenic shoots did root on this selection. Stiekema *et al.* (1988) reported that as little as 10 mg/l kanamycin would successfully prevent non transformed shoots from rooting, but that transgenic shoots would root on medium with kanamycin concentrations as high as 100 mg/l. Altering this parameter seemed to have the greatest effect on increasing transformation efficiency. Higgins *et al.* (1992) report that selection on 50 mg/l kanamycin was optimal compared to selection at 25 or 100 mg/l. Why reducing selection pressure like this should alter the ability to regenerate transgenic plants is not clear. They suggest that selection is a compromise between providing a competitive advantage to transformed cells and killing those with a relatively low expression of the NPT II gene. Higgins *et al.* (1992) reported that a large number of transformation events in potato (as visualised by GUS staining) were not converted into transgenic plants. The early stages in

regeneration, when transformed cells are converted into primordia, would appear to be inefficient and the major barrier to achieving high efficiency potato transformation. Recently Yenofsky *et al.* (1990) have reported that there is a mutation in some neomycin phosphotransferase genes (the selection marker in transformation vectors such as BIN19 that confers kanamycin resistance in plants) that reduces the activity of the gene resulting in these plants being less resistant to antibiotics. Other attempts to increase the transformation efficiency, such as using acetosyringone to promote infection by *Agrobacterium*, did not appear to alter the success rate. It has been reported that even the time of year, as well as the physiological state of the tissue explants, can affected the transformation efficiency (Visser *et al.*, 1989).

7.3.2. Transgene Expression

The low transformation efficiency makes it difficult to draw conclusions from the results of the transformations. Most of the plants regenerated showed either no change in gene expression or the changes in expression that were observed did not alter the accumulation of ANT protein. Whether this is because only plants in which protein accumulation was not significantly affected were regenerated is not clear.

The expression of the transgenes confirms a number of observations made by other researchers when introducing transgenes into plants, including the 'position effect', where the level of expression of the introduced transgene shows great variation between different transformants (for example, van der Krol *et al.*, 1990). The reason for this effect is not clear, but is assumed to be due to the adjacent DNA sequences or chromosomal structure at the site of insertion influencing the expression of the introduced gene. This position effect can be seen most clearly in plants transformed with maize ANT sense transgenes. The levels of transgene expression with the constitutive CaMV 35S promoter revealed a large variation between different transgenic plants, with no expression being detectable in some plants. Such an effect was also observed in plants where the transgene was under the control of the light induced ST-LS1 promoter, and the

transgene expression was highly variable. The variation in the level of inhibition of the endogenous ANT gene observed in different transformants harbouring the same antisense construct would also appear to support the 'position effect' argument. The phenomenon of 'co-suppression', where the introduction of extra copies of a sense gene results in the inhibition of endogenous gene expression was not observed.

The heterologous maize ANT cDNA seemed to be as efficient an antisense agent as the homologous potato cDNA, when under the control of the constitutive CaMV 35S promoter. Both antisense transgenes produced a range of ANT mRNA inhibition to a maximum level of 30 % of the wild type control. However even this level of inhibition did not result in a detectable change in the accumulation of ANT protein. The percentage similarity between the maize and potato ANT cDNAs is approximately 67%, which is lower than that of other heterologous genes used as antisense genes in other systems.

The number of transgenes integrated into the genome of the transgenic plants does not appear to affect the level of expression of the transgene. Plants with multiple copies of the transgene did not necessarily exhibit either higher levels of sense transgene expression or reduced levels of endogenous gene expression when antisense transgenes are introduced. This is similar to results obtained by van der Krol *et al.* (1988) and Visser *et al.* (1991) (where transgene expression was not related to transgene copy number) but in contrast to the correlation between transgene number and inhibition of expression found by Delauncy *et al.* (1988).

When the abundance of antisense message was investigated it could only be detected in plants expressing the heterologous maize ANT antisense gene. In these plants the gene was under the control of the CaMV 35S promoter. The abundance of this transcript was very low, and it was scarcely detectable when a double stranded probe specific to maize ANT was used, even in the plants where the expression of the antisense transcript appeared to be greatest. Using a strand specific probe for the potato antisense transcript it was not possible to detect antisense

transcripts in any of the plants investigated. The amount of antisense transcript does not necessarily correlate with the level of target gene inhibition observed e.g. whilst van der Krol *et al.* (1988) were able to detect an antisense transcript, Rodermel *et al.* (1988) could only detect the antisense transcript at very low abundance. Hamilton *et al.* (1990) were not able to detect an antisense transcript when the level of target sense transcript was greatly reduced, whilst Vaucheret *et al.* (1992) could not detect either sense or antisense transcripts. Hofgen and Willmitzer (1992) and Stockhaus *et al.* (1990) found a range of antisense transcript levels on screening a number of different transformants, including some where no antisense transcript was detected, although the steady state transcript levels of the target gene were inhibited.

As no antisense transcript is detected in any of the plants (except at very low level with the heterologous maize ANT cDNA) these results suggest that the site of antisense inhibition is in the nucleus, the antisense RNA interfering with the synthesis or processing of the target transcript and preventing export of mRNAs to the cytoplasm. If antisense duplexes are formed in the cytoplasm they may be rapidly degraded, although RNase protection assays were not performed to detect such duplexes. There may be a 'threshold' level of those mRNAs that do enter the cytoplasm below which translation is inefficient, which would explain why ANT mRNA levels could be reduced without affecting accumulation of protein. Another possible mechanism for preventing mRNA translation is that the antisense transcript is unstable in the cytoplasm and degraded to antisense RNA fragments which interact with the ANT mRNA to enhance the antisense effect (as suggested by van der Krol *et al.*, 1991).

7.3.3. Plants With Reduced Levels of ANT Protein

The only plants where an effect of the antisense ANT transgene was seen at both the RNA and protein level were those transformed with the transgene under the control of the ST-LS1 promoter. The level of ANT transcripts was greatly reduced in these plants and the level of ANT protein, in leaves, below the level of detection by immunoblotting. Although other plants were

generated in which the level of ANT expression was reduced these differences did not apppear to affect the accumulation of the protein. It might well be that the use of the tissue specific light inducible ST-LS1 promoter provided sufficient quantities of antisense RNA in the leaf tissue to reduce the level of the target ANT message below a threshold such that translation became inefficient, or that the antisense message is no longer intact, but is present in the cytosol as many very small fragments that affect the translation of ANT mRNA. The expression of the transgene seemed to follow the expected pattern as no change in ANT expression could be detected in the roots of these plants. Whilst these plants appeared to share the same phenotypic characteristics as control wild type plants, the rate of oxygen uptake in the dark was reduced in these plants in comparison to the wild type.

If the amount of ANT protein in the mitochondria has been reduced so that it is below the levels of detection and the function of these mitochondria in generating energy has been inhibited, how do these plant cells survive? Glycolysis in the cell cytosol is capable of generating ATP, but only yields 4 molecules of ATP per molecule of glucose, whereas oxidative phosphorylation in the mitochondrion can generate an extra 32 ATP molecules per molecule of glucose. Thus, if a cell has to rely solely on glycolysis to meet its energy requirements then the supply of energy will be greatly reduced (mitochondrial toxins such as cyanide are often lethal). In green cells glycolysis can use fixed carbon in the form of triose phosphates, exported from chloroplasts by the phosphate translocator, to increase the amount of ATP and NADH generated through this pathway (Flügge and Heldt, 1984). As the inhibition of ANT appears to be tissue specific, ATP may be being supplied to cells in these tissues from other tissues in the plant, where presumably the mitochondria are functioning correctly. Another possible source of ATP during the light, may be from the chloroplast. In the light chloroplasts can generate ATP by photophosphorylation in the thylakoid membranes. This ATP is primarily utilised within the plastid, but an ATP/ADP translocator has been identified, although it functions very slowly in exporting

ATP (Heldt, 1969; Heber and Heldt, 1981). However they reported that inhibiting photosynythesis had little effect on cytosolic ATP/ADP ratios, although inhibiting mitochondrial oxidative phosphorylation did (Stitt *et al.*, 1982) suggesting that chloroplasts are not the major sources of ATP for cytosolic reactions in normal plants. Chloroplasts in the leaves of transgenic plants with reduced ANT may export more ATP to compensate for reduced mitochondrial function.

7.3.4. Increase in the Expression of NAD Malic Enzyme

The steady state level of expression of NAD malic enzyme was greatly increased (five fold) in the transgenic plants compared to the wild type control. NAD malic enzyme is ubiquitous in plant mitochondria, but its role is poorly understood in most plants. It catalyses the reversible decarboxylation of malate in the mitochondrial matrix:

$NAD^+ + malate <--> pyruvate + CO_2 + NADH$

It has been suggested that it has an anaplerotic role (a 'filling up' function), by maintaining the level of TCA cycle intermediates (Palmer, 1976). The reaction catalysed by NAD malic enzyme, the reversible decarboxylation of malate, compensates for the drain on the TCA cycle as intermediates are removed for biosynthesis. Rustin *et al.* (1980) have suggested that under certain circumstances NAD malic enzyme is linked to the rotenone insensitive and cyanide insensitive (the alternative oxidase) pathways. Malate oxidation in mitochondria has better access to the alternative oxidase than other substrates (Gardestrom and Edwards, 1984), and that NADH produced by malic enzyme when malate is oxidised may or may not result in ATP production, depending on its fate (Lance *et al.*, 1985). The rotenone insensitive NADH dehydrogenase is able to bypass complex 1 of the electron transport chain without translocating protons across the inner mitochondrial membrane (Møller and Palmer, 1982). The alternative oxidase in the inner mitochondrial membrane is non phosphorylating as it does not generate a potential gradient (Moore and Bonner, 1982). Bahr and Bonner (1973) put forward the hypothesis that the alternative oxidase

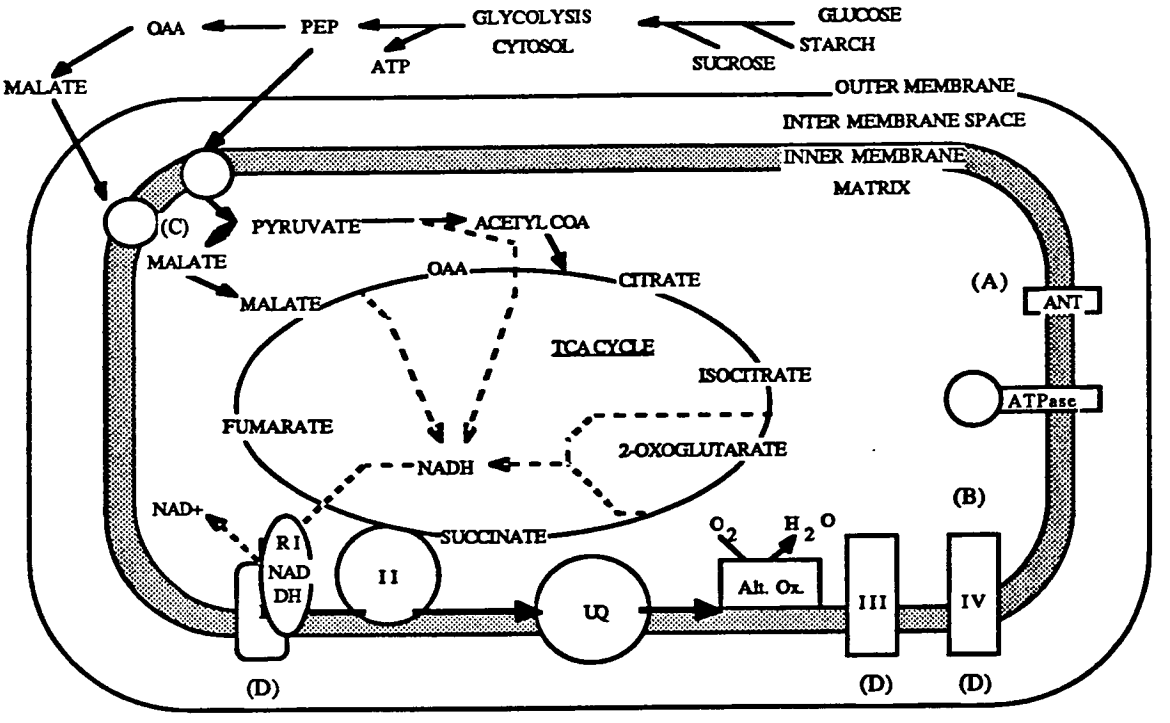


Fig.7.1. Changes in mitochondria with reduced amounts of ANT protein. The reduction on the amount of ANT protein (a) in mitochondria from leaves of transgenic plants with an light induced antisense ANT transgene results in:

(b) a decrease in the respiration rate of the mitochondria,

(c) an increase in the expression of NAD malic enzyme,

(d) no membrane potential is observed.

These observations suggest that the function of the mitochondria has been altered. If ANT is not present the ATP synthase is not as active and a proton gradient is not generated. The rotenone insensitive bypass of complex 1 and the alternative oxidase are active in association with the TCA cycle to allow production of biosynthetic intermediates. The activity of NAD malic enzyme is increased to maintain the TCA cycle.

allows the mitochondrial substrate oxidations to occur that are required to produce carbon skeletons for biosynthesis. If these two alternative pathways are activated it is possible to envisage a non-phosphorylating pathway which bypasses the phosphorylating pathway in mitochondria and maintains TCA cycle turnover (Lance *et al.*, 1985; Palmer and Ward, 1985).

If ANT is not present or is reduced in activity in these mitochondria then the function of the ATP synthase is reduced. The proton gradient that would be utilised by the ATP synthase is reduced. Oxygen consumption by these mitochondria is lowered. The non phosphorylating alternative oxidase is active to maintain the TCA cycle feeding biosynthetic intermediates to the cytosol, and NAD malic enzyme activity increases to allow the operation of the TCA cycle (summarised in Fig.7.1).

7.4. Future Directions

7.4.1. Are ANT Transgenes Lethal?

The very low rate of transformation achieved with potato makes it difficult to draw conclusions about the results of introducing sense or antisense ANT transgenes into potato. In the majority of the plants regenerated, either the expression of ANT mRNA was not altered or the changes in expression levels did not affect the accumulation of ANT protein. This may be because the introduction of ANT transgenes, especially constitutively expressed ones, is lethal to the plant and the regeneration of shoots only occurs if the transgene expression does not result in inhibition of the endogenous genes. The only plants that did show any difference in ANT protein were those in which the transgene was under the control of a light induced tissue specific promoter. By limiting the reduction of ANT protein in mitochondria to a specific tissue, leaves, plant regeneration may be possible as functional mitochondria are present in other tissues. To investigate this further a more efficient method of plant transformation would need to be used. *Agrobacterium tumefaciens* mediated potato transformation has been reported to be highly efficient and reproducible, but this has not been the case in this laboratory. Using a different strain of *Agrobacterium*

may increase the transformation efficiency, as the virulence of different strains can vary with the genotype of the plant to be transformed. Alternative methods of introducing transgenes into potato, such as *A. rhizogenes* mediated transformation (Ooms *et al.*, 1985, 1986) or biolistics may reveal more about the effect of introducing ANT transgenes on plant regeneration. A more effective strategy to investigate this possible lethal effect may be to use another plant species as the model system. A number of other plant species can be transformed with *Agrobacterium tumefaciens* and are regularly used as model systems for investigating effects of introduced transgenes. Probably the most widely used model plant species is *Nicotiania tabaccum* (tobacco), which is easily transformed with *Agrobacterium tumefaciens*. However, at present neither ANT cDNA clones or genomic clones have been isolated from this species. In any investigation into the effects of ANT transgenes on regeneration efficiency the endogenous ANT genes from tobacco would have to be identified.

In this study tissue specific promoters were used to specifically regulate the expression of transgenes in transgenic plants. An alternative to using plant promoters to specifically regulate transgenes in plants has been described by Gatz *et al.* (1991). They have demonstrated that a Tn*10*-encoded tetracycline (*tet*) repressor-operator system from bacteria can function in stably transformed plants to regulate the expression of a suitably engineered CaMV 35S promoter. In tobacco plants a transgene under the control of this promoter can be induced by 0.1 mg/l tetracycline and transgene expression was induced within 30 minutes of the application of 10 mg/l tetracyline. Using such a system in which the expression of ANT transgenes could be precisely regulated would have great advantages in determining the effects of the transgene.

Whilst the introduction of ANT transgenes may be in general lethal, plant metabolism has proved to be remarkably flexible (or 'plastic'). Tobacco plants completely lacking pyruvate kinase, thought to be a key enzyme in the glycolytic pathway, were morphologically normal, with similar respiration rates (Gottlob-McHugh *et al.*, 1992). This illustrates the difficulty of attempting

to modify a single step in a pathway, which a plant may be able to overcome.

7.4.2. Analysis of ST-LS1 Mutant

The initial aim of this project was to use genetic engineering and the ability to transfer genes between organisms to create plants with altered patterns of ANT expression to investigate the function of ANT, eventually at the biochemical level. The ST-LS1 plants contain greatly reduced levels of ANT protein in their leaves, and preliminary investigation shows that mitochondrial function is changed in leaf tissue. Areas of future research using these plants will be considered, and certain points highlighted considering the results presented in the preceding chapters. The characterisation of these plants and the effect of reduced ANT protein levels await further investigation at many different levels, from the whole plant to isolated mitochondria.

The results of investigating transgenic tobacco plants with reduced levels of *rbcS* expression illustrate the amount of information about plant metabolism that can be obtained from the inhibition of expression of a single gene (Rodermel *et al.*, 1988: Quick *et al.*, 1991a, b; Stitt *et al.*, 1991).

7.4.2.1. Growth and Morphology of Transgenic Plants

The morphology of the transgenic plants and their ability to form tubers appeared to be unchanged compared to control plants grown under the same conditions. The consequences of altering mitochondrial function, and presumably reducing energy supply in the plant, may result in detectable changes in plant growth, such as the leaf area ratio (leaf area per g plant dry weight), or the number and size of tubers per plant. The reduction of RUBISCO in transgenic tobacco plants did not seriously affect plant growth until almost half the RUBISCO was removed (Quick *et al.*, 1991). However, mutations in mitochondrial genes in other plant species, such as non-chromosomal stripe in maize, result in reduced growth and altered leaf development as sectoring of normal and mutant leaf tissue occurs (Newton and Coe, 1984; Roussell *et al.*, 1991).

Mitochondrial mutations (often the result of rearrangements of mitochondrial DNA) are associated with cytoplasmic male

sterility (CMS) in which plants fail to produce functional pollen. CMS has been reported in over 150 plant species (including potato), but only studied in a few species (Kaul, 1988). The effect of modifying mitochondrial function in potato may result in the induction of male sterility. However, at present, no flowers have been formed on the transgenic plants and this possibility has not yet been investigated.

7.4.2.2. Ultrastructural Examination of Mitochondria

The adenine nucleotide translocator plays a pivotal role in linking two separate compartments in the cell and is one of the most abundant proteins of the mitochondrial inner membrane. The effect of reducing the amount of ANT protein on mitochondrial structure awaits investigation. Are there ultrastructural changes between mitochondria from tissues where the antisense gene is expressed and tissues where the transgene is not? Increases in the intramitochondrial adenine nucleotide content (and thus the involvement of ANT) of mammalian mitochondria immediately after birth have been implicated in the reduction of mitochondrial volume and the development of functional mitochondria (Valcarce *et al.*, 1988; Valcarce and Cuezva, 1991).

7.4.2.3. Effect on Chloroplast Structure and Function

Mitochondria are just one organelle containing a subset of a plant's metabolic machinery. One of the unique features of plants is that plant mitochondria in green tissue must interact with another organelle, the chloroplast. The specific inhibition of mitochondrial ATP synthesis (using low concentrations of oligomycin) caused a reduction in the rate of photosynthesis in protoplasts, although isolated chloroplasts were insensitive to such concentrations of inhibitor (Ebbighausen *et al.*, 1987; Kromer *et al.*, 1988). Mutations in genes encoding components of the mitochondrial electron transport chain result in defective chloroplasts in maize plants with non-chromosomal stripe (Rousell *et al.*, 1991). The reduction in ANT protein may result in a similar reduction in chloroplast function which could be detected by electron microscopy.

7.4.2.4. Mitochondrial Function and Gene Expression

The results described in Chap.6 illustrate the potential of fluorescent dyes to measure the activity of mitochondria *in vivo*. Whilst this technique requires further refinement, the initial results (and experience that is being gained at the moment in Oxford) suggest that it should be possible to identify differences in mitochondrial activity, both between control and transgenic plants and within plant tissues. The apparent difference in the inhibition of mitochondrial function between the vascular bundle and the mesophyll in leaves of the transgenic plants suggests that transgene expression may differ within tissues of an organ. Northern blot analysis of ANT expression does not discriminate between differential expression of a gene within different parts of a tissue. Using *in situ* hybridization technology the expression of the antisense transgene and its effects on ANT expression can be investigated in more detail. Expression of genes linked to the ST-LS1 promoter is closely associated with the presence of chloroplasts (Stockhaus *et al.*, 1989). Thus ANT expression in the leaf main vein, where there are few chloroplasts compared to the mesophyll (which has a high number of chloroplasts, as illustrated by the autofluorescence of chlorophyll), should be very similar in both transgenic and control plants, if the expression of the ST-LS1 promoter follows the predicted pattern and the antisense transcript is not present. If this is the case and ANT expression is not changed in the main vein of transgenic leaves, then the signal detected in northern blot analysis of total leaf RNA may be due to expression in the main vein of the leaf and ANT expression may be totally inhibited in the mesophyll of the transgenic plants.

7.4.3. The Effects of Changing the Level of ANT Protein on Mitochondria

One of the reasons for using potato as the model system for this work was the ease with which functional mitochondria could be extracted from potato tubers for use in biochemical assays. The only transgenic plants that were recovered which show a dramatic difference in ANT protein were transformed with a light induced, leaf specific promoter. This prevents the use of transgenic tubers as a source of mitochondria with reduced levels

of ANT protein. It is possible to isolate mitochondria from leaves, but this is complicated by the presence of chloroplasts and the amount of tissue required to achieve a sufficient yield of mitochondria for biochemical assays is large. As the transgene is under the control of an inducible promoter, it offers the opportunity to manipulate the expression of the transgene. Cell suspension cultures have been used to study the effects of sucrose deprivation on mitochondrial function (Journet *et al.*, 1986). Establishing such cultures from transgenic plants would provide a controlled environment, where illumination of the cultures would induce expression of the transgene. By altering the quality or length of illumination of the suspension cultures, the functional levels of ANT protein in mitochondria could be manipulated, generating mitochondria that demonstrate a range of inhibition. Stockhaus *et al.* (1989) showed that ST-LS1 was weakly expressed in callus tissue containing chloroplasts. These cultures, in which the level of ANT has been manipulated, would provide an alternative to using the specific inhibitor of ANT function, carboxyatractyloside, for calculating the flux control coefficient of ANT. The effect of altering the level of RUBISCO in tobacco plants was studied by selfing progeny of the original transformants to obtain a series of plants with a range of RUBISCO inhibition. It may prove possible to follow the effect of progressive inhibition or increases in the amount of ANT protein and the effects on mitochondrial function following inhibition or 'derepression' of the gene.

7.4.3.1. Quantification of the Levels of ANT Protein

When total protein from leaves of the transgenic plants was immunoblotted it was not possible to detect ANT protein. Such a result does not mean that no ANT protein is present, merely that it is below the levels of detection by immunoblotting. Vignais *et al.* (1976) have developed a more accurate method of determining the level of ANT protein, by measuring the binding of radioactively labelled carboxyatractyloside, a specific inhibitor of ANT, to isolated mitochondria. This would provide an accurate quantifiable measurement of the reduction in ANT protein caused by the antisense transgene.

7.4.3.2. *In vivo* Measurement of ANT Activity

Nuclear magnetic resonance (NMR) spectroscopy offers the possibility of measuring the concentrations of ATP, ADP and NADH and the activity of enzymes such as ATP synthase and ANT *in vivo* (Roberts *et al.*, 1984; Masiakos *et al.*, 1991). ^{31}P saturation transfer NMR has been used to study the transfer of ^{31}P in both isolated mitochondria and plant tissues. Suspension cultures are especially amenable to NMR techniques to monitor metabolism, as demonstrated by Douce *et al.* (1989). Using NMR it may be possible to determine the activity of ANT and the ATP synthase in mitochondria with reduced levels of ANT protein.

7.4.3.3. Changes in Biochemical Function

With isolated mitochondria from the transgenic plants, changes in mitochondrial biochemistry resulting from the inhibition of ANT can be investigated. In the light of the increase in expression of NAD malic enzyme observed in the transgenic plants, investigation of the activity of this enzyme would be especially interesting. It is possible to assay the activity of this enzyme in isolated mitochondria by following the production of pyruvate (Day *et al.*, 1984). It is possible to use specific inhibitors of the electron transport chain to investigate changes in mitochondrial function. For example, if a proton gradient is not being generated, but the alternative oxidase is active, then this can be detected using salicylhydroxamic acid (SHAM), a specific inhibitor of the alternative oxidase.

7.4.3.4. Expression of Genes Encoding Other Mitochondrial Proteins

The reduction in ANT expression was accompanied by a rise in the expression NAD malic enzyme. If the function of mitochondria in transgenic plants is affected by inhibition of ANT, then the expression of other genes in addition to NAD malic enzyme may also be altered. The expression of genes encoding the subunits of the electron transport chain should be investigated, especially if inhibition of ANT does result in a reduction in the generation of ATP by ATP synthase. A number of mitochondrial enzymes complexes are the products of both

nuclear and mitochondrial genes. The mitochondrial F_1-ATP synthase is encoded by mitochondrial and nuclear genes. If the activity of the enzyme is inhibited by the reduction of ANT it would be of interest to see if the expression of these genes was affected.

7.5. Conclusion

The transgenic approach to study the adenine nucleotide translocator has not proved as simple as originally expected, but initial investigations of transgenic plants with reduced levels of ANT suggest that much about the role and function of this protein could be elicited from further study of these plants.

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