Approaching an RT-PCR Assay to Analyse Gene Expression in Chilling-Stressed *Rhododendron*: Partial Cloning of an Ascorbate Peroxidase Gene and Enzyme Activity Studies.

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Abstract

Production and regulation of active oxygen species are important responses to environmental stress in plant tissues. This study was concerned with development of a competitive RT-PCR assay to study changes in ascorbate peroxidase gene expression in chilled and non-chilled *in vitro* grown cultures of *Rhododendron ponticum*, *R. hatsugiri* and *R. impeditum*.

Oligonucleotides for PCR amplification of ascorbate peroxidase and glutathione reductase DNA sequences were designed using a sequence homology alignment of mRNA/DNA sequences from six distinct plant species. Ligation of PCR products into the pT-Adv plasmid vector and transformation into *Escherichia coli*, followed by partial sequencing, confirmed fragment identity. The subsequent design of *Rhododendron*-specific primers, and the construction of a cRNA competitor fragment by *in vitro* transcription for use in competitive RT-PCR, were also mediated by *E. coli* cloning. RT-PCR was developed using M-MLV reverse transcriptase and total RNA isolated from *R. ponticum*. The response of *in vitro* grown *R. ponticum* cultures upon exposure to chilling (4 and 2°C) and non-chilling (20°C) temperatures was investigated by competitive RT-PCR and enzyme activity studies. Ascorbate peroxidase enzyme activity and gene expression appeared to be closely correlated. Results indicated *in vitro* grown cultures of *R. ponticum* to be chilling sensitive at 2°C.

Contents

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Declaration

I declare that research presented in this thesis was conducted by myself under supervision. I also certify that no part of this thesis has been submitted previously for the award of a degree to a university.

Laura Heggie
June, 1999.

Abbreviations

ΔG	free energy change
ΔH	enthalpy energy change
ΔS	entropy energy change
2-D	2 dimensional
A	adenine
APX	ascorbate peroxidase
ATP	adenosine triphosphate
BLAST	basic local alignment search tool
bp	base pairs
BSA	bovine serum albumin
C	cytosine
cDNA	complementary DNA
CO_2	carbon dioxide
cRNA	complementary RNA
Cu/ZnSOD	copper/zinc superoxide dismutase
DEPC	diethylpyrocarbonate
DHAR	dehydroascorbate reductase
DMF	dimethyl formamide
DNA	deoxyribonucleic acid
dNTP	nucleotide triphosphate molecule
EDTA	ethylenediaminetetra-acetic acid
FeSOD	iron superoxide dismutase
G	glycine
GR	glutathione reductase
GSH	reduced glutathione
GSSG	oxidised glutathione
H^+	hydrogen atom
H_2O_2	hydrogen peroxide
HCl	hydrogen chloride
IAA	indole-3-acetic acid

IPTG	isopropyl thiogalactoside
kb	kilo bases
kcal	kilo calories
KCl	potassium chloride
$L^{\bullet}$	lipid radical
LB	Luria-Bertani
$LOO^{\bullet}$	lipid peroxyl radical
LOOH	lipid hydroperoxide
MDHAR	monodehydroascorbate reductase
Mg^{2+}	magnesium ion
$MgCl_2$	magnesium chloride
MnSOD	manganese superoxide dismutase
mol	molar
mRNA	messenger RNA
NaCl	sodium chloride
NADPH	nicotinamide adenine dinulceotide phosphate
$O_2^{\bullet}$	superoxide radical
O_3	ozone
$OH^{\bullet}$	hydroxyl radical
PCR	polymerase chain reaction
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT-PCR	reverse transcription PCR
SOD	superoxide dismutase
T	thymine
T_m	melting temperature
X-Gal	5-bromo-4-chloro-3-indolyl-β-galactopyranoside

MOPS	(3-[N-morpholino]propanesulphonic acid
MES	(2-[N-morpholino]ethanesulphonic acid
TBE	tris-borate buffer
TAE	tris-acetate buffer
TE	tris-EDTA buffer
DMSO	dimethyl sulphoxide
BLOTTO	bovine-lacto-transfer-technique optimiser
NaN_3	sodium azide
TRIR	total RNA isolation reagent

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Chapter One General Introduction

The environment elicits responses within every living organism and it has become clear that certain environmental stresses induce specific physiological and genomic responses in both plant and animal cells (Burdon, 1994). These include extremes of temperature, light, anaerobiosis, water stress, xenobiotics, heavy metals and pest and pathogen attack (Gilmour *et al.*,1988; Brown *et al.*, 1995; Iturbe-Ormaetxe, *et al.*, 1995; Sgherri & Navari-Izzo, 1995; Kirtikara & Talbot, 1996; Knörzer *et al.*, 1996; Mehedy *et al.*, 1996; de Paula *et al.*, 1996; Schwanz *et al.*, 1996; Weckx & Clijsters, 1996). The employment of molecular biological techniques has given an insight into how an organism perceives stress at a genetic level, and how it can activate appropriate defence mechanisms (Burdon *et al.*, 1994). Using such approaches in tandem with physiological studies will expand on the knowledge already obtained concerned with gene regulation and expression. This information can be used as a base for the genetic engineering of organisms. Many such studies have been undertaken in an attempt to analyse the benefits of over-expression of antioxidant systems in plants (McKersie *et al.*, 1993; Creissen *et al.*, 1996; Tanaka *et al.*, 1996; Allen *et al.*, 1997), to allow them to adapt more readily to hostile environments.

1.1 Oxidative Stress

One of the most important mechanisms by which a plant incurs damage due to environmental stress is the excess production of active oxygen species within its tissues. These include superoxide radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\bullet}$). It has been shown that not only does oxidative stress occur in plants exposed to high and low temperatures, but also in those exposed to high light intensities, drought, air pollutants and herbicides (Foyer and Mullineaux, 1994). Since

oxidative stress levels vary in response to different stimuli, organisms are able to adapt to fluctuating stresses by inducing the synthesis of antioxidant enzymes and damage removal or repair enzymes (Davies, 1995).

Superoxide radicals can inactivate various macromolecules within the cell directly. $O_2^{\bullet-}$ reacts with proteins that contain transition metal prosthetic groups, such as haem moieties and iron-sulphur groups, causing damage to amino acids and loss of protein and enzyme function (Davies, 1995). One of the most important superoxide reactions is with another superoxide radical, i.e. dismutation, resulting in production of hydrogen peroxide. This is an intermediate generated by many different oxidation pathways, and is a detrimental oxidant of many biological compounds. However, it is the conversion of $O_2^{\bullet-}$ and H_2O_2 to the hydroxyl and hydroperoxyl radicals (by the Haber-Weiss reaction, see Inzé and Van Montagu, 1995) which accounts for their main toxicity. These radicals will immediately react with lipids, proteins and DNA causing rapid cell damage and death.

Lipid peroxidation in particular, can be thought of as an indicator of cellular oxidative stress (Rice-Evans *et al.*, 1991). Membrane phospholipids are continually subject to oxidant challenges. Figure 1.1 illustrates peroxidation of unsaturated fatty acids. The process of peroxidation is initiated when an H^+ atom is abstracted by a previously formed peroxide radical, thus creating a carbon-centred lipid radical ($L^{\bullet}$). In an aerobic environment, oxygen will add to this radical, giving rise to a lipid peroxyl radical ($LOO^{\bullet}$). Once instituted, $LOO^{\bullet}$ can increase the peroxidation by obtaining an H^+ atom from neighbouring unsaturated fatty acids. The resulting lipid hydroperoxide (LOOH) can easily decompose into several reactive species: aldehydes, alkanes, lipid epoxides and alcohols (Davies, 1995). Peroxidised membranes become rigid, lose the

selectivity and, in severe cases, lose their integrity. Figure 1.2 illustrates the mechanisms related to oxidative stress, both in damage and defence.

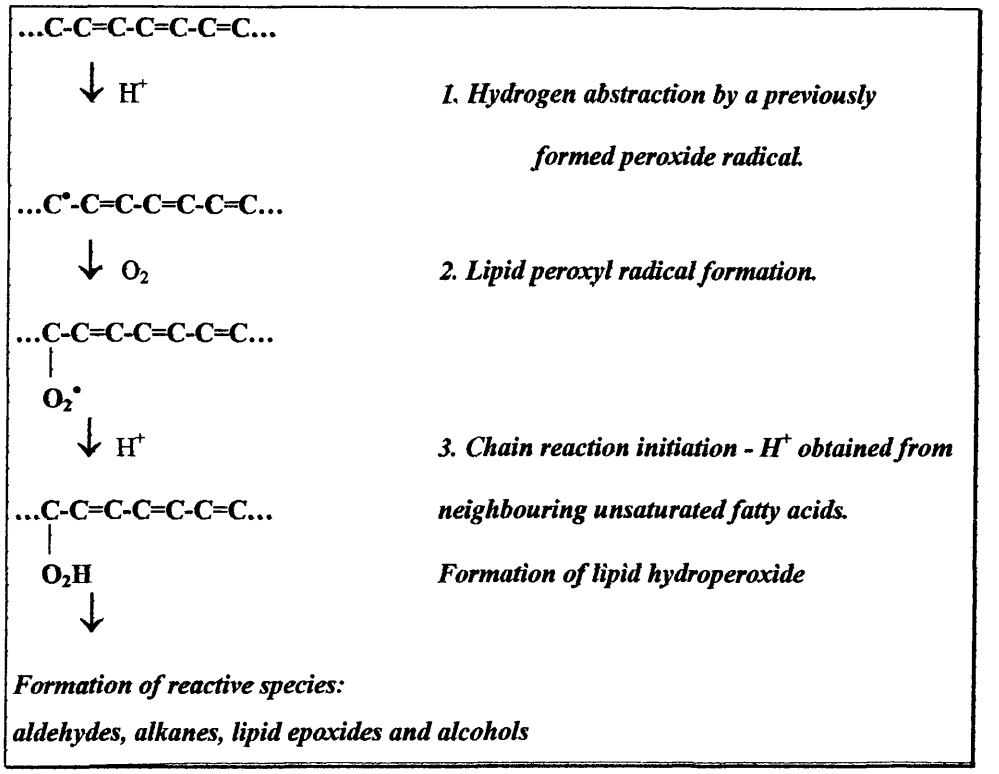

Figure 1.1 The mechanism of peroxidation of unsaturated fatty acids. The chain reaction causing peroxidation can be inhibited by abstraction of the H atom from another source such as α-Tocopherol (vitamin E) This is illustrated in Figure 1.2.

Several metabolic processes do, however, make use of active oxygen species in a profitable way. For example, a large and rapid increase in active oxygen species can be observed upon pest or pathogen attack. This burst is thought to be linked to the hypersensitive response associated with pathogen defences (Inzé and Van Montagu, 1995). Some potential sources of active oxygen species include chloroplasts and mitochondria. In plants, the major production site of superoxide radicals in chloroplasts is the reducing site of Photosystem I (Asada *et al.*, 1974).

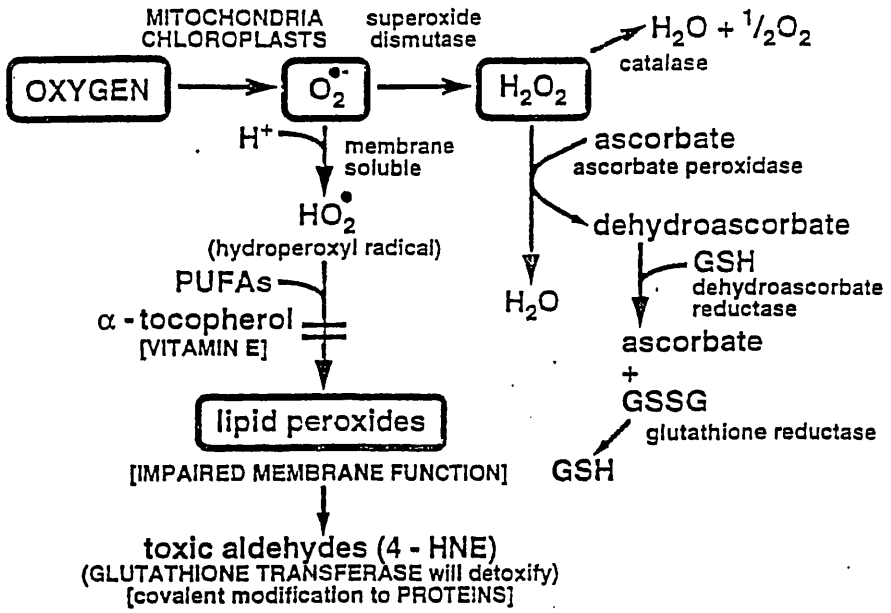

Figure 1.2 Summary of oxidative stress pathways in plants (adopted from Burdon, 1993).

1.2 Oxidative Defence Mechanisms

Organisms produce many antioxidant compounds to enable them to cope with oxidative stress, including α-tocopherol (vitamin E), ascorbate (vitamin C), β-carotene, ubiquinone, uric acid and glutathione (Figure 1.2). Some of these compounds are lipid soluble, others are aqueous, thus allowing antioxidant activity to be carried out in different parts of the cell (Davies, 1995). The enzymes which work in cohort with these compounds are of equal importance in the role of oxidative defence.

1.2.1 Superoxide Dismutase

Superoxide dismutase (SOD) is the first, and arguably most significant (Davies,1995), of the antioxidant enzymatic protection mechanisms employed in plant and animal cells. Its function is to dismutate superoxide radicals to hydrogen peroxide (Chatfield and Dalton, 1993; Inzé and Van Montagu, 1995). There are several different isoforms of superoxide dismutase, distinguishable by the metal compounds they contain. Generally plants contain manganese superoxide dismutase (MnSOD) located in the mitochondria, as well as cytosolic and chloroplastic copper/zinc superoxide dismutase (Cu/ZnSOD; Bowler *et al.*, 1994). It has been found that many plant species also contain FeSOD (iron-containing superoxide dismutase; Van Camp *et al.*, 1990; Bowler *et al.*, 1994).

1.2.2. Ascorbate - Glutathione Cycle

Whether the generation of hydrogen peroxide is from spontaneous or enzymatic (superoxide dismutase) dismutation from superoxide, its presence can be deleterious to plant tissues, and therefore it must be removed as quickly as possible. The cycle illustrated in Figure 1.3 is a series of redox reactions which occurs in plant and animal cells, becoming elevated when the organism is subjected to oxidative stress. Hydrogen peroxide is removed by ascorbate peroxidase, an important H_2O_2 'scavenger', using ascorbate as the electron donor, thus yielding monodehydroascorbate and water. Monodehydroascorbate is rapidly reduced by its reductase enzyme, otherwise it will spontaneously disproportionate into ascorbate and dehydroascorbate. The latter protein is recycled into ascorbate however, by utilising reduced glutathione as a reductant, which is in turn regenerated by glutathione reductase in an NADPH-dependent reaction.

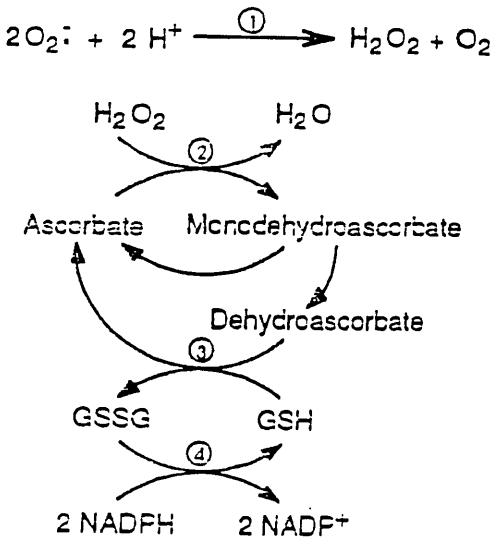

Figure 1.3. Ascorbate - Glutathione cycle detail (reproduced from Kangasjärvi *et al.*, 1994). Abbreviations: GSH, reduced glutathione; GSSG, oxidised glutathione. Enzymes catalysing the reactions are indicated by the following numbers: 1, superoxide dismutase; 2, ascorbate peroxidase; 3, dehydroascorbate reductase; 4, glutathione reductase.

1.2.3 Ascorbate Peroxidase

Plant peroxidases were among the first enzymes whose intermediates were identified. Their enzymatic and molecular characteristics have been well reported (Mittler *et al.*, 1991; Tanaka *et al.*, 1991; Chen *et al.*, 1992; Kubo *et al.*, 1992; Chatfield and Dalton, 1993; Koshiba, 1993). Many that have been isolated from plant tissues to date have low specificities with respect to the electron donor, and these enzymes are commonly referred to as guaiacol peroxidases. These are assumed to play a broad role in a wide range of biological activities, for example biosynthesis of lignin, ethylene production, degradation of Indole-3-Acetic Acid (IAA), wound healing and pathogen defence (Chen, Sano and Asada, 1992). In contrast, ascorbate peroxidase is a far more specific enzyme, playing a precise role within plant and animal tissues.

Ascorbate peroxidase (APX, EC 1.11.1.11) is present in all higher plant species and many cyanobacteria, suggesting a possible route of evolution (Gressel and Galun, 1994). It has been identified that chloroplastic APX is a component of the scavenging system of active oxygen species produced during oxidative stress. APX is the enzyme responsible for the catalysis of hydrogen peroxide reduction, utilising ascorbate (commonly known as vitamin C) as the electron donor:

$$2 \text{ ascorbate} + H_2O_2 \rightarrow 2 \text{ monodehydroascorbate} + 2\ H_2O$$

The existence of ascorbate peroxidases, different to 'plant' guaiacol peroxidases such as horseradish peroxidase, has been established in both higher and many C4 plants (Tanaka *et al.*, 1991). There are both enzymatic and molecular differences between the two types of peroxidase:

- in the absence of an electron donor, guaiacol peroxidases are not inactivated, but APX is rapidly so. (Nakano and Asada, 1987).
- guaiacol peroxidases are glycoproteins, ascorbate peroxidase is not.
- ascorbate peroxidase is inhibited by thiol reagents, guaiacol peroxidases are not.
- ascorbate peroxidase contains non-heme iron in addition to protoheme, but guaiacol peroxidases do not.
- ascorbate peroxidase has a high degree of specificity for ascorbate as the electron donor, especially apparent in the case of the chloroplast isozyme which will rapidly lose activity if ascorbate is absent (Takana *et al.*, 1991).
- there is little homology between the amino acid sequences of the amino-terminal region for ascorbate peroxidase and guaiacol peroxidases.

Results from Chen, Sano and Asada (1992) suggest that the amino acid sequence for APX obtained from the chloroplast in tea leaves have a higher degree of homology to the sequence of cytochrome C peroxidase in yeast than to guaiacol peroxidases in plants, thus indicating that these

enzymes, one from the chloroplast, one from the mitochondrion, may be part of the same family. It has been reported that cytochrome C peroxidase also acts as a hydrogen peroxide scavenger, as do glutathione peroxidases in mammals and NAD(P)H peroxidase in bacteria (Asada and Takahashi, 1987).

It has been identified that the action and specificity of ascorbate peroxidase differs amongst plant species. Nakano and Asada (1987) reported that spinach ascorbate peroxidase has a high specificity for ascorbate as its electron donor, whereas in *Euglena* (Shigeoka *et al.*, 1980) and root nodules (Dalton *et al.*, 1986), APX has a specificity for artificial electron donors. It is now known that spinach ascorbate peroxidase is localised in the chloroplasts, and immediately loses its activity when ascorbate is absent (Nakano and Asada, 1981, Nakano and Edwards, 1987). In root nodules and *Euglena*, however, ascorbate peroxidase will remain active if ascorbate is absent (Dalton *et al.*, 1987). It can therefore be assumed that there are different types of APX present in plants to cope with the different defence mechanisms present.

1.2.4 Glutathione Reductase

Glutathione (GSH) is a widely distributed thiol-containing tripeptide found in the majority of plant and animal tissues and is necessary for a wide range of cell functions (Connell and Mullet, 1986). These include acting as a protein disulphide reductant, (Candas *et al.* 1997), the detoxification of xenobiotics (herbicides and air pollutants), the prevention of lipid peroxidation and associated oxidative stress mechanisms (Meister and Anderson, 1983; Zeigler *et al.*, 1985; Alscher, 1989; Edwards *et al.*, 1991). For all these functions, whether enzymatic or non-enzymatic, glutathione must be used in its reduced form as it is itself a reductant. The reduction of the oxidised form of glutathione (GSSG) is

catalysed by the flavoprotein oxidoreductase, glutathione reductase (Connell and Mullet, 1986; Creissen *et al.*, 1994).

Glutathione reductase (GR, EC 1.6.4.2) utilises NADPH as the electron donor in the reaction to reduce oxidised glutathione:

$$GSSG + NAD(P)H + H^{+} \rightarrow 2GSH + NAD(P)^{+}$$

This reaction is believed to be the rate limiting step during acute oxidative stress, thus playing a central role in the intracellular antioxidative mechanism (Candas *et al.*, 1997).

Foyer and Halliwell (1976) found that the concentration of glutathione differs in different organelles in the plant cell, the highest concentration being in the chloroplast. This correlates with the hypothesis that reduced glutathione is involved in recycling ascorbate in the ascorbate - glutathione pathway (Halliwell *et al.*, 1981), and is involved in defence against oxidative stress, caused primarily by superoxide generation, which may occur in PS I. It is not surprising then, that researchers have found the highest activity of glutathione reductase in the chloroplast. Edwards *et al.* (1990) for example, have found that 70% of GR activity in pea leaves occurs in the chloroplast, and only 3% in mitochondria.

It could be that because the enzyme is present in different cell compartments, there are different isoforms of glutathione reductase. Several authors have found this to be the case in many different plant species. Creissen *et al.* (1994) found eight isoforms of GR in pea using 2-D gels, five of which were revealed to be contained in chloroplasts and three in mitochondria, when studied using Western blots. Spinach leaves were found to contain different isoforms of glutathione reductase when they were cold acclimated (Guy and Carter, 1984). It was shown that they possessed different characteristics, the cold acclimated isoform having a

different thermal inactivation window to the non-acclimated form, as well as displaying differing electrophoretic mobilities. Foyer *et al.* (1991) detected six isoforms of GR in tobacco, and Anderson *et al* (1990) have found two isoforms in eastern white pine. It could be concluded then, that GR is a ubiquitous enzyme in plants especially significant when the organism is subjected to oxidative stress. It is then that the roles of glutathione and glutathione reductase become important in the recycling of ascorbate, the electron donor necessary for the scavenging of H_2O_2.

1.3 Environmental Stimuli and Oxidative Stress

Organisms which are subjected to environmental stress are invariably exposed to conditions suitable for the production of active oxygen species. If a plant is to tolerate these conditions, it must have the ability to facilitate antioxidant mechanisms within its cells. Active oxygen species are manufactured in the cell by a number of different methods, depending on the external stimulus, and a plant must be able to perceive such differences to survive in hostile habitats.

1.3.1 Drought

One of an organism's fundamental requirements for survival is water. When a plant is suffering from water stress, one of its primary responses is to close its stomata. The advantage is, however, transient. Although further water loss will be prevented, there will be a lower influx of carbon dioxide and so a reduced rate of net photosynthesis (see Kaiser, 1987). This may not affect the perpetuation of the photosynthetic electron chain and because of the reduction in CO_2 fixation electrons will be transferred to oxygen, giving rise to superoxide radicals and other associated active oxygen species.

The oxidative effects of drought have been extensively studied in plant species, alone and in combination with other environmental factors. Baisak *et al.* (1994) studied the effects of water stress in primary leaves of wheat. It was observed that levels of the antioxidant enzyme, catalase, also responsible for the scavenging of H_2O_2 (Burdon *et al.*, 1994), increased with time, then declined in the control tissue. Water stress prevented the initial rise in catalase activity to varying degrees, depending on the degree of stress inflicted. It was also noted by Baisak *et al.* (1994) that water stress caused a rapid increase in superoxide dismutase activity whatever the degree of stress, but ascorbate peroxidase activity only increased in mild water stress situations, and was found to decline rapidly when the leaves were under severe stress. In contrast, glutathione reductase activity increased under all water stress conditions, as did the activity of SOD. This could however be due to the increase in the number of different isoforms noted for both these enzymes, a common response in plant tissues under stress (Edwards *et al.*, 1994).

These activity phenomena were also noted by Sgherri and Navari-Izzo (1995). Sunflower (*Helianthus annuus* L.) seedlings subjected to water deficit conditions were seen to induce antioxidant enzyme activities at the onset of moderate stress. By the later stages of the study, when severe conditions were noted, the antioxidant capacity of the seedlings declined and oxidative processes increased. Both Sgherri and Navari-Izzo (1995) and Baisak *et al.* (1994) conclude that the water status and degree of water stress inflicted on the plant is important in the activation of the antioxidative mechanism. However, in maize (*Zea mays* L.), observations by Brown *et al.* (1995) concluded that moderate drought had little effect on the antioxidant activities in leaf tissue although plant growth was inhibited.

Conversely, in a study of oxidative stress in oak (*Quercus rubor*) and pine (*Pinus pinaster*), Schwanz *et al* (1996) illustrated that all antioxidant

enzyme activities studied decreased during drought. However, if the plants were grown under elevated CO_2 (700µl l^{-1}), the enzymes studied were found to increase in activity, thus increasing survival rates. The enriched CO_2 atmosphere may allow the plants to compensate for reduced fixation due to stomatal closure during drought and water stress.

Because of the relatively uniform way that plants will respond to oxidative stress when induced by drought, Rensburg and Krüger (1993) suggested that oxidative stress could be used as a means of selection between drought tolerant and sensitive tobacco (*Nicotania tabacum*) cultivars. A progressive, highly significant, differential increase in glutathione reductase activity was observed in cultivars in relation to their drought tolerance as their leaf water potential decreased. Superoxide activity was also seen to increase by as much as 244% in tolerant cultivars, but only 161% in sensitive plants. Ascorbate peroxidase activity was noted to increase 300-400% in tolerant plants under stress, a substantially higher increase than catalase activity, suggesting APX is responsible for the scavenging of hydrogen peroxide during drought, and not catalase. Although both sensitive and tolerant cultivars exhibited high levels of lipid peroxidation when under stress, upon rehydration, levels decreased more quickly in tolerant plants. Rensburg and Krüger (1993) concluded that drought tolerant tobacco cultivars were capable to initiate an effective antioxidant system in response to drought-induced oxidative stress, and that the activities of APX and GR are useful indicators of tolerance.

1.3.2 Iron deficiency

The antioxidant mechanism in plants can also be a useful indicator of plant nutritional status. Iturbe-Ormaetxe *et al.* (1995) studied the activity of antioxidant enzymes in pea (*Pisum sativum*) plants deficient in iron.

The activities of glutathione reductase, monodehydroascorbate reductase and dehydroascorbate reductase were unaffected by the lack of free iron in tissue, but the activity of ascorbate peroxidase was seen to decrease by half. They suggest that the activity of APX is highly correlated with the amount of iron available in tissue. This may be because the hydroxyl radical is thought to be produced through the iron catalysed Haber-Weiss reaction (see Inzé and Van Montagu, 1995), in which ascorbate could replace O_2^- as the reductant of iron (Gutteridge and Halliwell, 1989).

1.3.3 Herbicides

Herbicides are known for their effectiveness by their different modes of action in plant tissues, for example, the production of detrimental active oxygen species. Oxyfluorfen and related ρ-nitrodiphenyl ether herbicides produce free radicals in the presence of light (Knörzer *et al.*, 1996), which in turn, inhibit the primary target enzyme of oxyfluorfen, protoporphyrinogen oxidase. This causes an accumulation of pigment intermediates, mainly protoporphyrin IX, (an excited form) leading to the generation of active oxygen species. The action of paraquat however, is quite different, the major target site being the chloroplast. Paraquat is thought to accept electrons from photosystem I, to produce paraquat radicals. These react with molecular oxygen forming superoxide radicals (Kirtikara and Talbot, 1996). Both paraquat and oxyfluorfen induce oxidative stress conditions.

Knörzer *et al.* (1996) studied the changes in antioxidant enzymes when soybean (*Glycine max*) cell suspensions were exposed to various concentrations of oxyfluorfen. They observed that all the major antioxidant enzymes, with the exception of DHAR, i.e. APX, catalase, MDHAR and GR, increased 40-70% when the cells were exposed to all concentrations of the herbicide. This may have led to ascorbate

concentrations decreasing 50% in stressed cells. It is interesting to note that there was a considerable increase in the activities of catalase and ascorbate peroxidase, suggesting that although the activity of ascorbate peroxidase was compromised due to the lack of its electron donor, the cells were able to compensate.

A study of paraquat-treated plants has revealed a different chain of antioxidative events. Kirtikara and Talbot (1996) observed ascorbate regeneration to be constant in paraquat-stressed tomato (*Lycopersicon esculerentum*) cultivars, possibly explaining the uniform ascorbate peroxidase activity noted. Again, glutathione reductase activity was found to increase, probably because of an increase in the number of isoforms, but perhaps also because of an imbalance in the reduced glutathione (GSH) : oxidised glutathione (GSSG) ratio. GSSG can also act as a pro-oxidant, and so constant regeneration of GSH, another important plant protein, is necessary.

1.3.4 Ozone

Kirtikara and Talbot (1996) studied the effects of ozone (O_3) in conjunction with paraquat in tomato cultivars, in an attempt to establish the target site of ozone damage. Results revealed two sets of distinct polypeptides for the treatments: those which increased and those which decreased during both types of stress, but for which there was no overlap. These studies also unveiled differing patterns for the antioxidant system for paraquat and ozone, suggesting that the target site of ozone is not the chloroplast.

Studies by Rao *et al.* (1996) again illustrated the different mechanisms by which plants activate antioxidative defence. In this case, ozone enhanced the activities of SOD, peroxidases, GR and APX, while modifying the

substrate affinity for both ascorbate peroxidase and glutathione reductase, a phenomenon not previously noted by any other workers. However, this may explain the increase in activities of plant peroxidases and catalase (Knörzer *et al.*, 1996) when exposed to elevated ozone levels.

1.3.5 Salinity

Salt is an important toxin encountered by many agricultural species, a prime example being rice, a crop, which although salt sensitive, is frequently grown in tidal swamps (Greenland, 1990). Fadzilla *et al.* (1997) studied the antioxidative responses of rice (*Oryza sativa*) shoot cultures when grown in a medium containing 0.35M sodium chloride. After only one day of exposure to elevated salinity, there were signs of oxidative stress primarily due to enhanced levels of superoxide dismutase activity, hydrogen peroxide and oxidised glutathione. However, by day eight, the concentration of GSSG had returned to that found in control cultures. This corresponds with the observed rise in glutathione reductase activity in the stressed cultures. The activities of APX and catalase remained similar to those of the controls. Similar activities were noted by Sheokand *et al.* (1995) in chickpea. Although plants initially displayed signs of oxidative stress, by day 14 of the study, antioxidant enzyme activities had returned to levels similar to controls. This suggests that although these species do display signs of oxidative sensitivity, and are classed as such, the plants have the antioxidant ability to deal with such stress and recover. Although there is evidence for metabolic changes occurring in plant tissue exposed to ionic stress (Binzel *et al.*, 1988), it is apparently unclear as to why oxidative stress may be induced by salinity.

1.3.6 Plant Disease Resistance

Oxidative stress and active oxygen species are known to exert powerfully damaging effects against living organisms. Perhaps that is why plants have been shown to induce oxidants in response to attack by pests and pathogens (Inzé and Van Montagu, 1995). This 'oxidative burst' seems to be generated by a signalling pathway, similar to that observed in mammalian neutrophils during immune responses (Mehedy *et al.*, 1996). Illustrated in Figure 1.4 is a possible model of the components involved in the oxidative burst. Elicitor receptors in plant plasma membranes are thought to activate G proteins and phospholipase C leading to ultimately, an active NADPH oxidase complex. NADPH oxidase produces $O_2^{\bullet-}$ which is known to be converted rapidly to hydrogen peroxide. H_2O_2 is thought to play a central role in interceding different aspects of disease resistance, e.g. participation in killing pathogen cells during defence responses (Apostol *et al.* 1989), oxidative cross linking of cell wall proteins to render them less digestible by pathogens (Mehedy *et al.*, 1996) and as a possible intracellular signal regulating defence-related gene expression (Mehedy *et al.*, 1996). All these mechanisms will vary depending on the sensitivity of the plant to attack and of the pathogen to H_2O_2.

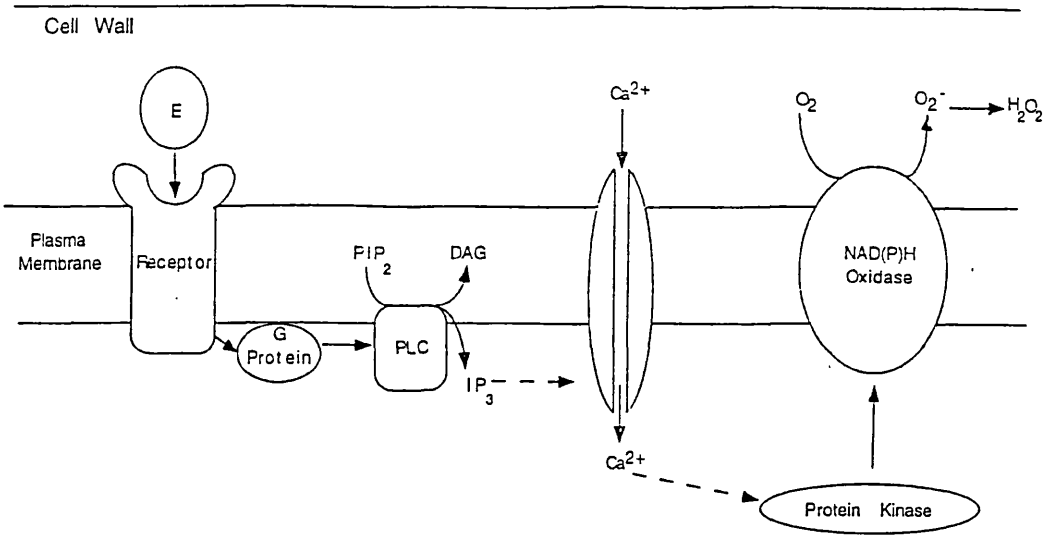

Figure 1.4. A model of possible components involved in the oxidative burst in plant cells involved in the hypersensitive response (taken from Mehdy *et al.*, 1996).

1.3.7 Senescence

Superoxide radicals are thought to play an important role in the senescence of plant leaves (Sandalio *et al.*,1987, Thompson *et al.*, 1987). When the senescence of pea leaves was studied by Pastori and del Rio (1994), it was found that antioxidant enzyme activities were depressed, and those systems which are known to increase active oxygen species, including superoxide dismutase, were found to increase considerably. This suggests that oxidative stress is necessary in certain plant functions but can be the cause of damage under other circumstances.

1.4 Low Temperature Injury

Referred to as winter damage by horticulturists, this term encompasses a whole range of circumstances associated with low temperatures. This does not only include damage incurred by frost and freezing, but also that caused by low water availability due to the restriction of free water frozen in the soil, desiccating effects caused by wind and injury caused by temperatures above freezing point.

Damage caused by frost is usually obvious, and plants not normally harmed by winter severities are said to be hardy, or have the ability to acclimate (Ougham *et al.*, 1992). Non-hardy plants usually blacken, wilt and shrivel when experiencing the first autumn frosts. Common long-term frost damage signs include foliar sunscald, stem or trunk splitting, winter burn of coniferous foliage and midwinter kill of dormant flower buds (Anisko and Lindstrom, 1995). If frosts occur particularly early in the autumn, these symptoms can also be seen on growing shoots of hardy species. For example, Polle *et al.* (1996) noted that Norway spruce seedlings could be severely damaged by unseasonal frosts, although the species is known to be very hardy.

The damage caused to both hardy and non-hardy plants by late spring frosts is of much greater importance. Frost damage to young leaves and flowers in a wide range of plants can severely retard normal plant growth. If these frosts occur immediately before bud break, then the buds may be killed (Kuroda *et al.*, 1993). Repeated spring frost damage can leave some trees and shrubs permanently dwarfed (Weiser, 1970).

Low temperature injury can be caused by chilling and freezing stress. Chilling stress is injury caused to plant tissues at temperatures above freezing, and forms the focal point for the present study.

1.5 Chilling Temperature Damage

Responses of plants to low temperature stress are dependent on the functions affected, i.e. development, growth rate and survival. Many plants originating from tropical regions are chilling sensitive, suffering damage when subjected to temperatures below 10°C (Levitt, 1980). Depending on the minimum temperature and its duration, plants may be partially damaged or killed, resulting in lower yield and quality at harvest, or in severe cases, complete crop failure. Low temperatures have been known to devastate the caneberry crops in the Northwest Pacific (Hummer *et al.*, 1995). Chilling temperatures are known to reduce grain set in rice, causing great economic loss to the industry. It has been speculated by Hale and Orcutt (1987) that a one degree Celsius drop in world mean temperature could result in a forty percent decrease in world rice production.

Species from temperate regions can not only withstand chilling temperatures, but are also able to increase their tolerance to freezing temperatures in response to exposure to low non-freezing temperatures. This process is known as cold acclimation. For example, non-acclimated wheat seedlings are killed when exposed to freezing at -5°C, whilst cold-acclimated seedlings can survive temperatures as low as -20°C (Thomashow, 1993). Such adaptation of plants to low temperatures could prove to be important in relation to climate change. Milder winters may lead to plants de-acclimating earlier in the season, thus exposing them to damage from late frosts. Plants that require a period of vernalisation may also be affected, and may no longer possess the ability to complete their natural life cycle. Considerable effort has therefore been directed at trying to obtain a better understanding of the mechanisms underlying cold tolerance. Physiological studies have shown that acclimation to cold is associated with a variety of changes within the plant, including alterations in protein, carbohydrate and lipid composition (Sakai and Larcher, 1987;

Biggs, 1996). In most cases, the precise role that any particular biological change has in the cold acclimation process is uncertain. It can be presumed that some contribute to the overall health of the plant and its fitness for low temperature survival, whilst others play a part in increasing the tolerance of the organism to low temperature exposure (Lin *et al.*, 1990).

It has been reported that changes in the composition of lipids can directly contribute to freezing tolerance (Steponkus *et al.*, 1988). As the temperature is lowered, lipids in the cell membrane change from a liquid crystalline condition to a solid state, the degree of which is determined by the ratio of saturated to unsaturated fatty acids (Quinn, 1988). This change occurs at a temperature which is equivalent to that which elicits chilling damage (O'Kane *et al.*, 1995). The development and degree of tolerance and the hardiness of plants to freezing temperatures appears to involve changes in the ratio between different classes of fatty acid, as noted by Biggs (1996) in several *Rhododendron* species. An increase in the amount of unsaturated fatty acids results in continuing membrane function at lower temperatures. However, change in the composition of membranes towards a higher unsaturated fatty acid content can result in an accompanying increase in membrane permeability due to lipid peroxidation. This results in a loss of solutes from the cell, causing an ionic and pH imbalance. Dysfunction of membrane-based systems, such as photosynthesis and respiration, may also occur. Ionic and pH imbalances also lead to the accumulation of toxic by-products. Levitt (1980) stated that aerobic respiration is disrupted during chilling in sensitive species, and observed an accumulation in the products of glycolysis, accompanied by a reduction in the formation of ATP. Burdon *et al.* (1994) also noted that chilling induces an increase in the production of superoxide radicals and hydrogen peroxide, toxins involved in the peroxidation of lipids, and concluded that fatty acid ratios may not be the

only important mechanism involved in chilling and freezing damage, but that oxidative stress may also be significant.

1.6 Acclimation

1.6.1 Cold-Induced Proteins

An ability to increase cold tolerance in response to low non-freezing temperatures, known as cold acclimation, is essential for plants to survive in temperate winter climates (Chen, 1994). Cold acclimation is associated with a variety of changes in plant tissue, including alterations in carbohydrate, protein and lipid composition (Thomashow, 1993), thought to be governed by genetic control. In most cases, it is unclear how important a given change is to the observed increase in cold tolerance. Genetic studies have shown that the ability of a plant to cold-acclimate is a quantitative trait, and that changes in gene expression occur during cold acclimation (Thomashow, 1993). It remains uncertain how many genes are involved in the process and what their identities and function are.

Even in a single plant, individual tissues can demonstrate differences in cold hardiness, as illustrated by Chen *et al* (1983). Cold-acclimated winter rye and winter wheat plants were exposed to 2°C for 15 days, and the LT_{50} (the lethal temperature for 50% death) determined for crown, leaf and root tissues according to ion leakage. In both species, the crown tissue was found to be the most hardy, followed by leaves and then roots. These results concurred with previous results from Weiser (1970) who noted that in woody species, 'wood' cells (xylem, parenchyma and pith) are several degrees less resistant to cold that those of bark (cambium, phloem, cortex and epidermis), an expected phenomenon due to the physical structure of woody stems.

More recently, bark and xylem tissues were the focus of study in genetically-related (sibling) deciduous and evergreen peach (*Prunus persica* L. Batsch; Arora *et al.*, 1992). Seasonal changes in cold hardiness and proteins were characterised. It was found that evergreen trees, while retaining their leaves and displaying signs of shoot elongation under suitable conditions, achieved only half the level of hardiness in winter, in contrast with deciduous trees, whilst in both genotypes, xylem tissue obtained maximum supercooling, i.e. ability to tolerate cold. However, the mechanism by which plants achieve this acclimation to cold and freezing temperatures must be considered.

Identification and cloning of the genes responsible for cold acclimation and the resultant proteins involved in this process may lead to a better understanding of these mechanisms. Changes in gene expression occur during cold acclimation in many plant species. Gilmour *et al.* (1988) studied the abilities of two varieties of *Arabidopsis thaliana* (Landsberg and Columbia) to cold-acclimate. After initial exposure of both varieties to 4°C for 24 hours, plants were able to survive temperatures down to -6°C. After 8-9 days exposure to 4°C, the LT_{50} for both varieties was as low as -10°C. *In vitro* translation of poly (A^+) RNA isolated from control and cold-treated Columbia plants showed that the low temperatures induced changes in the mRNA population. Two of the polypeptides produced (160 and 47 kilodaltons in size) were only synthesised at low temperatures (Gilmour *et al.*, 1988).

Polypeptides of a similar size were also isolated by Lin *et al.* (1990) in *Arabidopsis thaliana* and wheat. Analysis by Southern and northern blotting, and cDNA cloning of the genes encoding these polypeptides, indicated that wheat and *A. thaliana* had a similar Cold Related (*cor*) gene, with a polypeptide of 47 kilodaltons, (*cor*47). They suggested that these *cor* polypeptides have a fundamental role in the ability of plants to cold-acclimate, and that they may act as cryoprotectants. Taking account

of the evolutionary distance between *A. thaliana* and wheat, it could be postulated that the mechanism of acclimation is a highly conserved one (Lin *et al*, 1990) and may not be specifically confined to cold stress.

It has been suggested by several authors that cross-resistance may be an important factor in stress acclimation (Lin *et al.*, 1990; Thomashow, 1993; Chen, 1994; Anisko and Lindstrom, 1995). Anisko and Lindstrom (1995) found that reducing the water supply to evergreen azaleas in late summer induced cold acclimation regardless of previous plant husbandry, allowing plants to remain tolerant to early autumn frosts. Drought and cold stress are thought to be closely linked because plant cells become severely dehydrated during cold and freezing temperatures. Supposed functions of '*cor*' genes and proteins include cryoprotection, altered lipid metabolism, protein protection and alteration and desiccation tolerance. Identification of such genes expressed in cross-resistance and determination of their regulation and function would facilitate an understanding of mechanisms involved in plant survival.

1.6.2 Acclimation and Antioxidants

None of the *cor* proteins studied to date have been identified as antioxidant proteins, but from current literature, their involvement in gene regulation induced in response to cold stress cannot be ruled out, as low temperature is a well known instigator of oxidative stress. Generally, cold-acclimated conifer species such as *Pinus sylvestris* and *Picea abies* are subject to an increase in the efficiency of photosystem II, which is assumed to provide a mechanism for dissipation of excess excitation energy of the photosynthetic apparatus, causing the production of active oxygen species (Schoner *et al.*, 1989). Antioxidant related genes will be triggered, thus increasing the activity of antioxidant metabolites. This was illustrated by Anderson *et al.* (1992), who found that antioxidant

two to four fold from summer to winter, whilst related enzymes increased two to 122 fold during the same period. These results clearly indicate the supposed protective function ascorbate peroxidase and glutathione reductase have against photo-oxidative winter injury.

Researchers have been intrigued by antioxidative acclamatory responses of plants. Hakam and Simon (1996) studied the activities of oxygen-scavenging enzymes in two populations of the C_4 grass species *Echinochloa crus-galli* L. Five-week-old plants were taken from two sites, Mississippi (warm climate) and Quebec (cold climate) and subjected to cold-induced photoinhibition. The activities of ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase were all significantly higher in the Mississippi population, suggesting their poorer ability to acclimate to low temperatures.

McKersie *et al.* (1993) facilitated the transformation of alfalfa (*Medicago sativa* L) with the gene encoding superoxide dismutase to determine the response of control and transformed plants to chilling. Transgenic plants which overexpressed superoxide dismutase were found to exhibit a more rapid recovery and regrowth after chilling than their control counterparts, strengthening the hypothesis that antioxidants play a protective role in plants subjected to chilling and freezing temperatures. Results obtained from a study of seasonal fluctuations of antioxidant enzymes in Norway spruce (*Picea abies*) by Polle *et al.* (1996) illustrate further the protective role of antioxidant enzymes in cold acclimation. When seedlings were exposed to an artificial frost (-5°C) in spring, some displayed an increase in all the antioxidant-related enzymes and survived. During the following autumn, plants which had been exposed to the artificial frost had lower enzyme activities in comparison to control plants, but with no compromise to health, indicating a possible memory effect of unseasonal frost, thus leading to an improved ability to acclimate.

Plants are constantly subjected to a changing environment and must be able to adapt in order to stand any chance of survival. One of the ways this is achieved is by initiating antioxidant mechanisms in response to oxidative stress. Although the ultimate symptoms of oxidative stress are similar, a plant's response depends very much on which environmental stimulus induces the increase in active oxygen species. Indeed, evidence shows that there is no clear consensus amongst authors as to whether different stress induced pathways initiate antioxidants in the same way.

This study provides an insight into ascorbate peroxidase and glutathione reductase activities, both physiological and genetic for the former enzyme, in *Rhododendron* exposed to chilling temperatures. Understanding such functions will provide a base for plant genetic modifications, allowing plant adaptation to a more hostile environment.

1.7 *Rhododendron* as an Experimental Species

Belonging to the family *Ericaceae, Rhododendron* is a genus of evergreen, semi-evergreen and deciduous shrubs ranging from a dwarf habit to tree-like stature. Members of this genus are grown mainly for their beauty and flowers. The scientific reason for their study is more fundamental. *Rhododendron* is one of the largest genera in the plant kingdom, containing over 900 species (Davidian, 1982), 320 of which are found at the main centre of diversity, in north west Yunnan, south east Tibet and western Szechwan, between 22° and 30°N (Sakai *et al.*, 1986). *Rhododendron* species are found in most areas of the globe, ranging from the tropics to the Arctic circle. The diverse range in habit, genotype and geographic situation amongst *Rhododendron* species means that they supply an excellent tool for the detailed study of plant response to various environmental influences, such as extremes of temperature.

Cold tolerance in *Rhododendron* is directly related to the severity of their native climate, many species being hardy to between -20 and -25°C. For example, *Rhododendron* species found inhabiting the timberline in eastern Himalaya and high altitudes of north west Yunnan, have been found to be tolerant to such low temperatures (Sakai *et al.*, 1981). Many of the dwarf species originating from northern altitudes are classed as hardy (Davidian, 1982). Sakai *et al.* (1986) found species such as *Rhododendron parviflorum*, *R. aureaum* and *R. impeditum* to be very hardy. Leaves were shown to display tolerance to temperatures as low as -60°C, flower buds to -34°C, vegetative buds to -60°C and xylem to -50°C. Larger *Rhododendron* species, which display tolerance to temperatures as low as -30°C, for example *R. maximum* and *R. carolinianum*, are found in the Appalachian mountains of eastern USA. However, even the hardiest species are found to be restricted to forested habitats or those protected by snow (Sakai and Larcher, 1987).

The injuries incurred by *Rhododendron* species due to low temperature stress include bark splitting (caused by mechanical action of ice crystal formation within the xylem and phloem) foliar damage (such as chlorosis and necrosis), tissue desiccation due to a restriction of free water in the soil system and flower bud death. This may be explained in several ways: different moisture loss rates, differences in the ability of roots to extract free water from near-frozen or frozen soil (Cameron and Dixon, 1997) and different rates of water movement through frozen or damaged tissues.

A phenomenon of many of the larger *Rhododendron* species is thermotropic leaf movement after temperatures decrease during winter months. It is thought that the change in the angle of the leaf may help to protect against injury caused by the combination of low temperatures and high light intensities (Biggs, 1996), which can cause photo-oxidation or photoinhibition, leading to the peroxidation of membrane lipids, and eventually cell death. Antioxidant enzymes provide another mechanism by which *Rhododendron* species can tolerate chilling and oxidative stress, although these metabolic pathways have yet to be studied.

Biggs (1996) investigated the acclimatory responses of several *Rhododendron* species and monitored the physiological responses to exposure to low temperatures. It was noted that there was an increase in unsaturated fatty acid content as the temperature was lowered, as is typical of cold tolerant plant species. As previously mentioned, it is unsaturated fatty acids which are more susceptible to lipid peroxidation, but little is known of the extent of peroxidation in *Rhododendron* tissue.

The hardiness ranking for *Rhododendron* was given by Davidian (1982, 1989, 1992), referring to the tolerance to cold in the UK.

H4 Hardy anywhere in the British Isles

H3 Hardy in the west, east and south and inland, but requires shelter

H2 Suitable for sheltered gardens on the west coast

H1 Usually a greenhouse plant

The three species chosen for the current study were previously used in cold stress research by Biggs (1996) and Cameron and Dixon (1997) preferred for their different characteristics and diverse habitats. Choosing species such as these, with their original habitats in diverse areas of the globe, and such distinct phenotypes, would indicate woody plants' differing behaviour when exposed to chilling. This would provide an extended picture of how woody species may behave when responding to temperature extremes. Details of the species are given below:

Rhododendron ponticum. This, one of the most common *Rhododendron* species found in the British Isles. It can vary considerably in its habit, being either a broad upright, lax or compact shrub of 1-4.5m, or of tree-like stature up to 7.6m in height. It bears oblong, dark green, shiny leaves and funnel-shaped mauve flowers in June and July. It has been merited a hardiness rating of H4.

R. ponticum was first described by Linnaeus in 1762 (Davidian, 1992). Its distribution ranges from north Anatolia, Turkey, the Caucasus and Lebanon and south-east Bulgaria, to Spain and Portugal. Its habitats include pine, mixed deciduous, alder, laurel, beech and spruce forests, as well as scrub-land. *R. ponticum* can characteristically be found forming thickets at elevations from sea-level to between 1,200 and 1,700m.

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Rhododendron impeditum was first discovered by Forrest in 1910 (Davidian, 1982) in mid-west and north-west Yunnan and south-west Szechwan. Its natural habitat is open peaty pasture, alpine meadows, rocky slopes and cliffs at elevations of 3,600-4,880m. *R. impeditum* is a low-growing shrub, its height rarely beyond 700mm with characteristic short, thick branchlets, densely covered in small, dark green leaves, bearing small purple flowers in April - May. Davidian (1982, 1989, 1992) gives the species a hardiness rating of H3-H4.

Rhododendron **cv. 'Hatsugiri'**, commonly known as an evergreen Azalea, was first introduced to Britain from Japan in 1920. It has a low growing habit, less than 800mm, with dense foliage cover and characteristic pink-red flowers borne in May and June. The parentage of this cultivar included *R. kiusianum*, *R. kaempferi* and *R. obtusum*, This plant also merits a hardiness rating of H4.

1.8 Objectives of this Study

1. To establish methods for *Rhododendron* micropropagation and maintenance, suitable for high quality DNA extraction.

2. To establish protocols for DNA isolation

3. To identify conserved regions of ascorbate peroxidase and glutathione reductase genes in related species, and design primers suitable for their amplification in *Rhododendron* species.

4. To amplify sequences for ascorbate peroxidase and glutathione reductase.

4. To clone and partially sequence an ascorbate peroxidase gene from *Rhododendron* species, to confirm the design of the primers.

5. To clone and partially sequence a glutathione reductase gene from *Rhododendron* species, to confirm the design of the primers.

6. To establish a protocol for isolation of high quality RNA suitable for use in a reverse transcription-PCR assay to study the change in quantity of mRNA in chilled and unchilled plants.

7. To establish a protocol for extraction and analysis of the specific activity of ascorbate peroxidase and glutathione reductase.

8. To apply the molecular and enzymatic assays to *Rhododendron ponticum* to study their responses to chilling temperatures.

Chapter 2 Optimisation of PCR for the amplification of ascorbate peroxidase and glutathione reductase sequences

2.1 Introduction

Since the advent of the Polymerase Chain Reaction (PCR) in 1985 (Saiki, *et al.*), it has been utilised, improved and modified to answer many molecular questions. PCR provides a rapid, straightforward means of producing microgram amounts of DNA from a few picograms of starting material. DNA polymerases carry out the synthesis of a complementary strand of DNA (cDNA) in the 5′ to 3′ direction using a single-stranded template, initiated at a specified double-stranded DNA region, i.e. a primer extension reaction, a practise commonly used for labelling and sequencing techniques (Taylor, 1991). In PCR however, two primers are used in the reaction, each complementary to opposite strands of the target region of DNA from a total DNA (genomic DNA) preparation. The primer sites are arranged such that the extension reaction directs new strand synthesis toward the other, resulting in *de novo* synthesis of the target region flanked by the two primers (Figure 2.1).

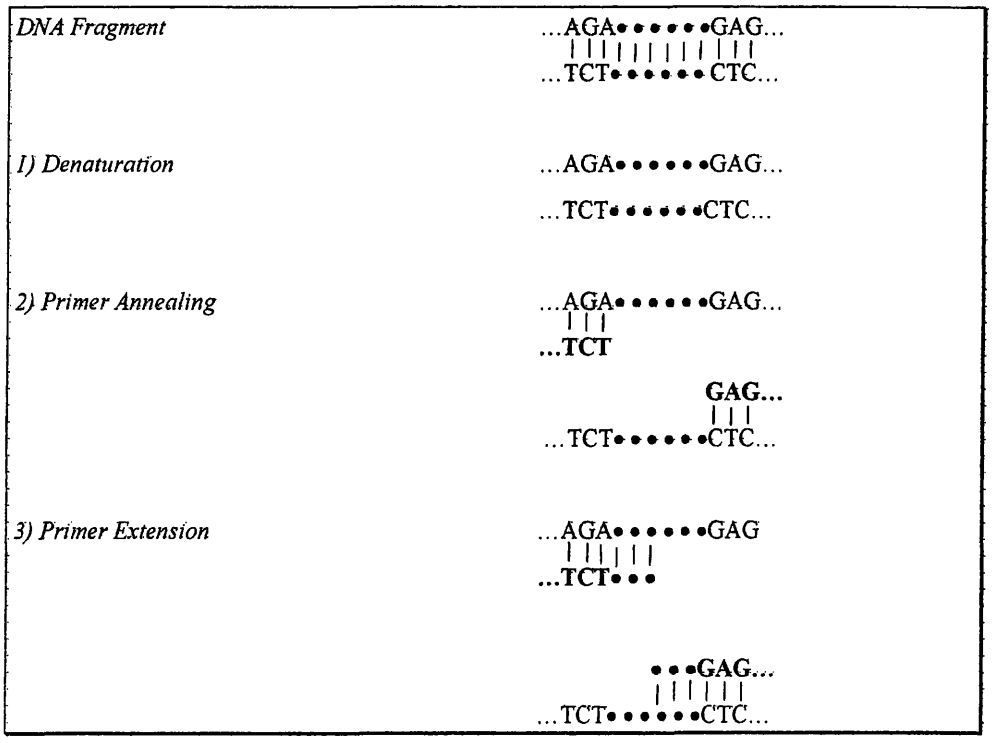

Figure 2.1 Polymerase Chain Reaction. This cycle is repeated for many molecules at an exponential rate

However, no standard procedure exists for a fail-safe method of DNA amplification. For any application of PCR therefore, alterations must be made to the basic principles of the technique for success. There are many contributing factors to the success or failure of a reaction, all of which must be considered singularly and in combination for any experimental problem. Of crucial importance to this project was the establishment of a suitable approach for the development of primers capable of amplifying previously uncharacterised genomic regions. This required an understanding of the importance of primer design characteristics.

2.1.1 Primer design

The design and composition of primers used in PCR is paramount to the success of the reaction. There are many parameters to be considered in the selection of suitable primers pairs for any specific application. These include the stability of the primer at the 5' and 3' termini (which can be measured by the length of the DNA target duplex, the GC/AT ratio of the primer sets, the duplex formation free energy (ΔG)or melting temperature (T_m) (Dieffenbach *et al.*, 1993 Rychlik, 1995)), primer self-compatibility and the formation of a stable duplex with the specific target DNA site. Generally, the more DNA sequence that is available, the better the chance of finding a suitable primer pair for PCR (Rychlik, 1995). Dieffenbach *et al.* (1993) also state that a comparison of all available related sequences will determine DNA regions of least heterogeneity, and that these should be the starting points for primer selection.

It need not be that all primer criteria are met to obtain success. Alteration of the reaction conditions themselves will improve specificity, as discussed in section 2.4. Depending on the desired size of the amplification product, the primers may be designed manually (for short,

200-400bp products), or by using dedicated computer software. The parameters for either method must be considered with equal importance, aiming to create a balance between specificity and efficiency of amplification (Dieffenbach *et al.* 1993).

To ensure correct annealing, primers that are chosen must have a unique sequence within the region to be amplified (Rychlik, 1993). It is important that the 3' end of the primer is closely matched as this is where extension commences. It is also beneficial if the primer has a G/C sequence or 'clamp' at the 3' end to ensure annealing, because the triple hydrogen bond gives extra stability. These parameters are easily, and in some cases automatically, checked by computer-aided design.

It is important that the primers are designed with no self homology (Dieffenbach *et al.*, 1993). This can lead to primer-dimer formation, an artefact which can compete against the target product for reaction reagents and lead to false positives in PCR analysis. Because of this, it is very important that any complementarity between the 3' ends of the primer pair is removed. Problems can also arise when a hairpin-loop forming primer is used. Although this may be suitable in some cases (Rychlik, 1995), they become problematic because the 3' end has doubled back on itself to form a stable duplex, causing internal primer extension and rendering the primer useless in PCR. Because duplex extension continues in a 5' to 3' direction, hairpins at the 5' end will not significantly effect the reaction.

As previously mentioned, the triplet of hydrogen bonds between the G and C bases gives greater stability than the twin hydrogen bonds between the A and T bases. Due to this phenomenon, many authors recommend that a G/C content of 40-60% within the primers will render them suitably stable for PCR (Innes and Gelfand, 1990; Kidd and Ruano, 1995). Alternatively,

Rychlik (1995) recommends that the G/C content should be close to or higher than the G/C content of the target sequence if possible. All authors agree however, that the optimum primer length for PCR is 18-25 nucleotides for DNA amplification, although in some cases, it is recommended that shorter primers be used for the amplification of complementary DNA (cDNA; produced by reverse transcription of messenger RNA), to reduce the formation of non-specific primer/template interactions (Dieffenbach *et al.*, 1993).

Melting temperature must be considered when designing primers. This is defined as the dissociation temperature of the primer-template duplex (Dieffenbach, *et al.*, 1993). The most accurate way to calculate this is by the nearest neighbour method, using the formula:

$$T_m\text{primer} = \Delta H[\Delta S + R \ln (c/4)]\text{-}273.15^{\circ}\text{C} + 16.6 \log_{10} [K^+]$$

Where ΔH and ΔS are the enthalpy and entropy energy for helix formation respectively, R is the molar gas content and c is the concentration of the primer (Breslauer *et al.*, 1986). This calculation is often substituted by the simplified free energy values (ΔG) equation, also based on a formula derived form Breslauer *et al.* (1986):

$$\Delta G = \Delta H - T\Delta S$$

where ΔH and ΔS are the enthalpy and entropy of duplex formation respectively, and T is the temperature in degrees Kelvin. Table 2.1 gives the ΔG values for all nucleotide pairs, which are substituted into the formula to calculate internal stability of a possible primer pair.

	Second Nucleotide [ΔG (kcal/mol)]			
First (5') nucleotide	dA	dC	dG	dT
dA	-1.9	-1.3	-1.6	-1.5
dC	-1.9	-3.1	-3.6	-1.6
dG	-1.6	-3.1	-3.1	-1.3
dT	-1.0	-1.6	-1.9	-1.9

Table 2.1: Free Energy values of a Nearest Neighbour Nucleotide (Rychlik, 1995). The values are given for each nucleotide pair, and can be used to calculate the internal stability of possible oligomers.

The combination of GC content and melting temperature is an important one to consider. Oligo primers of 20 bases and 50% GC content can be said to have T_m values in the range of 56-62°C, providing a suitable thermal window for efficient annealing. If there is a relatively poor GC content and melting temperature match, the primers will be much reduced in efficiency and specificity. Loss of specificity occurs at a lower T_m value. A primer with a higher T_m than its partner will have a greater chance of mispriming, however if the T_m value is too high, the primer with the lower value may not function at all.

Consideration of both the melting temperature and internal stability is important to ensure specificity of the product. Such specificity is especially important when the target DNA concentration is low in comparison with non-target DNA such as in the case of single copy sequences.

If using computer-aided primer design, the internal stability of the primer pairs can be represented graphically. Figure 2.2, (Rychlik, 1995), shows the internal stability profiles of successful and unsuccessful primer pairs. The low stability of specific primers improves the possibility of PCR being successful, without a preliminary series of optimisation

experiments. It has been observed that oligonucleotides with 3' terminals less stable than -9 kcal/mol are more likely to be specific.

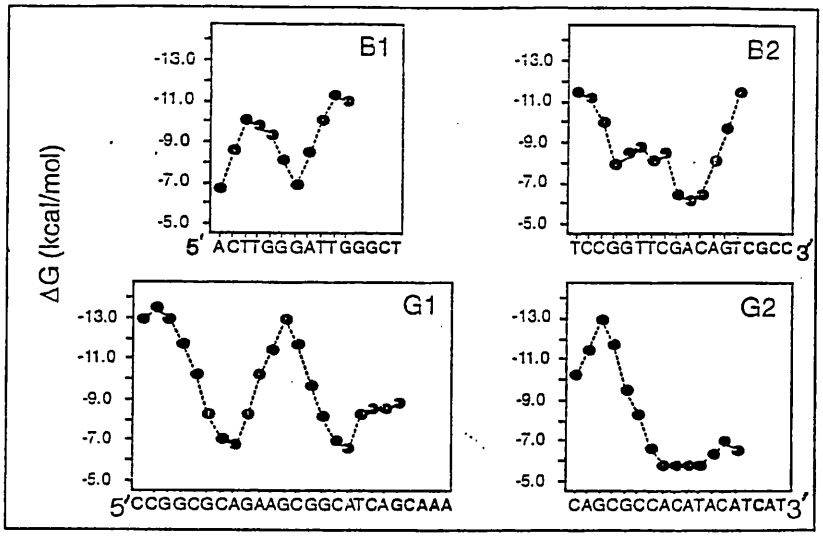

Figure 2.2: Internal stability of successful (G1 & G2) and unsuccessful primer pairs (B1 &B2) (Rychlik, 1995)

The length of the PCR product and the placement of the primers within the target sequence influences the efficiency of amplification. In some cases, this is also dependent on the template material used. Some clinical samples prepared from fixed tissues will yield DNA only suitable for generation of relatively small products (Green *et al.*, 1991; cited in Dieffenbach *et al.*, 1993). The criteria for the size of the product are usually determined by the application of PCR. For example, to study the expression of genes by quantitative PCR (a method used to quantify amounts of mRNA obtained from tissue samples), the product must be large enough that a competitive fragment can be constructed, and both easily distinguished by gel electrophoresis.

When designing primers for use in Reverse Transcription PCR (transcribing single stranded transient mRNA molecules from a given sample to a stable cDNA molecule suitable for use in PCR), it is best to have the primers span an intron site to easily distinguish any contaminating DNA, and have the primers placed in a region of coding

mRNA, rather than non-coding mRNA which may have similarities with many other mRNA sequences.

It is not only the parameters for primer design that will influence the potential success of PCR, but the reaction conditions themselves. Optimising the PCR reaction mix is equally as important as having the primers balanced in efficiency and specificity.

2.1.2 Polymerase chain reaction conditions

PCR is an enzymatic, chemical method of exponentially increasing the concentration of particular nucleic acid sequences, relative to others in the mixture. Because of the myriad of uses of PCR technology, no single protocol will be suitable for all applications. The first step must be to match a series of parameters to a particular situation. Generally, for successful amplification, there must be a suitable target sequence with enough information such that oligonucleotide primers can be synthesised to anneal to the target site. A suitable DNA polymerase must be used to catalyse the extension of the annealed primers, with a supply of free oligonucleotides to allow this extension to occur. The thermal profile used for the reaction must incorporate the correct temperatures for DNA double-strand denaturing, primer annealing and strand extension.

More specifically, within a given PCR, reaction conditions must be modified to obtain optimum amplification. These include the specific temperatures of the thermal cycle, the concentration of the primers and template used, the concentration of free nucleotides within the reaction mix and the concentration of free magnesium ions necessary to satisfy enzymatic requirements (Bloch, 1990; Kidd and Ruano, 1995).

Many problems will be encountered when beginning a new PCR application. These include little or no product detected, the presence of non-specific background bands due to mispriming and the formation of primer-dimers that compete with the product in amplification (Innes and Gelfand, 1990; Kidd and Ruano, 1995). To overcome such problems, several parameters are modified to improved the specificity, efficiency and yield of PCR.

To optimise PCR, the basic principles behind the reaction must be understood. All PCR reactions begin with the denaturation of the DNA template at 95°C. As the temperature is lowered to that required by the primers, they will anneal, or hybridise, to the template. This is important during the first few cycles because the primers have to effectively scan the template to find the correct complementary region with which to anneal (Kidd and Ruano, 1995). Following annealing, the temperature is raised to 72°C, and DNA polymerase binds to the primer-template complex and, by utilising free oligonucleotides (dNTPs), will extend the primer along the template strand. By repeating this cycle of denaturation, primer annealing and extension, there becomes an exponential increase in the amount of target DNA.

The temperature allowed for primer annealing depends on the length, concentration and composition of the primer. Innes and Gelfand (1990) recommend that a suitable annealing temperature is 5°C lower than the T_m of the primer. Kidd and Ruano (1995) suggest however, that a range of temperatures should be tested to find the optimum for a particular primer set. Primer annealing will take place at temperatures between 40 and 75°C, although temperatures toward the higher end of this spectrum will result in an increased reaction specificity (Innes and Gelfand, 1990). Some researchers report two-step PCR to be more successful in some situations. PCR by this method uses only the denaturation step and one

temperature for both annealing and extension (Kim and Smithies, 1988), thus increasing the stringency and specificity of the reaction.

The time and temperature allowed for extension depends on the concentration and length of the target sequence. Most authors recommend an extension temperature of 72°C (Innes and Gelfand, 1990; Saiki, 1990; Kidd and Ruano, 1995). At the end of PCR, a final extension time of 10 minutes is added, to ensure all extension and double stranding has occurred.

As PCR technology has advanced, so too have the reagents used within the reaction. When it was first utilised in 1985 (Saiki *et al.*), the enzyme catalyst was the bacterial DNA polymerase, Klenow fragment polymerase I (pol I) of *Escherichia coli*. This enzyme is not thermoresistant so must be added at the beginning of each annealing cycle. This was rapidly replaced following the isolation of the thermostable DNA polymerases from *Thermus aquaticus*, (*Taq* polymerase) and *T. thermophilus*, (*Tth* polymerase). Using a thermoresistant enzyme allows complete amplification of the product without disturbance, thus giving greater reliability, precision, convenience and productivity (Bloch, 1991). It has also led to the use of rapid-cycling automated thermal cyclers.

The recommended concentration of *Taq* DNA polymerase in PCR amplifications is between 1 and 2.5 units per 100µl reaction when other parameters are optimal. (Innes and Gelfand, 1990; Perkin Elmer, 1993; Kidd and Ruano, 1995). However, a range of concentrations can be tested to define the optimum for a specific application.

Among the other reaction components the correct concentration of magnesium chloride can be highly influential to the outcome. Excluding the requirement DNA polymerase has for free magnesium ions, other factors in the reaction are affected including primer annealing efficiency,

product specificity and primer dimer formation. Free nucleotides have an affinity for Mg^{2+} ions, thus a higher concentration of dNTPs may require a higher concentration of magnesium chloride (Taylor, 1991). Because of these phenomena, the concentration should be between 0.5 and 2.5mM magnesium chloride (Innes and Gelfand, 1990).

Using the above criteria for primer design and PCR, studies were undertaken to provide a suitable system for the amplification of ascorbate peroxidase and glutathione reductase DNA sequences from *Rhododendron ponticum*, *R. hatsugiri* and *R. impeditum*. Also necessary was the establishment of a suitable plant culture system in an adaptable environment, which could be used as a source of material for high quality extractions of DNA, RNA and proteins.

2.2 Materials and Methods

2.2.1 Rhododendron micropropagation

Before beginning the DNA work, a suitable source of plant material had to be established. This was achieved by using *Rhododendron* micropropagation which allowed a large number of plants to be produced in a relatively short time, in an environment which was relatively easy to manipulate.

For the culture of micropropagules of *Rhododendron ponticum, R. impeditum* and *R. hatsugiri,* the procedure described by Kenyon *et al.* (1995) was used. Shoot tips of approximately 20mm in length were taken from stock plants and all the leaves removed. These shoots were surface sterilised in 2% w/v sodium hypochlorite solution for between 20 and 30 minutes and rinsed three times in sterile distilled water to remove all the bleach solution. The shoots were trimmed to eliminate oxidised tissue and the apical bud removed to promote axillary bud growth. The sections were placed in 60 ml sample jars containing 25 ml Anderson *Rhododendron* stage I medium (Appendix 1), pH 5.7, previously autoclaved for 15 minutes at 121°C (Anderson, 1980). Cultures were incubated under fluorescent light (280μmol/m^2.s) with an 18 hour daylength and a constant temperature of 20°C. When established shoots had formed, these were excised from the main stem and transferred to fresh Anderson *Rhododendron* Stage II medium (Shoot Multiplication medium, Appendix 1) and grown under the same conditions. Micropropagules were subcultured every 4-6 weeks onto fresh Stage II medium, and served as a source of material for DNA, RNA and protein extractions.

2.2.2. Inter-species sequence comparisons

A *Genbank* database search at the National Centre for Biotechnology Information (NCBI) database (website: www.ncbi.nlm.nih.gov/) revealed no sequence data for either ascorbate peroxidase or glutathione reductase in any *Rhododendron* species. The approach used to obtain sequence sites suitable for primer design was similar to that applied by Diffenbach *et al* (1993), in which related sequences from other plant species were pooled to identify highly conserved sequence regions.

A total of six species were studied for ascorbate peroxidase and glutathione reductase; *Arabidopsis thaliana* (thale cress), *Glycine max* (soybean), *Spinacia oleracea* (spinach), *Pisum sativum* (pea), *Oryza sativa* (rice) and *Nicotiana tabacum* (tobacco).

The sequences were manually aligned to identify regions of conservation. Once these had been determined (APX Figure 2.3; GR Figure 2.4), the areas of greatest homology were investigated as possible sites for PCR primer annealing.

2.2.3 Oligo 5.0 primer analysis software.

Oligo 5.0 (National Biosciences, Inc.) is a software tool which searches for and selects oligomers from a sequence data file suitable for use as primers or probes in PCR, sequencing, site-directed mutagenesis and hybridisation techniques. The program calculates hybridisation temperatures and secondary structures of the oligomers based on the nearest neighbour free energy change (ΔG) values (see Section 2.1).

One of the many functions of Oligo 5.0 is to perform a search for primers suitable for PCR. There are many parameters within the search program

which are automatically set to select suitable primer pairs. These include stringency values against intra-primer hairpin loops, non-compatible primer pairs and false priming. The program will also search for regions with a GC clamp and matches the melting temperature with oligomer length to produce optimal primer pairs. Parameters directly relating to the sequence are manually entered into the program.

2.2.4 Primer design

The mRNA sequence file for *Pisum sativum* ascorbate peroxidase was loaded into the Oligo 5.0 Primer Analysis software. The primers and probes option was chosen and specific search ranges entered according to the regions of sequence conservation identified. The area of sequence to be analysed for upper and lower primers were entered by oligonucleotide number, illustrated in Table 2.2. The desired length of the product was also entered, however this was primarily dictated by the primer search ranges. Once these had been completed, the program was ready to run. The same search strategy was used for *Arabidopsis thaliana* glutathione reductase mRNA and the areas of conservation and primer analysis can be found in Figure 2.4.

Sequence Range	Ascorbate Peroxidase	Glutathione Reductase
Set A Upper Primer	185-215	360-400
Lower Primer	740-770	885-910
Set B Upper Primer	145-180	620-655
Lower Primer	620-650	1180-1200
Set C Upper Primer	185-215	915-930
Lower Primer	490-520	1501-1530

Table 2.2. Search ranges entered into Oligo 5.0 for primer design and analysis. These areas are marked as bold in Figure 2.3 (Ascorbate Peroxidase) and Figure 2.4 (Glutathione Reductase)

Primers which fell within the desired regions and were recommended for PCR by the programme were selected for further investigation and were custom synthesised (Cruachem Ltd.)

2.2.5 DNA extraction (method 1)

For the extraction of DNA from *Rhododendron* species, a protocol derived from Paterson *et al.*, (1993) was used. Fresh tissue (40 mg) was homogenised in liquid nitrogen and added to 500 µl DNA extraction buffer (0.35 M glucose, 0.1 M Trizma-HCl, 0.005 M EDTA, 2% (w/v) polyvinylpyrolidone (PVP40) in a total volume of 1 litre, pH 7.5, and autoclaved at 121°C for 15 minutes. Immediately before use, 0.1% (w/v) diethyldithiocarbamic acid (DIECA), 0.1% (w/v) filter-sterilised ascorbic acid and 0.2% v/v β-mercaptoethanol were added).

Following incubation on ice for 10 minutes, the sample was centrifuged at 7000 *g* for 20 minutes and the pellet saved. This was resuspended in 600 µl nuclei lysis buffer (0.1 M Trizma-HCl, 1.4 M NaCl, 0.02 M EDTA, 2% (w/v) hexadecyl triammonium bromide (CTAB), 2% (w/v) PVP40, pH adjusted to 8.0 and autoclaved at 121°C for 15 minutes. Prior to use, 0.1% (w/v) DIECA, 0.1% (w/v) ascorbic acid and 0.2% v/v β-mercaptoethanol were added), and incubated at 65°C for 30 minutes, with regular agitation to keep the sample homogenous.

The mixture was cooled to 35°C, and incubated for a further 10 minutes following the addition of 5 µl RNase (100 µl/ml). Chloroform:octanol (24:1; 600 µl) was added, and the mix inverted approximately 50 times. Following centrifugation at 7000 *g* for 5 minutes, the aqueous layer, containing DNA, was transferred to a fresh tube and the chloroform:octanol step repeated. Again, the aqueous layer was

transferred to a fresh tube and 450 µl ice-cold isopropanol was added and thoroughly mixed to precipitate the DNA. Precipitation was further aided by incubation at -20°C for 30 minutes. The sample was centrifuged at 10,000 *g* for 5 minutes to pellet the DNA. which was subsequently washed in 70% (v/v) ethanol. The centrifugation step was repeated to repellet the DNA, which was then air-dried for 30 minutes to remove any traces of ethanol. The DNA pellet was then resuspended in sterile Molecular Biology Grade water (BDH) and stored at -20°C.

2.2.6 DNA extraction (method 2)

This method followed the Nucleon Phytopure™ Plant DNA Extraction kit (Scotlab Bioscience). *Rhododendron* tissue (0.1g) was homogenised in liquid nitrogen and added to 600 µl Reagent 1. When fully homogenous, RNase (100 µl/ml) was added and mixed thoroughly. After incubation at 37°C for 30 minutes, 200 µl reagent 2 was added and the mixture rehomogenised and incubated at 65°C for 10 minutes, with regular agitation throughout.

Following a further incubation period of thirty minutes on ice, 500 µl ice-cold chloroform and 100 µl silica suspension were added to the mixture. This was incubated at room temperature for 10 minutes with regular agitation, and then centrifuged at 1,300 *g* for 10 minutes. The upper, aqueous layer was transferred to a fresh tube. To this, 450 µl ice-cold isopropanol was added, and incubated at -20°C for 30 minutes to precipitate the DNA. The mix was centrifuged at 4000 *g* for 5 minutes to pellet the DNA, which was then briefly washed in 70% (v/v) ethanol and recentrifuged at 4000 *g*. The pellet was air-dried for 15 minutes and resuspended in sterile Molecular Biology Grade water and stored at -20°C.

Using both these techniques, DNA was extracted from *Rhododendron ponticum, R. impeditum* and *R. hatsugiri.*

2.2.7 Measurement of DNA concentration

UV densitometry was used as a means to calculate the concentration of DNA extracted from a tissue sample. DNA solution (2 µl) was added to an Eppendorf tube containing 2 µl ethidium bromide (5 µg/ml) and incubated for 10 minutes at room temperature. The samples were pipetted as discrete dots onto an Ultraviolet transilluminator (300 nm) and photographed using the IS-500 Gel Documentation System. This photograph was saved onto disk and the image transferred to the Windows Bandleader programme. Using a grid to isolate each dot, the programme calculated the fluorescence reading for each sample. Using 10 ng bacteriophage lambda (λ) DNA as a standard, the amount of DNA for each sample could be calculated using the following equation:

$$x = (S \times 10\text{ng}/\mu\text{l}) \div \lambda$$

where S is the sample densitometry reading, 10 is the amount of λ DNA standard and λ is the densitometry reading of λ DNA standard.

2.2.8 Polymerase Chain Reaction

The basic parameters used as a starting point for primer tests were as follows: the reaction mix consisted of PCR buffer (10 mM Tris-HCl, 50 mM KCl; Perkin Elmer), 1.5 mM $MgCl_2$ (Perkin Elmer), 20 pmoles upper primer, 20 pmoles lower primer, 2.5 mM dNTPs (Perkin Elmer), 1 unit *Taq* DNA Polymerase (Perkin Elmer) and 10 ng *Rhododendron* DNA, in a total volume of 25 µl. The thermal cycling programme was set at 95°C for 5 minutes initially, followed by 1 minute at 94°C, 2 minutes at 55°C and 3 minutes at 72°C for a total of 30 cycles, followed by a final extension

cyclogene thermal cycler. In pre-tests, all four machines gave similar results.

As mentioned in section 2.1.2, the main PCR conditions to review when optimising the reaction are the temperatures used for template-specific annealing of primers and the concentration of the primers used. A series of annealing temperatures were tested to improve the specificity of the reaction: 45, 50, 52, 55, 56, 57 and 58°C. These temperatures were initially used with all primer sets and each *Rhododendron* DNA sample. Four primer concentrations were also used as a means of optimising the PCR: 20, 30, 35 and 40 pmoles were tested.

2.2.9 Gel electrophoresis

Agarose gels were used to analyse PCR products and DNA extractions. Agarose, 1% (w/v) (Seakem Le, Flowgen) was melted in 0.5× Tris Borate EDTA (TBE) buffer (Sigma) and poured into the appropriate gel casting tray: Horizon 58 (minigel), Horizon 11.14 (midigel) or Horizon 2025 (maxigel) (Gibco BRL). Once set, the gel was immersed in TBE buffer and the wells loaded with DNA sample. Prior to loading, the DNA sample was mixed with DNA loading buffer (0.0025% (w/v) bromophenol blue, 6 mM EDTA, 30 % (v/v) glycerol) to enable the samples to sink into the wells. The gel was electropherised at a voltage suitable to the gel size, and when the run was completed, the gel was immersed in ethidium bromide stain (5 μg/ml) for 30 minutes, then destained in distilled water for 30 minutes. The gel was viewed on an UV transilluminator and photographed using the IS-500 Gel Documentation System.

2.3 Results and Discussion

2.3.1 Rhododendron micropropagation

Micropropagation of *R. ponticum*, *R. hatsugiri* and *R. impeditum* resulted in successful shoot growth and culture maintenance. Plant culture by this method provided a ready source of sterile material in an easily controlled environment, with no apparent somaclonal variation.

2.3.2 Inter-Species sequence comparisons

As stated in section 2.2.1, the mRNA sequence for ascorbate peroxidase in *Pisum sativum* was used in a Genbank database search. Comparisons show that the gene has a relatively high level of sequence conservation across the range of plant species chosen. Table 2.3 shows the percentage homology of APX in pea with five other plant species, whilst Figure 2.3 illustrates the regions of conservation in the gene throughout the six species.

Species	% Homology with APX in *Pisum sativum*
Glycine max	85.6
Nicotiana tabacum	77.7
Spinacia oleracea	77.4
Arabidopsis thaliana	75.5
Oryza sativa	72

Table 2.3. Ascorbate peroxidase percentage homology results between the species studied

The same procedure was followed for glutathione reductase using the mRNA sequence in *Arabidopsis thaliana*. The percentage homology

results are shown in Table 2.4. Again, a relatively high percentage of homology was found between the species, as is illustrated in Figure 2.4.

Species	% Homolgy with GR in *Arabidopsis thaliana*
Pisum sativum	76.9
Glycine max	76.8
Nicotiana tabacum	76.8
Oryza sativa	65.9
Spinacea oleracea	63.9

Table 2.4. Glutathione reductase percentage homology results between the species studied

2.3.3 Primer design

Figures 2.3 and 2.4 also depict the areas of the sequence from which the primers were designed. Because they were selected from conserved regions of the gene and displayed reasonable melting temperature and internal stability profiles (Figure 2.5), it was felt they were more likely to anneal to the *Rhododendron* template DNA, whilst remaining specific to the gene. To ensure this further, alternative nucleotides were incorporated into the primer sequences for those areas where there was a choice of two bases in the sequence. Table 2.5 shows the possible nucleotide alternatives used in primer design.

Figure 2.3. Ascorbate peroxidase sequence homology. * indicates identical bases within all sequences, bold type within the sequence indicates the regions chosen as parameters for Oligo 5.0 primer design and analysis. Cyan typescript represents the primer sets A, B and C synthesised. Alternative bases that were used in the primer sequences are represented in red type.

```
                                                  ***  ****   *** *
Pea1   GAATTCGGCTTGTGCTCTCCTCGTGTCACTAGGGTTTAACTTCTTCGTTTTTGCTTCTTA 60
Syb11                                                 TTCCATTTTCTCTCTCA 27

         **** ********  ** ****  **    ** **    ** **     * ***
Pea61  GATTTCGAGAATCGTTTGCTATGGGAAAATCTTACCCAACTGTTAGTCCCGATTACCAGA 120
Syb28  CTTTTCAAGAATCGTAAGCTATGGGAAAGTCTTACCCAACTGTGAGTGCTGATTACCAGA 87
Tob79                 TTGCTATGGGTAAGTGCTATCCCACTGTAAGCGAGGAGTACCTCA 124
Spn43                   GCCATGGGAAAGAGCTACCCAACTGTCAGTGAGAACTACCAGA 86
Ath52                                  TACCCAACCGTGAGCGAAGATTACAAGA 80
Ric37                   GCCATGGCTAAGAACTACCCCGTCGTGAGCGCCGAGTACCAGG 80
                                         B
       *  *  * ** **      *   ** **  *R* WY* **Y**W******   ** ** *
Pea121 AGGCCATTGAAAAGGCTAAGAGGAAGCTCAGAGGTTTTATCGCTGAGAAGAAATGCGCTC 180
Syb88  AGGCCGTTGAGAAGGCGAAGAAGAAGCTCAGAGGCTTCATCGCTGAGAAGAGATGCGCTC 147
Tob125 AGGCTGTTGACAAATGTAAGAGGAAACTCAGAGGACTCATTGCTGAGAAGAATTGCGCTC 184
Spn87  AATCTATTGAAAAGGCCCGGAGAAAGCTCAGGGGTTTGATCGCAGAGAAGCAATGTGCTC 147
Ath81  AGGCTGTTGAGAAGTGCAGGAGGAAGCTCAGAGGTTTGATCGCTGAGAAGAACTGTGCAC 140
Ric81  AGGCCGTCGAGAAGGCCAGGCAGAAGCTGCGCGCCCTCATCGCCGAGAAGAGCTGCGCCC 140
                  A + C
       *  * **  * **WY* ** ******** ** ** ** *  *           *   * *
Pea181 CTCTAATTCTCCGTTTGGCATGGCACTCTGCTGGTACTTTTGATTCCAAGACAAAGACTG 240
Syb148 CTCTAATGCTCCGTTTGGCATGGCACTCTGCTGGAACCTTTGCAAGGGCACGAAGACCGG 207
Tob185 CTCTTATGCTCCGTCTTGCATGGCACTCTGCTGGTACCTATGATGTGTGCTCCAAAACTG 244
Spn148 CTCTTATGCTTCGTCTTGCATGGCACTCTGCTGGTACCTTTGATTGTACTTCAAAAACTG 207
Ath141 CCATCATGGTCCGACTCGCATGGCACTCTGCTGGAACTTTCGATTGTCAATCAAGGACTG 200
Ric141 CTCTCATGCTCCGCCTCGCGTGGCACTCGGCGGGGACGTTCGACGTGTCGTCGAAGACCG 200

         *  ** *  *   *    *         *  *          *     *     *
Pea241 GTGGTCCTTTCGGAACAATTAAGCACCAAGCTGAGCTTGCTCATGGTGCTAACAACGGTC 300
Syb208 TGGACCCTTCGGAACCATCAAGCACCCTGCCGAACTGGCTCACAGCGCTAACAACGGTCT 267
Tob245 GAGGTCCATTCGGTACCATGAGGCTCAAGGCTGAGCAAGGACATGGAGCAAACAATGGTA 304
Spn208 GAGGTCCCTTTGGTACAATGAAGCACCAGGCAGAGCTGGCTCATGGGGCTAACAATGGGC 267
Ath201 GAGGTCCATTCGGAACAATGAGGTTTGACGCTGAGCAAGCTCATGGAGCCAACAGTGGTA 260
Ric201 GGGGCCCGTTCGGGACGATGAAGACCCCGGCGGAGCTGTCGCACGCCGCCAACGCGGGGC 260

       *           *  *  *   *   *     * *         *
Pea301 TTGATATCGCGGTTAGGCTGTTGGAGCCTATTAAGGAGCAATTCCCTATTGTGAGCTATG 360
Syb268 TGACATCGCTGTTAGGCTTTTGGAGCCACTCAAGGCGGAGTTCCCTATTTTGAGCTACGC 327
Tob305 TTGACATTGCTATAAGACTCTTGGAGCCCATTAAGGAGCAGTTTCCTATCCTCTCATATG 364
Spn268 TTGTTATTGCTGTTAGGCTGTTGGAACCCATCAAGGAACAATTCCCCGAAATTACTTATG 327
Ath261 TCCACATTGCTCTTAGGTTGTTGGACCCCATCAGGGAGCAACATCTTCCCTACCATCTCT 320
Ric261 TGGACATCGCGGTGCGGATGCTCGAGCCCATCAAGGAGGAGATACCCACCATCTCCTACG 320

            *              *   *  *                *  *  *      * *
Pea361 CTGATTTCTACCAGTTGGCTGGTGTTGTTGCTGTTGAGATTACCGGTGGACCTGAAGTTC 420
Syb328 CGATTTCTACCAGTTGGCTGGCGTTGTTGCCGTTGAGGTCACGGGTGGACCTGAAGTTCC 387
Tob365 GTGATTTCTATCAATTAGCTGGAGTTGTTGCTGTTGAAGTTACTGGAGGACCTGATGTTC 424
Spn328 CTGACTTTTACCAGCTGGCTGAGTTTGTGGCCGTTGAAGTTACTGGAGGACCTGAAGTTC 387
Ath321 TTTGCTGATTTCAGCTTGCTGGTGTTGTGGCCGTTGAAGTTACTGGTGGCCCTGACATTC 380
Ric321 CCGATTTCTACCAGCTTGCCGGAGTTGTGGCCGTCGAGGTGTCCGGTGGACCTGCCGTCC 380

         *    **  *       *   *        *           *        **
Pea421 CTTTCCACCCTGGTAGGGAGGACAAGCCTGAGCCACCACCTGAGGGTCGCTTGCCTGATG 480
Syb388 ATTCCACCCTGGAAGAGAGGACAAGCCTGAGCCACCACCAGAGGGTCGCTTGCCCGATGC 447
Tob425 CCTTTCACCCTGGTAGAGAGGACAAGACAGAGCCACCCGTTGAAGGTCGCTTGCCTGATG 484
Spn388 CCTTCCACCCAGGCAGAGAGGACAAGCCAGAGCCACCCCAGGAAGGACGTCTCCCTGATG 447
Ath381 CTTTCCACCCTGGAAGAGAGGACAAGCCCCAACCACCTCCAGAGGGTCGTCTTCCTGATG 440
Ric381 CCTTCCACCCAGGAAGGGAGGACAAACCTGCACCCCCACCTGAGGGCCGTCTTCCTGATG 440

                    C
       *    * ** *     *  R     Y     S**      *     ***  *
Pea481 CCACTAAGGGTTCTGACCATTTGAGGGATGTGTTTGGAAAGGCTATGGGGCTTAGTGATC 540
Syb448 CACTAAGGGTTCTGACCATTTGAGAGATGTGTTTGGCAAAGCTATGGGGCTTACTGACCA 507
Tob485 CTACCAAGGGTTCTGACCACTTGAGAGATGTGTTTGTGAAGCAAATGGGTCTATCTGATA 544
Spn448 CCACCAAGGGTTGTGACCATTTGAGAGATGTCTTCATCAAGCAAATGGGTCTTACTGACC 507
Ath441 CTACCAAGGGTTGTGACCATTTGAGAGATGTCTTTGCTAAGCAGATGGGCTTATCTGACA 500
Ric441 CTACCAAGGGTTCTGACCACCTAAGGCAGGTCTTCGGTGCGCAGATGGGCTTGAGTGATC 500
```

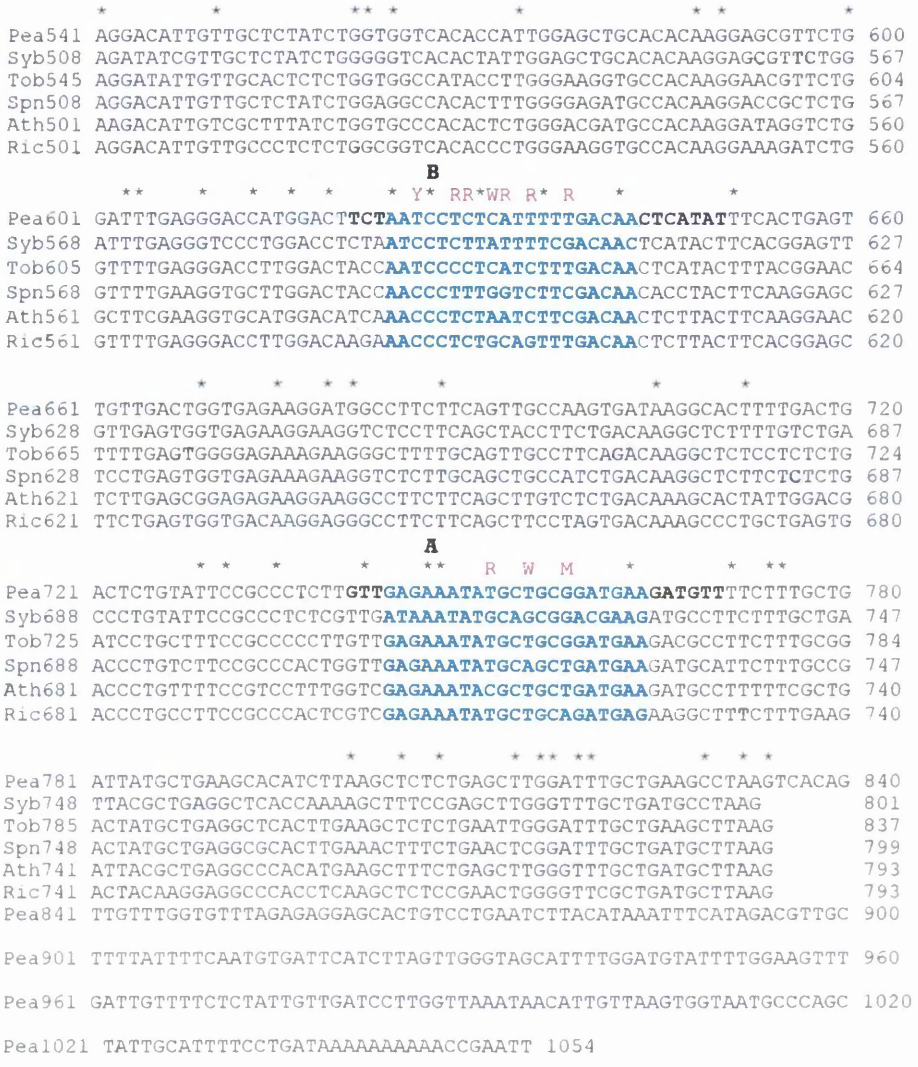

Figure 2.4. Glutathione reductase sequence homology. * indicates identical bases within all sequences, bold type within the sequence indicates the regions chosen as parameters for Oligo 5.0 primer design and analysis. Cyan typescript represents the primer sets A, B and C synthesised. Alternative bases that were used in the primer sequences are represented in red type.

```
Ath1 AGAAGAAGAATCGTGGAGATGGCTTCGACCCCGAAGCTTACCAGTACAATTTCATCATCT 60

Ath61 TCTCCATCTCTTCAATTCCTCTGCAAAAAACTCCCAATCGCAATTCATCTACCATCATCT 120

Ath121 TCTTCCTCTAGCTTTCTCTCGCTTCCTAAAACCCTAACCTCTCTCTATTCTCTCCGTCCC 180

Ath181 CGTATCGCCCTACTCTCAAACCACCGCTATTACCACTCTCGCCGGTTTTCTGTTTGTGCC 240

            ** * ** ** ** **  *    ***** ** ** ** **  * ***** *****
Ath241 AGTACCGATAATGGAGCTGAATCAGACCGCCACTACGATTTTGATCTCTTCACTATCGGT 300
Pea266     CCCAAAACGGCGCCGACCCCGCCCGCCAGTATGACTTCGACCTTTTCACCATCGGC 316
Tob209          AATGGCGCTGACGCTCCTCGCCACTACGACTTTGACTTATTCACCATCGGT 260
Syb247                            CCACTACGACTTCGACCTCTTCACCATCGGC 278

         ** ** ***** ** **  * **    ** ** **  * *  *  ** ** **    **
Ath301 GCCGGAAGCGGCGGCGTCCGCGCCTCTCGCTTCGCCACTAGCTTCGGTGCATCCGCCGCC 360
Pea317 GCTGGAAGCGGAGGCGTCCGTGCTTCCCGCTTTGCCTCCAATTTCGGCGCTTCTTCTGCT 376
Tob261 GCTGGTAGCGGTGGTGTTAGGGCTTCTCGTTTTGCGTCTAATTTTGGGGCTTCTGTTGCT 320
Syb279 GCAGGCAGCGGCGGGGTCCGAGCCCGTCGCTTCGCCGCCAACTACGGCGCTTCTGTCGCC 338
       A
         * **Y*****Y*****Y**Y***** ** ** **  *  * ** *  *   * ** **
Ath361 GTTTGCGAGCTTCCTTTTTCCACTATCTCTTCCGATACTGCTGGAGGCGTTGGAGGAACG 420
Pea377 GTCTGCGAGCTCCCTTTCTCTACTATCTCCTCCGATACCACCGGTGGTGTCGGCGGCACC 436
Tob321 GTTTGTGAGCTCCCTTTCTCCACTATTTCTTCTGATTCCACTGGTGGCGTTGGTGGCACG 380
Syb339 ATCTGCGAGCTTCCTTTCTCCACTATCTCCTCCGAAACCACCGGAGTCG---GCGGAACC 398

         ****** *    *         ** *  ** *                  **         *
Ath421 TGTGTATTGAGAGGATGTGTACCAAAGAAGTTACTTGTGTATGCATCCAAATACAGTCAT 480
Pea437 TGTGTAATACGGGGATGTGTCCCTAAGAAATTGCTAGTCTATGCCTCAAAATTCTCTCAT 496
Tob381 TGTGTACTTGTGGATGCGTACCCAAGAAATTACTCGTGTACGCGTCAAAATATTCTCACT 440
Syb399 TGTGTAATACGAGGATGTGTGCCGAAGAAGTTGCTGGTTTATGCATCTAAATTTTCTCAT 458

         ** ***** ** **   *** ** ** ****  *****    ** **      *** ***
Ath481 GAGTTTGAAGACAGTCATGGATTTGGTTGGAAGTATGAGACTGAGCCTTCTCATGATTGG 540
Pea497 GAATTTGAAGAAAGCAATGGTTTTGGATGGAGATATGACAGTGAACCTAAGCATGACTGG 556
Tob441 GAGTTTGAGGAAAGTTGTGGTTTTGGATGGAACTATGATGTGGAACCTAGATTTGATTGG 500
Syb459 GAATTTGAAGAAAGTAATGGTTTCGGATGGAGATATGACAGTGAGCCCAAGCATGATTGG 518

         *  *   * ** ** ** ** ** ** ***** *****  * ** ** ** ** ******
Ath541 ACTACTTTGATTGCTAACAAGAATGCTGAGTTACAGCGGTTGACTGGTATTTATAAGAAT 600
Pea557 AGTAGTTTGATTGCTAATAAAAACGCCGAGTTGCAGCGGCTTACTGGTATCTATAAGAAT 616
Tob501 AGCACCCTCATTGCCAATAAAAATGCCGAGTTGCAGCGCCTCACGGGTATTTACAAGAAT 560
Syb519 AGTAGTTTCATAGCTAATAAAAATGCTGAGTTGCAGCGTCTTACTGGCATCTACAAGAAT 578
                                  B
         *   ***  ** **    ** *   **********YMRW*****R *  * **  * **
Ath601 ATACTGAGCAAAGCTAATGTCAAGTTGATTGAAGGTCGTGGAAAGGTTATAGACCCACAC 660
Pea617 ACTTTGAAAAATGCCGGTGTTAAGTTGATTGAAGGCCGTGGAAAGATTGTAGATGCTCAC 676
Tob561 ATTCTGAAGAATGCTGGTGTCACTCTGATTGAAGGGAGAGGAAAGGTTGTGGATCCTCAT 620
Syb579 ATTTTGAACAATGCTGGGGTCAAGCTGATTGAAGGCCATGGAAAGATGATAGATCCTCAC 638

         ** ** ***** ***** **  * **  *  * *   * **  *  ** ******* **
Ath661 ACTGTTGATGTAGATGGGAAAATCTATACTACGAGGAATATTCTGATTGCAGTTGGTGGA 720
Pea677 ACAGTTGATGTTGATGGGAAGTTATATTCAGCGAAACACATTTTAGTTTCAGTTGGAGGT 736
Tob621 ACGGTGGATGTGGATGGAAAACTCTACTCGGCTAAGAACATACTGATTTCAGTTGGGGGA 680
Syb639 ACGGTTGATGTTAATGGGAAGCTATATTCAGCCAAACACATTTTAGTTGCAGTTGGAGGT 698

         ** ** ** ** ** ** ***** ** *  ** *  ** ** ***** ***** ** ***
Ath721 CGTCCTTTCATTCCTGACATTCCAGGAAAAGAGTTTGCTATTGATTCTGATGCCGCGCTT 780
Pea737 CGACCCTTCATTCCTGATATTCCTGGAAAGGAATATGCAATAGATTCAGATGCTGCCCTT 796
Tob681 CGCCCATTTATCCCAGACATTCCTGGTAGCGAATATGCTATAGATTCCGATGCTGCCCTT 740
Syb699 CGCCCCTTCATTCCTGATATCCCTGGGAAGGAATTAGCAATAGATTCAGATGCTGCCCTT 758

         ***** **  * ** ***   ** ** ** ** ** ** ** ** ***** **  * **
Ath781 GATTTGCCTTCCAAGCCTAAGAAAATTGCAATAGTTGGTGGTGGCTACATAGCCCTGGAG 840
Pea797 GATTTACCATCAAAGCCTCAGAAGATAGCTATTGTTGGTGGGGGTTACATTGCCTTGGAG 856
Tob741 GATTTGCCAACGAAGCCTAACAAAATTGCCATTGTCGGAGGCGGTTACATTGCACTTGAA 800
Syb759 GATTTACCAACAAAACCTGTGAAAATAGCCATTGTTGGTGGTGGTTACATTGCCTTGGAG 618
```

```
                                                                  A
          ***** ** ***** *****  * **  **** ** ***** ******   *********
Ath841    TTTGCGGGGATCTTCAATGGTCTTAACTGTGAAGTTCATGTATTTATAAGGCAAAAGAAG 900
Pea857    TTTGCTGGTATCTTTAATGGTTTGAAAAGTGAAGTTCATGTATTTATAAGACAAAAGAAG 916
Tob801    TTTGCTGGAATCTTCAATGGCTTGAAAAGTGAGGTCCATGTTTTTATAAGACAAAAGAAG 860
Syb619    TTTGCTGGTATCTTTAATGGTTTGAAAAGTGAGGTTCATGTATTTATACGGCAAAAGAAG 678
                          C
          **MR**K*Y**************  R*Y**R*****Y****  **  * *** *    ***
Ath901    GTGCTGAGGGGATTTGATGAAGATGTCAGGGATTTCGTTGGAGAGCAGATGTCTTTAAGA 960
Pea917    GTTTTGCGGGGATTTGATGAAGAGATTAGAGATTTTGTTGCAGAAAATATGGCTCTGAGA 976
Tob861    GTTTTGAGAGGATTTGATGAAGAAATTAGGGATTTTGTTGGTGAACAGATGTCACTGAGA 920
Syb679    GTTTTGCGGGGATTTGATGAAGAGATTAGAGATTTTGTTGAAGAACAAATGTCTGTAAGA 738

          ** ***** ** ** ** ** ** ** ***  *** **    **    *  ***** **
Ath961    GGTATTGAGTTTCACACTGAAGAATCCCCTGAAGCCATCATCAAAGCTGGAGATGGCTCG 1020
Pea977    GGTATTGAATTCCATACTGAGGAGTCTCCTGTAGCTATCACTAAGGCAGCTGATGGTTCG 1036
Tob921    GGAATTGAGTTCCATACTGAGGAGTCGCCTCAGGCTATTGTAAAGTCAGCGGATGGCTCA 980
Syb739    GGTATTGAATTCCATACCGAGGAGTCTCCTCAAGCTATCACGAAGTCAGCGGATGGCTCA 798

            * *** * ***** * **  ** ** *  ** ** ** ** *** *  * ***** **
Ath1021   TTCTCTCTGAAGACCAGCAAGGGAACTGTTGAGGGATTTTCGCATGTTATGTTTGCAACT 1080
Pea1037   CTCTCTTTAAAGACCAACAAAGGTACTGAGGAAGGTTTCTCTCATATTATGTTTGCCACT 1096
Tob981    CTGTCTTTAAAGACTAGCAGAGGAACAGTTGAAGGTTTCTCTCATATCATGTTTGCAACG 1040
Syb799    TTCTCTTTAAAGACCAACAAAGGTACAGTGGACGGTTTCTCACATATTATGTTTGCTACA 858
Ric909                                                TGTTCTGTTTGCTACA 924
Spn844                                                TGTACTCTTTGCTACT 859

          **  *    ** **  *  *     *     * **       ** **  ** *
Ath1081   GGTCGCAAGCCCAACACAAAGAACTTAGGGTTGGAGAATGTTGGCGTTAAAATGGCGAAA 1140
Pea1097   GGACGCTCACCTAATACTAAGGATTTGGGCCTGGAGTCTGTTGGTGTGAAAGTGGCTAAA 1156
Tob1041   GGAAGAAGACCTAATACAAAGAATTTAGGATTAGAGACAGTGGGAGTGAAAATGACAAAG 1100
Syb859    GGACGCAGACCTAATACTCAGAACTTAGGCCTGGAGTCTGTTGGTGTGAAACTAGCTAAA 918
Ric925    GGTCGCACACCAAACTCCCAGAGGTTGAACTTGGAAGCTGCTGGTGTTGAAGTTGATAAT 984
Spn860    GGGCGTTCTCCTAACACAAAAAGGCTGAATCTGGAAGCTTTAGGTGTAGAACTTGACCGA 919
                                                                B
            *** *  *  ******* ** ** **     **     **    KW**W*****WY**
Ath1141   AATGGAGCAATAGAGGTTGACGAATATTCACAGACATCTGTTCCATCCATCTGGGCTGTT 1200
Pea1157   GATGGATCAATAGAGGTTGATGAATACTCTCAAACATCGGTTCCTTCTATTTGGGCAATT 1216
Tob1101   AATGGAGCCATAGAGGTTGATGAGTATTCTCGTACATCAGTACCATCAATTTGGGCAGTT 1160
Syb919    GATGGAGCTATAGAGGTTGATGAATACTCTCAAACATCAGTTTATTCAATTTGGGCAGTT 978
Ric985    ATTGGAGCTATAAAGGTTGATGATTATTCTCGTACATCAGTCCCAAATATATGGGCTGTG 1044
Spn920    ACAGGAGCTGTCAAGGTTGACGAGTATTCAAGAACGAGTGTACCTAGCATATGGGCTATT 979

          ** ****  **  *  *  * **  * ** ** ** **  ******* *        **
Ath1201   GGGGATGTTACTGACCGAATCAATTTGACTCCAGTTGCTTTGATGGAGGGAGGTGCATTG 1260
Pea1217   GGAGATGCTACAAATAGAGTAAATCTCACTCCAGTTGCTTTGATGGAGGGAGTGGCATTA 1276
Tob1161   GGAGATGTTACTGATAGAATTAATTTAACTCCAGTTGCTTTGATGGAGGGAGGAGCATTG 1220
Syb979    GGAGATGTTACAAACAGGATAAATCTCACCCCAGTTGCTTTGATGGAGGGAGGAGCATTA 1038
Ric1045   GGTGATGTAACGAACCGGATAAATTTAACACCTGTTGCACTGATGGAGGCTACCTGCTTT 1104
Spn980    GGTGATGTTACTAATCGAATGAACCTTACTCCTGTGGCTTTGATGGAAGGAACTTGTTTC 1039

             *****  * **        *    *  ** ** ***** **    * *   *     **
Ath1261   GCTAAAACTTTGTTTCAAAATGAGCCAACAAAGCCTGATTATAGAGCTGTTCCCTGCGCC 1320
Pea1277   GCAAAAACTTTGTTTCAGAATGAGCCGACAAAACCTGATTATAGGGCTATACCTTCTGCT 1336
Tob1221   GCAAAAACTATTTTCGCTCATGAACCCACAAAACCAGATTATAGGAATGTACCAGCTGCG 1280
Syb1039   GTAAAAACGCTGTTTCAGGATAACCCAACAAAACCTGATTATAGAGCTGTTCCTTCTGCT 1098
Ric1105   TCTAAAACTGTGTTTGGTGGCCAGCCAACTAAACCTGATTACAGAGATGTACCTTGTGCT 1164
Spn1040   GCTAAAACTGTATTTGGGGGCCAGAATAGCAAACCGGATTACAGCAATATAGCATGTGCC 1099

          ** **   *   ** **  *        ** **  * *  **  * ** **     *  *
Ath1321   GTTTTCTCCCAGCCACCTATTGGAACAGTTGGTCTAACTGAAGAGCAGGCCATAGAACAA 1380
Pea1337   GTGTTTTCCCAACCACCAATTGGAGGAGTTGGTCTTACAGAGGAACAGGCTGCTGAACAA 1396
Tob1281   GTATTTTCCCAACCACCTATTGGACAAGTTGGTCTAATGGAAGAACAGGCCATCAAAGAG 1340
Syb1099   GTATTTTCTCAACCACCAATTGGACAAGTTGGTCTTACTGAGGAACAGGCTGTACAACAA 1158
Ric1165   GTTTTCTCCATCCCACCACTATCAGTAGTGGGCTTGAGTGAACAGCAGGCTTTGGAGGAA 1224
Spn1100   GTCTTCAGCATACCTCCTCTAGCCGTGGTGGGCCTAAGTGAAGAACAAGCTATAGAGCAA 1159
              ** **  *      * *  ** *    **     *     **
Ath1381   TATGGTGATGTGGATGTTTACACATCGAACTTTAGGCCATTAAAGGCTACCCTTTCAGGA 1440
Pea1397   TATGGTGATATTGACGTTTTCACAGCAAATTTTAGGCCGATGAAGGCCACCCTCTCTGGG 1456
Tob1341   TTTGGTGACGTTGATGTTTATACAGCAAATTTTAGGCCCTTAAAGGCTACTATTTCTGGT 1400
Syb1159   TATGGAGATATTGACATCTTCACTGCAAATTTAGGCCGCTGAAAGCTACTCTCTCTGGGT 1218
Ric1225   GAAGGCGATGTTCTTGTTTACACTTCCAGCTTCAACCCAATGAAGAACAGCATATCCAAA 1284
Spn1160   AATGGTGATATTTTGGTCTTCACGTCATCATTCAACCCAATGAAAAATACTATTTCTGGA 1219
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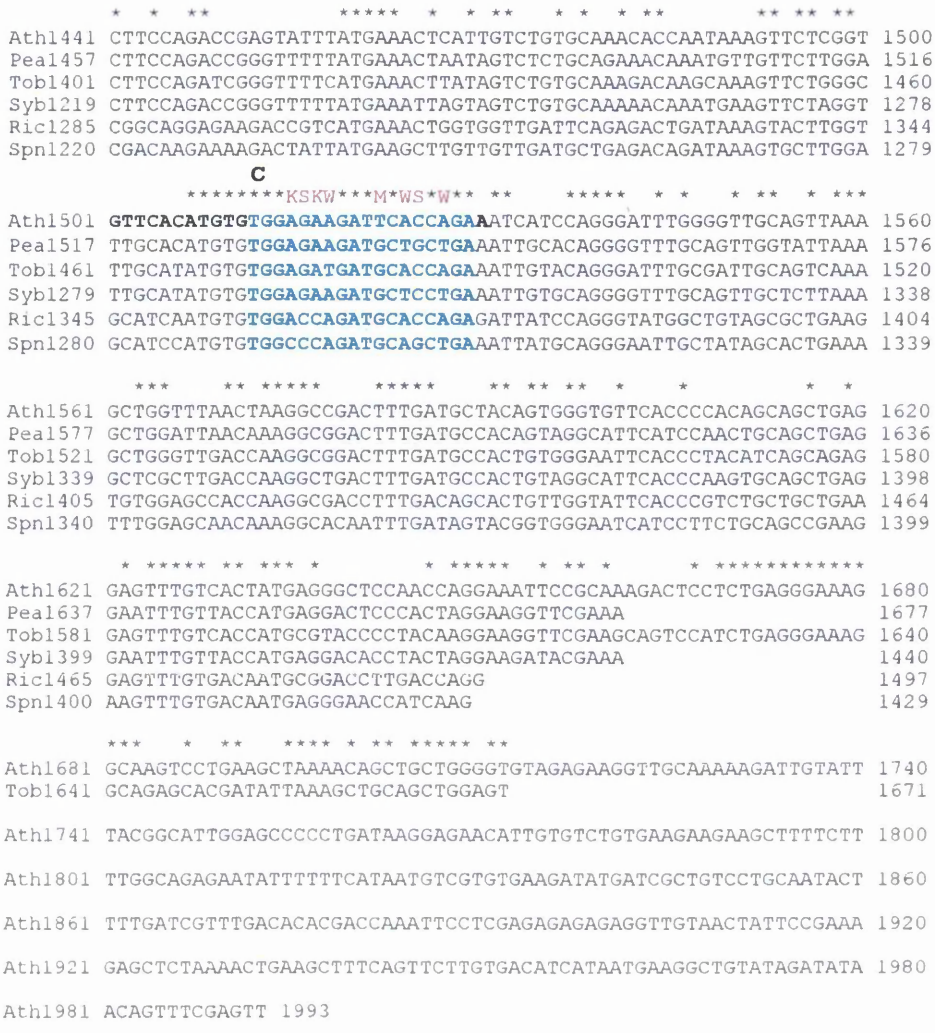
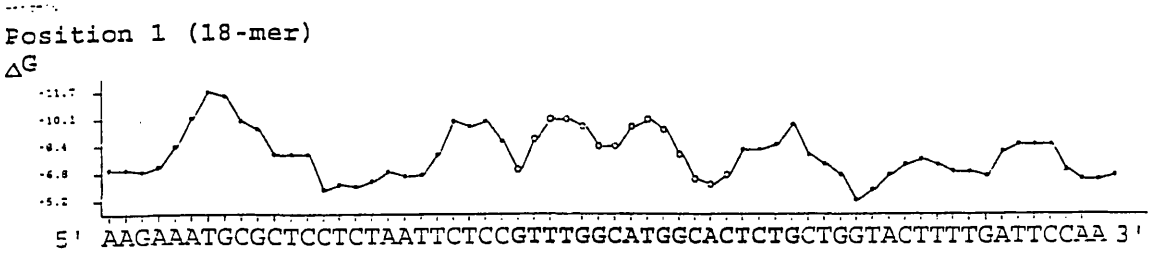

Ath1441 CTTCCAGACCGAGTATTTATGAAACTCATTGTCTGTGCAAACACCAATAAAGTTCTCGGT 1500
Pea1457 CTTCCAGACCGGGTTTTTATGAAACTAATAGTCTCTGCAGAAACAAATGTTGTTCTTGGA 1516
Tob1401 CTTCCAGATCGGGTTTTCATGAAACTTATAGTCTGTGCAAAGACAAGCAAAGTTCTGGGC 1460
Syb1219 CTTCCAGACCGGGTTTTTATGAAATTAGTAGTCTGTGCAAAAACAAATGAAGTTCTAGGT 1278
Ric1285 CGGCAGGAGAAGACCGTCATGAAACTGGTGGTTGATTCAGAGACTGATAAAGTACTTGGT 1344
Spn1220 CGACAAGAAAAGACTATTATGAAGCTTGTTGTTGATGCTGAGACAGATAAAGTGCTTGGA 1279
C
Ath1501 GTTCACATGTGTGGAGAAGATTCACCAGAAATCATCCAGGGATTTGGGGTTGCAGTTAAA 1560
Pea1517 TTGCACATGTGTGGAGAAGATGCTGCTGAAATTGCACAGGGGTTTGCAGTTGGTATTAAA 1576
Tob1461 TTGCATATGTGTGGAGATGATGCACCAGAAATTGTACAGGGATTTGCGATTGCAGTCAAA 1520
Syb1279 TTGCATATGTGTGGAGAAGATGCTCCTGAAATTGTGCAGGGGTTTGCAGTTGCTCTTAAA 1338
Ric1345 GCATCAATGTGTGGACCAGATGCACCAGAGATTATCCAGGGTATGGCTGTAGCGCTGAAG 1404
Spn1280 GCATCCATGTGTGGCCCAGATGCAGCTGAAATTATGCAGGGAATTGCTATAGCACTGAAA 1339
Ath1561 GCTGGTTTAACTAAGGCCGACTTTGATGCTACAGTGGGTGTTCACCCCACAGCAGCTGAG 1620
Pea1577 GCTGGATTAACAAAGGCGGACTTTGATGCCACAGTAGGCATTCATCCAACTGCAGCTGAG 1636
Tob1521 GCTGGGTTGACCAAGGCGGACTTTGATGCCACTGTGGGAATTCACCCTACATCAGCAGAG 1580
Syb1339 GCTCGCTTGACCAAGGCTGACTTTGATGCCACTGTAGGCATTCACCCAAGTGCAGCTGAG 1398
Ric1405 TGTGGAGCCACCAAGGCGACCTTTGACAGCACTGTTGGTATTCACCCGTCTGCTGCTGAA 1464
Spn1340 TTTGGAGCAACAAAGGCACAATTTGATAGTACGGTGGGAATCATCCTTCTGCAGCCGAAG 1399
Ath1621 GAGTTTGTCACTATGAGGGCTCCAACCAGGAAATTCCGCAAAGACTCCTCTGAGGGAAAG 1680
Pea1637 GAATTTGTTACCATGAGGACTCCCACTAGGAAGGTTCGAAA 1677
Tob1581 GAGTTTGTCACCATGCGTACCCCTACAAGGAAGGTTCGAAGCAGTCCATCTGAGGGAAAG 1640
Syb1399 GAATTTGTTACCATGAGGACACCTACTAGGAAGATACGAAA 1440
Ric1465 GAGTTTGTGACAATGCGGACCTTGACCAGG 1497
Spn1400 AAGTTTGTGACAATGAGGGAACCATCAAG 1429
Ath1681 GCAAGTCCTGAAGCTAAAACAGCTGCTGGGGTGTAGAGAAGGTTGCAAAAAGATTGTATT 1740
Tob1641 GCAGAGCACGATATTAAAGCTGCAGCTGGAGT 1671
Ath1741 TACGGCATTGGAGCCCCCTGATAAGGAGAACATTGTGTCTGTGAAGAAGAAGCTTTTCTT 1800
Ath1801 TTGGCAGAGAATATTTTTTCATAATGTCGTGTGAAGATATGATCGCTGTCCTGCAATACT 1860
Ath1861 TTTGATCGTTTGACACACGACCAAATTCCTCGAGAGAGAGAGGTTGTAACTATTCCGAAA 1920
Ath1921 GAGCTCTAAAACTGAAGCTTTCAGTTCTTGTGACATCATAATGAAGGCTGTATAGATATA 1980
Ath1981 ACAGTTTCGAGTT 1993

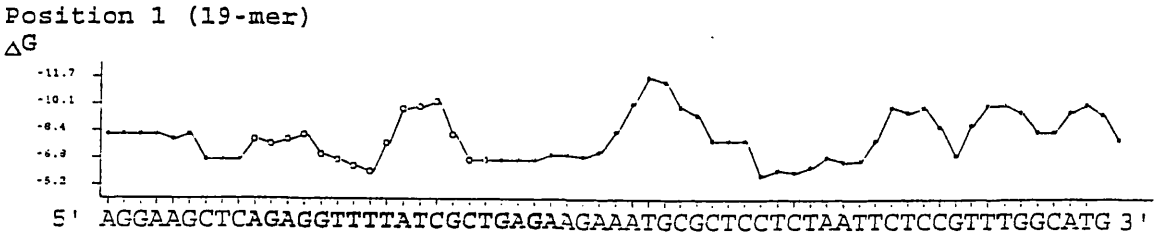
Figure 2.5. Internal Stability profiles of ascorbate peroxidase and glutathione reductase primers chosen for PCR optimisation and analysis. These profiles were obtained from Oligo 5.0 Primer Analysis Software. An indication of a successful primer in PCR is low stability at the 3' end (Rychlik, 1995). 2.5A: APX set A profiles; 2.5B: APX set B profiles; 2.5C: APX set C profiles; 2.5D: GR set A profiles; 2.5E: GR set B and 2.5F: GR set C internal stability profiles. Primer regions are indicated by bold type.

2.5A: Ascorbate Peroxidase Set A Upper Primer

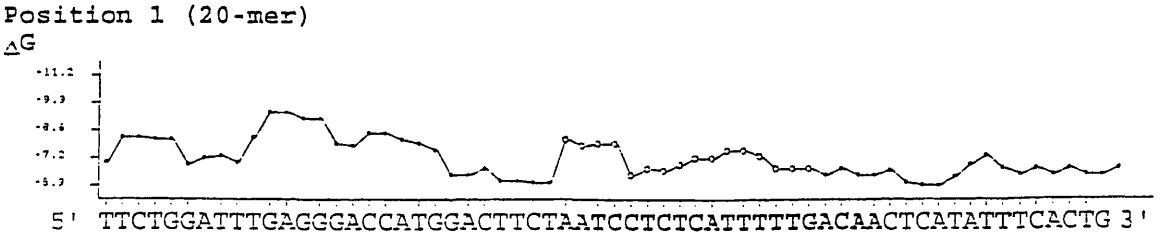


Ascorbate Peroxidase Set A Lower Primer

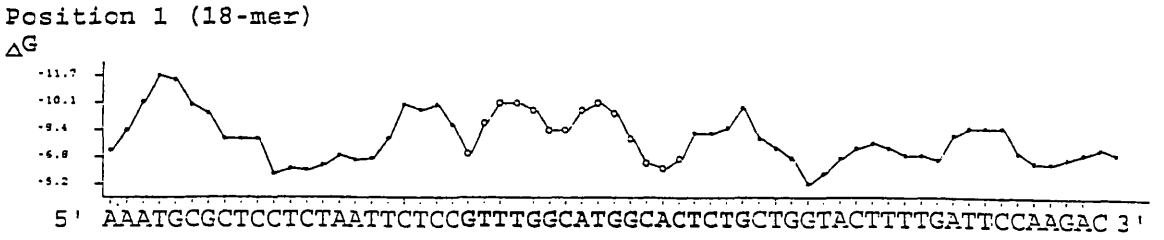
2.5B: Ascorbate Peroxidase Set B Upper Primer



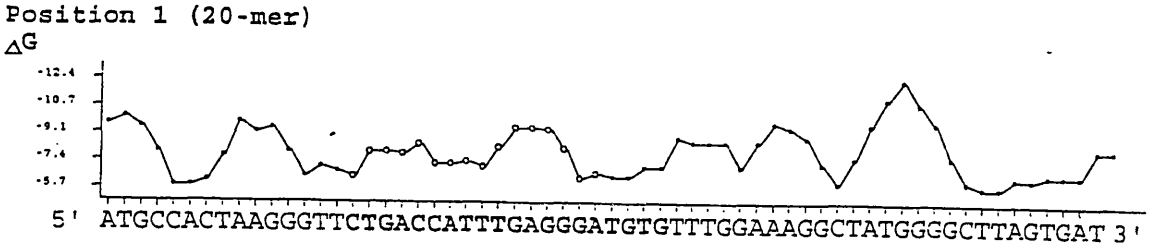
Ascorbate Peroxidase Set B Lower Primer



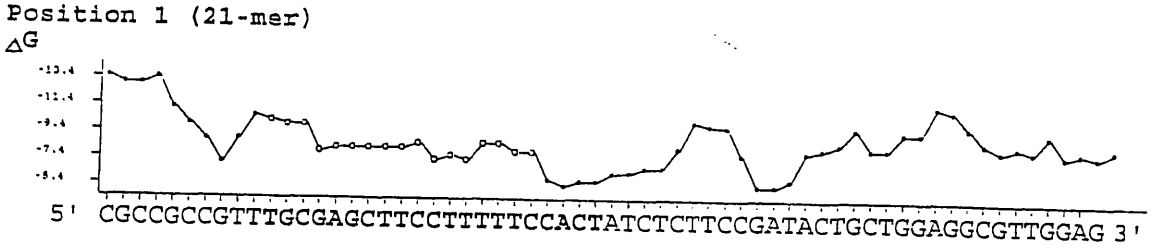
2.5C: Ascorbate Peroxidase Set C Upper Primer



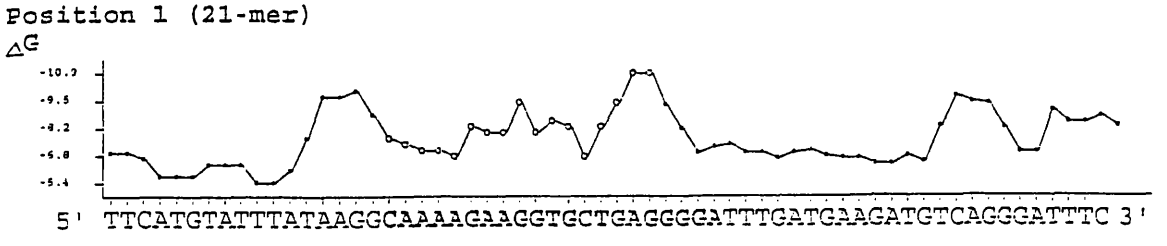
Ascorbate Peroxidase Set C Lower Primer



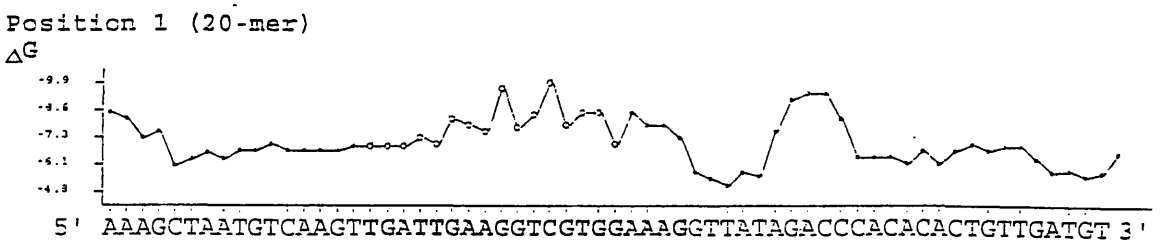
2.5D: Glutathione Reductase Set A Upper Primer



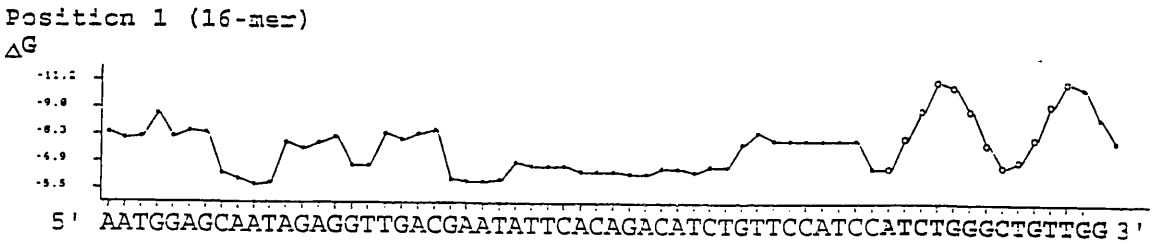
Glutathione Reductase Set A Lower Primer



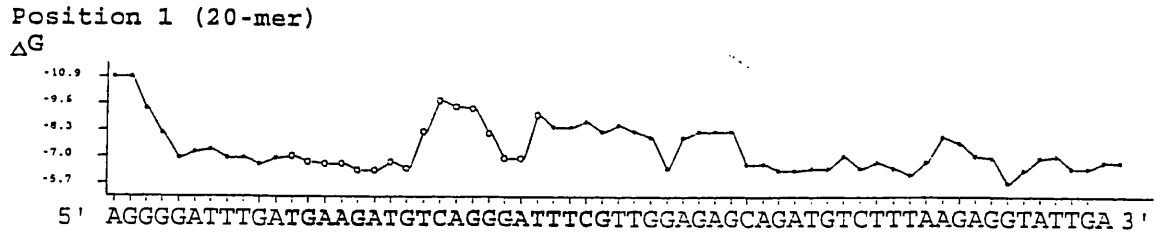
2.5E: Glutathione Reductase Set B Upper Primer



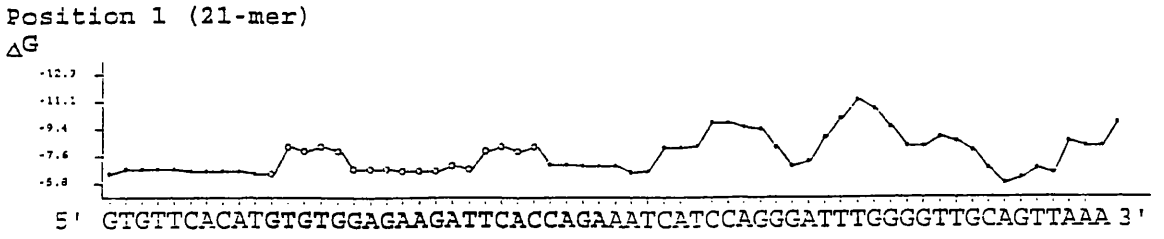
Glutathione Reductase Set B Lower Primer



2.5F: Glutathione Reductase Set C Upper Primer



Glutathione Reductase Set C Lower Primer



Base Pairs	Represented by:
C+G	S
C+A	M
C+T	Y
A+G	R
A+T	W
G+T	K

Table 2.5. Possible base alternatives used in primer design. If sequence homology differed due to a choice of two bases, an alternative nucleotide could be incorporated into the primer sequence, so that both bases were represented in the final primer sequence, thus increasing the likelihood of annealing.

Thus, the final sequences for all the primers synthesised are shown in Table 2.6.

APX Set A Upper Primer	**5′ GWYTGGCATGGCACTCTG 3′**
APX Set A Lower Primer	**5′ TTCATCMGCWGCRTATTTCTC 3′**
APX Set B Upper Primer	**5′ AGRGGWYTTATYGCWGAGA 3′**
APX Set B Lower Primer	**5′ TTGTCRAARARWARRGGYTT 3′**
APX Set C Upper Primer	**5′ GWYTGGCATGGCACTCTG 3′**
APX Set C Lower Primer	**5′ ASACATCYCTCAARTGGTCA 3′**
GR Set A Upper Primer	**5′ TGYGAGCTYCCTTTYTCYACT 3′**
GR Set A Lower Primer	**5′ TCCYCKCARMACCTTCTTTTG 3′**
GR Set B Upper Primer	**5 TGATTGAAGGYMRWGGAAAR 3′**
GR Set B Lower Primer	**5′ CCAAYWGCCCAWATWK 3′**
GR Set C Upper Primer	**5′ TGAAGATRTYAGRGATTTYG 3′**
GR Set C Lower Primer	**5′ TCWGSWCMATCWKSKCCA 3′**

Table 2.6. Ascorbate Peroxidase and Glutathione Reductase Primer Sequences. Three primer pairs for each gene were synthesised, with the incorporation of base pair representatives to increase the possibility of primer annealing to the correct gene sequences.

2.3.4 DNA extractions

Extractions of DNA were performed using both techniques outlined in sections 2.2.5 and 2.2.6. Figure 2.6 shows *R. ponticum* genomic DNA samples from the Nucleon Phytopure™ kit were less degraded (e.g. lane 8) than those obtained using the technique from Paterson *et al* (1993), for example lane 5. Similarly for the other species, data not shown, the Nucleon kit provided a higher quality of DNA and was therefore used for all subsequent extractions. In addition, as illustrated in section 2.3.6, no difference was observed in amplification of the DNA extracted by either method.

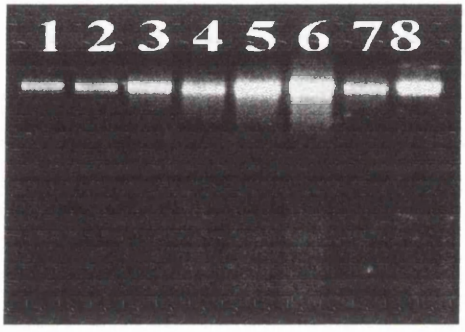


Figure 2.6. *Rhododendron* DNA extracted by Paterson *et al* (1993) technique (Lanes 1-6) and Nucleon Phytopure™ Plant DNA extraction kit (Scotlab Bioscience, lanes 7-8, *R. ponticum*). Lanes 1-2: *R. impeditum*, lanes 3-4: *R. hatsugiri*, Lanes 5-6: *R. ponticum*. A comparison of the quality of DNA can be made using lanes 5 and 8 which contain similar quantities of DNA.

2.3.5 DNA concentration

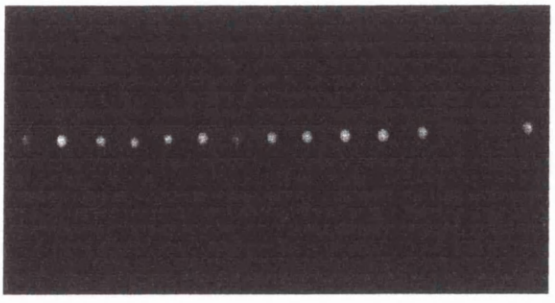


Figure 2.7. Fluorescent DNA dots viewed on the UV transilluminator. Using a computer grid system, each dot was placed in a square, and the densometric reading of the dot obtained. The dot positioned on the far right of the figure was the 10ng λ DNA standard.

The fluorescent dots obtained by incubation of genomic DNA and 5 μg/ml ethidium bromide are shown on Figure 2.7. These dots were analysed densometrically by the Bandleader Windows programme and the value compared against that of a known standard, in this case 10 ng/μl λ DNA. Using the formula stated in section 2.2.7, the quantity of DNA in an extract was calculated. All DNA samples were standardised to the same concentration to be used in Polymerase Chain Reaction.

2.3.6 PCR optimisation

The conditions used for Polymerase Chain Reaction are stated in Section 2.2.8. The reaction mix was constant throughout the annealing temperature experiments, and was adjusted accordingly with water for the primer concentration experiments.

Results for the annealing temperature experiments and primer concentrations for ascorbate peroxidase are shown in Figure 2.8a-f and Figure 2.10a-d respectively. It is clear by analysis using gel electrophoresis, that an annealing temperature of 55°C was best for APX primer sets A (Figure 2.8) and C (Figure 2.9), with a concentration of 20 pmoles. As illustrated, the range of annealing temperatures tested had a marked effect on the PCR result. Authors have stated that the higher the annealing temperature (Innes and Gelfand, 1990; Saiki, 1990; Kidd and Ruano, 1995; Rychlik, 1995), the greater the specificity of the primers and this has also proved to be the case here.

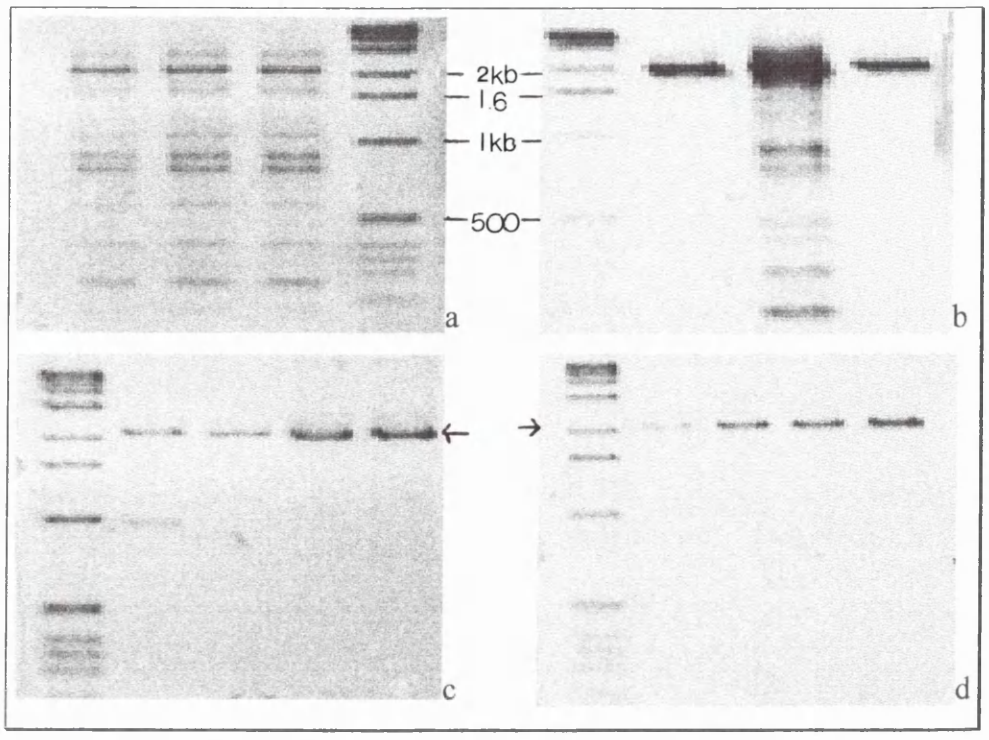


Figure 2.8a-d. Manipulation of primer annealing temperature in ascorbate peroxidase PCR *using R. ponticum* genomic DNA replicates. A: annealing temperature 45°C, B: annealing temperature 50°C, C: annealing temperature 52°C, D: annealing temperature 55°C. In all cases 1kb ladder (Gibco BRL) was used. 55°C yielded a discrete product of 2.1kb using primer set A.

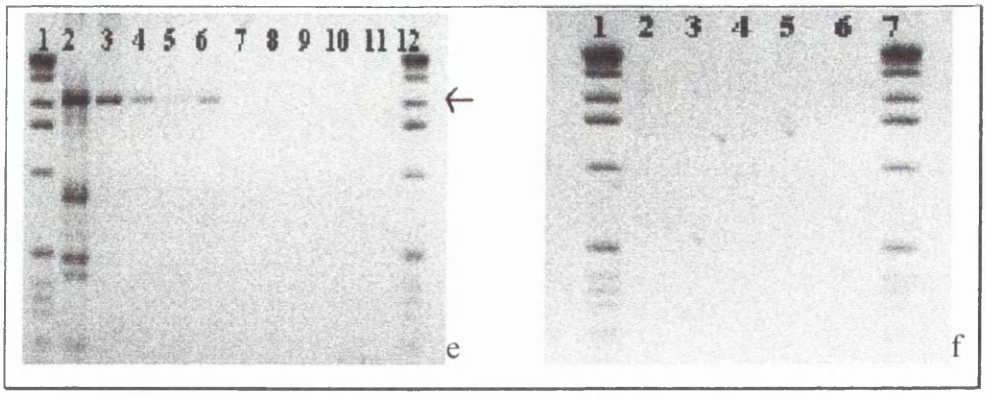


Figure 2.8e-f. Manipulation of primer annealing temperature (58°C) in ascorbate peroxidase PCR using *R. ponticum* genomic DNA (Figure 7E, lanes 2-6), *R. impeditum* DNA (Figure 7E, lanes 7-11) and *R. hatsugiri* DNA (Figure 7F), *n*=5.

At an annealing temperature of 45°C (Figure 2.8a) there are many bands appearing on the gel, indicating a low specificity of the primers to the specified template region. As the temperature is increased to 55°C (Figures 2.8d and 2.9b and c) there is an increase in the specificity of the reaction, yielding only one product of the expected size. To ensure that this temperature was ideal, annealing temperatures were tested as high as 58°C(Figures 2.8e and f), upon which few PCR samples yielded a product in all three *Rhododendron* species.

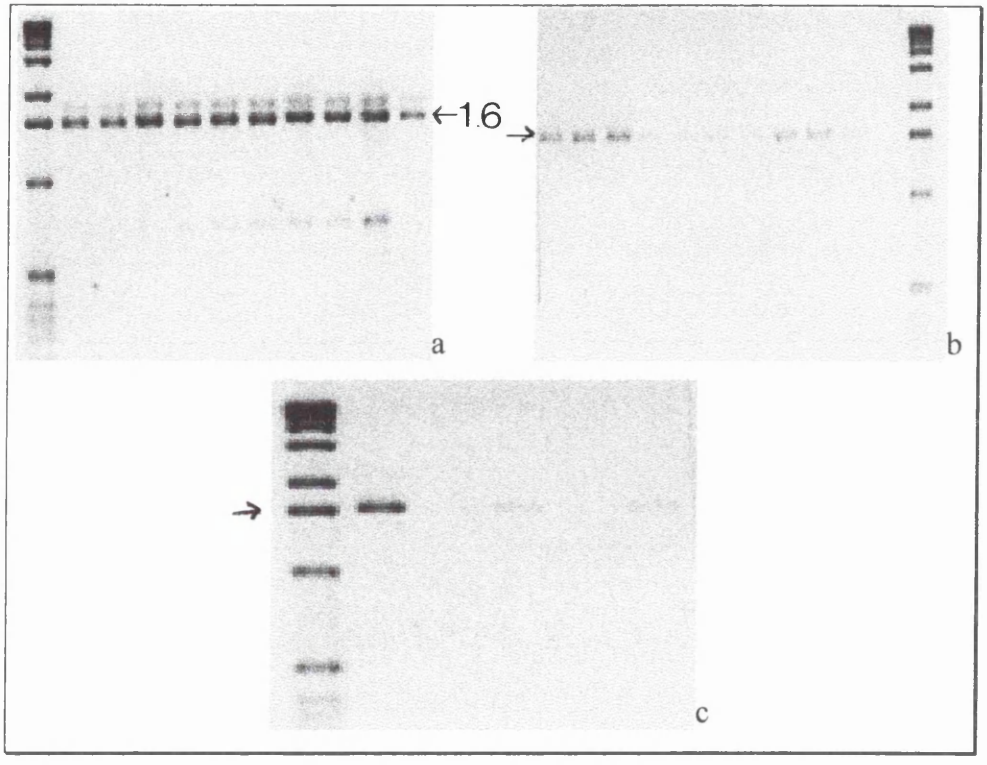


Figure 2.9. Ascorbate peroxidase set C amplification, annealing temperature 55°C. 8A: *R. ponticum* genomic DNA replicates amplified, 8B: *R. hatsugiri* genomic DNA amplified, 8C: *R. impeditum* genomic DNA amplified. A discrete product of 1.6kb was obtained at this annealing temperature for *R. hatsugiri* and *R. impeditum*.

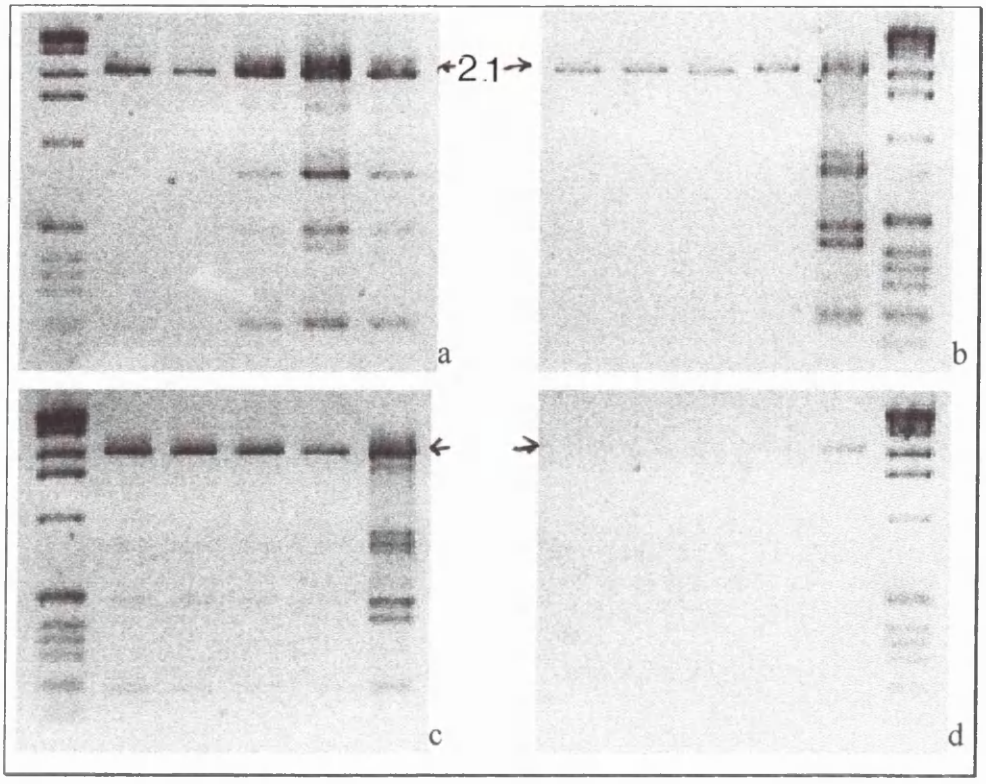


Figure 2.10. Manipulation of Primer Concentration. Figures A-D are replicates *of R. ponticum* DNA amplified with ascorbate peroxidase primers set A. 9A: 20 pmoles each primer used in the reaction, 9B: 30 pmoles, 9C: 35 pmoles, 9D: 40 pmoles. Each figure illustrates 5 replicates of the reaction and 1kb ladder (Gibco BRL).

As illustrated in Figure 2.10, four different concentrations of primer set A were also tested as a means to optimise PCR: 20, 30 35 and 40 pmoles. For 20, 30 and 35 pmole reactions (Figures 2.10 a, b and c respectively), there was little difference in the result, all yielding the expected product of approximately 2kb in size with some spurious banding for all samples. The result for the 40pmole reaction (Figure 2.10d) however indicates very little or no PCR product whatsoever. Disruption of optimal PCR conditions due to the addition of excess primer may have lead to a depletion in amplification efficiency.

Using both these primer sets for all three *Rhododendron* species gave a discrete product of approximately 2 kb in size at 55°C, 20 pmoles each primer. This product was much larger than the size estimated from the mRNA sequence. It was thought that this product was most likely to be a

fragment of the ascorbate peroxidase gene following a study of the DNA sequence of APX in *Pisum sativum.* With primer sets A and C superimposed onto the *P. sativum* sequence, they would theoretically yield a product of 1.7 kb (Appendix 2). This is because both primer sites were designed to span at least one intron. Allowing for the differing sizes of plant genomes, the given PCR product was expected to be approximately this size or greater.

Primer set B for ascorbate peroxidase yielded no product throughout all the optimisation experiments. This was to be expected when the physical properties of the primers themselves were noted. Upon studying the internal stability profiles (Figure 2.5), it can be seen that this was not an ideal primer pair. Looking at the lower primer, the 3′ end is the most stable area, contradicting the rules of PCR primer design. It is possibly for this reason that set B proved to be unsuccessful in PCR. As mentioned by Rychlik (1995), instability at the 3′ end of a specific primer will improve the chances of successful PCR, and this is not the case with primer set B.

PCR optimisation for glutathione reductase DNA yielded similar results. In this case, it was only primer set A which yielded a discrete product of approximately 500 bp (Figures 2.11. and 2.12). It was estimated that this would be the most successful primer pair due to the suitable internal stability profiles of the primer set. Figure 2.11 shows the results of GR primer concentration experiments. 40 pmoles of each primer yielded the greatest amount of product whilst maintaining product specificity (Figure 2.11d), thus was subsequently used in glutathione reductase PCR. Lower concentrations of primers (Figure 2.11a-c) resulted in little or no PCR product. The size of the glutathione reductase fragment is much smaller than that obtained for ascorbate peroxidase, because there is much less distance between the exon sites in the DNA sequence for glutathione reductase in *Arabidopsis thaliana* (Appendix 2).

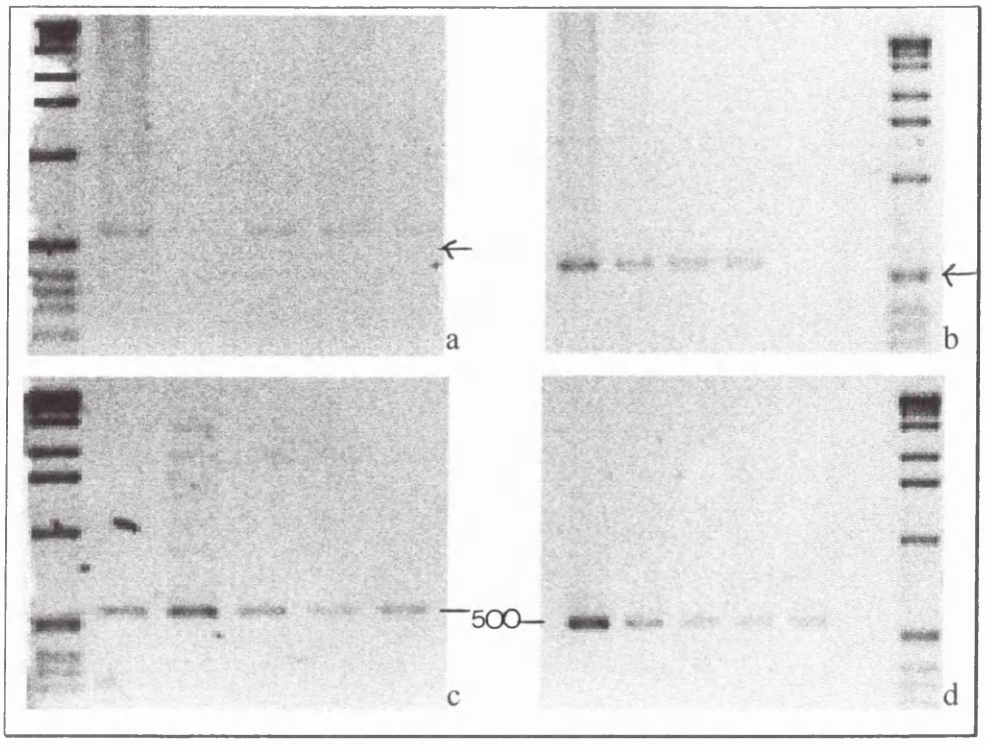


Figure 2.11. Manipulation of glutathione reductase primer concentration. In each case, results for the amplification of *R. ponticum* genomic DNA are shown. 10A: 20 pmoles each primer (Set A) used in the reaction, B: 30 pmoles, C: 35 pmoles, D: 40 pmoles used in PCR.

The annealing temperature experiments indicated that 55°C was also the optimal temperature for primer annealing. Illustrated in Figures 2.12a, b and c are PCR results of annealing temperatures 45, 50 and 52°C respectively. These indicate that due to smearing and multiple bands, these temperatures are too low to yield a discrete product of 500bp.

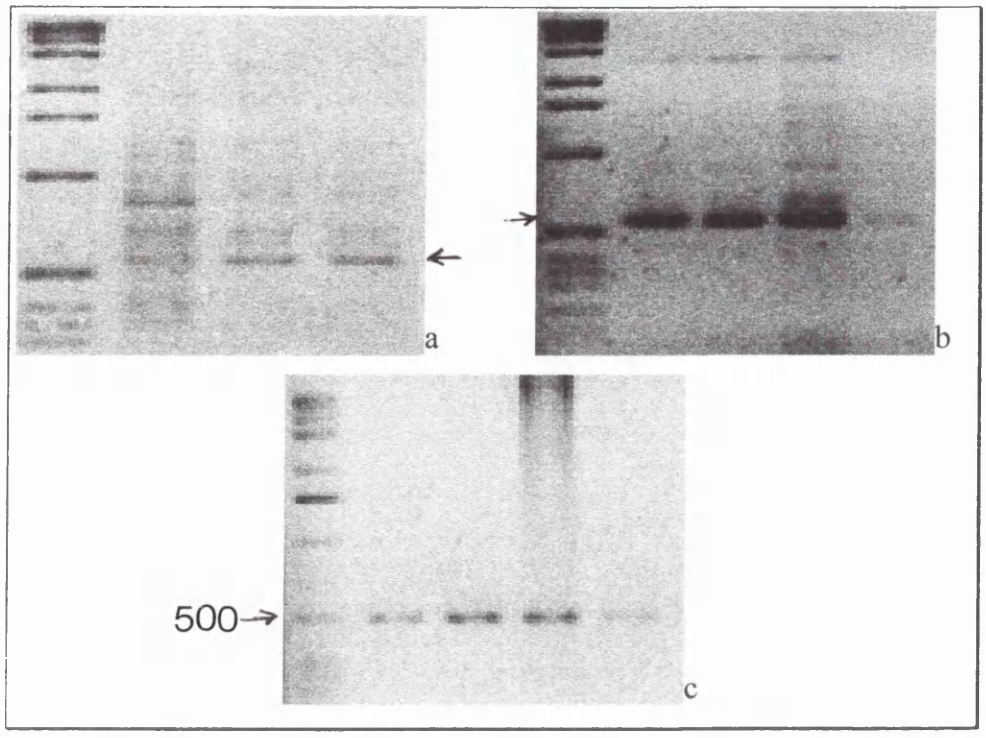


Figure 2.12. Manipulation of glutathione reductase set A primer annealing temperature. In each case, results are shown for the amplification of *R. ponticum* genomic DNA. 11A: 45°C used in the thermal cycle, 11B: 50°C, 11C: 52°C used in the thermal cycle.

2.4 Conclusions

- A suitable system for the production of maintenance of Rhododendron micropropagules was established as a source of plant material for the extraction of high quality DNA, RNA and proteins.

- Inter-species comparisons of six diverse plant species revealed highly conserved mRNA regions for ascorbate peroxidase and glutathione reductase. These areas were suitable for gene-specific PCR primer design.

- Analysis of these regions by Oligo 5.0 Primer Analysis software indicated several suitable primer pairs for APX and GR gene sequences.

- Primer pairs were synthesised and applied in PCR optimisation experiments.

- DNA Isolation using Nucleon Phytopure™ Plant DNA extraction kit (Scotlab Biosceience) resulted in high quality preparations, suitable for PCR.

- A range of optimisation experiments showed an annealing temperature of 55°C and primer concentration of 20 pmoles (APX) and 40 pmoles (GR) to be optimal for discrete PCR products (2.1 kb, APX primer set A; 1.6 kb, APX primer set C and 500 bp GR primer set A).

- Primer pairs which had been designed with too much stability at the 3′ ends performed poorly in PCR.

- PCR product sizes of 2.1kb for APX and 500bp for GR in *Rhododendron* were accepted as gene-specific following comparative studies with DNA sequences in *Pisum sativum* (APX) and *Arabidopsis thaliana* (GR). PCR

product sizes of approximately 1.8kb and 600bp respectively were estimated from these DNA sequence data.

Chapter 3 Ascorbate Peroxidase and Glutathione Reductase: Cloning and Sequencing

Chapter 3 Ascorbate Peroxidase and Glutathione Reductase: Cloning and Sequencing

3.1 Introduction

3.1.1 T/A cloning introduction

Generation of PCR products expected to be ascorbate peroxidase and glutathione reductase necessitated their cloning and sequencing to confirm their identity. A number of strategies are available for cloning PCR products but it was decided to attempt T/A cloning in the first instance. This approach exploits the terminal transferase activity of the thermostable DNA polymerase, *Taq*. During the Polymerase Chain Reaction (PCR), the enzyme adds a single deoxyadenosine (A) to the 3′ end of many reaction products (Clark, 1988; Mead *et al.*, 1991), irrespective of the template sequence. Many polymerases studied exhibited this phenomenon, both from pro- and eukaryotic organisms, suggesting that this is a possible evolutionary remnant of activity used by an ancestral enzyme prior to the advent of template-directed synthesis. The rate at which the non-templated addition takes place is thought to be slower than template-mediated synthesis, although this is dictated by the particular polymerase (Clark, 1988). The biological significance of transferase activity in DNA polymerases is unclear at present, but the phenomenon is easily exploited for blunt-end cloning.

The pT-Adv plasmid vector illustrated in Figure 3.1, with its 3′ T overhangs, enables direct cloning of reaction products. The transferase activity of the enzyme is non-specific, theoretically allowing ligation of any PCR product into the plasmid, without any prior sequence knowledge. The plasmid includes sites for T7 RNA Polymerase, and flanking M13 forward and reverse primer sites for direct sequencing, as well as a T7

promoter for RNA transcription and translation. Several restriction sites have also been incorporated into the plasmid structure for ligation analysis by restriction digestion. The vector also contains the *lacZα* gene, providing a simple blue/white visual assay for rapid identification of positive transformants.

The *lacZα* gene codes for part of the enzyme β-galactosidase. This enzyme is one of a series involved in the breakdown of lactose to glucose and galactase, and is normally coded by the gene *lacZ*. The strain of *E. coli* used in T/A cloning has a modified *lacZ* gene, lacking a segment referred to as *lacZα*. This E. coli strain can only synthesise β-galactosidase when the cells contain a plasmid, such as pT-Adv, that carries the *lacZα* gene segment. Cloning with pT-Adv involves the inactivation of the gene by insertion of a DNA sequence.

The reaction used to test for the production of this enzyme involves a lactose analogue, known as X-Gal (5-bromo-4-chloro-3-indolyl-β-galactopyranoside) which is broken down by β-galactosidase to a product that is blue in colour. If X-Gal and an enzyme inducer (IPTG; isopropyl-thiogalactoside) is added to the growth medium, only those cells which synthesise β-galactosidase will be blue in colour.

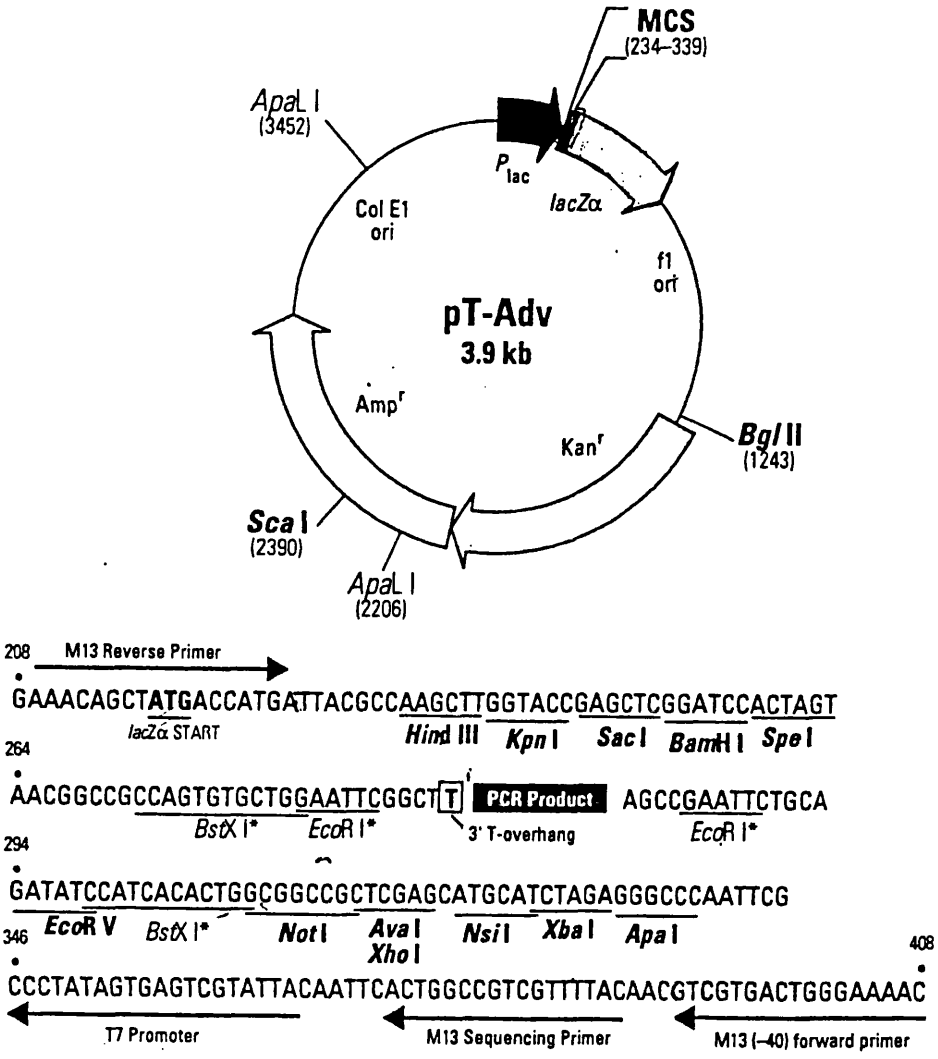


Figure 3.1. Plasmid Map indicating restriction sites and the Multiple Cloning Site (MCS) of pT-Adv (Clontech Laboratories Inc., 1997). Unique restriction site are shown in bold. Restriction sites with asterisks (*) are only present in the MCS and can be used to excise the inserted product. The MCS illustrated above represents the vector sequence after modification and linearisation for ligation (The 3' T overhang preceding the insert is added to the vector during modification).

3.1.2 Transformation into competent E. coli cells

Cloning involves the maintenance and amplification of the recombinant plasmid in bacterial cells which requires a bacterial transformation process. The majority of bacterial transformations are based on results from a study by Mandal and Higa (1970, cited by Sambrook *et al*, 1989),

which showed that bacteria which were chilled with calcium chloride and briefly heated could be transfected with bacteriophage λ DNA. Such treatment induces a transient state of 'competence' in bacterial cells allowing them to take up DNA from a variety of sources. The efficiency of DNA uptake can be improved by treating the cells with a number of reagents, such as DMSO and reducing agents, but it is not known how these compounds act. Improving the strain of the bacteria to be transfected also improves the efficiency of transformation. In this study, TOP10F' *E. coli* (Promega) cells were used.

3.1.3 DNA Sequencing using dye terminators

DNA sequencing is a molecular technique utilising primer extension reactions. Traditional techniques use radio-labelled dideoxynucleotides which terminate the reaction when bound to the DNA strand. Separate reactions are carried out for each nucleotide base and the products separated by polyacrylamide gel electrophoresis. The DNA sequence is then read from an autoradiograph of the gel. This technique is time consuming and accommodates manual reading errors.

The majority of automated sequencing reactions performed at present use dye-labelled terminators as well as free nucleotides found in a normal DNA extension reaction (Perkin Elmer 1997). These terminators have a specific fluorescent dye attached to them denoting a particular dideoxynucleotide base, A (green), T (red), C (blue) or G (black). Dideoxynucleotides do not contain the hydroxyl group at the 3'position of the sugar component, necessary for attachment of the next nucleotide (Brown, 1989). When a dideoxynucleotide is incorporated into the extending DNA, chain termination occurs. The use of dye terminators in sequencing reactions allows all four bases to be sequenced in the same reaction tube. They comprise an energy donor (fluorescein) and acceptor

dye (rhodamine) that are connected by an energy transfer linker. Sequencing reactions can be catalysed by AmpliTaq® or Sequenase® DNA Polymerase (Perkin Elmer, 1997) and the reaction proceeds under the following conditions: denaturation at 96°C for 30 seconds, primer annealing at 50°C for 15seconds, and extension at 60°C for four minutes, for a total of 25 cycles. (Perkin Elmer, 1995). The completed reaction is still separated by polyacrylamide gel electrophoresis, however band florescence in the automated sequencer is read by an argon laser (Lee *et al.*, 1997) and interpreted by computer to create a chromatogram of dye fluorescence and an accurate translation of the DNA sequence.

3.1.4 Sequence homology

In order to correctly identify a gene, thus providing some information about its structure and function, a comparison of the sequenced fragment in question and known gene sequences must be made. This is routinely carried out by sequence homology studies, performed by many different software packages. The majority of these are based upon a measure of chance similarities between sequences (Karlin and Altschul, 1989). BLAST (Basic Local Alignment Search Tool, Altschul *et al.*, 1990) offers a new approach to sequence similarity searches, accessed through the NCBI (National Centre for Biotechnology Information) database.

BLAST, developed by Altschul *et al.* (1990), has the ability to identify alignments with a high measure of local similarity, as opposed to global similarity, which may include vast regions of the sequence with low similarity. Hence, unconserved regions of a gene are not taken into account,resulting in greater accuracy. This precision is achieved by utilising the Maximal Segment Pair (MSP) measure, defined as the highest scoring pair of identical length segments chosen from two sequences. Statistical studies carried out by Altschul *et al.* (1990) confirm

the significance of the alignments given by the programme. The algorithm used is simple (compiling a list of high-scoring words, scanning the database for hits and extending hits) reliable and can be applied to any number of situations; DNA and protein sequence analysis, gene identification searches, the analysis of multiple regions of similarity in long DNA sequences. Also noteworthy of this tool, is the relative speed at which the homology is calculated in comparison to other software packages (Altschul *et al.*, 1990).

Manual gene analysis can also be carried out to obtain more information about the newly sequenced gene. Alignment of sequences of the same gene in the same family of plant species, such as *Rhododendron*, as well as unrelated species, can give an indication of possible evolutionary differences and sequence conservation. Structural information such as the intron sites in the gene can also be useful in identification and determining function.

Using such a variety of molecular biological techniques as outlined in this chapter, one can study specific genes and their function. Ligation of a gene sequence of interest into a plasmid vector allows the manipulation of the sequence in many molecular techniques; sequencing, gene expression studies and RNA transcription and translation for example. Such information can help to increase the foundation of knowledge of genetic studies.

3.2 Materials and Methods

3.2.1 PCR amplification

PCR was carried out as stated in Section 2.2.8, using genomic DNA from the three experimental *Rhododendron* species, *R. ponticum*, *R. impeditum* and *R. hatsugiri*. Initially, Primer set APX A was used in the reaction but met with limited success, thus APX set C was used in latter cloning attempts. Details of both primer sets can be found in Table 3.1. The cycle used consisted of an initial denaturation step of 95°C for 5 minutes, 1 minute at 94°C, 2 minutes at 55°C and three minutes at 72°C, for a total of 30 cycles, and a final extension of 10 minutes at 72°C, before being held at 4°C, until the ligation mix was ready. An aliquot of the reaction mix was removed after PCR for analysis by gel electrophoresis (refer to Section 2.2.10), to confirm successful fragment amplification.

A control DNA template and primers supplied with the kit were also amplified for ligation into the pT-Adv vector. The control primers amplify a 700bp fragment which should produce about 80% white colonies on IPTG/X-Gal selective media following transformation.

APX Set A Upper Primer	**5′ GWYTGGCATGGCACTCTG 3′**
APX Set A Lower Primer	**5′ TTCATCMGCWGCRTATTTCTC 3′**
APX Set C Upper Primer	**5′ GWYTGGCATGGCACTCTG 3′**
APX Set C Lower Primer	**5′ ASACATCYCTCAARTGGTCA 3′**

Table 3.1. Ascorbate peroxidase primers used in PCR amplification.

3.2.2 Purification of PCR product from agarose gels

The desired PCR fragment was cut from the 1% (w/v) agarose gel using a scalpel and placed in a 1.5ml Eppendorf and incubated at -80°C for 30 minutes. Once thawed, the gel and liquid were transferred to a Wizard™ Minicolumn (Promega) and centrifuged at top speed in a MicroCentaur for one minute. To the liquid, 0.1 volume of 3M sodium acetate and 2 volumes 96% (v/v) ice-cold ethanol were added and the DNA precipitated at -20°C for one hour. After centrifugation at 4000*g* for 5 minutes, the pellet was washed in 70% (v/v) ethanol and centrifuged at 4000*g* for a further 5 minutes. The pellet was allowed to air-dry and resuspended in 20µl sterile distilled water.

3.2.3 Ligation into pT-Adv plasmid vector

A 10 µl ligation reaction was prepared by adding the following components in the order listed:

PCR Product:	2µl
10× T4 Ligation Buffer*	1µl
pT-Adv vector (25ng/µl)	2µl
Sterile Molecular Biology Grade H_2O	3µl
T4 DNA Ligase	1µl

* Working solution of ligation buffer contained 50 mM Tris-HCl (pH 7.8), 10 mM Mg Cl_2, 10 mM dithiothreitol, 1 mM ATP, 25 µg/ml Bovine Serum Albumin (BSA).

The reaction mixture was incubated overnight at 14°C, and then stored at -20°C until ready to proceed to transformation. Ligation was confirmed by gel electrophoresis and by growth of white colonies in transformed *E. coli* culture plates.

3.2.4 Transformation into E. coli

The tubes containing the ligation reactions were centrifuged briefly and placed on ice. For each ligation reaction, one tube of competent TOP10F' *E. coli* cells (Clontech) were thawed on ice.

β-mercaptoethanol (2 μl) was pipetted into each tube of competent cells and mixed by gently stirring the mix with the pipette tip. To this, 2 μl of ligation reaction was added and mixed by stirring gently with the pipette tip. The tubes were then incubated on ice for 30 minutes, before treating with heat shock for exactly 30 seconds at 42°C. The tubes were removed and placed on ice for exactly two minutes and then 250 μl of SOC medium (Appendix 1) was added at room temperature. The cultures were incubated horizontally at 37°C for 1 hour at 225 rpm in a rotary shaking incubator, then kept on ice until ready to be plated.

LB agar plates, containing 100 μg/ml ampicillin*, 40 μl (40 mg/ml in DMF) X-Gal and 40 μl (100 mM) IPTG, which had been prepared earlier, were used to culture the cells. Plates were spread with either 50 μl or 200 μl cell suspension, and left to incubate at 37°C overnight. The plates were transferred to 4°C to allow full colour development of the untransformed colonies.

* The first attempts at transformation selection resulted in growth of many small white colonies around the colonies of smaller size. These are ampicillin sensitive satellites, which do not contain the plasmid, so higher concentrations of antibiotic were tested until one suitable was found at which no small colonies were present.

3.2.5 Analysis of transformation

Any pure white single colonies found were isolated into 5 ml LB broth, containing 100 μg/ml ampicillin, and incubated overnight at 37°C at 225 rpm in a rotary shaking incubator. The culture was streaked out on ampicillin/X-Gal/IPTG plates and incubated at 37°C overnight to obtain single colonies. A single colony from each plate was isolated and incubated overnight in 5ml LB broth as before. These cultures were used for plasmid purification and subsequent analysis.

3.2.5.1 Plasmid Purification

Wizard™ Plus Minipreparation DNA Purification System (Promega).

E. coli cell cultures (3 ml) were pelleted by centrifugation for 1-2 minutes at 10 000 *g* in a microcentrifuge, and all the supernatant media carefully removed. The pellet was completely resuspended in 200 μl Cell Resuspension Solution (50 mM Tris-HCl, pH 7.5; 10 mM EDTA; 100 μg/ml RNase A) by vortexing. To this, 200 μl of Cell Lysis Solution (0.2M NaOH; 1% (w/v) SDS) was added and mixed by inversion until the lysate became clear. An aliquot (200 μl) of Neutralisation Solution (1.32 M potassium acetate) was mixed with the lysate by inversion and centrifuged at 10 000 *g* for 5 minutes. The supernatant was removed and transferred to a fresh 1.5 ml Eppendorf. 1ml of the Wizard™ Minipreps. DNA Purification resin was added to the supernatant and mixed by inversion. The mix was left to incubate while the minicolumns were prepared.

The plunger from a 3 ml disposable syringe was removed and a Wizard™ Minicolumn attached to the luer-lock extension of the barrel. The DNA/resin mix was pipetted into the barrel and the plunger carefully inserted. The mix was then pushed into the column by the syringe plunger.

The syringe was removed from the Minicolumn and the plunger withdrawn and set aside while the Minicolumn was reattached to the barrel. Column Wash (2ml; 80 mM potassium acetate; 8.3 mM Tris-HCl, pH 7.5; 40 μM EDTA; 55% (v/v) ethanol) was pipetted into the barrel and pushed through the column with the plunger as before. The Minicolumn was detached from the syringe and transferred to a 1.5 ml Eppendorf. This was centrifuged at top speed in a microcentrifuge for 20 seconds to dry the column.

The Minicolumn was transferred to a fresh Eppendorf, and 50 μl of sterile distilled water applied to the Minicolumn and left to incubate for one minute at room temperature. The DNA could then be eluted from the column by spinning at top speed in a microcentrifuge for 20 seconds. The recovered plasmid DNA was stored at -20°C until needed.

UltraClean™ Mini Plasmid Preparation Kit (MO BIO Laboratories, California)

An overnight *E. coli* culture (2 ml) was centrifuged at top speed in a MicroCentaur (MSE) for 30 seconds and all traces of supernatant removed. The pellet was thoroughly resuspended in 50 μl Cell Suspension solution (Tris-HCl, EDTA and RNase A- concentrations not stated in published protocol). To this, 100 μl Cell Lysis solution (containing SDS and NaOH) and 325 μl Binding solution (containing potassium acetate and binding agent™) was added and mixed by inversion and the homogenous mixture centrifuged at top speed for one minute. The supernatant was transferred to a spin filter and centrifuged for a further 30 seconds. The liquid collected from centrifugation was discarded and 300 μl Ethanol Wash solution (ethanol, Tris-HCl and NaCl) added to the spin filter. Following centrifugation for 30 seconds at top speed, the filter was fitted to a fresh centrifuge tube, and 50 μl sterile distilled water was

added, and the filter centrifuged for a further 30 seconds to elute the plasmid DNA, which was stored at -20°C until ready to use.

3.2.5.2 PCR amplification

PCR was carried out as stated in Section 2.2.8. Initially the primer pair APX A was used for ascorbate peroxidase, GR A for glutathione reductase. The cycle used consisted of an initial denaturation step of 95°C for 5 minutes, 1 minute at 94°C, 2 minutes at 55°C and three minutes at 72°C, for a total of 30 cycles, and a final extension of 10 minutes at 72°C, before being held at 4°C. The samples were mixed with gel loading buffer (0.0025% (w/v) bromophenol blue, 6 mM EDTA, 30% (v/v) glycerol) and analysed electrophoretically, as stated in Section 2.2.10.

3.2.5.3 Restriction analysis

The plasmid vector used contains several restriction enzyme recognition sites (Figure 3.1). These can be used to analyse the success of transformation. In this case *Eco*RI was used to excise the known size PCR fragment from the plasmid. The *Eco*RI sites flank either side of the PCR insert, so analysis by restriction should yield two fragments, one of approximately 2 kb (APX insert; GR insert, 500 bp) and one of 3.9 kb (plasmid).

A 20 µl restriction reaction was prepared by adding the following components in the order listed:

*Eco*RI Reaction Buffer 3(Gibco BRL)*	2µl	
Sterile Molecular Biology Grade Water		8µl
Plasmid Preparation		8µl
*Eco*RI Restriction Enzyme (Gibco BRL)	2µl	

* Reaction buffer contained 50 mM NaCl, 100 mM Tris-HCl, 10 mM $MgCl_2$, 0.025% (v/v) Triton X-100 (pH 7.5 @ 25°C).

The reaction mix was incubated at 37°C overnight, then the enzyme inactivated by a 2 minute incubation at 85°C. Before analysis by gel electrophoresis, the samples were precipitated by sodium acetate. To the reaction mix, 0.1 volume 3 M sodium acetate and 2 volumes of 96% (v/v) ethanol were added, and the DNA left to precipitate at -20°C for 30 minutes. The DNA was pelleted by centrifugation at 4000 *g* for 5 minutes and subsequently washed in 70% (v/v) ethanol and centrifuged as before. Once the pellet had been air-dried to remove any trace of ethanol, it was resuspended in 20 µl sterile molecular biology grade water. The sample could then be analysed by gel electrophoresis.

3.2.6 Storage of bacterial cultures

The transformed *E. coli* cultures obtained were stored using several different methods, to maintain a supply of transformed organisms, without mutation: single colony plates, stab cultures and glycerol cultures.

3.2.6.1 Stab cultures

Bijoux bottles were half filled with molten LB agar and autoclaved for 15 minutes at 121°C. When cooled, the bottles were stored at room temperature until needed. To store *E. coli*, a single, well isolated fresh colony was picked with a sterile inoculating needle, and stabbed several times in the LB agar to the bottom of the bottle. The bottles were labelled and stored in the dark at room temperature.

3.2.6.2 Glycerol Cultures

Sterile glycerol (150 µl) was added to 850 µl *E. coli* culture and thoroughly mixed by vortex in a 1.5 ml Eppendorf tube. The Eppendorf tubes were sealed with parafilm before instant freezing in liquid nitrogen, and transferred immediately to -80°C for long-term storage. Cultures were easily rejuvenated by thawing to room temperature and incubating in 5 ml LB broth overnight at 37°C and 225 rpm in a rotary shaking incubator.

3.2.7 Preparation of plasmid for sequencing

Plasmid preparations were generated as outlined in Section 3.2.5.1, and sent in dry ice packaging to Oswell Laboratories (in the first instance) and later to Cambridge Bioscience for autosequencing of the fragment ends using the M13 forward and reverse sequencing primers.

3.2.8 Sequence analysis

Fragment sequences were entered into the NCBI BLAST (Basic Local Alignment Search Tool) homology search database as a means of identification. Once the origin of the fragments had been established, a series of gene analyses could be performed; inter-species comparisons with the original sequences used in primer design, conservation of the gene within the *Rhododendron* genus, comparison of primer sequences between the original sequence and those found in *Rhododendron* and possible intron sites in the *Rhododendron* DNA sequence.

3.3 Cloning of Ascorbate Peroxidase - Results and Discussion

3.3.1 Cloning attempt one

To successfully ligate a PCR product into a plasmid vector, the fragment must be discrete and abundant. To achieve these criteria, following PCR and gel electrophoresis (shown in Figure 3.2), the product band was cut and purified from the gel as descibed in section 3.2.2.

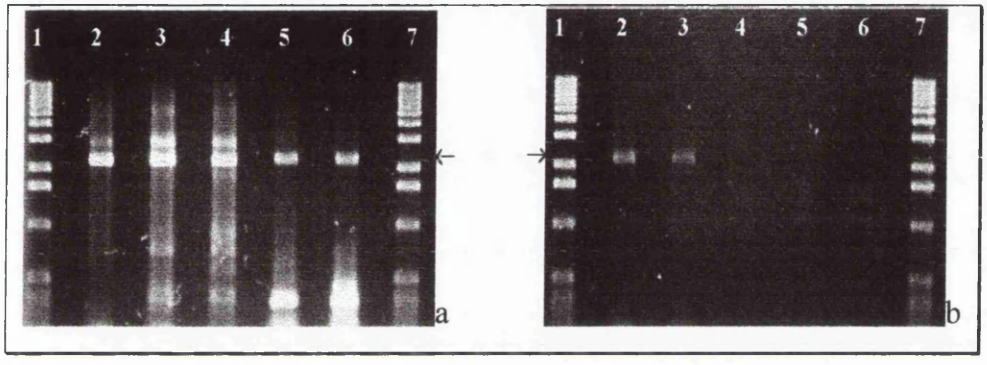


Figure 3.2 A and B. PCR products of 2.1kb, suspected to be that of ascorbate peroxidase were cut from the gel and purified. 1A: lanes 1 and 7 are 1kb ladder (Gibco BRL), lanes 2-4 represent amplification using APX A primers and *R. ponticum* genomic DNA, lanes 5-6 using *R. hatsugiri* genomic DNA. 3.2B: lanes 1 and 7 are 1kb ladder, lanes 2-3 represent amplification using APX A primers and *R. impeditum* genomic DNA, lanes 4-6 using *R. ponticum* genomic DNA. All DNA was sourced from *Rhododendron* micropropagules, except DNA used in PCR lanes 4-6, Figure 3.2B, which were obtained from whole plant leaf tissue.

The control DNA template and primers supplied with the kit had also been used in PCR, and were found to amplify a product of 700 bp, as stated by the protocol. This result is shown in Figure 3.3. Because of the clarity and abundance of the product, there was no need to purify the product from the gel, and the PCR reaction mix was used directly in the cloning protocol.

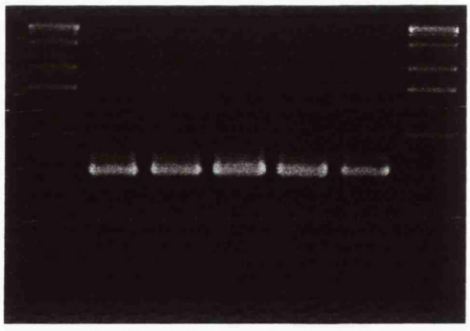


Figure 3.3. Gel electrophoresis of PCR product using control DNA template and primers supplied in AdvanTAge PCR Cloning Kit (Clontech). Lanes 1 and 7 are 1kb ladder (Gibco BRL), lanes 2-6 are replicates of the reaction. Lane 5 sample was used in ligation and transformation. The expected 700 bp product was obtained.

Following ligation and transformation, the samples were plated out onto IPTG/X-Gal/ampicillin selective media. Growth of the cultures revealed that only the control PCR had been successful in procedure, in which approximately 70% of the cultures were white. Several colonies were isolated and grown in LB/ampicillin broth overnight, before plasmid purification and storage.

It was clear from this result that the cloning procedure was successful in the laboratory environment and the reagents were suitable and free from contamination. The lack of success in the *Rhododendron* samples could be due to a variety of reasons. The purification of the PCR product from the agarose gel may well have degraded the dA nucleotide additions necessary for this type of blunt-end cloning (Clark, 1988). The PCR product must be as pure as possible before proceeding to ligation and transformation, and it was this aspect which became a central focus in the second attempt at cloning the ascorbate peroxidase fragment from *Rhododendron* species.

3.3.2 Cloning attempt two

Repeated PCR of genomic DNA from the three *Rhododendron* species yielded little or no result, therefore fresh DNA was extracted from the three species using the Nucleon Phytopure™ Plant DNA Extraction kit (Scotlab Bioscience), the protocol of which is outlined in section 2.2.6. Figure 3.4 shows the high quality of the genomic DNA extracted from the micropropagule tissue. The PCR products obtained and used directly in ligation are shown in Figure 3.5. The sample in lane 2 was used for *R. ponticum*, lane 7 for *R. hatsugiri* and lane 12 for *R. impeditum* ascorbate peroxidase cloning.

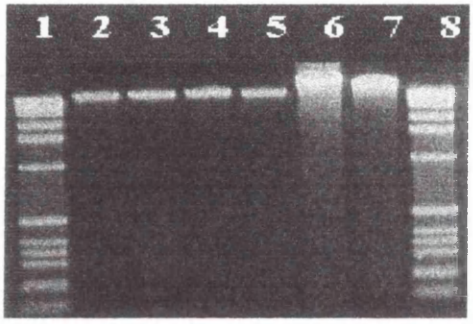


Figure 3.4. Gel Electrophoresis of Rhododendron genomic DNA using the Nucleon Phytopure™ Plant DNA Extraction kit (Scotlab Bioscience). Lanes 1 & 8 are 1 kb ladder (Gibco BRL). Lanes 2-3: duplicate extractions of *R. ponticum*, lanes 4-5: duplicate extractions of *R. hatsugiri*, lanes 6-7: duplicate extractions of *R. impeditum*.

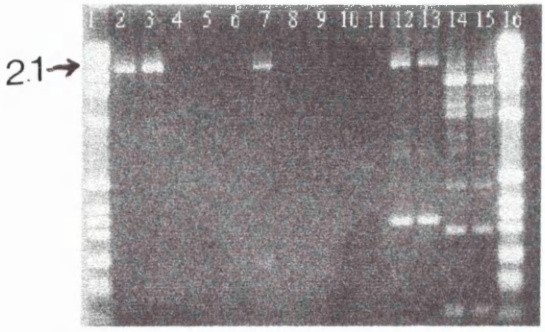


Figure 3.5. Gel electrophoresis of PCR using APX primer set A and *Rhododendron* genomic DNA. Lanes1 & 16 are 1kb ladder (Gibco BRL). Lanes 2-3, 8-9: *R. ponticum* genomic DNA amplified, lanes 4-5, 12-13: *R. impeditum*, lanes 6-7, 10-11: *R. hatsugiri*, lanes 14-15: *Pisum sativum* genomic DNA amplified as positive control.

Visualisation of the IPTG/X-Gal/ampicillin plates revealed several pure white colonies in *Rhododendron* samples from each species. Many of the colonies were observed to be white with blue centres, caused by only partial disruption of the *lacZ* gene. This may be due to partial ligation of the smaller bands generated in the original PCR reaction (Figure 3.5) or the PCR product having lost ligation efficiency. The white colonies were isolated and the plasmids purified for further analysis.

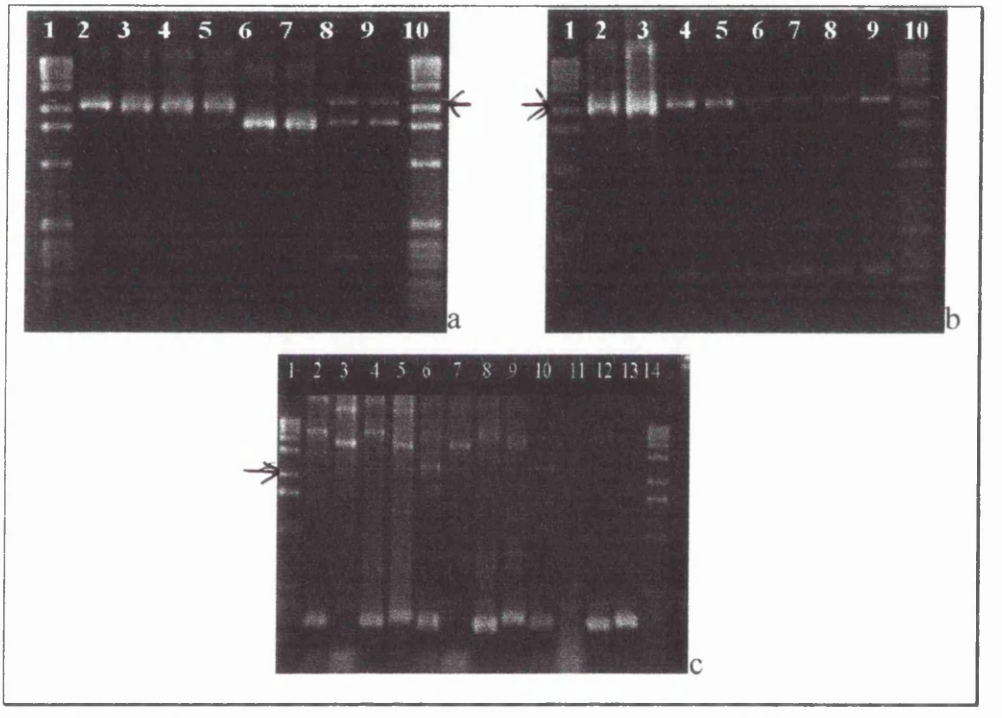


Figure 3.6. PCR products visualised by gel electrophoresis of plasmids from transformed colonies. 3.6A: *R. ponticum* fragments. Lanes 1 and 10 are 1kb ladder (Gibco BRL), lanes 2-9 are duplicate samples of plasmid samples amplified with APX A primers. Lanes 8 & 9 show an upper band of expected size, 2.1kb. 3.6B: *R. hatsugiri* fragments, all lanes as above. 3.6C: *R. impeditum* fragments, all lanes as above. *R. impeditum* shows no fragment of expected size, 2.1kb.

PCR analysis of 1:100 dilution of plasmid samples with APX inserts *from R. ponticum* and *R. hatsugiri* revealed, upon visualisation by gel electrophoresis, a product of 2.1kb, a size corresponding to that of the original PCR product, which is shown in Figure 3.6, suggesting successful

preparations is another questionable factor in this case, although the first 90 or so bases sequence well, corresponding to the plasmid run-in sequence, so this explanation is unlikely.

When studying the entire cloning protocol, the most plausible reason for problems in the sequencing is the quality of the clone itself. When studying the PCR products which were used as the plasmid insert, only fragments using *R. hatsugiri* have a single product band (lane 7, Figure 3.5), and thus gave rise to a successful clone coding the correct gene sequence for ascorbate peroxidase. To confirm this hypothesis, the cloning protocol was repeated only with *R. hatsugiri* fragments, and sequenced by Cambridge Bioscience Autosequencing (Figure 3.7b).

Figure 3.7a. Ascorbate peroxidase *R. ponticum* fragment sense strand

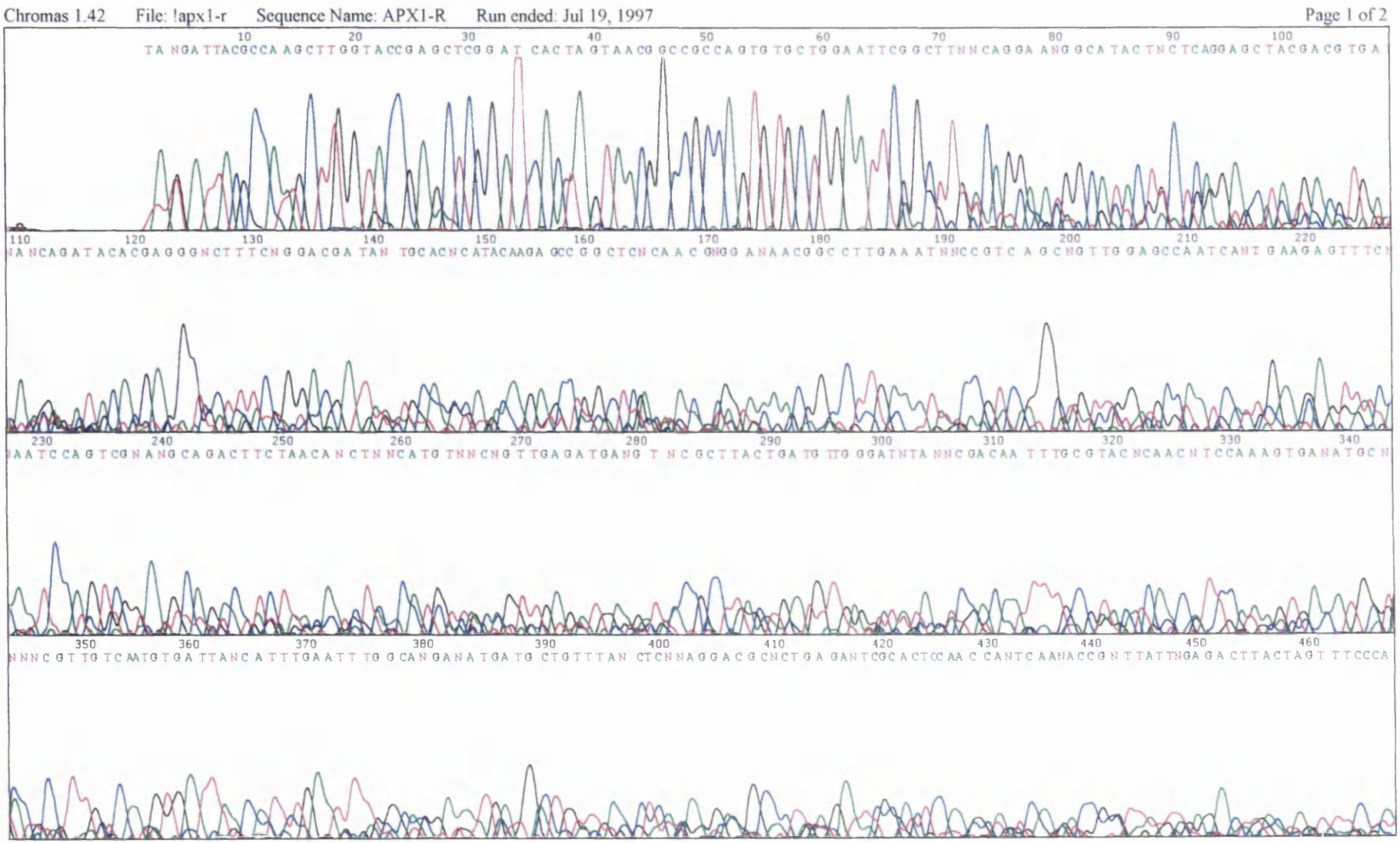


Figure 3.7a. Ascorbate peroxidase *R. ponticum* fragment antisense strand.

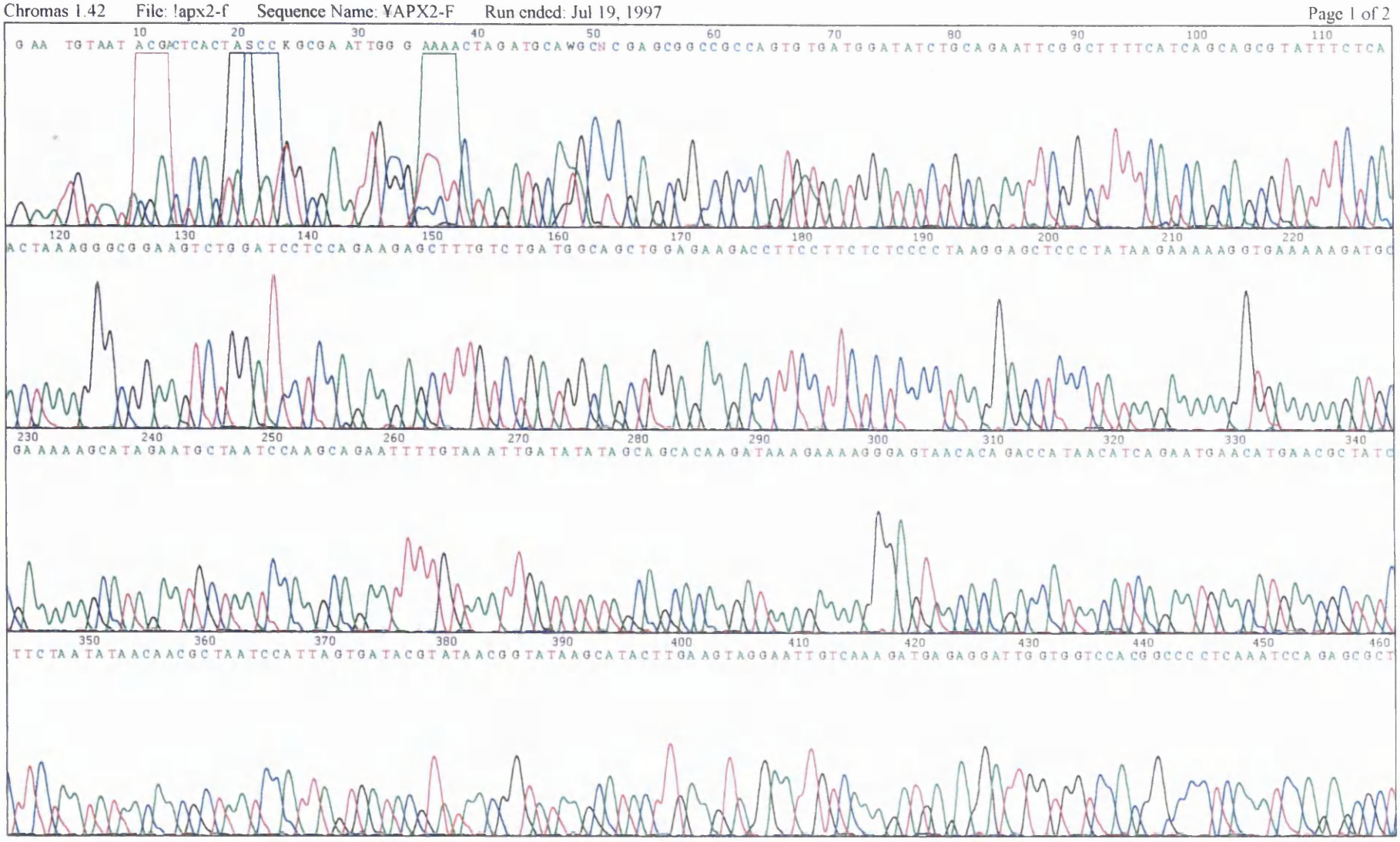


Figure 3.7b. Ascorbate peroxidase R. hatsugiri fragment sense strand

Figure 3.7b. Ascorbate peroxidase R. hatsugiri fragment antisense strand

Figure 3.7b. Repeated sequence reaction by Cambridge Bioscience of *R. hatsugiri* ascorbate peroxidase sense strand.

Figure 3.7c. Ascorbate peroxidase R. impeditum fragment sense strand

Figure 3.7c Ascorbate peroxidase R. impeditum fragment antisense strand

Figure 3.7d Ascorbate peroxidase R. hatsugiri replicate sense strand

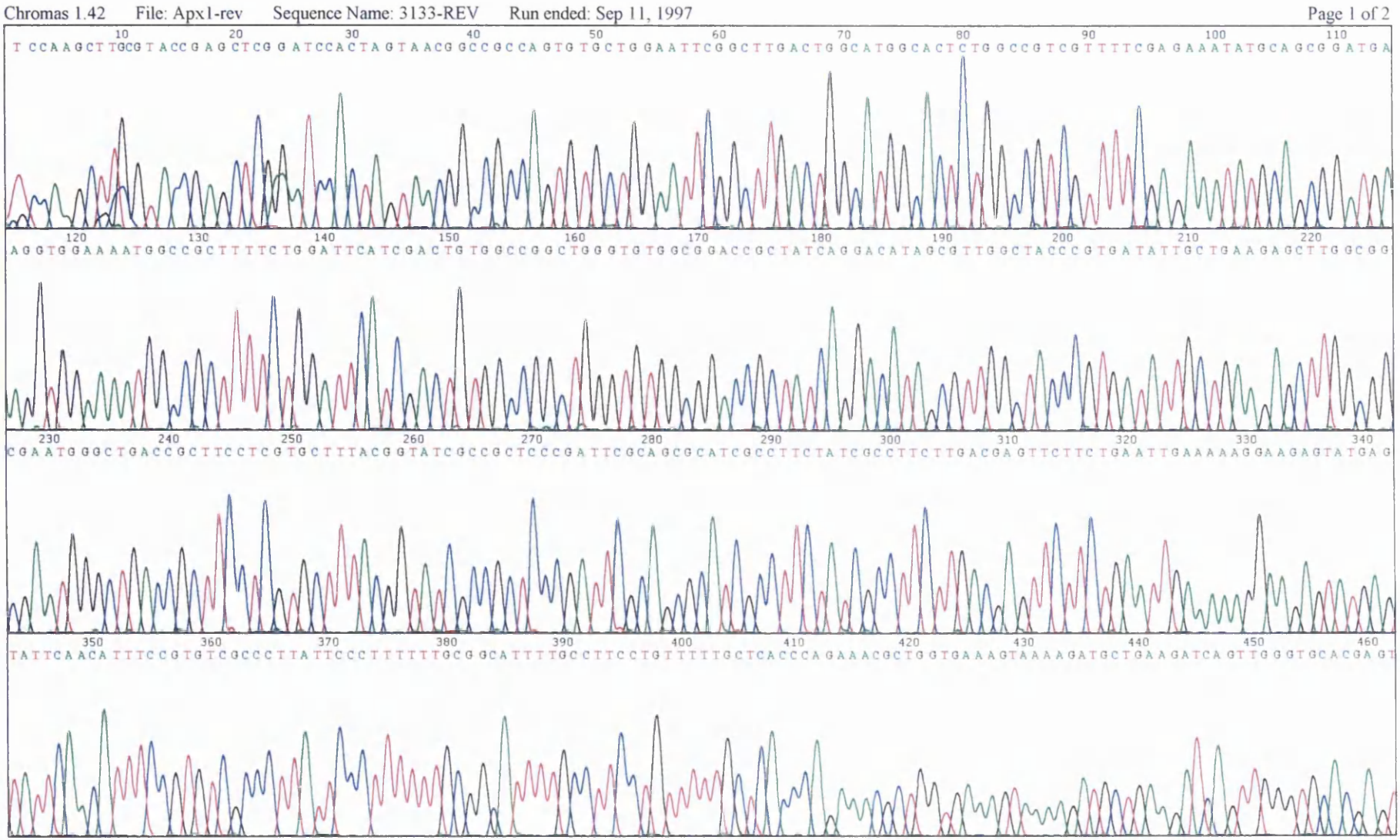


Figure 3.7d Ascorbate peroxidase R. hatsugiri replicate antisense strand

3.3.3 Cloning attempt three

Ligation and transformation were performed using the 2.1kb PCR product generated from the amplification of genomic *R. hatsugiri* DNA and ascorbate peroxidase primers A. Figure 3.8 illustrates analysis of 1:100 dilutions of the cloned plasmids by PCR and gel electrophoresis. Lanes 11-12 have a band of the approximate fragment size, but it is unclear as to whether it relates to the insert fragment. Restriction digestion of the plasmids using the restriction enzyme *Eco*RI, illustrated in Figure 3.9, yields a band at 2.1kb in lane 8, corresponding to the original PCR product band size, which is shown in lane 13. Thus this plasmid sample was used for further analysis by sequencing at Cambridge Bioscience Autosequencing.

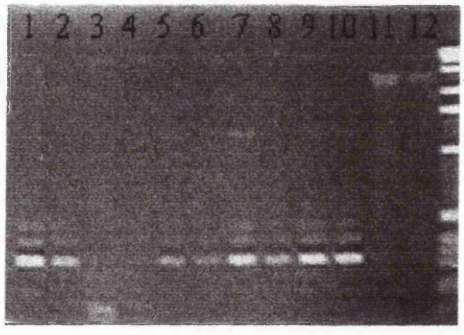


Figure 3.8. PCR analysis of *R. hatsugiri* plasmid fragments. Lane13: 1 kb ladder (Gibco BRL). Lanes 1-12 are dulpicate replicates of 1:100 dilutions of plasmid with *R. hatsugiri* APX fragment insert. Lanes 11 & 12 show a product which may be the insert size of 2.1kb.

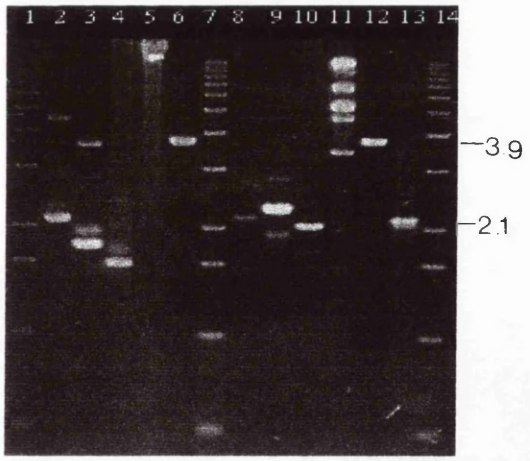


Figure 3.9. Restriction Digestion of *R. hatsugiri* cloned plasmids using *Eco*RI. Lanes 1, 7 and 14 are 1kb ladder (Gibco BRL). Lanes 2-4 replicates of undigested cloned plasmid, lane 5: undigested λ DNA, lane 6: undigested pT-Adv plasmid vector (control). Lanes 8-10 are digested replicates of cloned plasmids, lane 11: digested λ DNA, lane 12: digested pT-Adv plasmid vector control, lane 13 original PCR fragment control (undigested) as size comparison.

BLAST homology searches of the sequences generated revealed homology with plasmid vectors (Appendix 3), thus it could be concluded that ligation of the PCR insert was unsuccessful.

3.3.4 Cloning attempt four

In each case of repeating the cloning procedure, initial amplification of genomic DNA proved to be challenging, despite PCR optimisation, as outlined in Chapter 2. Fresh genomic DNA extractions from all three *Rhododendron* species were prepared, and ascorbate peroxidase primer set A used in PCR amplification. Figures 3.10a and b illustrate the limited success of the reaction i.e. in Figure 3.10b there are no PCR products for *R. hatsugiri* and *R. impeditum.* Indeed, after reamplification of the products (Figures 3.10c and d), results were little improved.

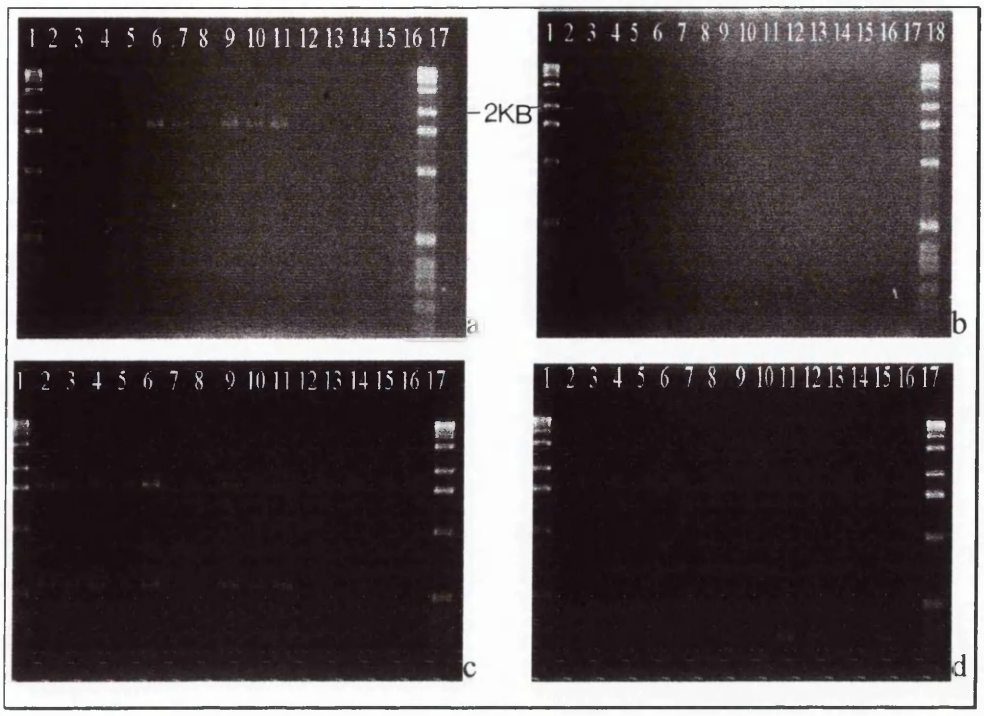


Figure 3.10A-D. PCR Amplification of *Rhododendron* DNA. 8A: Lanes 1 and 17 are 1kb ladder (Gibco BRL), lanes 2-11 are *R. ponticum* DNA amplified with APX primers A. Lanes 12-16 are *R. hatsugiri* DNA 3.10B: lanes 2-6 are *R. hatsugiri* DNA and lanes 7-16 are *R. impeditum* Lane 17, water control. 3.10 C & D: lanes as above, but PCR products have been reamplified under same PCR conditions.

Ligation reactions were carried out with the products from Figure 3.10A (*R. ponticum* fragments), and analysed by gel electrophoresis, illustrated in Figure 3.11. This analysis indicated that ligation of the PCR fragment had not taken place, because the samples are approximately the same size as the original plasmid vector, shown in lane 6. The control ligation reaction, using the control PCR product of 700 bp (Figure 3.3) had been successfully ligated into the vector, as indicted by the higher molecular weight of the control band.

Figure 3.11. Gel electrophoresis of ligation reactions. Lanes 1 and 7 are 1kb ladder (Gibco BRL), lanes 2-4 are replicates of *R. ponticum* PCR ligation into pT-Adv plasmid vector, lane 5: PCR control fragment ligation and lane 6: pT-Adv vector unligated.

The cloning protocol was halted at this stage and PCR methods reconsidered.

3.3.5 Cloning attempt five

Several reasons necessitated the alteration in PCR from APX primer set A to APX primer set C. Amplification of a smaller fragment, set C generating a product of 1.6 kb in size, may increase the efficiency of the ligation reaction. The efficiency of primer set A annealing was questionable, and set C had performed well in PCR optimisation. Fresh DNA extractions from all three *Rhododendron* species were also prepared.

Figure 3.12 illustrates the success of PCR using APX set C and the fresh DNA extracts. All three species amplified well, and duplicate samples of the PCR reaction mix were used directly in the ligation reactions (*R. ponticum*, lanes 6 & 11, Figure 3.12A; *R. hatsugiri*, lane 16, Figure 3.12A and lane 6, Figure 3.12B; *R. impeditum* lanes 10 and 11, Figure 3.12B).

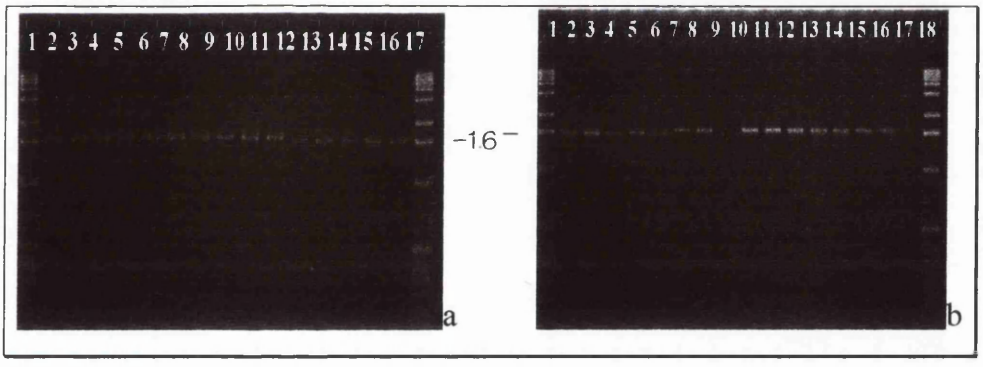


Figure 3.12. 3.12A: lanes 1 and 17 are 1kb ladder (Gibco BRL). Lanes 2-11: *R. ponticum* DNA replicates amplified using APX primer set C, lanes 12-16: *R. hatsugiri* DNA replicates amplified using APX primer set C. 3.12B: Lanes 1 and 18 are 1kb ladder, lanes 2-6: *R. hatsugiri* replicates amplified with APX primer set C, lanes 7-16: *R. impeditum* DNA replicates amplified with APX primer set C, lane 17: water control.

Ligation reactions were analysed by gel electrophoresis, but results remained inconclusive because of band smearing, as shown in Figure 3.13. The transformations were carried out, and it was noted after incubation at 37°C overnight, that there were a number of pure white

colonies present on the IPTG/X-Gal/ampicillin selective plates. Figure 3.14 illustrates one of the transformation plates, the blue and white colonies clearly visible.

Figure 3.13. Gel electrophoresis of ligation reactions. Lanes 1 and 8 are 1kb ladder (Gibco BRL). Lanes 2-7 are duplicates of ligations using *R. ponticum*, *R. hatsugiri* and *R. impeditum* respectively.

Figure 3.14. Sample of transformation plates observed after overnight incubation at 37°C. All of the colonies present on the plate had been transformed with the vector plasmid. Those colonies which were white in colour contained the plasmid vector that carried the PCR insert.

Samples of the pure white colonies were isolated and grown in LB/ampicillin broth, and the plasmid DNA purified from the cultures by Wizard™ Minipreparation system (Promega). Digestion of the transformed plasmid with the restriction enzyme *Eco*RI, whose recognition sites flank the PCR insert (Plasmid vector map, Figure 3.1) yielded two bands, one of 3.9 kb (the size of the plasmid vector) and one of 1.6 kb (the size of the PCR insert), as shown in Figure 3.15.

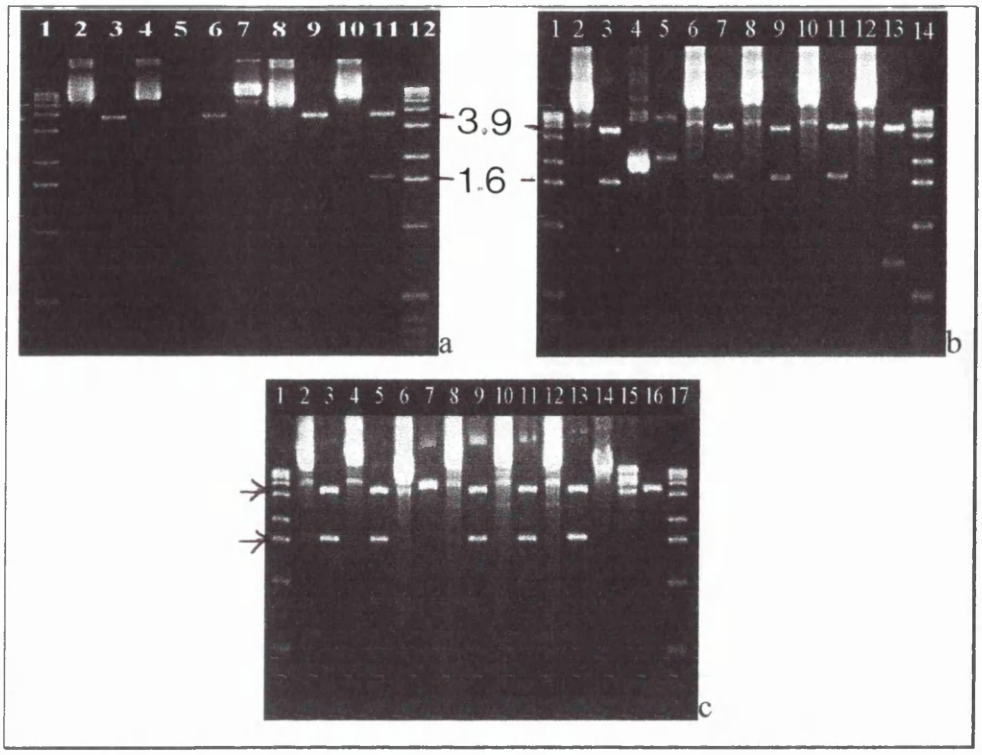


Figure 3.15. Digestion of the transformed plasmids with *Eco*RI. 3.15A: Lanes 1 and 12 are 1 kb ladder (Gibco BRL), lanes 2-11: undigested and digested transformed plasmids with *R. ponticum* APX inserts, (loaded alternately undigested/digested), lane 11 illustrating successful cloning, showing two bands of 3.9kb (plasmid) and 1.6kb (insert). Figures 3.15B &3.15C show digestions of plasmids with *R. hatsugiri* and *R. impeditum* APX inserts respectively. 3.15B: lane 13, PCR control transformation band of 700bp. 14C: lanes 14 and 15 show undigested and digested λ DNA control, lane 16 pT-Adv vector.

The single successful *R. ponticum* clone was sequenced with four samples each of *R. hatsugiri* and *R. impeditum*. The sequence was easily determined from the resulting chromatograms, Figure 3.16, and BLAST analysis confirmed that the *R. ponticum* insert, three *R. hatsugiri* inserts and two *R. impeditum* inserts that had been ligated into the plasmid vector and subsequently cloned, were significantly homologous to previously published ascorbate peroxidase mRNA sequences.

Figure 3.16. Ascorbate peroxidase R. ponticum sense strand. Example of successful chromatogram profile.

3.3.6 Ascorbate peroxidase sequence analysis

The attainment of the insert sequences allowed a number of sequence analyses to be performed. Figures 3.17A -D show the sense and antisense strand comparisons between the replicates of *R. hatsugiri* and *R. impeditum*. The dT end of the plasmid vector is marked in each case. These figures illustrate the accuracy of the PCR/cloning procedure and the sequencing system used at Cambridge Bioscience Autosequencing, as all replicates are identical.

Far more interesting however, is the comparison of the fragment ends between the three *Rhododendron* species. Illustrated in Figure 3.18A and B, a high degree of homology between the three species can clearly be seen in both the sense (Figure 3.18A) and antisense(Figure 3.18B) strands. Indeed, for the first 200 or so bases in the sense strand, the sequences are identical. There are some insertions and/or deletions further into the sequence, which may be due to PCR amplification by *Taq* DNA polymerase. It is known that *Taq* polymerase has a relatively high percentage of base misincorporation. Using the *lac* 10Zα fidelity assay, it was observed that *Taq* DNA polymerase yielded 16% mutated products (Stratagene, © 1997). Amplification with the proof-reading DNA polymerase, *Pfu*, would reduce mutated products to as low as 2%. It cannot be ruled out, however, that such differences may be evolutionary. This assumption can be made due to the former comparisons of replicates of the same species, in which such mutations have not occurred. It can be safely assumed by examination of the sequence chromatograms that the differences were not a sequencing anomaly. Such phenomena were also noted in the antisense strand.

Figure 3.17a. ***R. hatsugiri*** **-reverse sequence alignment. The alignments show the reading frame errors of** ***Taq*** **DNA polymerase in three samples which should be identical.**

```
15a TTTTNAAANCCCGCTCGGATNCACTAGTAACGGCCCGCCAGTGTGCTGGA
20a *****AAATCCAGCTCGGNTTCACTAGTAACGGCCGCC*AGTGTGCTGGA
15e TTTTTGANACCCCGCTCGGATCCCTAGTAACGGCCCGCCAGTGTGCTGGA

15a ATTCGGCTTGACTGGCATGGCACTCTGCTGNTACCTACGATGTGAAAACA
20a ATTCGGCTTGTCTGGCATGGCACTCTGCTGGGACCTACGATGTGAAAACA
15e ATTCGGCTTGATTGGCATGGCACTCTGCTGGGACCTACGATGTGAAAACA

15a AAAACAGGATTGNCTTTCGGGACGATAAAGCACCCAGCAGAGCTGGCTTA
20a AAAACAGGAGGGCCTTTCGGGACGATAAAGCACCCAGCAGAGCTGGCTTA
15e AAAACAGGAGGGCCTTTCGGGACGATAAAGCACCCAGCAGAGCTGGCTTA

15a CGAAGCAAACAACGGCCTTGAAATAGCCGTCAGGCTGTTGGAGCCAATCA
20a CGAAGCAAACAACGGCCTTGAAATAGCCGTCAGGCTGTTGGAGCCAATCA
15e CGAAGCAAACAACGGCCTTGAAATAGCCGTCAGGCTGTTGGAGCCAATCA

15a AGGAGCAGTTTCCTATCCTGTCTTACGCAGACTTCTACCAGGTAACCTGT
20a AGGAGCAGTTTCCTATCCTGTCTTACGCAGACTTCTACCAGGTAACCTGT
15e AGGAGCAGTTTCCTATCCTGTCTTACGCAGACTTCTACCAGGTAACCTGT

15a TGCTGTTGATAGGATATAATGGTTACCAATTTGATTTTTATGCTCAACAT
20a TGCTGTTGATAGGATATAATGGTTACCAATTTGATTTTTATGCTCAACAT
15e TGCTGTTGATAGGATATAATGGTTACCAATTTGATTTTTATGCTCAACAT

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20a ACGTGTTCCTCTTTGTTATGTGATTCTCAATTGAAGTTGGCTGGAATTGT
15e ACGTGTTCCTCTTTGTTATGTGATTCTCAATTGAAGTTGGCTGGAATTGT

15a TGCTGNTGAAGTCACAGGAGGCCCTGAGATTCCTTTCCACCCAGGCAGAC
20a TGCTGTTGAAGTCACAGGAGGCCCTGAGATTCCTTTCCACCCAGGCAGAC
15e TGCTGTTGAAGTCACAGGAGGCCCTGAGATTCCTTTCCACCCAGGCAGAC

15a CGGTTAGTGAGACACTGAGACTTACTACTTCTTTCTTTTCTTCTTATTGT
20a CGGTTAGTGAGACACTGAGACTTACTACTTCTTTCTTTTCTTCTTATTGT
15e CGGTTAGTGAGACACTGAGACTTACTACTTCTTTCTTTTCTTCTTATTGT

15a AATATGTAAAACTCACTTGGTTCTTCCTTTTTCCGTCACAAGGGTGGAAA
20a AATATGTAAAACTCACTTGGTTCTTCCTTTTTCCGTCACAAGGGTGGAAA
15e AATATGTAAAACTCACTTGGTTCTTCCTTTTTCCGTCACAAGGGTGGAAA

15a ATTGAAAGAANGAAAAATGGAAAAATGATAATTACAACTTTTGGCCCGAG
20a ATAGAAAGAAAGAAAAATGGAAAAATGATAATTACAACTTT*GGCC*GAG
15e ATAGAAAGAAAGAAAAATGGAAAAATGATAATTACAACTTT*GGCC*GAG

15a TGATTGTAAGGGATATTAACAGGGCCCGATGCTGGCTTTGCCCTTCACGC
20a TGATTGTAAGG*ATAT*AACAGGGCC*GATGCTGGCTTTGCC*TTCACGC
15e TGATTGTAAGG*ATAT*AACAGGGCC*GATGCTGGCTTTGCC*TTCACGC

15a ATAGGTAGTTTGGGGTTCANTCCTTGGAACTGGGCCCAAATTGNGAGTAA
20a AGAGGTAGTTTGGGGTCATTCGCT*GGAACTGGGCCAAATTGTGATGTAA
15e AGAGGTAGTTTGGGGTCCATTCGCTGGAACTGGGCCAAATTGNGATGTAA

15a TTGATGCCAATAACTNAATGGCTGAAATGGAANTAGGGCGGCG
20a TGATGCAATAATNAATGGCTGAAATGGAAGTANGCCGACT
15e TGATGCCATAATAAATGGCTGAAATGGAAGTAGCC
```

Figure 3.17b. ***R. hatsugiri*** **-21 sequence alignment. The alignments show the reading frame errors of *Taq* DNA polymerase in three samples which should be identical.**

```
15a TNACTATNCGGCGAATTGGGCCCTCTAGATGCATGCTCGAGCGGCCGCCA
20a TCACTATANGGCGAATTGGGCCCTCTAGATGCATGCTCGAGCGGCCGCCA
15e GTACTATCCGGCGAATTGGGCCCTCTAGATGCATGCTCGAGCGGCCGCCC

15a GTGTGATGGATATCTGCAGAATTCGGCTTAGACATCTCTCAAGTGGTCAG
20a GTGTGATGGATATCTGCAGAATTCGGCTTAGACATCTCTCAAGTGGTCAG
15e GTGTGATGGATATCTGCAGAATTCGGCTTAGACATCCCTCAAGTGGTCAG

15a CACCTGATATTAAGAAATGAAAAAAAAAATGGAAAGGCAACTGATAATCT
20a CACCTGATATTAAGAAATGAAAAAAAA*TGGAAAGACAACTGATAATCT
15e CACCTGATATTAAGAAATGAAAAAAAA*TGGAAAGACAACTGATAATCT

15a CAGTCATTAGTTCATAGAAAAGTACCATACAAATAAGAACAAGAGACTTA
20a CAGTCATTAGTTCATAGAAAAGTACCATACAAATAAGAACAAGAGACTTA
15e CAGTCATTAGTTCATAGAAAAGTACCATACAAATAAGAACAAGAGACTTA

15a CCTTTTTTGGCATCAGGCAAGCGGCCTTCCGGAGGTGGTTCAGATTTGTC
20a CCTTTTTTGGCATCAGGCAAGCGGCCTTCCGGAGGTGGTTCAGATTTGTC
15e CCTTTTTTGGCATCAGGCAAGCGGCCTTCCGGAGGTGGTTCAGATTTGTC

15a CTGAGATCAATGAATATTTTTAGTTAATATGAACACAATACCATCTCAAG
20a CTGAGATCAATGAATATTTTTAGTTAATATGAACACAATACCATCTCAAG
15e CTGAGATCAATGAATATTTTTAGTTAATATGAACACAATACCATCTCAAG

15a CCATGACACAAGCATTGCTCCACGAAATTTTCATGGTTCAAAGCTGTAGT
20a CCATGACACAAGCATTGCTACACGAAATTTTCATGGTTCAAAGCTGTAGT
15e CCATGACACAAGCATTGCTACACGAAATTTTCATGGTTCAAAGCTGTAGT

15a CAAAGTTCTCGGACAATACTGTCAGTACACAAGGACTAGGCAAGAGTGGC
20a CAAAGTTCTCGGACAGTACTGTCAGTACACAAGGACTAGGCAAGAGTGGC
15e CAAAGTTCTCGGACAGTACTGTCAGTACACAAGGACTAGGCAAGAGTGGC

15a AAAGATGGTTTCATGAATCATGTCACCAAAATTGTTGATGCTCAAGAACT
20a AAAGATGGTTTCATGAATCATGTCACCAAAATTGTTGATGCTCAAGAACT
15e AAAGATGGTTTCATGAATCATGTCACCAAAATTGTTGATGCTCAAGAACT

15a TTTTCTCCCATCGTATTACTAAGATGAAGTAGTGCACGCAAACTATTAAC
20a TTTTCTCCCATCGTATTACTAAGATGAAGTAGTGCACGCAAACTATTAAC
15e TTTTCTCCCATCGTATTACTAAGATGAAGTAGTGCACGCAAACTATTAAC

15a ATAGTTAACGTCAGCATTGGATCTCTGTCACCAGATCAAATAGGTCTAAA
20a ATAGTTAACGTCAGCATTGGATCTCTGTCACCAGATCAAATAGGTCTAAA
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20a TTAAGCTTAACAAAAAATGTCATCAGAAGCAACTAGATTCTAACTTACTG
15e TTAAGCTTAACAAAAAATGTCATCAGAAGCAACTAGATTCTAACTTACTG

15a ACCCAAAAGATAAAA**CACACCTAGACTCTACTCAAGCTGTATTTGGAT
20a ACCCAAAAGATAAAAANCACACCTAGACTCTACTCAGCTGTATTTGNATC
15e ACCCAAAAGATAAAAA*CACACCTAGACTCTAGCTCAGGCTGTTATTTGT

15a CTCTAACTCTAAAGGATCAACCAAAGCCTCCACATGAATCTGCTGACATT
20a TCTTAGCTCTAAAGGATCAACCAAGCCTACCACATGAATCTGCTGACATT
15e ATCTCTTAGCTCTAAAGGATCAACCAAAGCCTACCACATGAATCTGCTGA

15a GCTGAGTACGTTTGGCTAGTT
20a GCTGAGTACGTTTGGCTAGTTN
15e CATTGCTGAGTTACCGTTTGGCTAGTTN
```

Figure 3.17c *R. impeditum* -reverse sequence alignment. The alignments show the reading frame errors of *Taq* DNA polymerase in three samples which should be identical.

```
24a NCTTTCTTGGTACCNAGCTCGGATCCACTAGTAACGGCCGCCAGTGTGCT
25a *NCTTNTTGATACCCCGCTCGGATCCACTAGTAACGGCCGCCAGTGTGCT

24a GGAATTCGGCTTACACATCTCTCAAATGGTCAGCGCCTCACATTAAGAAA
25a GGAATTCGGCTTACACATCTCTCAAATGGTCAGCGCCTGACATTAAGAAA

24a TCGAAAAACAAAATTAGAAAGACAACTGATAATCTTAGTCATGAGTG*TA
25a TCGAAAAACAAAATTAGAAAGACAACTGATAATCTTAGTCATGAGTGCTA

24a AGAAACGTACCACACATATAAGAACAAGAGACTTACCTTTTTTGGCATCA
25a AGAAACGTACCACACATATAAGAACAAGAGACTTACCTTTTTTGGCATCA

24a GGCAAGCGGCCTTCCGGAGGTGGTTCAGATTTGTCCTGAGATCAATGAAT
25a GGCAAGCGGCCTTCTGGAGGTGGTTCAGATTTGTCCTGAGATCAATGAAT

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24a TGCTACACGAAATTTTCTTAGTTCAAAGCTGTAGTTGAAGTTCTCGGACT
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24a AATGACATGCACGGTGCCTTTCGGCCATTGTAATACTAAGATGAAGTAGT
25a AATGACATGCACGGTGCCTTTCGGCCATTGTAATACTAAGATGAAGTAGT

24a GCATGCAAACTTTTAACATAGCTAACATCAGAGCATTGGATCTCTGTCAC
25a GCATGCAAACTTTTAACATAGCTAACATCAGAGCATTGGATCTCTGTCAC

24a CAGACCAAATAGGATTAAATTAAGCTAACAAAAAATATCANAAGCAACTA
25a CAGACCAAATAGGATTAAATTAAGCTAACAAAAAATATCAGAAGCA*CTA

24a GATTCTGACTTACTAACCCAAAAGAAAAAAACACACCTAGACTCTAACTC
25a GATTCTGACTTACTAACCCAAAAGGAAAAAAACACACCTAGACTCTACTC

24a ANGCTGGTATT*GNATCTTCTTAGCTCTGAAGGATCAACCCAAGCCT*CC
25a CAGCTTTTATTTGNATCTTCTTAGCTCTTAAGGATCAACCCAAGCCTACC

24a ACATGAATCTGCTGCCATGNTGATTACGTTTGGCTAGTTN
25a ACATGAATCCNTTGCATTGCTGATTACGTTTGGCTAG
```

Figure 3.17d *R. impeditum* -21 sequence alignment. The alignments show the reading frame errors of *Taq* DNA polymerase in three samples which should be identical.

```
24a CATTATCATATACGGCGAATTGGGCCCTCTAGATGCATGCTCGAGCGGCC
24h CTTNNTNNTATCCGGCGAATTGTGCCCTCTAGATGCATGCTCGAGCGGCC
25a NTACTCACTATANGGCGAATTGGGCCCTCTAGATGCATGCTCGAGCGGCC

24a GCCAGTGTGATGGATATCTGCAGAATTCGGCTT**GACTGGCATGGCACT
24h GCCAGTGTGATGGATATCTGCAGAATTCGGCTTTGGCATGGCACTCTGCT
25a GCCAGTGTGATGGATATCTGCAGAATTCGGCTTGTTTGGCATGGCA**CT

24a CTGCTGGGACCTACGACGTGAAAACAAAAACAGGAGGGCCTTTCGGGACG
24h CTGCTGGGACCTACGTCGTGAAAACAAAAACAGGAGGGCCTTTCGGGACG
25a CTGCTGGGACCTACGACGTGAAAACAAAAACAGGAGGGCCTTTCGGGACG

24a ATAAAGCATCCAGCAGAGCTGGCTCACGAAGCAAACAACGGCCTTGAAAT
24h ATAAAGCATCCAGCAGAGCTGGCTCACGAAGCAAACAACGGCCTTGAAAT
25a ATAAAGCACCCAGCAGAGCTGGCTCACGAAGCAAACAACGGCCTTGAAAT

24a AGCCGTCAGGCTGTTGGAGCCAATCAAGGAGCAGTTTCCGATCCTGTCTT
24h AGCCGTCAGGCTGTTGGAGCCAATCAAGGAGCAGTTTCCGATCCTGTCTT
25a AGCCGTCAGGCTGTTGGAGCCAATCAAGGAGCAGTTTCCGATCCTGTCTT

24a ACGCAGACTTCTACCAGGTAACCTGTTGCAGTTGATATGATATACTGGTT
24h ACGCAGACTTCTACCAGGTAACCTGTTGCAGTTGATATGATATACTGGTT
25a ACGCAGACTTCTACCAGGTAACCTGTTGCAGTTGATATGATATACTGGTT

24a ACTGATTTAATTTTTATGCTCAACATATGTTGTCGGCGTATTATTAGTCC
24h ACTGATTTAATTTTTATGCTCAACATATGTTGTCGGCGTATTATTAGTCC
25a ACTGATTTAATTTTTATGCTCAACATATGTTGTCGGCGTATTATTAGTCC

24a ACATTTTGCGTACTCTAACGTCCAAAATGACATGTTCCCCTTTCTTATGT
24h ACATTTTGCGTACTCTAACGTCCAAAATGACATGTTCCCCTTTCTTATGT
25a ACATTTTGCGTACTCTAACGTCCAAAATGACATGTTCCTCTTTCTTATGT

24a GATTATCAATTGAAGTTGGCTGGAATTGTTGCTGTTGAAGTCACAGGAGG
24h GATTATCAATTGAAGTTGGCTGGAATTGTTGCTGTTGAAGTCACAGGAGG
25a GATTATCAATTGAAGTTGGCTGGAATTGTTGCTGTTGAAGTCACAGGAGG

24a CCCTGAGATTCCTTTCCACCCAGGCAGACCGGTTAGTGAGAGTTATTACT
24h CCCTGAGATTCCTTTCCACCCAGGCAGACCGGTTAGTGAGAGTTATTACT
25a CCCTGAGATTCCTTTCCACCCAGGCAGACCGGTTAGTGAGAGTTATTACT

24a TCTTTCTTTGCTTCTTATCGTAATATGTAAAACTCACTTGGTTCTTCCTT
24h TCTTTCTTTGCTTCTTATCGTAATATGTAAAACTCACTTGGTTCTTCCTT
25a TCTTTCTTTGCTTCTTATCGTAATATGTAAAACTCACTTGGTTCTTCCTT

24a TT*CTGTCACAAGGGTGGAAAATAGAAAGGAAAAAAATAAAGGAAGGAAA
24h TTTCTGTCACAAGGGTGGAAAATAGAAAGGAAAAAAATAAAGGAAGGAAA
25a TTTCTGTCACAAGGGTGGAAAATAGAAAGGAAAAAAATAAAGGAAGGAAA

24a AATGAGAATTACAACTTTGGCCGAGTGATTGTAAGGATATAACAGGGCC*
24h AATGAGAATTACAACTTTGGCCGAGTGATTGTAAGGATATAACAGGGCCC
25a AATGAGAATTACAACTTTGGCCGAGTGATTGTAAGGATATAACAGGGCCC

24a GATGCTGGCTTTGCGCTCCACGCAGAGGTAGTTTGGGGTCCATTCGCTAG
24h GATGCTGGCTTTGCGCTCCACGCAGANGTAGTTTGGGGTCCATTCGCTAG
25a GATGCTGGCTTTGCGCTTCACGCAGANGTAGTTTGGGGTCCATT*GCTAG

24a ACTGTGCCAAATTCTGATGTAATGATGCNN
24h ACTG*GCCAAATTCTGATGTAATGATCAATAN
25a ACTAGACTGNGCCAAATTCTGATGTAATGATG
```

Figure 3.18a -Rev Sequence similarity *R. ponticum* (10h), *R. hatsugiri* (15a) and *R. impeditum* (25b) Upper Primer APX C highlighted (*Arabidopsis thaliana*)

```
10h TTGTGATACCCCGCTCGGATCCACTAGTAACGGCCGCC-AGTGTGCTGGA
15a TTTTNAAANCCCGCTCGGATNCACTAGTAACGGCCCGCCAGTGTGCTGGA
25b CTGCTTGGTACCGNCTCGGATCCCTAGTAACGGCCGCC-AGTGTGCTGGA

10h ATTCGGCTTGACTGGCATGGCACTCTGCTGGGACCTACGACGTGAAAACA
15a ATTCGGCTTGACTGGCATGGCACTCTGCTGNTACCTACGATGTGAAAACA
25b ATTCGGCTTGATTGGCATGGCACTCTGCTGGGACCTACGACGTGAAAACA

10h AAAACAGGAGGGCCTTTCGGGACGATAAAGCACCCATCAGAGCTGGCTCA
15a AAAACAGGATTGNCTTTCGGGACGATAAAGCACCCAGCAGAGCTGGCTTA
25b AAAACAGGAGGGCCTTTCGGGACGATAAAGCATCCAGCAGAGCTGGCTCA

10h CGAAGCAAACAACGGCCTTGAAATAGCAGTCAGGCTGTTGGAGCCAATCA
15a CGAAGCAAACAACGGCCTTGAAATAGCCGTCAGGCTGTTGGAGCCAATCA
25b CGAAGCAAACAACGGCCTTGAAATAGCCGTCAGGCTGTTGGAGCCAATCA

10h AGGAGCAGTTTCCAATCCTGTCTTATGCAGACTTCTACCAGGTAACCTGT
15a AGGAGCAGTTTCCTATCCTGTCTTACGCAGACTTCTACCAGGTAACCTGT
25b AGGAGCAGTTTCCGATCCTGTCTTACGCAGACTTCTACCAGGTAACCTGT

10h TGCAGTTGATATGATATACTGGTTACTGATTTGATTTGATTTTTATGCTC
15a TGCTGTTGATAGGATATAATGGTTACCAATTTGA-----TTTTTATGCTC
25b TGCAGTTGATATGATATACTGGTTACTGATTTAA-----TTTTTATGCTC

10h AACATATATTGTCGGCGTATTATTAGTCCACATTTTGCGTACTCTAACGT
15a AACATATGTTGTTGGCGTATT---AGTCCATATTTTGCGTATTCTAACGT
25b AACATATGTTGTCGGCGTATTATTAGTCCACATTTTGCGTACTCTAACGT

10h CCAAAATGACATGTTCCTCTTTGTTATGTGATCATCAATTGAAGTTGGCT
15a CCAAGATGACGTGTTCCTCTTTGTTATGTGATTCTCAATTGAAGTTGGCT
25b CCAAAATGACATGTTCCCCTTTCTTATGTGATTATCAATTGAAGTTGGCT

10h GGAATTGTTGCTGTTGAAGTCACAGGAGGCCCTGAGATTCCTTTCCACCC
15a GGAATTGTTGCTGNTGAAGTCACAGGAGGCCCTGAGATTCCTTTCCACCC
25b GGAATTGTTGCTGTTGAAGTCACAGGAGGCCCTGAGATTCCTTTCCACCC

10h AGGCAGACCGGTTAGTGAGAC---------TACTACTTCTTTCTTTGCTT
15a AGGCAGACCGGTTAGTGAGACACTGAGACTTACTACTTCTTTCTTTTCTT
25b AGGCAGACCGGTTAGTGAGAG--------TTATTACTTCTTTCTTTGCTT

10h CTTATCGTAATATGTAAAACACACTTGGTTCTTCCTTTTTCTGTCACAAG
15a CTTATTGTAATATGTAAAACTCACTTGGTTCTTCCTTTTTCCGTCACAAG
25b CTTATCGTAATATGTAAAACTCACTTGGTTCTTCCTTTTTCTGTCACAAG

10h GGTGGAAAATAGAAAGAAAAAAAAAGGACAAA-TGAGAAATGAGAAATAA
15a GGTGGAAAATTGAAAGAANGAAAAATGGAAAAATGATAATTACAAC----
25b GGTGGAAAATAGAAAGGAAAAAAATAAAGGAAGGAAGAATGAGAATTACA

10h AACTTTGGCC-GAGTGATTGTAAGG-ATAT-AACAGGGCC-GATGCTGCC
15a --TTTTGGCCCGAGTGATTGTAAGGGATATTAACAGGGCCCGATGCTGCC
25b A-CTTTGGCC-GAGTGATTGTAAGG-ATAT-A-CAGGGCC-GATGCTGGC

10h TTTGCCCTTCACGCANANGTAGTTTGAGGTCCATTTGCTAGAACTGNGC-
15a TTTGCCCTTCACGCATAGGTAGTTTGGGGTTCANTCCTTGGAACTGGGCC
25b TTTGCGCTTCACGCAGAAGTAGNTTGGGGNNCATTCGCTAGAACTGNGCC

10h CAAATTCTGATGTAATGGATGC-AATAACTTAATGGCTTAAATGGAAGTGGG
15a CAAATTGNGA-GTAATTGATGCCAATAACTNAATGGCTGAAATGGAANTAGGGCGGCG
25b CAAAT-CTGATGTAATGATGCAATAATNAATGGCTTAAATGGAAGAAGGG
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Figure 3.18b -21 Sequence similarity *R. ponticum* (10h), *R. hatsugiri* (15a) and *R. impeditum* (25b) Lower Primer APX C highlighted (*Pisum sativum*).

```
10h TCACTATCNGGCGAATTGGGCCCTCTAGATGCATGCTCGAGCGGCCGCCA
15a TNACTATNCGGCGAATTGGGCCCTCTAGATGCATGCTCGAGCGGCCGCCA
25b TCACTATAGGGCGAATTGGGCCCTCTAGATGCATGCTCGAGCGGCCGCCA

10h GTGTGATGGATATCTGCAGAATTCGGCTTAGACATCTCTCAAATGGTCAG
15a GTGTGATGGATATCTGCAGAATTCGGCTTAGACATCTCTCAAGTGGTCAG
25b GTGTGATGGATATCTGCAGAATTCGGCTTAGACATCTCTCAAGTGGTCAG

10h GGCCTGACATTAAGAAATCGAAAAACAAAATTAGAAAGACAACTGATAAT
15a CACCTGATATTAAGAAATGAAAAAAAAAA-T-GGAAAGGCAACTGATAAT
25b CGCCTCACATTAAGAAATCGAAAAACAAAATTAGAAAGACAACTGATAAT

10h CTTAGTCATGAGTGCTTAGAAACGTACCGCACATATAAGAACAAGAGACT
15a CTCAGTCATTAGTTCATAGAAAAGTACCATACAAATAAGAACAAGAGACT
25b CTTAGTCATGGGT-GTAAGAAACGTACCACACATATAAGAACAAGAGACT

10h TACCTTTTTTGGCATCAGGCAAGCGGCCTTCCGGAGGTGGTTCAGATTTG
15a TACCTTTTTTGGCATCAGGCAAGCGGCCTTCCGGAGGTGGTTCAGATTTG
25b TACCTTTTTTGGCATCAGGCAAGCGGCCTTCCGGAGGTGGTTCAGATTTG

10h TCCTGAGATCAATGAATATTTTTAGTTAATATGAACGAAATACCATCTCA
15a TCCTGAGATCAATGAATATTTTTAGTTAATATGAACACAATACCATCTCA
25b TCCTGAGATCAATGAATATTTTTAGTTAATATGAATGCAATACCATCTCA

10h AACCATGACACAAGAATTGCTACACGAAATTTTCTTGGTTCAAAGCTGTA
15a AGCCATGACACAAGCATTGCTCCACGAAATTTTCATGGTTCAAAGCTGTA
25b AACCATGACACAAGAATTGCTACACGAAATTTTCTTAGTTCAAAGCTGTA

10h GTCGAAGTTCTTGGACTGTACACAAGGACTAGGAAAGAGTGGCAAAGATG
15a GTCAAAGTTCTCGGACAATACTGTCAGTACACAAGGACTAGGCAAGAGTG
25b GTTGAAGTTCTCGGACTGTACACAAGGACTAGGCAAGAGTGGCAAAGATG

10h GTTTCATGAATCACAGTAATTAAGATTGTTGATGCTCAAGTAATTTTTCT
15a GTTTCATGAATCATGTCACCAAA*ATTGTTGATGCTCAAGAACTTTTTCT
25b GTTTCATGAATCACAGTAATTAAGATTGTTGATGCTCAAGTACTTTTTCT

10h CAGGCTGTCAAGCCACAAATGACATGCACGGTGCCTTTCGGCCATT-GT-
15a CCCATCGTATTACTAAGATGAAGTAGTGCACGCAAACTATTAACATAGTT
25b CAGGCTGTCCAGCCACAAATGACATGCACGGTGCCTTTCGGCCATT-GT-

10h AATACTAAGAT-GAAGTAGTGCATGCAAACTATTAACATAGCTAACATCA
15a AACGTCAGCATTGGATCTCTGTCACCAGATCAAATAGGT--CTAAATT--
25b AATACTAAGAT-GAAGTAGTGCATGCAAACTTTTAACATAGCTAACATCA

10h GAGCATTGGATCTCTGTCACCAGATCAAATAGGTCTAAATTAAGCTAACA
15a *AAGCTTAACAAAAAATGTCATCAGAAGCAACTAGATTCTAACTTACTGA
25b GAGCATTGGATCTCTGTCACCAGACCAAATAGGATTAAATTAAGCTAACA

10h AAAAATATCAGAAGCAACTAGATTCTG-CTTACTAACCCAAAAGAAAAAA
15a CCCAAAAGATAAAA-CACACCTAGACTCTACTCAAGCTGTATTTGGATCT
25b AAAAATATCAGAAGCAACTAGATTCTGACTTACTAACCCAAAAGAAAAAA

10h ACACACCTAGACTCTACTCAGCTGGTATTTGGATCTTTCTTAGCTCTAAA
15a CTAACTCTAAAGGATCAACCAAAGCCTCCACATGAATCTGCTGACATTGC
25b ACACACCTAGACTCTAACTCAAGCTGGTATTTGTATCTTCTTAGCTCTGA

10h GGATCAACCAAAGCCTACCACATGAAT
15a TGAGTACGTTTGGCTAGTT
25b AGGATCAACCAAAGCCTACCACATGAAT
```

BLAST homology searches permit a diagrammatic representation of the regions of homology with published ascorbate peroxidase sequences, in this case pea (*Pisum sativum*, Mittler and Zilinskas, 1991). Figure 3.19 illustrates the full mRNA sequence of ascorbate peroxidase in pea. The primer sequences used to amplify ascorbate peroxidase in *Rhododendron* are highlighted, and show only a three base difference at the beginning of the primer region for the sense primer, and only a two base difference in the antisense primer. The stars represent bases which are identical in the *Rhododendron* sequence.

Figure 3.19. Pea Ascorbate peroxidase mRNA complete sequence / *Rhododendron* homology (* = sense strand homology with *Rhododendron*, * = antisense strand homology with *Rhododendron*).
Coloured sequence represents the primer sequences - upper (U) and lower (L).

Of the region highlighted in Figure 3.19, the *Rhododendron ponticum* fragments have 79% homology with ascorbate peroxidase mRNA in pea (refer also to BLAST homology searches, Appendix 2). It may be deduced from Figure 3.19 that APX primer set C was suitable for successful amplification of ascorbate peroxidase in *Rhododendron* species, and such primer sites may be suitable for the amplification of mRNA by Reverse Transcription PCR.

The *R. ponticum* APX fragment was derived from genomic DNA, and so to further interpret the sequence, the *Rhododendron* fragments were joined, the antisense strand being reversed and complemented, thus representing the partial DNA sequence of APX in *Rhododendron.* This is depicted in Figure 3.20. It can be postulated when studying this diagram, that there is at least one, if not two intron sites in the known sequence. There is no gene conservation with known published sequences between 243 bp and 392 bp in the sense strand, and again after 458 bp. The first gap in homology may well be an intron, as after 458 bp, however the latter is less certain because of a reduced resolution of the electrophoresis. The small gap in homology in the antisense strand is also worthy of noting, but may however, be an evolutionary difference rather than an intron.

Successful cloning and sequencing of a small part of the ascorbate peroxidase gene in *Rhododendron* has yielded information about gene conservation between quite diverse species such as pea and *Rhododendron*, as well as PCR primer suitability. Specific sequence data for *Rhododendron* species which was previously unknown, can allow the design and generation of *Rhododendron*-specific primers for amplification of ascorbate peroxidase mRNA in *Rhododendron ponticum.*

Figure 3.20. *R. ponticum* fragments joined together to make singular sense strand the - antisense sequence has been reversed and complementary sequence made to generate single sense strand (* = sense strand homology with *Pisum sativum*, * = antisense strand homology with *Pisum sativum*). Coloured sequence represents the primer sequences - upper (U) and lower (L).

3.4 Cloning of Glutathione Reductase - Results and Discussion

3.4.1 Cloning attempt one

The same disciplines were adhered to in the cloning of this enzyme. DNA extractions of *Rhododendron ponticum*, *R. hatsugiri* and *R. impeditum* were prepared and amplified using glutathione reductase primer set A, and thermal cycle conditions stated in section 2.2.8. Figure 3.21 illustrates the gel electrophoresis analysis of PCR. Lane 10 shows the only successful amplification with *R. impeditum* genomic DNA, with a products size of approximately 500 bp, and its reaction mix was subsequently used directly for replicate ligation reactions.

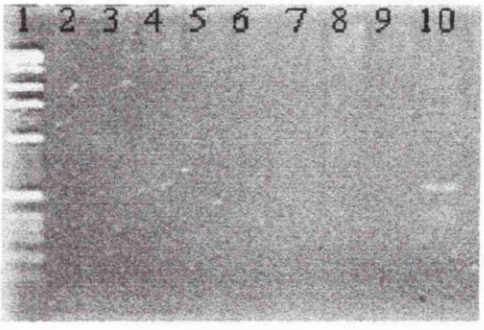

Figure 3.21. Gel electrophoresis of PCR using glutathione reductase primer set A. Lane 1: 1kb ladder (Gibco BRL). Lanes 2-4: *R. ponticum* genomic DNA amplified, lanes 5-7: *R. hatsugiri* genomic DNA amplified, lanes 8-10 *R. impeditum* genomic DNA amplified.

Following transformation and incubation of the *E. coli* cultures, inspection of the colonies revealed several pure white colonies, which were isolated for plasmid purification by Wizard™ Minipreparation System (Promega). Plasmid DNA was analysed by gel electrophoresis, both before and after digestion with the restriction enzyme *Eco*RI (Figure 3.22). Bands of 3.9 kb and 500 bp are visible on the gel, indicating successful ligation and transformation of the insert and pT-Adv plasmid vector, thus these plasmid samples were sequenced by Cambridge Bioscience Autosequencing.

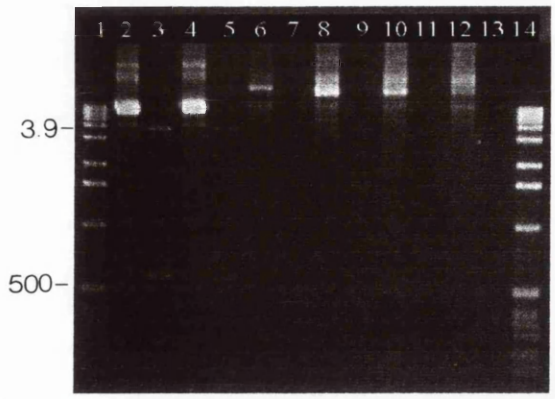

Figure 3.22:GR plasmids digested with *Eco*RI. Lanes 1 and 14 are 1kb ladder (Gibco BRL), lanes 2 & 4 are samples of undigested plasmid *with R. impeditum* GR insert, lanes 3 & 5 are the former plasmids digested with *Eco*RI. Lanes 6-13 are APX inserts digested with *Eco*RI.

BLAST homology searches (Appendix 3) revealed that the inserts contained in the plasmid vector were not homologous to any published gluathione reductase sequence. As noted by the uniform chromatograms (Figure 3.23), the sequencing process was successful. Further consideration must therefore be given to the PCR protocol.

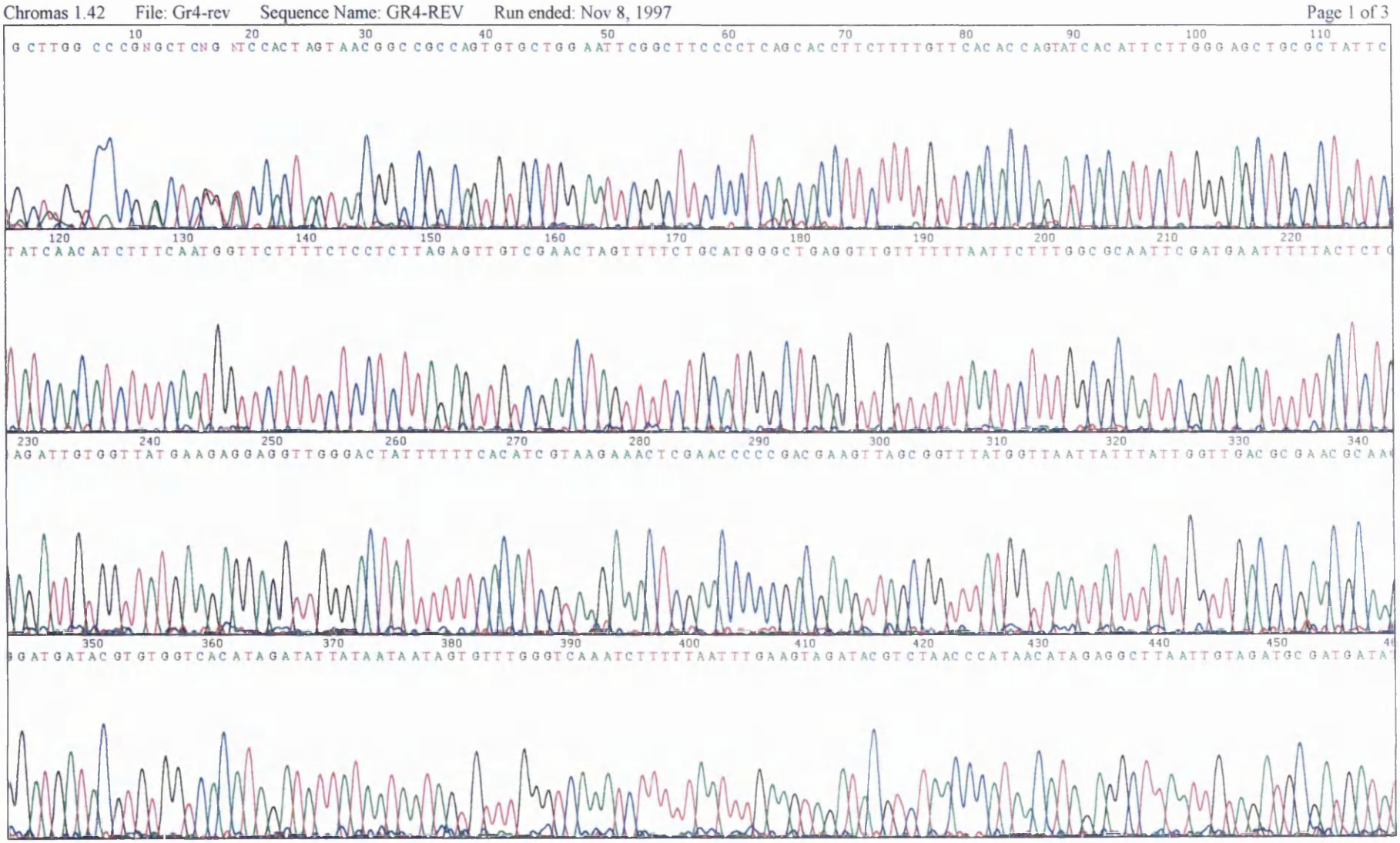

Figure 3.23a. Glutathione reductase *R. impeditum* replicate sense strand

Figure 3.23a Glutathione reductase *R. impeditum* replicate antisense strand

Figure 3.23b. Glutathione reductase *R. impeditum* replicate sense strand

Figure 3.23b. Glutathione reductase *R. impeditum* replicate antisense strand

3.4.2 Cloning attempt two

For successful Competitive Reverse transcription PCR, an important criterion is the distinction between the amplification of the control and target sequences. If the control fragment is to be made from DNA, it is best if the primers span at least one intron site. Because previous PCR studies with glutathione reductase involved a fragment of only 500bp in length, and did not represent a fragment of the gene, new primers were designed, synthesised and tested using methods described in chapter 2, to meet this criterion. Table 3.2 shows the primer sequences designed and tested.

GR 1 Upper Primer	**5′ CTATCGGTGCCGGAAGC 3′**
GR 2 Lower Primer	**3′ TGAACACCCACTGTAGCATCA 5′**
GR A Upper Primer	**5′ GCGAGCTTCCTTTTTCCACTA 3′**
GR B Lower Primer	**3′ AACCAGCTTTAACTGCAACCC 5′**

Table 3.2. Glutathione Reductase Primer sequences used in PCR for subsequent product cloning (attempt two).

PCR tests revealed successful amplification using GR A & B primers, illustrated in Figure 3.24. The final PCR conditions consisted of a reaction mix containing 10× PCR Buffer (10 mM Tris-HCl, 50 mM KCl, Perkin Elmer), 1.5 mM $MgCl_2$, (Perkin Elmer), 20 pmoles upper primer, 20 pmoles lower primer (synthesised by Cruachem), 2.5 mM dNTPs (Perkin Elmer), 1 unit *Taq* Polymerase (Perkin Elmer) and 10ng *Rhododendron* genomic DNA, in a total volume of 25 µl. The thermal cycle programme was set at 95°C for 5 minutes initially, followed by 1 minute at 94°C, 2 minutes at 55°C and 3 minutes at 72°C for a total of 30 cycles, and a final extension time of 10 minutes at 72°C. No results were gleaned from primers GR 1 & 2 throughout the tests. PCR reaction mixes using *R. ponticum* genomic DNA yielding a product of approximately 2.1kb, shown in lanes 2-6 of Figure 3.24, were used directly in ligation.

Figure 3.24. Gel Electrophoresis of PCR amplification using primers GR A & B. Lanes 1 and 17 are 1kb ladder (Gibco BRL), lanes 2-6: amplification using *R. ponticum* DNA, lanes 7-11: amplification using *R. hatsugiri* DNA, lanes 12-16: amplification using *R. impeditum* DNA.

Incubation of the transformed *E. coli* cultures on selective IPTG/X-Gal/ampicillin LB plates revealed unsuccessful cloning using this protocol. Due to time constraints on the project, work was unable to continue with the glutathione reductase gene. There are again several reasons why the cloning procedure for glutathione reductase was unsuccessful. The non-specific addition of dA to the ends of the PCR product, may in this case have been unsuccessful. The nucleotide addition may also be particularly unstable, therefore if the PCR mix was not used immediately, the dA ends may have been lost, rendering the ligation reaction inefficient.

3.5 Conclusions

- Cloning by T/A overhang method proved to be a delicate technique to apply because of the vulnerability of the single base overhang. However, this approach was efficient when successful.

- A high degree of accuracy is necessary at every stage of the cloning procedure, particularly with the quality and age of the PCR fragment to be ligated into the pT-Adv plasmid vector.

- Initial cloning attempts for both genes possibly failed for several reasons:
  - poor PCR product quality,
  - inadequate concentrations of product for successful ligation,
  - multiple PCR products within the sample (APX cloning attempt two).

- Correction of the above criteria resulted in successful cloning of a possible 'ascorbate peroxidase' fragments for *Rhododendron ponticum*, *R. hatsugiri* and *R. impeditum* and a possible 'glutathione reductase' fragment for *R. impeditum*.

- DNA sequencing and gene homology studies of the fragments from *Rhododendron* species revealed the 1.6 kb product to be ascorbate peroxidase.

- The 500 bp product cloned from *R. impeditum* was shown not to be a fragment of glutathione reductase, illustrating the importance of PCR primer design.

- Generation of new primers enabled the amplification of a larger product to be synthesised, however the sensitivity of the cloning procedure and time constraints prevented sequencing to identify the fragment.

Chapter 4 Competitive Reverse Transcription - Polymerase Chain Reaction Development and Application for Ascorbate Peroxidase Gene

4.1 Introduction

Polymerase Chain Reaction (PCR) allows sensitive, selective detection and amplification of a target DNA fragment. Combined with its flexibility, these properties have led to the utilisation of the technique in DNA sequencing, cloning and mutagenesis (Ohan and Heikkila, 1993). In theory, PCR also offers an attractive means of detecting and analysing mRNA, provided an initial reverse transcription (RT) step is incorporated. Because of the sensitivity of the technique, RT-PCR has many potential advantages over established mRNA analyses, including sensitivity, rapidity and generation of complementary DNA (cDNA) samples that can be stored and used for the study of various genes (Phillips, Sarang and Gibson, 1993). Characterisation of mRNAs previously undetectable by conventional methods such as northern, dot or slot blots, hybridisation and RNase protection assays (Gilliland, *et al.*, 1990; Wang and Mark, 1990; Ohan and Heikkila, 1993) and disease diagnosis (Ohan and Heikkila, 1993) also contribute to RT-PCR becoming an invaluable technique. The sensitivity of the technique allows successful amplification with relatively small amounts of mRNA. As little as 60 pg total RNA (of which mRNA comprises less than 5%) can be used successfully, as demonstrated by Ohan and Heikkila (1993).

4.1.1 RT-PCR criteria

In theory, the design of the reverse transcription reaction is relatively straightforward, with only the primers and transcriptase enzyme to be decided upon. The efficiency of first strand cDNA synthesis is paramount to the success and reproducibility of the reaction. The quality of the RNA preparation used is an important contributor to the success of RT-PCR, less degraded preparations generating concise results (Philips, *et al.*, 1993).

4.1.2 Reverse transcription primers

There are three types of DNA primer which can be used to initiate reverse transcription: a) random hexanucleotides, b) oligo (dT) primers and c) target-specific primers (Ohan and Heikkila, 1993; Philips, *et al.*, 1993). Random hexamers will generate cDNA from all RNA present in the reaction mix and are particularly useful for amplification of RNA larger than 2 kilobases (kb). Oligo (dT) primers are best applied when study of poly (A) RNA of 2-3kb in size is desired. Both these primer types can however, result in the coamplification of non-specific templates (Baier *et al.*, 1993). Target specific primers (the antisense primer of DNA PCR primer pairs) are less often used in the majority of RT-PCR applications because optimal conditions must be determined for each mRNA. If used, design should be such that the primers span an intron site, thus contaminating DNA can be distinguished by size (Wang *et al*, 1989).

4.1.3 Reverse transcription enzymes

As with PCR, reverse transcription PCR must be tailored to suit specific applications and RNA. The type of transcriptase enzyme used is an important factor in the success of mRNA studies. At present, there are two specific transcriptase enzymes commonly in use, and more recent studies have determined the success of a DNA polymerase in reverse transcription reactions.

Avian Myeloblastosis Virus Reverse Transcriptase (AMV) is a DNA polymerase which can use DNA, RNA or RNA:DNA hybrids as a template, and requires the presence of an oligomer for template annealing and free magnesium in the reaction mix for optimum transcription. Because of its high RNase H activity, a phenomenon which causes cleaving of the extending strand if the enzyme pauses during transcription,

AMV is best suited for producing relatively short cDNA structures. The high optimal temperature for enzyme efficiency (42°C) reduces problems of RNA secondary structure allowing increased efficiency of cDNA synthesis (Ohan and Heikkila, 1993).

Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV) is an RNA-dependent DNA polymerase requiring a DNA primer and an RNA template to synthesise a complementary DNA strand. This enzyme has a weaker inherent RNase H activity, an important factor in obtaining longer cDNA products (Promega Corporation, 1998) However, because of its lower optimal temperature for activity (37°C), M-MLV reverse transcriptase is less efficient when the RNA template exhibits secondary structures.

Thermus thermophilus DNA polymerase (*Tth* polymerase) has more recently been utilised in RT-PCR applications (Myers and Gelfand, 1991) because it has dual activity (RT and DNA polymerase) and is heat stable. In such cases, the same enzyme is used for both the reverse transcription and polymerase chain reaction, improving the efficiency of the overall procedure and providing more scope for automation. *Tth* polymerase also has the ability to read RNA secondary structures, regions best avoided when using AMV and M-MLV reverse transcriptase, because the higher optimal activity temperature allows for secondary structure denaturation.

4.1.4 mRNA quantification using RT-PCR

Reverse transcription PCR has made possible the study of rare and previously undetectable RNA transcripts (Gilliland *et al.*, 1990). However, the quantification of the amount of mRNA in tissue or cells has been a more challenging procedure, restricted mainly by the amplification process. Because amplification is an exponential process, small

differences in variables at the beginning of the reaction can lead to dramatic variation between replicate samples. Such variations include the concentration of the enzyme, dNTPs, magnesium, DNA and primers, all which can be alleviated to a certain extent by the use of master mixes (Wang *et al.*, 1989; Gilliland *et al.*, 1990; Chen *et al.*, 1993; Clontech, 1993).

Such reaction characteristics make the quantification of mRNA difficult. However, the use of internal standards in RT-PCR can aid the collection of accurate quantitative data. Standards can be generated by different means - endogenous 'housekeeping' genes which are expressed at a constant rate in cells or tissues (Clontech, 1993), or exogenous standards which are added to the mix (Wang *et al*, 1989; Gilliland *et al*, 1990), sometimes referred to as Competitive RT-PCR. The use of 'housekeeping' genes, such as β-actin (Philips *et al*, 1993), to quantify mRNA is restrictive as data can only ever be semi-quantitative because of differences in primer pairs for the target and standard mRNA (Wang *et al*, 1989).

It is possible to determine initial amounts of mRNA in competitive RT-PCR by using a known dilution series of control fragment with constant amounts of target mRNA in reverse transcription. A graph of the ratio of final yield of competitor:target product yield versus known concentration of initially added control fragment will determine the amount of starting mRNA in the reaction. A ratio of 1:1 is thought to be representative of the initial amount of target template (Gilliland *et al*, 1990; Ohan and Heikkila, 1993). Alternatively, the amount of mRNA can be determined by extrapolation against the standard curve created from the internal standard (Wang *et al*, 1989).

4.1.5 Competitive RT-PCR

In the present study, an attempt was made to use a specifically prepared exogenous template as the internal standard in reverse transcription PCR, in a truly competitive fashion because the control and target are competing for the same primers and reaction reagents in the same tube. One method of quantifying mRNA by PCR is by using a synthetic RNA molecule as the competitor (Wang *et al*, 1989), which consists of a linear arrangement of target sense primer sequences, followed by the complementary sequences to the antisense primers in the same order. This type of competitor can allow the quantification of several target mRNA species without altering reaction conditions.

Ideally, the competitor fragment would have the same sequence as the target mRNA, with a small mutation (introduction of a restriction site, for example) to discern one from the other. Thus the efficiency of reverse transcription or amplification would not be compromised by sequence differences or size (Clontech, 1993). Alternatively, the DNA fragment generated by PCR with the same primers may be used as the competitor fragment (Gilliland *et al*, 1990). Because target-specific primers are designed to span an intron site (Wang *et al*, 1989), it is feasible to generate a synthetic transcipt (RNA) molecule from genomic DNA PCR to create a competitor which will amplify with comparable efficiency as the target mRNA sequence. However, using such an approach calls for reaction optimisation for each mRNA studied.

Although RT-PCR offers a very powerful tool to study and quantify mRNA and gene expression in a wide variety of biological situations, it can prove to be a technically demanding procedure. The efficiency of first strand synthesis is paramount to the success of the reaction, thus the quality and quantity of initial RNA is important. As the quality of RNA decreases, so does the efficiency of the reaction, likewise as the quantity

of the RNA increases, the efficiency of first strand synthesis decreases (Philips *et al*, 1993). Consideration of all reaction parameters must be given in order to achieve success. The type of primers, enzyme and internal standard used all contribute to the accuracy and efficiency of reverse transcription PCR. Developing an assay to glean information regarding mRNA quantification to study mechanisms such as chilling and oxidative damage, for a woody species such as *Rhododendron*, can only serve to increase our understanding of genetic stimuli and responses.

4.2 Materials and Methods

4.2.1 Design of Rhododendron-specific APX primers and PCR optimisation

Using the ascorbate peroxidase data obtained from the cloned *Rhododendron ponticum* fragments (Figure 3.20), APX primer set C was altered to be specific to the *Rhododendron* species, named APX primer set R and illustrated in Table 4.1. Once synthesised by Cruachem, the primers were used to amplify *R. ponticum* genomic DNA to produce a discrete product of 1.6 kb. Primers were tested initially using a 52°C annealing temperature.

APX Set R Upper Primer	**5′ ACGACGTGAAAACAAAAACA 3′**
APX Set R Lower Primer	**5′ ATTCGGCTTAGACATCTCTCA 3′**

Table 4.1 Rhododendron-specific ascorbate peroxidase primers

As mentioned previously in section 2.1.2, several PCR conditions must be reviewed to attain an individual PCR product, including annealing temperatures (52 and 55°C), primer concentration (5, 10, 15 and 20 pmoles), the availability of free magnesium (0.375, 0.75, 1,125 and 1.5 mM $MgCl_2$) in the reaction and the concentration of free nucleotides (0.625, 1.25, 1.875 and 2.5 mM dNTPs). Because of the significantly high homology of the two primer sets, primer mismatch tests were also performed, using upper primer C and lower primer R, and vice versa. Once a discrete product of 1.6 kb had been obtained and PCR optimised, reverse transcription tests could begin.

4.2.2 Extraction of RNA

4.2.2.1 Total RNA Isolation Reagent (Advanced Biotechnologies)

This protocol is based on a patent pending guanidine salt/phenol extraction solution available from Advanced Biotechnologies.

Fresh plant tissue (100 mg) was ground in liquid nitrogen with sterile quartz sand using a mortar and pestle. To this, 1ml of Total RNA Isolation Reagent (14 M guanidine salts, urea and phenol, concentrations not given; Advanced Biotechnologies, TRIR) was added and the mixture allowed to freeze in the mortar and pestle. Once the homogenised mixture had thawed, it was transferred to a 1.5 ml Eppendorf tube, 200 µl of chloroform was added and the mix was shaken vigorously for 15 seconds before a 5 minute incubation on ice. The homogenate was then centrifuged at 12,000 *g* for 15 minutes at 4°C.

The upper, aqueous phase was transferred to a fresh Eppendorf tube and an equal volume of isopropanol added. This mixture was incubated at -20°C for 30 minutes to allow full precipitation of the RNA. Samples were centrifuged at 12,000 *g* for 10 minutes at 4°C, and the supernatant removed. The white pellet was washed twice in 75% (v/v) ethanol, by vortex and centrifugation at 7,500 *g* for 5 minutes at 4°C. The pellet was dried briefly in a laminar flow cabinet before resuspension in 50 µl DEPC-treated water*. The samples were ready for analysis by TAE (Tris-Acetate electrophoresis buffer; 0.04 M Tris-Acetate, 0.01M EDTA, pH 8.0) gel electrophoresis and RT-PCR.

* All chemical reagents were treated with 0.1% (v/v) Diethylpyrocarbonate (DEPC) overnight then autoclaved. All glassware had been baked at 200°C before use to eliminate contamination with RNase enzymes.

4.2.2.2 RNA extraction using QIAGEN® column

This protocol is derived from the QIAGEN® Plasmid Purification Kit. Nucleic acids are extracted from fresh plant tissue and bound to the QIAGEN Anion-Exchange Resin in the column under high salt and pH conditions. DNA, proteins, dyes and low molecular weight impurities are removed by a column wash and the RNA eluted in a low salt buffer and precipitated by isopropanol, giving an RNA sample free from DNA contamination.

Fresh tissue (300 mg) was ground to a fine powder in liquid nitrogen and sterile quartz sand using a mortar and pestle. Twelve ml of a two-phase system containing an equal volume of RNA Lysis buffer (1% (v/v) Triton X-100; 500 mM MOPS pH 7.0, 50 mM EDTA, 2 M urea, 5% (v/v) β-mercaptoethanol, pH adjusted to 7.0,) and Ultrapure buffer-saturated phenol (pH 7.0, Gibco BRL) was added to the mortar and pestle and the mixture allowed to freeze. Once thawed at room temperature, the homogenous mixture was transferred to a sterile 50 ml centrifuge tube and vortexed for 30 seconds before shaking at room temperature for 25 minutes.

The mixture was centrifuged at 3500 *g* (Mistral 2000R, MSE) for 15 minutes at 4°C and the aqueous phase retained. Following a second extraction with buffer-saturated phenol (pH 7.0) the mixture was centrifuged at 3632 *g* (4°C) for 15 minutes, and the aqueous layer transferred to a fresh centrifuge tube. An equal volume ice-cold chloroform was added this, and the mixture vortexed for 30 seconds. Following centrifugation for 15 minutes at 3632 *g* (4°C), the aqueous nucleic acid layer was transferred to a fresh centrifuge tube and adjusted to contain 350 mM NaCl using 5 M NaCl and stored on ice.

The QIAGEN-tip 100 column was equilibrated with 5 ml Equilibration Buffer A (400 mM NaCl, 50 mM MOPS pH 7.0, 15% (v/v) ethanol, 0.15% (v/v) Triton X-100) before the nucleic acids were adsorbed onto the column. The column was washed with 5 ml Buffer A, before adding 5 ml Elution Buffer R (400 mM NaCl, 50 mM MOPS pH 7.0; 15% (v/v) ethanol, 0.15% (v/v) Triton X-100). The eluted RNA was captured in 0.8 volume isopropanol and incubated at -20°C for one hour (samples were stored at -70°C at this stage). The RNA was pelleted by centrifugation at maximum speed in a microcentrifuge for 30 minutes at 4°C. The pellet was washed in ice-cold 70% (v/v) ethanol and air-dried in a laminar flow cabinet for 5-10 minutes before resuspension in 50 µl DEPC-treated sterile water.

4.2.3 Determination of RNA concentration and visualisation by gel electrophoresis

A 10 µl aliquot of RNA sample was diluted in a total of 400 µl sterile DEPC-treated water. The diluted sample was placed in a quartz cuvette and the OD_{260} measured against a DEPC-water blank. A solution of RNA with an OD_{260} of 1.0 contains approximately 40 µg RNA per ml.

1% (w/v) agarose gels were used to analyse PCR products and DNA extractions. 1% (w/v) agarose (Seakem Le, Flowgen) was melted in 0.5× Tris Acetate EDTA (TAE) buffer (Sigma) and poured into the appropriate gel casting tray, Horizon 58 (minigel); Horizon 11.14 (midigel) or Horizon 2025 (maxigel) (Gibco BRL). Once set, the gel was immersed in TAE buffer and the wells loaded with 4 µl RNA sample which had been incubated for 15 minutes at 65°C with 10 µl RNA sample buffer (64% (v/v) formamide, 26 mM MOPS, 6.45 mM sodium acetate, 0,6 mM EDTA) and 2 µl RNA loading buffer (50% (v/v) glycerol, 1 mM EDTA, 0.4% (w/v) bromophenol blue, 1 mg/ml ethidium bromide). The gel was

electrophorised at a voltage suitable to the gel size and viewed on the UV transilluminator and photographed using the IS-500 Gel Documentation System.

4.2.4 Generation of cRNA competitor

The PCR competitor was produced by *in vitro* transcription using T7 RNA polymerase. This was procured in kit form as the RiboMAX™ Large Scale RNA production system (Promega).

4.2.4.1 Preparation of the DNA template

The plasmid samples used were obtained as described in Chapter 3, by the Wizard™ Plus Minipreparation DNA Purification System (Promega). The DNA template was linearised using the restriction enzyme *Bam*HI (Gibco BRL). Plasmid (2 µl) was mixed with 2 µl enzyme and 2 µl of reaction buffer 2 (150 mM NaCl, 10 mM Tris-HCL, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.9 @ 25°C; Gibco BRL) and added to 14 µl nuclease-free water to a total volume of 20 µl. This reaction mix was incubated for 4 hours at 37°C, then heated to 85°C for 2 minutes to deactivate the enzyme. Once the samples had been quick-chilled on ice for 5 minutes, the linearised template was precipitated from the reaction mix by sodium acetate, as outlined in Section 3.2.5c, and resuspended in 15 µl nuclease-free water, then, 6 µl of this was used for analysis by gel electrophoresis and the remaining 9 µl for *in vitro* transcription.

4.2.4.2 In vitro transcription

The following reaction was prepared at room temperature, the components added in the order shown:

5× T7 Reaction Buffer (Promega)[1]	4μl
rNTPs (25 mM ATP, CTP, GTP, UTP) (Promega)	6μl
Linear DNA template (50-100 μg)	8μl
T7 Enzyme Mix (Promega)[2]	2μl
Nuclease-Free Water to a final volume of	20μl

[1] T7 Reaction buffer contained 400 mM HEPES-KOH, pH 7.5, 120 mM $MgCl_2$, 10 mM spermidine and 200 mM DTT.

[2]T7 Enzyme Mix contained RNA Polymerase, rRNasin® Ribonuclease Inhibitor and Yeast Inorganic Pyrophosphatase.

The reaction was thoroughly mixed by gentle pipetting and incubated at 37°C for 4 hours.

4.2.4.3 Removal of DNA template after transcription

One unit per microgram of template RQ1 RNase-free DNase (Promega) was added to the reaction mix and incubated for 15 minutes at 37°C. The synthesised RNA was extracted with one volume of TE-saturated phenol: chloroform: isoamyl alcohol (25:24:1; pH 4.5) by vortexing for one minute before centrifugation at top speed in a microcentrifuge for 2 minutes. The upper, aqueous layer was transferred to a fresh tube and one volume of chloroform: isoamyl alcohol (24:1) added. The sample was vortexed and centrifuged as before, and the aqueous layer transferred to a fresh Eppendorf tube.

To precipitate the RNA, 0.1 volumes of 3 M sodium acetate (pH 5.2) and 1 volume of isopropanol were added to the sample and incubated at -20°C for 30 minutes, before centrifugation at top speed for 10 minutes. The supernatant was removed and the pellet washed with excess 70% (v/v) ethanol. The pellet was dried briefly in a laminar flow cabinet before resuspension in DEPC-treated (nuclease-free) water to a volume identical to that of the starting volume of the reaction. The samples were stored at -70°C.

4.2.5 Reverse Transcription

4.2.5.1 Reverse transcription using AMV reverse transcriptase (Promega)

A 20 µl reaction mix was prepared by adding the following components in the order listed:

Magnesium Chloride	4µl
AMV Reverse Transcription 5× Buffer*	2µl
dNTP mixture	2µl
Recombinant RNasin® Ribonuclease Inhibitor (Promega)	0.5µl
AMV Reverse Transcriptase (Promega)	0.65µl
Oligo $(dT)_{15}$ Primer (Control)/ Ascorbate Peroxidase Downstream Primer	0.5µg
1.2 kb Kanamycin Positive Control RNA / Substrate RNA	1µg
Nuclease-Free Water to a final volume of	20µl

*AMV Reverse Transcription buffer contained 250 mM Tris-HCl (pH 8.3 @ 25°C), 250 mM KCl, 50 mM $MgCl_2$, 2.5 mM spermidine and 50 mM DTT.

Following incubation at 42°C for 30 minutes, the reaction mix was heated to 99°C for 5 minutes and quick-chilled on ice to deactivate the enzyme and prevent further transcription. The mix was then used immediately in PCR, outlined in section 4.2.6.

4.2.5.2 Reverse transcription using M-MLV RNase H minus reverse transcriptase

All the reagents used in the reverse transcription reaction, with the exception of the primer, were obtained from Promega.

A 20 µl reaction was prepared by adding the following reagents in the order listed:

Magnesium Chloride	4µl
M-MLV Reverse Transcription 5× Buffer*	2µl
dNTP mixture	2µl
Recombinant RNasin® Ribonuclease Inhibitor	0.5µl
M-MLV Reverse Transcriptase (RNase H minus)	0.8µl
Oligo $(dT)_{15}$ Primer (Control)/ Ascorbate Peroxidase Downstream Primer	0.5µg
1.2 kb Kanamycin Positive Control RNA / Substrate RNA	1µg
Nuclease-Free Water to a final volume of	20µl

*M-MLV Reverse Transcriptase buffer contained 250 mM Tris-HCl (pH 8.3 @ 25°C), 375 mM KCl, 15 mM $MgCl_2$ and 50 mM DTT.

This reaction mix was incubated for 60 minutes at 37°C. Once completed, the sample was heated to 99°C for 5 minutes, then quick-chilled on ice for 5 minutes to deactivate the M-MLV Reverse Transcriptase and prevent it from binding to the cDNA.

4.2.6 Polymerase chain reaction

A 25 µl reaction mix was prepared immediately after construction of the single-stranded cDNA fragment by combining the following reagents:

Magnesium Chloride (Promega)	1µl
Reverse Transcription 5× Buffer (Promega)*	2µl
APX Upstream Primer	50 pmol
APX Downstream Primer	50 pmol
Taq DNA Polymerase (Perkin Elmer)	2.5 units
First Strand DNA Reaction	5µl
Nuclease-Free Water to a final volume of	25µl

*The type of reverse transcription buffer used corresponded to the enzyme used in the reverse transcription assay.

The thermal cycle programme used for PCR was set at 95°C for 5 minutes initially, followed by 1 minute at 94°C, 2 minutes at 55°C and 3 minutes at 72°C, for a total of 30 cycles, and a final extension period of 10 minutes at 72°C. The reaction was carried out in either a Perkin Elmer 480 thermal cycler, or a Techne PHC-3 thermal cycler / cyclogene thermal cycler. The products of the reaction were analysed by gel electrophoresis.

4.2.7 Reverse transcription-polymerase chain reaction optimisation

As with previous PCR applications using genomic DNA, the optimisation of PCR to successfully amplify complementary DNA sequences was necessary. Primer (5, 10, 15 and 20pmoles) magnesium chloride (0, 0.375, 0.75, 1,125 and 1.5 mM $MgCl_2$) and dNTP (0.625, 1.25, 1.875 and 2.5 mM dNTPs) concentrations were adjusted in both reverse transcription and PCR to obtain a product of 1.6 kb for the competitor fragment and

approximately 800 bp for the target cDNA, and only those two bands when the templates were combined in the reaction tubes. A suitable ratio of competitor:target cDNA was also established through a series of competitor dilutions and concentrations.

Because of limited PCR success in samples which had been transcribed with AMV reverse transcriptase, the PCR enhancing agent, Dimethyl Sulphoxide [DMSO, Frackman *et al.*, 1997) was added to the PCR mix (0, 1, 5 and 10% (v/v)] to increase the specificity of the reaction. Because no single additive is guaranteed to improve the result, the non-fat milk cocktail BLOTTO (Bovine Lacto Transfer Technique Optimiser) was also added to both the reverse transcription and polymerase chain reactions in an attempt to rule out reaction inhibition by plant compounds, such as polyphenolics (De Boer *et al.*, 1995). BLOTTO (10% (w/v) skimmed milk powder, 0.2% (w/v) NaN_3) was added at a concentration of 2% (v/v), to both the reverse transcription and PCR reactions, only the PCR reaction or not at all, and results analysed by gel electrophoresis.

4.3 Results and Discussion

4.3.1 Design of Rhododendron-specific APX primers and PCR optimisation

As with previous PCR optimisation experiments, annealing temperature was the initial variable to be altered. Figure 4.1 illustrates the initial PCR using the newly designed *Rhododendron*-specific primers using *R. ponticum, R. hatsugiri* and *R. impeditum* genomic DNA and an annealing temperature of 52°C. Multiple banding on the gel suggests little specificity of the primers, hence the annealing temperature was increased to 55°C. No result was given by this PCR analysis, as was also noted for PCR tests with magnesium chloride variations (0.375; 0.75; 1.125 and 1.5 mM), and dNTP concentrations (0.625, 1.25, 1.875 and 2.5 mM).

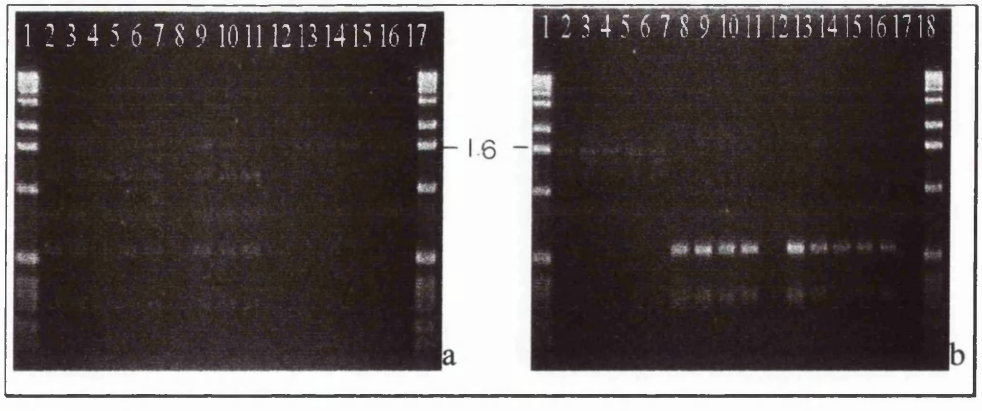

Figure 4.1. PCR amplification of genomic *Rhododendron* DNA using *Rhododendron*-specific APX primers. 4.1A: Lanes 1 & 17 are 1kb ladder (Gibco BRL), lanes 2-11: *R. ponticum* genomic DNA used, lanes 12-16: *R. hatsugiri* DNA used. 4.1B: Lanes 1 & 17 are 1kb ladder, lanes 2-6: *R. hatsugiri* DNA, lanes 7-16: *R. impeditum* DNA.

PCR amplification with the primer set mismatches (the reaction mix consisted of PCR buffer (10 mM Tris-HCl, 50 mM KCl; Perkin Elmer), 1.5 mM $MgCl_2$ (Perkin Elmer), 20 pmoles upper primer, 20 pmoles lower primer (synthesised by Cruachem), 2.5 mM dNTPs (Perkin Elmer), 1 unit

Taq DNA Polymerase (Perkin Elmer) and 10 ng DNA, in a total volume of 25 μl) gleaned a product of 1.6 kb, the expected fragment size, when the upper *Rhododendron*-specific primer and the lower primer from set C were used, as depicted in Figure 4.2a. Figure 4.2b illustrates the result of mismatches using the upper APX C primer and the lower *Rhododendron*-specific primer, which produced a band of less than 500 bp in size, possibly an artefact of PCR.

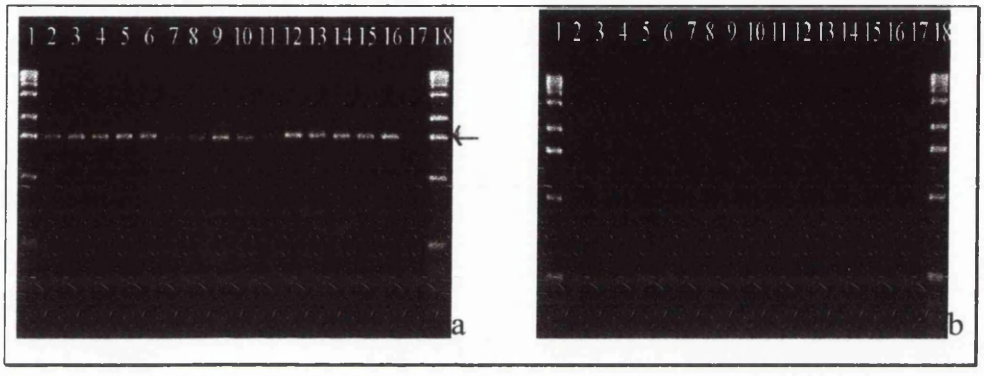

Figure 4.2. PCR amplification of Rhododendron genomic DNA using primer set mismatches. 4.2A: PCR using upper primer APX R, lower primer APX C. Lanes 1 & 18 are 1kb ladder (Gibco BRL), lanes 2-6: using *R. ponticum* DNA, lanes 7-11: using *R. hatsugiri* DNA, lanes 12-16: *R. impeditum* DNA. Lane 17 water control. 4.2B: PCR using mismatch upper primer APX C and lower primer APX R. Lanes as 2A.

Although Figure 4.2a shows a successful PCR amplification with a discrete product of 1.6kb for *R. ponticum* and *R. impeditum*, *R. hatsugiri*, the gels displayed evidence of a secondary product of larger size when amplifying *R. hatsugiri* DNA (see lanes 7-11) thus further PCR optimisation was necessary.

Magnesium chloride, dNTP and primer concentrations were all scrutinised as before, and the results are shown in Figures 4.3-4.5. Magnesium chloride concentration was noted to have a significant effect on PCR as shown in Figure 4.3. As the concentration of magnesium chloride decreases, so does the amount of PCR product. Illustrated in

Figure 4.3b, a drop in concentration to 1.125mM greatly reduces the amount of PCR product in comparison with 1.5mM $MgCl_2$ (Figure 4.3a). Reactions with 0.75 and 0.375mM $MgCl_2$ resulted in no detectable amplification (results not shown).

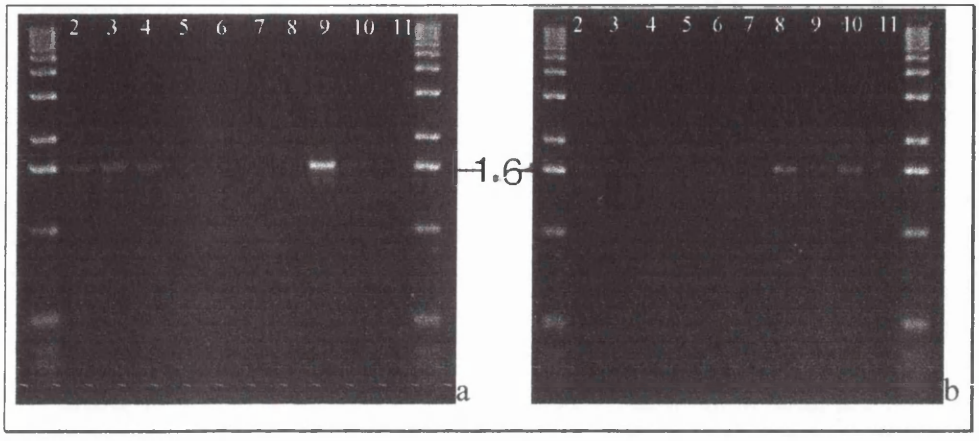

Figure 4.3. PCR with $MgCl_2$ variation. 4.3A: PCR using APX primer R (upper) and C (lower) and 1.5mM $MgCl_2$. Lanes 1 & 12 are 1kb ladder (Gibco BRL), lanes 2-4: *R. ponticum* DNA, lanes 5-7: *R. hatsugiri* DNA, lanes 8-10: *R. impeditum* DNA. Lane 11 water control. 4.3B: PCR using APX primer R (upper) and C (lower) and 1.125mM $MgCl_2$. Lanes as 4.3A.

Figure 4.4 illustrates the effect of altering the dNTP concentration in the PCR reaction mixture. Decreasing the amount of dNTPs available from 2.5mM (Figure 4.4a) to 0.625mM (Figure 4.4d) in the reaction simply results in less product amplification. This characteristic is also noted in experimentation of primer concentration alteration (Figure 4.5). Reducing the primer concentration from 20pmoles (Figure 4.5a) to 15pmoles (Figure 4.5b) and 10pmoles (Figure 4.5c) decreases the amount of amplified product, with 5pmoles primer (Figure 4.5d) giving no product whatsoever. However, in all the optimisation experiments, the larger secondary product was still amplified when PCR was successful using *R. hatsugiri* DNA.

Because discrete PCR products had been obtained for *R. ponticum* and *R. impeditum*, the primer mismatch APX R (upper) APX C (lower) were deemed of suitable specificity to use in reverse transcription PCR.

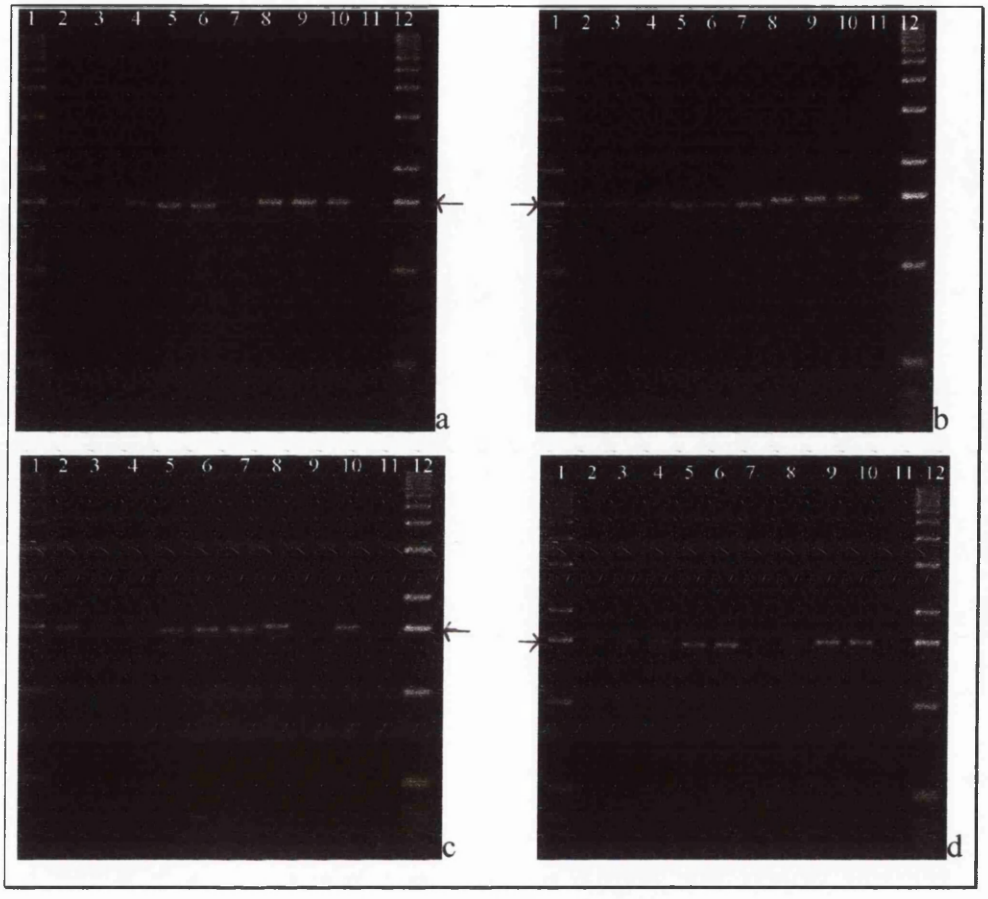

Figure 4.4. PCR with dNTP variation. 4.4A: PCR using APX primer R (upper) and C (lower) and 2.5mM dNTPs. Lanes 1 & 12 are 1kb ladder (Gibco BRL), lanes 2-4: *R. ponticum* DNA, lanes 5-7: *R. hatsugiri* DNA, lanes 8-10: *R. impeditum* DNA. Lane 11 water control. 4.4B: PCR using APX primer R (upper) and C (lower) and 1.875mM dNTPs. Lanes as 4.4A. 4.4C: PCR using 1.25mM dNTPs, lanes as above, 4.4D: PCR using 0.625mM dNTPs, lanes as above.

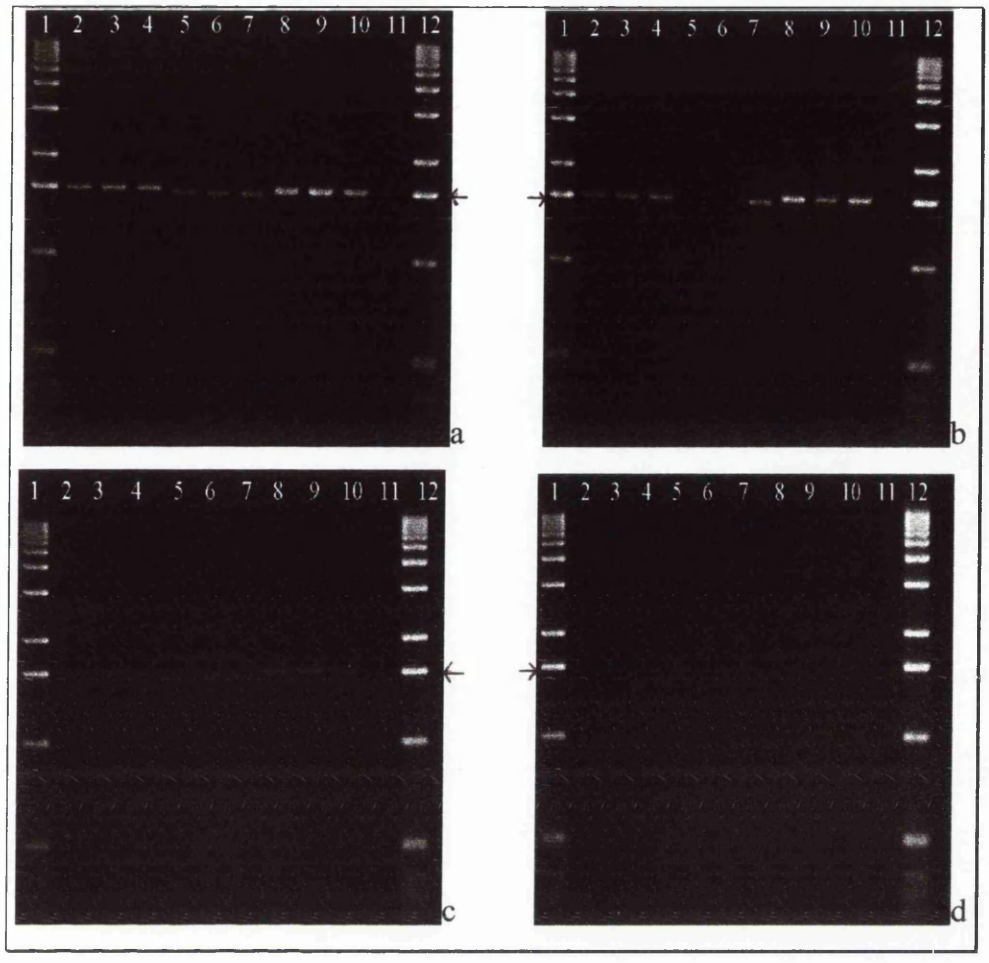

Figure 4.5. PCR with primer variation. 4.5A: PCR using APX primer R (upper) and C (lower) and 20pmoles each primer. Lanes 1 & 12 are 1kb ladder (Gibco BRL), lanes 2-4: *R. ponticum* DNA, lanes 5-7: *R. hatsugiri* DNA, lanes 8-10: *R. impeditum* DNA. Lane 11 water control. 4.5B: PCR using APX primer R (upper) and C (lower) and 15pmoles each primer. Lanes as 5A. 4.5C: PCR using 10 pmoles each primer, lanes as above, 4.5D: PCR using 5 pmoles each primer, lanes as above.

4.3.2 RNA Extraction - Total RNA Isolation Reagent (TRIR)

As illustrated by Figure 4.6, the RNA preparations obtained appeared as a smear with low molecular weight material when electrophorised on a 1% (w/v) TBE agarose gel, with no high molecular weight nucleic acids, suggesting the samples are free of DNA. Although the RNA extracted appeared degraded, the preparations were used in initial reverse transcription experiments. TAE gels (1% w/v agarose) were subsequently used to visualise RNA preparations because of the inconclusive nature of the TBE gel analysis.

Figure 4.6. 1% (w/v agarose) TBE gel electrophoresis of RNA preparations using TRIR. Lanes 1 & 2 are extracts prepared from *R. ponticum*, lanes 3 & 4 are preparations from *R. impeditum*.

4.3.3 RNA Extraction - QIAGEN® column

TAE (1% w/v agarose) gel electrophoresis analysis of initial total RNA extractions carried out at the RNA laboratory, SAC Edinburgh, (see Figure 4.7a), indicates high quality total RNA preparations. Both lanes 1 and 2 clearly indicate the presence of two ribosomal RNA bands suggesting RNA to be of reasonable quality with some degradation. Extractions by this method were subsequently used in all RT-PCR procedures. Some loss in quality was noted in the RNA preparations performed at the Molecular Ecology Laboratory at SAC Auchincruive

(Figure 4.7b). This is possibly due to the lack of facilities available and sharing of equipment, although aseptic techniques were used throughout.

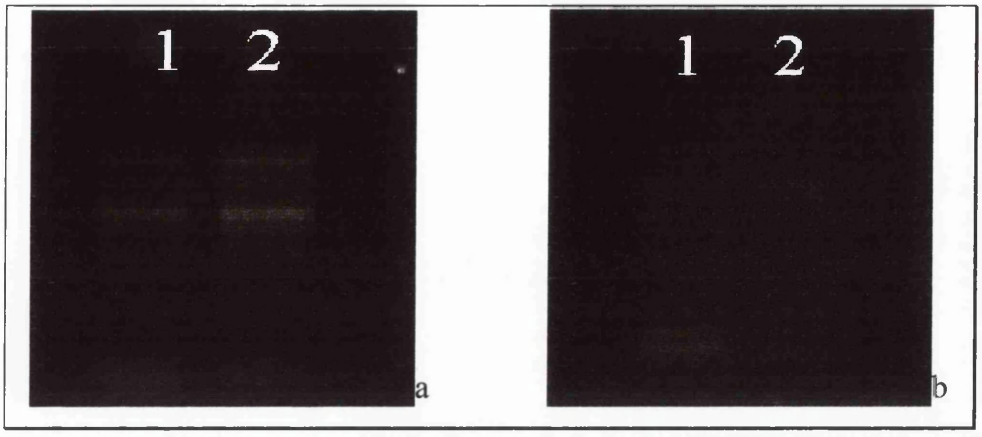

Figure 4.7. 1% (w/v agarose) TAE gel electrophoresis of RNA extracts prepared using QIAGEN column protocol. 4.7A: Lane 1: *R. impeditum* extraction, lane 2:*R. ponticum* extraction performed in the RNA laboratory, SAC Edinburgh. 4.7B: RNA extractions as 4.7A, but performed in the Molecular Ecology laboratory, SAC Auchincruive.

4.3.4 Generation of complementary RNA competitor by In vitro transcription

Before transcribing the desired PCR insert, the plasmid must first be linearised by digestion with a restriction enzyme, in this case *Bam*HI. As indicated on the pT-Adv plasmid vector map (see Figure 3.1, Chapter 3), *Bam*HI has only one recognition site in the plasmid construct, therefore will serve to cleave the plasmid once, creating a linear fragment. Figure 4.8 depicts gel electrophoresis analysis of *R. ponticum* APX insert plasmid samples following digestion with the restriction enzyme. The sample shown in lane 5 was successfully digested, producing a fragment of 5.5 kb in size (3.9 kb plasmid plus 1.6 kb insert), and was subsequently used for *in vitro* transcription of the fragment.

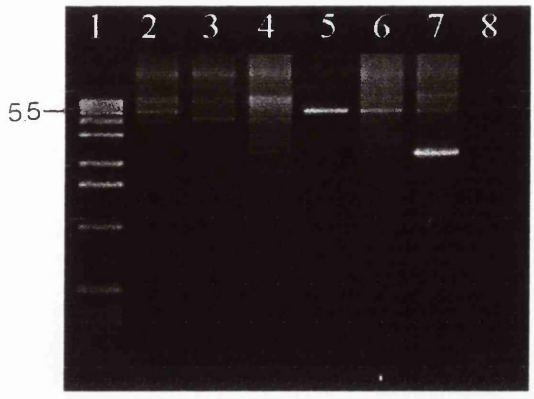

Figure 4.8. *Bam*HI digestions. Lane 1: 1kb ladder (Gibco BRL), lanes 2-8 are replicate samples of plasmid containing APX PCR fragment of 1.6kb. Lane 5 depicts successful cleavage of pT-Adv plasmid with the *R. ponticum* APX insert using BamHI with a discrete band of 5.5kb.

The success of *in vitro* transcription can be viewed as an RNA sample on a 1% TAE gel, as is illustrated in Figure 4.9. The *Rhododendron* PCR fragment gave rise to an RNA fragment similar in appearance to the kanamycin positive control RNA (Promega). Further confirmation of success of *in vitro* transcription was necessary by reverse transcription-PCR of the sample and control.

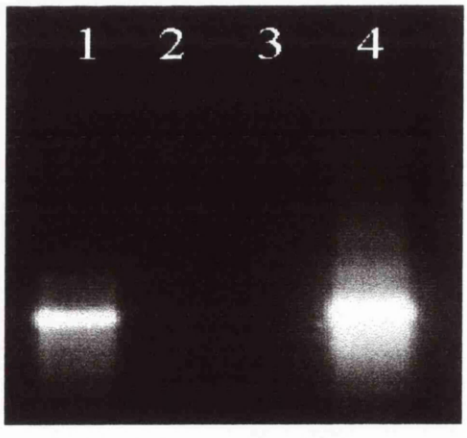

Figure 4.9. *In vitro* Transcribed RNA, *R. ponticum* APX fragement and kanamycin control. Lane 1: *in vitro* transcript created from APX PCR fragment ligated into pT-Adv plasmid vector, lanes 2 and 3 blank, lane 4: kanamycin positive control RNA (Promega).

4.3.5 Reverse transcription PCR using AMV reverse transcriptase

Initial attempts to reverse transcribe RNA samples extracted by the Total RNA Isolation Reagent (TRIR, Advanced Biotechnologies) protocol gave poor results. This was probably due to the poor quality of the RNA extracted from plant tissue (Figure 4.6). The kanamycin positive control RNA supplied with reverse transcription reagents (Promega), however performed well in RT-PCR.

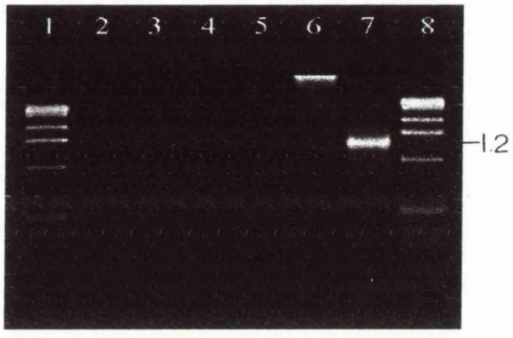

Figure 4.10. 1% TBE gel electrophoresis of RT-PCR using TRIR extracted RNA. Lanes 1 and 8 are 1kb ladder (Gibco BRL), lanes 2 & 3: *R. ponticum* total RNA used, lanes 4 & 5: *R. impeditum* total RNA used. Lane 6 represents first strand synthesis of 1.2kb kanamycin control, lane 7 RT-PCR of 1.2kb kanamycin control.

RNA extracted using the QIAGEN column protocol initially performed much better in RT-PCR using AMV reverse transcriptase. Figure 4.11 represents gel electrophoresis analysis of this reaction. Lane 5 illustrates the RT-PCR result using total RNA from *R. ponticum*, indicating successful, although unspecific, amplification.

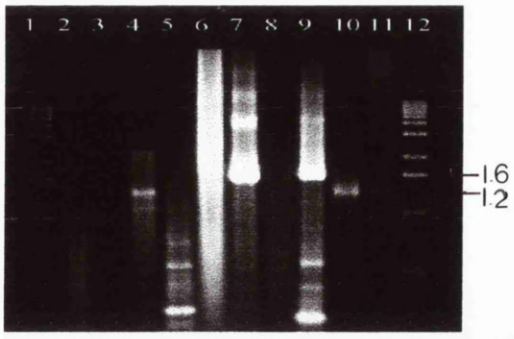

Figure 4.11. AMV RT-PCR. Lanes 1 and 12 are 1kb ladder (Gibco BRL), lanes 2-3: *R. impeditum* RNA (QIAGEN method) RT-PCR, lanes 4-5, RNA from *R. ponticum*, the first lane of each representing first strand synthesis. Lanes 7-9 are duplicates of *in vitro* transcription samples, the first of each representing first strand synthesis, second RT-PCR product, predominant band 1.6kb. Lanes 10 & 11: Kanamycin positive control RNA, first strand synthesis and RT-PCR product, 1.2kb.

The results from the RNA extracts were inconclusive at this stage, although the predominant bands present in duplicate samples of the *in vitro* transcript samples were 1.6kb in size, comparable to the original plasmid ascorbate peroxidase PCR insert size. The presence of multiple bands in these samples is probably due to overloading of the reaction with RNA, hence AMV RT-PCR optimisation was initiated.

Firstly, serial dilutions of the amounts of total RNA from *R. ponticum* and *R. impeditum* (Figure 4.12a) and *in vitro* transcript (Figure 4.12b) were tested, alongside a serial dilution of RNA / *in vitro* transcript mix; i.e. RNA target template and competitor fragment (Figure 4.12c). Apart from successful amplification of the competitor fragment of 1.6 kb, there was little to conclude from *R. ponticum* or *R. impeditum* RT-PCR. Addition of the PCR enhancing agent DMSO did little to improve the result of the reaction for either RNA samples (Figure 4.13a) or competitor fragment (Figure 4.13b) and indeed is decreased further the specificity of the reaction.

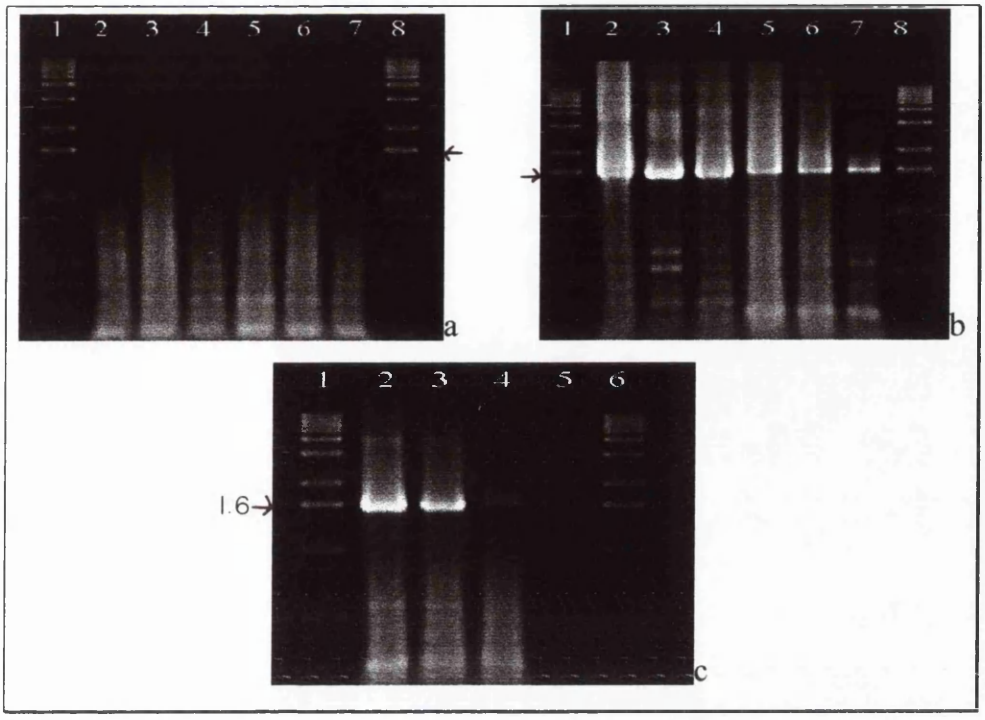

Figure 4.12. RT-PCR serial dilutions of RNA. 4.12A: lanes 1 and 8 are 1kb ladder (Gibco BRL), lanes2-4: 1:10, 1:100 and 1:1000 dilutions of *R. impeditum* RNA used in RT-PCR, lanes 5-7: 1:10, 1:100 and 1:1000 dilutions of *R. ponticum* RNA used in RT-PCR. 4.12B: lanes 1& 8 are 1kb ladder, lanes 2-7: 1:10, 1:100 and 1:1000 dilutions of *in vitro* transcription duplicates. 4.12C: lanes 1 and 6: 1kb ladder, lanes 2-5: 1:10, 1:100 and 1:1000 dilutions of *R. ponticum* RNA and *in vitro* transcript (competitor fragment) in same reaction tube.

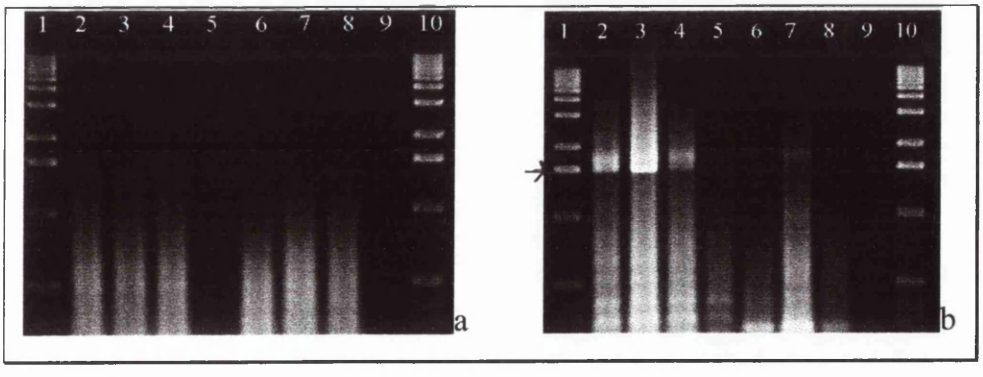

Figure 4.13. Addition of DMSO to Polymerase Chain Reaction. 4.13A: Lanes 1 and 10 are 1kb ladder (Gibco BRL), lanes 2-5: *R. impeditum* RNA with 0, 1, 5 and 10% DMSO added to PCR mix, lanes 6-9: *R. ponticum* RNA with 0, 1, 5 and 10% DMSO added to PCR mix. 4.13B: lanes 1 and 10 are 1kb ladder, lanes 2-9 are duplicates of competitor fragment with 0, 1, 5 and 10% DMSO added to PCR mix.

Adjusting the reaction temperature at which AMV reverse transcriptase optimally functions was tested in an attempt to improve target specificity. Temperatures of 42°C (the recommended enzyme optimum), 50°C and 58°C were used, illustrated by Figures 4.14a, b and c respectively. At 58°C with 20 pmoles primer (lane 6, Figure 4.14c) the RNA sample had been reduced to a single produced of 1.6 kb, most probably due to DNA contamination. The addition of too much competitor fragment caused similar results for all treatments. Little difference was noted in any of the reaction temperatures regarding primer concentration in reverse transcription (lanes 2-5, Figures 4.14a-c).

Reducing the amount of primers available in PCR as well as reverse transcription did little to improve results (Figure 4.15a-d), and no specific product was noted for RNA amplification in any of the tested reverse transcription temperatures, 42°C (Figure 4.15a); 50°C (Figure 4.15b) and 58°C (Figure 4.15c). The *in vitro* transcription competitor fragment RT-PCR however, did show an increase in specificity, with only one dominant product of 1.6 kb being observed (Figure 4.15c). Figure 4.15d illustrates that non-specific banding present in the RNA samples was not due to primer-dimer formation at 42°C as lanes 2-5 are blank.

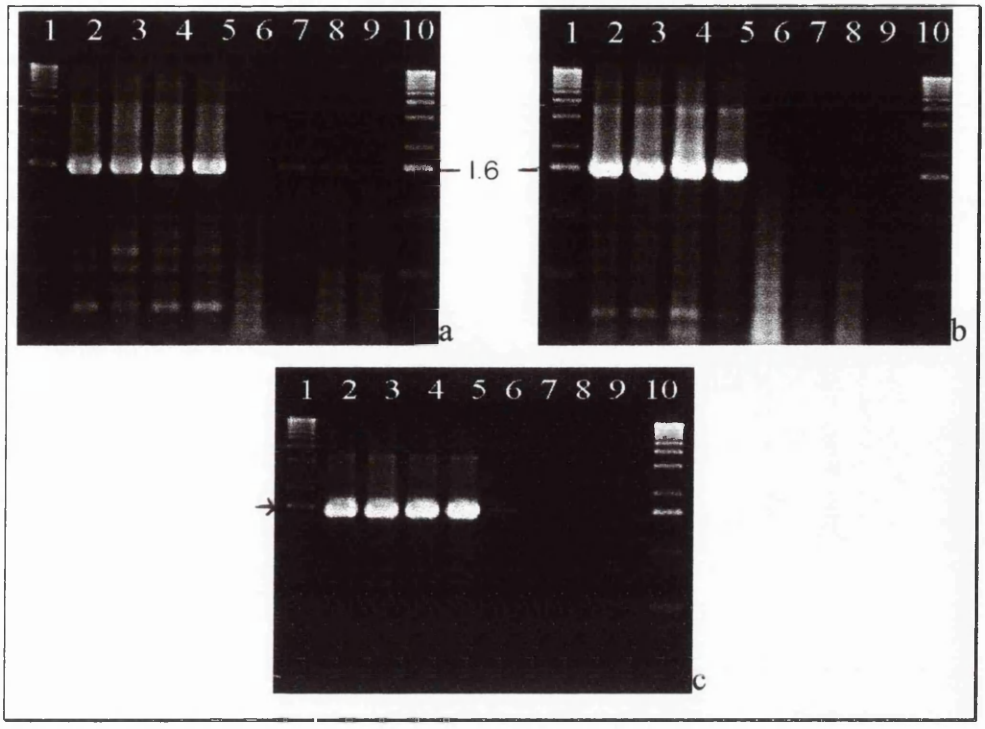

Figure 4.14. AMV Reverse Transcription reaction temperature variation, and primer concentration variation in RT only. 4.14A: RT reaction temperature 42°C. Lanes 1 and 10 are 1kb ladder (Gibco BRL), lanes 2-5 are competitor fragment 1:1000 dilution transcribed using 0.5μg, 0.375μg, 0.25μg and 0.125μg lower primer (respectively) added to reverse transcription. Lanes 6-9: *R. ponticum* amplified with same primer concentrations as competitor. 4.14B: RT reaction temperature 50°C. Lanes and samples as 4.14A. 4.14C: reaction temperature 58°C. Lanes and samples as 4.14A.

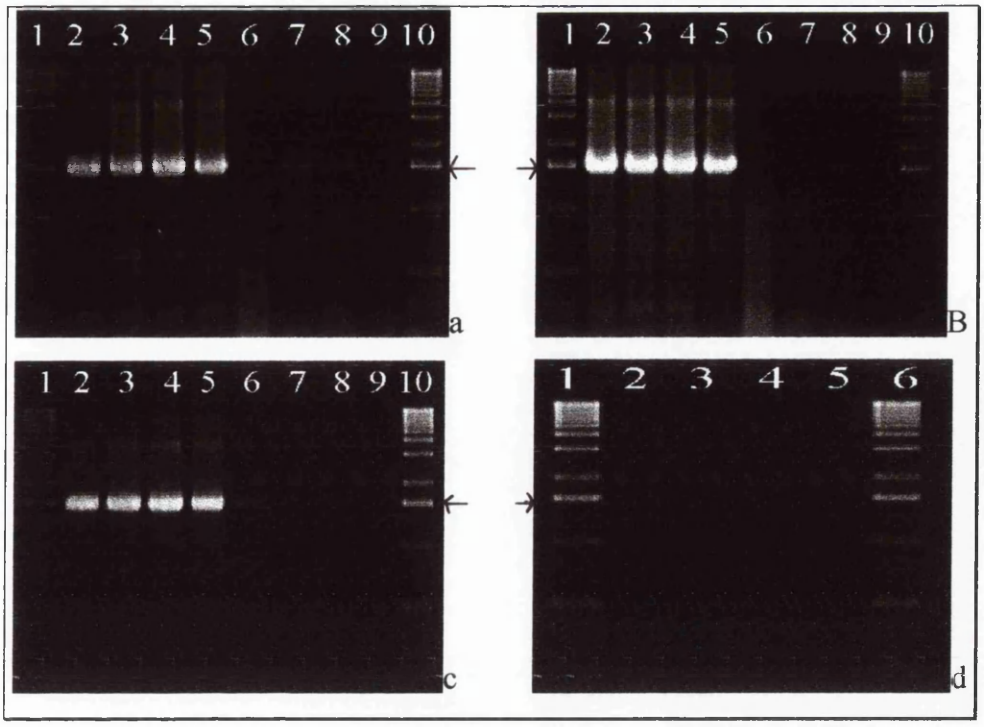

Figure 4.15. AMV Reverse Transcription reaction temperature variation. 4.15A: RT reaction temperature 42°C. Lanes 1 and 10 are 1kb ladder (Gibco BRL), lanes 2-5 are competitor fragment 1:1000 dilution transcribed using 0.5μg, 0.375μg, 0.25μg and 0.125μg lower primer (respectively) added to reverse transcription and 20, 15, 10 and 5 pmoles primer in PCR. Lanes 6-9: *R. ponticum* amplified with same primer concentrations as competitor. 4.15B: RT reaction temperature 50°C. Lanes and samples as 4.15A. 15C: reaction temperature 58°C. Lanes and samples as 4.15A. 4.15D: water controls at 42°C, reaction mixes as RNA and competitor fragments.

The universally poor result of RT-PCR tests observed in Figures 4.14 and 4.15 may have been due to plant compounds co-extracted with RNA from *Rhododendron*, inhibiting the success of both reverse transcription and PCR. Addition of 2% (w/v) BLOTTO to the reaction, a compound known to attenuate the inhibition of PCR due to such compounds (De Boer, *et al.*, 1995) did not however improve reaction results. As shown in Figure 4.16, RNA samples which had BLOTTO present in the RT-PCR reaction resulted in no amplification (Figure 4.16a), likewise with BLOTTO added to PCR only (Figure 4.16b) and one *in vitro* transcript duplicate generated a product of approximately 500 bp (Figure 4.16c, lanes 6-9), and was subsequently discarded from further use in experimentation

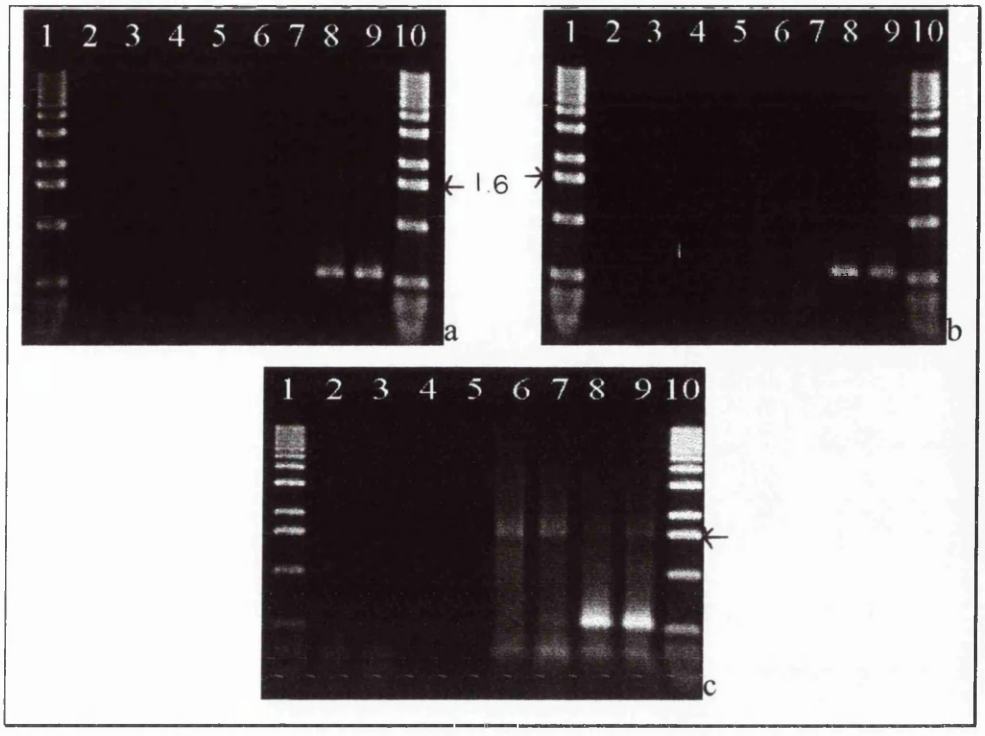

Figure 4.16. Addition of 2% BLOTTO. 4.16A: Addition of 2% BLOTTO to reverse transcription and PCR mixes. Lanes 1 & 10 are 1 kb ladder (Gibco BRL). Lanes 2-3: duplicates of *R. ponticum* RNA, lanes 4-5: duplicates of *R. impeditum* RNA, lanes 6-9 replicates of *in vitro* transcript competitor fragment. 4.16B: Addition of 2% BLOTTO to PCR mix only. Lanes as 4.16A. 16C: no addition of 2% BLOTTO, RT-PCR mixes as original. Lanes as 4.16A.

As Figure 4.16c illustrates, the original RT-PCR reactions were also functioning poorly. Reverse transcription may not be optimised due to the enzyme used in the reaction, thus tests were initiated using M-MLV reverse transcriptase as the reaction catalyst.

4.3.6 Reverse transcription PCR using M-MLV RNase H minus reverse transcriptase

Initial results using M-MLV reverse transcriptase showed a predominant product of approximately 800 bp for both *R. ponticum* and *R. impeditum* cDNA amplification (Figure 4.17b) in comparison to AMV reverse transcriptase reactions (Figure 4.17a). The competitor fragment of 1.6 kb also showed an increase in amplification as compared to reverse transcription using AMV reverse transcriptase. All subsequent RT-PCR protocols were performed using M-MLV reverse transcriptase. However, a degree of optimisation was necessary to improve results using this enzyme.

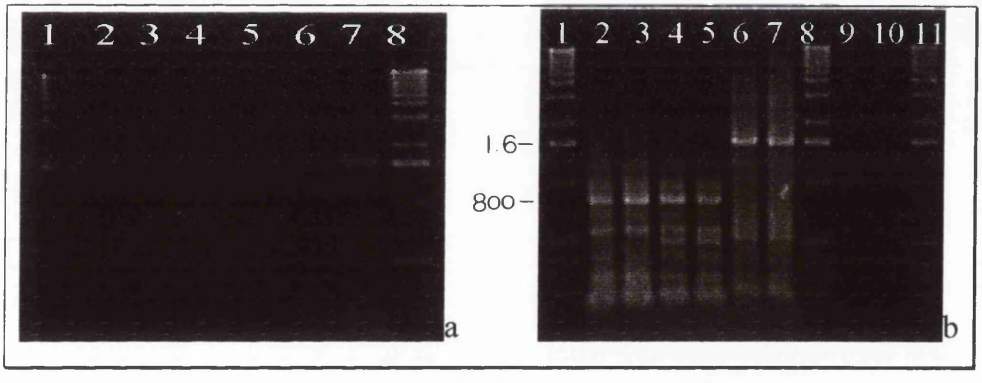

Figure 4.17. Comparison of RT-PCR using AMV and M-MLV Reverse Transcriptases. 4.17A: RT-PCR using AMV RT. Lanes 1 and 8 are 1kb ladder (Gibco BRL), lanes 2-3: *R. ponticum* RNA, lanes 4-5: *R. impeditum* RNA, lanes 6-7, competitor fragment, 1.6kb. 4.17B: RT-PCR using M-MLV RT. Lanes as 4.17A, lanes 9 & 10 are water controls using AMV (lane9) and M-MLV (lane 10) respectively. Lane 11, 1kb ladder.

As with AMV reverse transcriptase, optimisation tests by adjustment of annealing temperature (55-58°C, Figure 4.18a-d), primer concentration (5, 10, 15 and 20 pmoles, Figure 4.18a-d) and $MgCl_2$ concentration (1.5, 1.125, 0.75 and 0.375 mM, Figure 4.19) in the PCR mix. The dNTP concentration (2.5, 1.875, 1.25 and 0.625 mM, Figure 4.20 A & B) was adjusted in the reverse transcription mix.

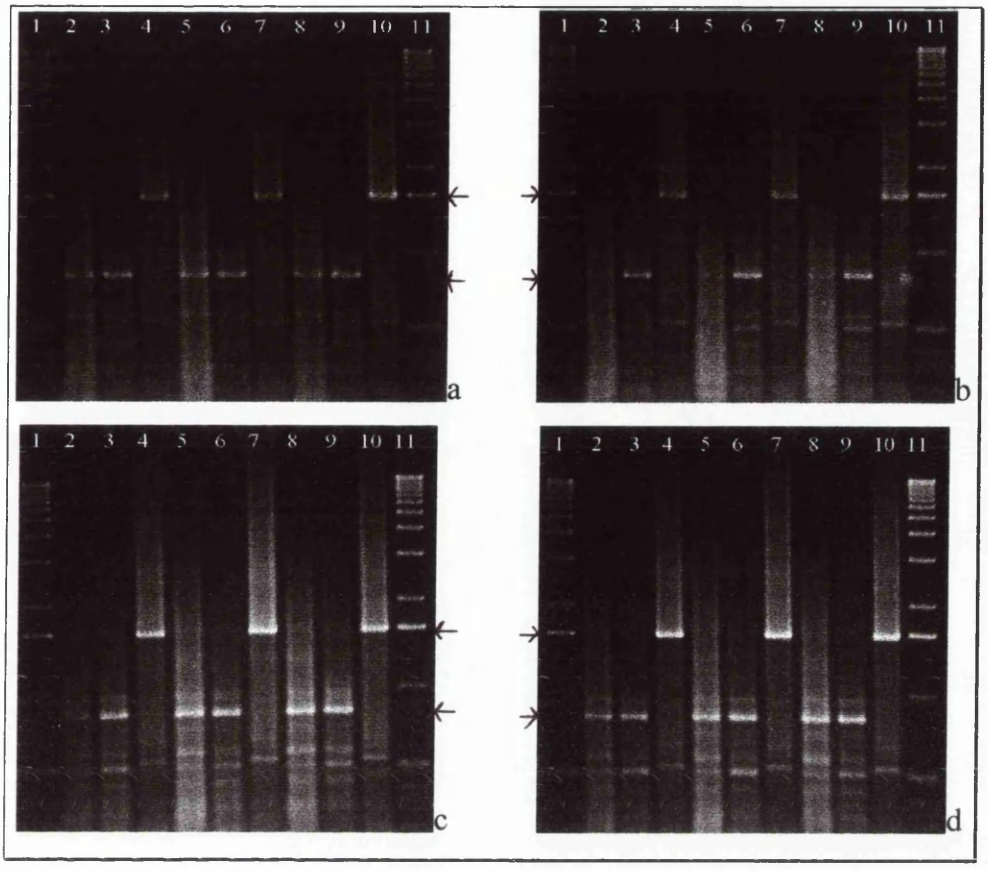

Figure 4.18. Primer concentration and annealing temperature variation. 4.18A: 55°C annealing temperature. Lanes 1 & 11 are 1kb ladder (Gibco BRL). Lanes 2-4: *R. ponticum, R. impeditum* and competitor fragment respectively, 20pmoles primers used in PCR. Lanes 5-7: as lanes 2-4, but 15pmoles primer. Lanes 8-10, 10pmoles primer. 4.18B: 56°C annealing temperature, lanes as 4.18A. 4.18C: 57°C annealing temperature, lanes as 4.18A. 4.18D 58°C annealing temperature, lanes as 4.18A.

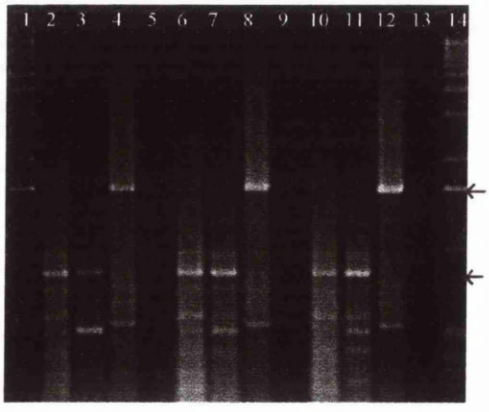

Figure 4.19. $MgCl_2$ variation in PCR mix. Lanes 1 & 14 are 1kb ladder (Gibco BRL). Lanes 2-5: *R. ponticum, R. impeditum*, competitor fragment and water control respectively, 1.5mM $MgCl_2$ used in PCR. Lanes 6-9: 0.75mM $MgCl_2$ used in PCR, lanes as 2-5. Lanes 10-13: no $MgCl_2$ used in PCR mix, lanes as 2-5.

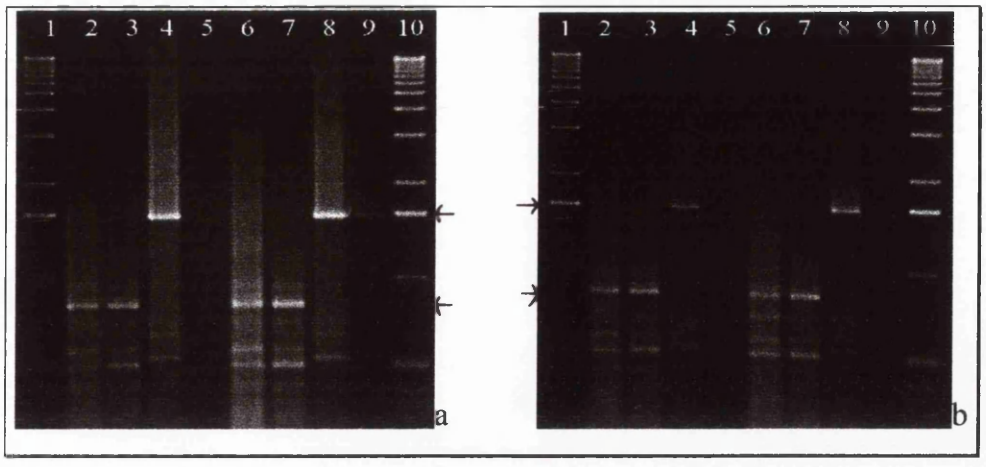

Figure 4.20. dNTP variation in RT mix. 4.20A: Lanes 1 & 10 are 1kb ladder (Gibco BRL), lanes 2-5: *R. ponticum, R. impeditum*, competitor fragment and water control respectively, 2.5mM dNTPs used in RT. Lanes 6-9: 1.875mM dNTPs used in RT, lanes as 2-5. 4.20B: Lanes 1 & 10 are 1kb ladder, lanes 2-5 as 4.20A, 1.5mM dNTPs used in RT, lanes 6-9: 0.625mM dNTPs used in RT.

Figures 4.18-4.20 show that there is little difference in the final result of the PCR optimisation tests. Although the presence of a predominant product of approximately 800 bp may suggest successful amplification of *Rhododendron* RNA, there is evidence of smaller, non-specific PCR products. There are several reasons for the lack of specificity: the quality of the RNA extract prepared under laboratory conditions, poor primer design, or poor first strand cDNA synthesis (Philips *et al.*, 1993).

Variations of the amounts of cDNA added to the PCR reaction as well as a reduction in the number of amplification cycles is shown in Figure 4.21. Here, 1µl cDNA was added, as opposed to 5 µl originally, and 20 thermal cycles used instead of 30, achieved a single RNA product of 800 bp. This however may be considered a false positive result, because other non-specific products will still be amplified, but to such a small degree as not to be readily visible upon gel electrophoresis analysis.

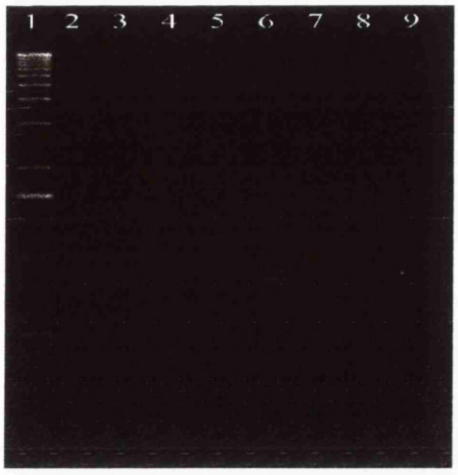

Figure 4.21. PCR amplification of 1µl *Rhododendron* cDNA, using only 20 thermal cycles. Lane 1: 1kb ladder (Gibco BRL) Lanes 2-5: *R. ponticum, R. impeditum*, competitor fragment and water control respectively, 1µl cDNA used in each case. Lanes 6-9: replicates of lanes 2-5.

4.3.7 Development of competitive RT-PCR assay to study ascorbate peroxidase gene expression

Despite multiple products in *Rhododendron* cDNA amplification, tests were carried out using the competitor (1.6 kb) fragment and the target RNA in the same tube to develop the competitive RT-PCR assay.

Figure 4.22 illustrates initial tests using target RNA:competitor fragment ratios. Whilst Figure 4.22a shows the amplification results of *Rhododendron* RNA alone, Figure 4.22b depicts the amplification of competitive RT-PCR. Ratios of 2.5:1 (*R. ponticum,* lane 2, Figure 4.22b) and 1:1 (*R. impeditum* lane 7, Figure 4.22b) proved successful, amplifying products of 1.6 kb and 800 bp, the expected size of the cDNA molecule for ascorbate peroxidase in *Rhododendron* species.

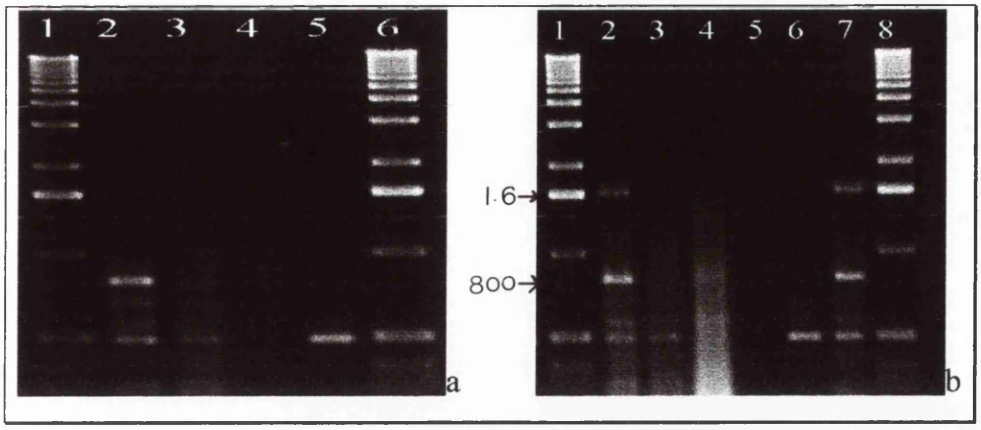

Figure 4.22. Competitive RT-PCR tests. 4.22A: lanes 1 and 6 are 1kb ladder (Gibco BRL). Lanes 2-3: 12.5 and 10µl *R. ponticum* RNA used in RT respectively, lanes 4-5: 12.5 and 10µl *R. impeditum* RNA used in RT. 4.22B: Target RNA:Competitor fragment ratios. Lanes 1 & 8 are 1 kb ladder, lanes 2-4: 12.5, 10 and 5µl *R. ponticum* RNA used with 5µl competitor. Lanes 5-7: 12.5, 10 and 5µl *R. impeditum* RNA used with 5µl competitor.

Replication of the reaction illustrated in Figure 4.23 (lanes 9-10) indicated that these ratios were optimal for competitive RT-PCR. The smaller bands which appeared throughout all the results are most likely due to the partially degraded quality of the RNA preparations used in the reactions (Figure 4.7b), which despite repeated attempts, could not be improved upon.

The final reaction conditions derived from these optimisation tests were as follows: reverse transcription was carried out at 37°C for one hour, using 0.5µg lower (reverse) primer; and PCR using 1.5mM $MgCl_2$, 2.5mM dNTPs, 20 pmoles forward and reverse primers, annealing temperature 55°C for 2 minutes for 30 thermal cycles.

Figure 4.23. Replication of Competitive RT-PCR ratios. Lanes 1 & 11 are 1kb ladder (Gibco BRL), lanes 2-4: 12.5, 10 and 5µl *R. ponticum* RNA only, lanes 5-7: 12.5, 10 and 5µl *R. ponticum* RNA and 1µl competitor fragment added, lanes 8-11 12.5, 10 and 5µl *R. ponticum* and 5µl competitor added.

With these ratios firmly determined and repeated with these cDNA samples, the RT-PCR assay could be used to study the changes in amounts of mRNA found in *Rhododendron ponticum* micropropagules when exposed to 4°C, a temperature known to induce chilling damage and oxidative stress (O'Kane *et al.*, 1996). The assay at this stage of development however, would provide semi-quantitative data because of the presence of smaller non-specific products, which would also be competing in the amplification reaction. *R. ponticum* micropropagules exposed to 20°C or 4°C were studied over a period of 14 days, sampling at days 0, 2, 4, 6, 8, 10 and 14 and total RNA extracted for competitive RT-PCR.

Experimental naïvety led to the failure of this first chilling study. Because the establishment of the assay had been implemented by using ratios of amounts of RNA preparation and competitor fragment, nothing was known of the absolute concentrations of total RNA present in either samples or competitor. For every fresh RNA extract that was used in the assay, as was so for each sampling day, the concentration of RNA would change in the reaction mix, thus rendering the ratios useless and any comparable studies redundant. Figure 4.24 illustrates this well. Figure 4.24a shows limited success of the assay for day 0 samples, when no

micropropagules had been exposed to 4°C. However, by day 14 (Figure 4.24b), when there may be more RNA present in the extracts because of chilling stress increasing total RNA in tissues, or an increased proficiency in the extraction technique, the assay failed to amplify the competitor fragment.

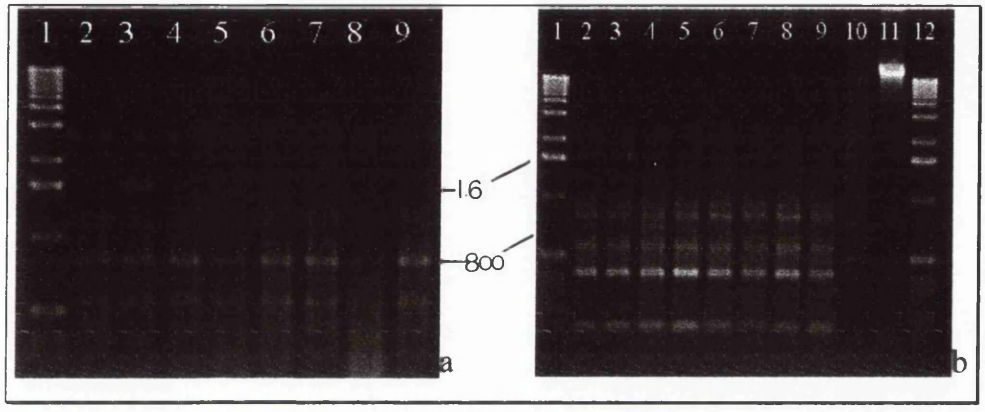

Figure 4.24. Competitive RT-PCR chilling experiment. 4.24A: Day 0 R. ponticum samples. Lane 1: 1kb ladder (Gibco BRL). Lanes 2-5: 20°C samples, lanes 6-9: 4°C samples. 4.24B: Day 14 R. ponticum samples. Lane 1: 1kb ladder. Lanes 2-5: 20°C samples, lanes 6-9: 4°C samples.

Figure 4.25 illustrates the difference between competitive RT-PCR using volume ratios (Figure 4.25b) and concentration ratios (Figure 4.25b). Determination of the concentrations of the RNA extracted, and of the competitor fragment achieved more comparable results through competitive RT-PCR. Tests using various amounts of RNA resulted in 2 ng total RNA and 6 ng competitor fragment being used in subsequent assays. The assay results are not of sufficient quality to glean absolute quantitative data due to a high degree of background amplification, but could possibly provide comparable data of a semi-quantitative nature, allowing comparisons between treatment days, indicating possible changes in ascorbate peroxidase gene expression.

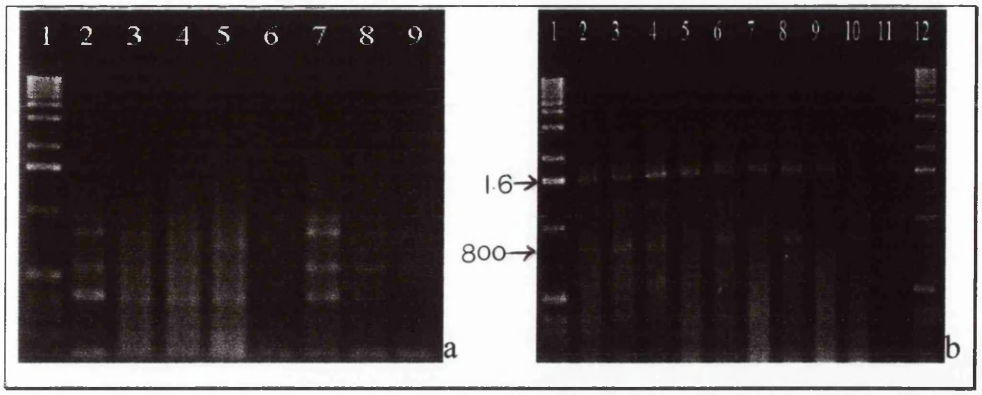

Figure 4.25. Competitive RT-PCR using volume ratios and concentration ratios. 4.25A: Day 8 samples. Lane 1: 1kb ladder (Gibco BRL), lanes 2-5: *R. ponticum* samples exposed to 20°C, lanes 6-9: samples exposed to 4°C. Volume ratio used 2.5µl:1µl RNA: competitor. 4.25B: Day 8 samples. Lane 1: 1kb ladder, lanes 2-9 as 25A, concentration ratio used 1ng:3ng RNA: competitor.

4.4 Conclusions

- PCR primer design based on homologous sequence data allowed specific amplification of APX sequences from *R. ponticum, R, hatsugiri* and *R. impeditum.*

- Good quality total RNA extracts were obtained from *R. ponticum* micropropagules using a protocol incorporating QIAGEN® columns.

- A competitor cRNA fragment was created by *in vitro* transcription of the cloned *R. ponticum* APX product.

- RNA samples (both plant and synthetic cRNA) were repeatedly and successfully amplified by reverse transcription PCR using M-MLV reverse transcriptase and *Taq* DNA polymerase.

- A ratio of 1:3 total plant RNA : cRNA competitor was found to repeatedly amplify two fragments suitable for comparable mRNA studies by competitive RT-PCR.

Chapter 5 *Rhododendron ponticum* Response to Chilling Stress at a Molecular and Physiological Level.

5.1 Introduction

Many environmental conditions, e.g. flooding, drought, ozone and temperature extremes are well known to induce oxidative stress in plants (Foyer and Mullineaux, 1994). Abscisic acid and salt stress in rice, for example, induce an up-regulation of the novel genes RAB21 and SalT (Mundy and Chua, 1988), thought to be involved in intracellular transport (Drew *et al.*, 1993). Attack from pathogens will also induce a stress response, perhaps attributed to the hypersensitive response (see Mehdy *et al.*, 1996).

One major area of research that provokes interest, however, is the relationship between the stress of temperature extremes and oxidative stress mechanisms in plant and animal systems. Because of this, there is now a greater appreciation for the two important groups of stress proteins: the heat shock proteins and the antioxidant proteins. This chapter deals specifically with antioxidant enzymes and their role in chilling-stressed plant tissue.

5.1.1 Oxidative stress and temperature extremes in plants

Extremes of temperature are widely known to lead to an increase in cellular lipid peroxidation, a phenomenon particularly marked following exposure to low temperatures (Levitt, 1980; Biggs, 1996). This raises the question of what actually causes the increase in peroxidation, as it is often the indicator of cellular oxidative stress (Rice-Evans *et al.*, 1991).

Investigations on chilled soybeans revealed increased levels of superoxide generation ($O_2^{\bullet}$), hence also hydrogen peroxide (H_2O_2) by dismutation (Bell and Burdon, unpublished observations, see Burdon, 1993). It is possible that during these reactions, the superoxide radical can combine

with protons in the membrane lipids to yield hydroperoxy radicals ($HO_2^{\bullet}$) which are capable of initiating the lipid peroxidation reaction. Peroxidised lipids do not only interfere with membrane fluidity and function. The lipid radicals formed during the peroxidation process may also damage neighbouring membrane proteins and affect their activity.

5.1.2 Superoxide production in plants

The increases in cellular lipid peroxidation that occur upon chilling stress may be an outcome of increased superoxide production. It is thought that the sources of superoxide generation in plants include the mitochondria and chloroplasts, the main site within the latter being the reducing site of Photosystem I (PS I) (Asada *et al.*, 1974). The electron which is generated in Photosystem II by the oxidation of water is transported to PS I, causing the reduction of NADP. Superoxide molecules are generated by the self-oxidation of the thylakoid-bound primary electron acceptor of PS I. When the thylakoids are illuminated by a flash of light for a single complete reaction, one molecule of superoxide is generated. The superoxide anions released from the thylakoid membranes are disproportionated by CuZn-superoxide dismutase yielding hydrogen peroxide. Superoxide dismutases are found in either the chloroplast (localised in the stroma), mitochondria or the cytosol (Asada *et al.* 1974).

Asada (1992) has since established the role of ascorbate peroxidase in scavenging the hydrogen peroxide in the chloroplasts, and in the regeneration of ascorbate from the primary oxidation product of the peroxidase reaction (monodehydroascorbate) and from its disproportionation product (dehydroascorbate). Monodehydroascorbate is reduced into ascorbate by electron donation from NADPH, whilst dehydroascorbate is recycled into ascorbate by utilising reduced glutathione as a reductant. Both reductants are supplied through PSI. The

oxidised glutathione subsequently produced is recycled back to its reduced form by the action of the second key antioxidant enzyme, glutathione reductase.

If the levels of the enzymes produced by these mechanisms do not become elevated during oxidative stress, whether induced by drought, waterlogging, ozone, paraquat or chilling, then the plant may suffer detrimentally from the outcome of excessive toxic chemicals in its tissues and organs. Superoxide generation can lead to the peroxidation of lipids and impaired membrane function (Burdon *et al.* 1994), ultimately causing death, if it cannot be scavenged by antioxidant systems.

The aim of the study was to determine possible differences in the activities of ascorbate peroxidase and glutathione reductase between plants exposed to chilling temperatures and plants grown at 20°C. Observations of the levels of mRNA in plants exposed to such experimental regimes, in conjunction with physiological data, i.e. the specific activities of the enzymes, may give an indication of antioxidant defence mechanisms induced in *Rhododendron ponticum* on exposure to chilling stress. Because of the diverse habitat range of *R. ponticum*, an insight into environmental stress mechanisms of such a tolerant plant would be of particular interest in aiding the understanding of defence mechanisms and could aid plant improvement by genetic modification. Using tissue cultured material would provide a clean source of plant material in an easily controllable environment.

5.2 Materials and Methods

5.2.1 Ascorbate peroxidase assay - 1

The protocol used was adapted from the methods of Berkowitz and Gibbs (1992) and Nakano and Asada (1980). It is based on the oxidation of ascorbate by ascorbate peroxidase.

Shoot samples (0.3 g) were ground in excess liquid nitrogen using a mortar and pestle and the resulting powder transferred to a 50 ml Coring centrifuge tube. Extraction buffer (2 ml; 50 mM MES (pH 6.2), 0.33 M sorbitol, 10 mM NaCl, 1 mM $MgCl_2$, 2 mM EDTA, 0.5 mM KH_2PO_4 and 1 mM ascorbate) was added to the powder and the mixture vortexed for 10 seconds. The homogenate was filtered through two layers of muslin and the filtrate retained. This was centrifuged at 750 *g* for 5 minutes at 4°C and the supernatant retained for protein determination (Section 5.2.4).

The pellet was resuspended in 2 ml HEPES-sorbitol buffer (50 mM HEPES (pH 7.6), 0.33 M sorbitol, 10 mM NaCl, 1 mM $MgCl_2$, 2 mM EDTA and 0.5 mM KH_2PO_4) and gently layered onto 2 ml 40% (v/v) percoll This was centrifuged at 2,500 *g* for 10 minutes at 4°C. The pellet obtained was resuspended in 0.5 ml HEPES-sorbitol buffer and centrifuged at 2,500 *g* for 15 minutes to remove all traces of percoll. The resulting pellet was resuspended in 100 µl HEPES-sorbitol buffer and the mixture sonicated for one minute in 6×10 second bursts to fully lyse the chloroplasts, immediately prior to performing the enzyme activity assay.

The assay reaction solution used contained 50 mM potassium phosphate (pH 7.0), 0.5 mM ascorbate and 0.1 mM EDTA, 979 µl of which was placed in a 1 ml quartz cuvette. To this, 20 µl enzyme extract and 1 µl 10mM hydrogen peroxide were added to initiate the reaction. The change in absorbance at 290 nm at 22°C was monitored over three minutes.

Ascorbate peroxidase activity can be expressed in μmoles ascorbate oxidised per hour per mg protein:

$$\text{Specific Activity (units/mg protein)} = \frac{(\Delta A/\text{min} \times \text{ml reaction} \times 10^6 \mu\text{mol/mol} \times 60 \text{ min/hour})}{(E \times \text{mg protein} \times 1000 \text{ml/litre})}$$

where E = molar extinction coefficient of ascorbate (2.8×10^3 $M^{-1}cm^{-1}$).

5.2.2 Ascorbate peroxidase assay - 2

The following protocol was derived from the method of Sen Gupta (1993) and also measures the rate of oxidation of ascorbate.

Plant tissue (0.3-0.5 g) was ground in liquid nitrogen with a mortar and pestle. Homogenisation buffer (10 ml; 50 mM HEPES (pH 7.0), 1 mM ascorbate, 1 mM EDTA, 1% v/v Triton X-100 and 1% w/v polyvinylpyrrolidone) was added and the mixture allowed to freeze. Once thawed at room temperature, the homogenate was transferred to a clean Coring centrifuge tube and vortexed for 10 seconds. Following centrifugation at 3600 *g* for 15 minutes at 4°C, the supernatant (enzyme extract) was retained for use in protein determination (Section 6.2.4) and enzyme activity.

The reaction solution contained 50 mM HEPES (pH 7.0), 1 mM EDTA, 1 mM ascorbate (974 μl) and 25 μl enzyme extract which was placed into a quartz cuvette. The reaction was initiated by the addition of 1 μl 10 mM hydrogen peroxide and the change in absorbance at 290 nm monitored over 3 minutes at 22°C.

Results were expressed as previously stated in section 5.2.1.

5.2.3 Glutathione reductase assay

This protocol, based on the method stated by Sen Gupta, (1993), measures the rate of oxidation of NADPH by glutathione reductase.

Shoot samples (0.1 g) were ground in excess liquid nitrogen with a mortar and pestle. Extraction buffer (1 ml; 50 mM potassium phosphate (pH 7.0), 0.1 mM EDTA, 1% w/v polyvinylpyrrolidone) was added to the frozen ground tissue and the mixture left to thaw at room temperature and transferred to a clean 1.5 ml Eppendorf tube. After vortexing (10 seconds), the homogenised mixture was centrifuged at 12,000 *g* for ten minutes at 4°C. The supernatant was transferred to a fresh Eppendorf tube and used for the determination of enzyme activity and protein content, the latter using Bradford's Reagent (Section 5.2.4).

The assay reaction mixture contained 0.1 M Tris-HCl (pH 7.8); 20 mM EDTA and 0.5 mM oxidised glutathione (GSSG). The reaction was initiated by the addition of 150 μM NADPH and 25 μl enzyme extract, and its progression monitored by the rate of change in absorbance at 340 nm over 3 minutes at 22°C. Initial experiments with this protocol gave inconsistent absorbance readings, some samples showing no enzyme activity whatsoever. Therefore the protocol was subject to a degree of modification.

The amount of substrate used in the reaction, i.e. the amount of NADPH, was altered to achieve a consistent result between similarly treated samples. A series of concentrations were tested, ranging from the recommended 50 mM to 250 mM.

Glutathione reductase activity was expressed in terms of μmoles NADPH oxidised per hour per mg protein:

$$\text{Specific Activity (units/mg protein)} = \frac{(\Delta A/\text{min} \times \text{ml reaction} \times 10^6 \mu\text{mol/mol} \times 60 \text{ min/hour})}{(E \times \text{mg protein} \times 1000\text{ml/litre})}$$

where E = molar extinction coefficient of NADPH (6.2×10^3 $M^{-1}cm^{-1}$).

5.2.4 Protein determination by Bradford's Reagent

Protein concentration was determined according to the method of Bradford (1976). A modified Bio-Rad Protein Assay kit was used. The assay is based on the chromogenic reaction of protein with Coomassie Brilliant Blue G-250 in the reagent. The protocol was followed as stated:

The dye reagent (450 ml solution of dye, phosphoric acid and methanol) was supplied as a five-fold concentrate, therefore was diluted in four volumes of HPLC grade water (BDH). The mixture was filtered through Whatman No. 1 paper and stored at room temperature in a glass bottle. Fresh solution was prepared every two weeks. Several dilutions of Bovine Serum Albumin (BSA) were prepared containing between 0.2 and 1.4mg/ml for construction of a protein standard curve, which was prepared every time the assay was performed.

BSA standard (0.1 ml), appropriately diluted samples and sample buffer (blank) were placed in clean, dry test-tubes. Diluted reagent (5 ml) was added to these and mixed by inversion several times. After an incubation time of ten minutes, the samples absorbencies were read at 595 nm versus the reagent blank. The OD_{595} of the standards were plotted against the concentration of BSA. Protein concentrations of the unknown samples were estimated from the standard curve.

5.2.5 Competitive reverse transcription polymerase chain reaction (RT-PCR)

The assay used to study the amount of mRNA in a given sample was derived and is described in full in Chapter 4, using Qiagen Column RNA extraction, as outlined in Section 4.2.2.2. Densitometry readings, obtained in a similar manner as DNA concentration determination (Section 2.2.7), were used to calculate the percentage of mRNA in the RT-PCR. Readings were obtained for the control fragment, the background and the mRNA fragment in successful RT-PCR lanes. The background reading was subtracted from both fragment readings and the sample reading calculated as a percentage of the control, whether it be greater or less than 100%.

5.2.6 Chilling experiment 1. A Comparison of Plants Held at 20°C and 4°C

Rhododendron ponticum plant tissue cultures were established and maintained as stated in Section 2.2.1. For experimental purposes, quadruplicate cultures for each sampling day were either transferred to a Sanyo Fitotron growth cabinet held at 4°C, or left in the growth room held at 20°C. The light intensity remained the same for both growth environments. All callus tissue was removed from the cultures before enzyme and nucleic acid extractions were initiated. All the cultures used in this experiment had been subcultured three to four weeks previously. Micropropagules were removed each sampling day for use in ascorbate peroxidase specific activity (Section 5.2.2), glutathione reductase specific activity (Section 5.2.3) and RT-PCR (Section 5.2.5) assays.

5.2.7 Chilling Experiment 2 - A Comparison of Plants Held at 20°C and 2°C

Rhododendron ponticum plant tissue cultures were established and maintained as stated in Section 2.2.1. For experimental purposes, quadruplicate cultures for each sampling day were either transferred to a Sanyo Fitotron growth cabinet held at 2°C, or left in the growth room held at 20°C. The light intensity remained the same for both growth environments. All callus tissue was removed from the cultures before enzyme and nucleic acid extractions were initiated. All the cultures used in this experiment had been subcultured three to four weeks previously. Micropropagules were removed each sampling day for use in ascorbate peroxidase activity (Section 5.2.2), glutathione reductase activity (Section 5.2.3) and RT-PCR (Section 5.2.5) assays.

5.3 Results

5.3.1 Ascorbate peroxidase assay 1

Although Figure 5.2 illustrates a significant difference between the two temperature treatments, when the assay was performed, the change in absorbance of the reaction mixture increased over the three minute observation, suggesting a reduction of ascorbate. Many researchers (Nakano and Asada, 1980; Rensburg and Krüger, 1994; Franck, *et al.*, 1996; Kirtikara and Talbot, 1996; Rao, Paliyath and Ormond, 1996) state that the OD_{290} should decrease during the experiment, thus indicating that the assay performed was not specific for ascorbate peroxidase in *Rhododendron* tissue, but was perhaps also detecting one of the other antioxidant enzymes such as dehydroascorbate reductase. Due to time constraints, a second protocol was tested to determine the activity of ascorbate peroxidase in *Rhododendron* tissues.

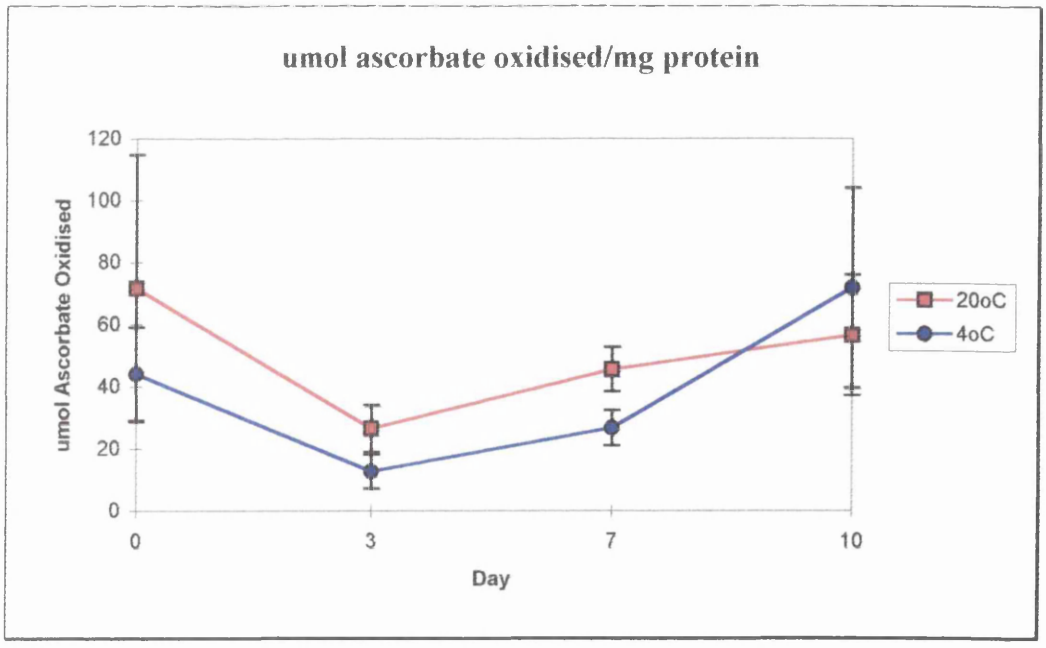

Figure 5.1. The activity profile of ascorbate peroxidase using Assay 1 (adapted from Nakano and Asada, 1980 and Berkowitz and Gibbs, 1992). A significant difference between treatments is observed after 3 and 7 days exposure to 20 and 4°C. (*n=3*)

5.3.2 Ascorbate peroxidase assay 2

This protocol achieved constant decreases in OD_{290} during the three minute test period for both chilling experiments, and it is this protocol which was used to determine the activity profiles illustrated in Figures 5.4 and 5.8.

5.3.3 Glutathione reductase assay - optimisation

Figure 5.2 illustrates the effects of NADPH concentrations on glutathione reductase activity in the assay. As the amount of NADPH added was increased, the reproducibility of the reaction increased, with all four samples of *Rhododendron ponticum* behaving similarly at 250 μM NADPH, noted by the small degree of error between the replicates. Subsequently, it was this concentration that was used in all following experiments.

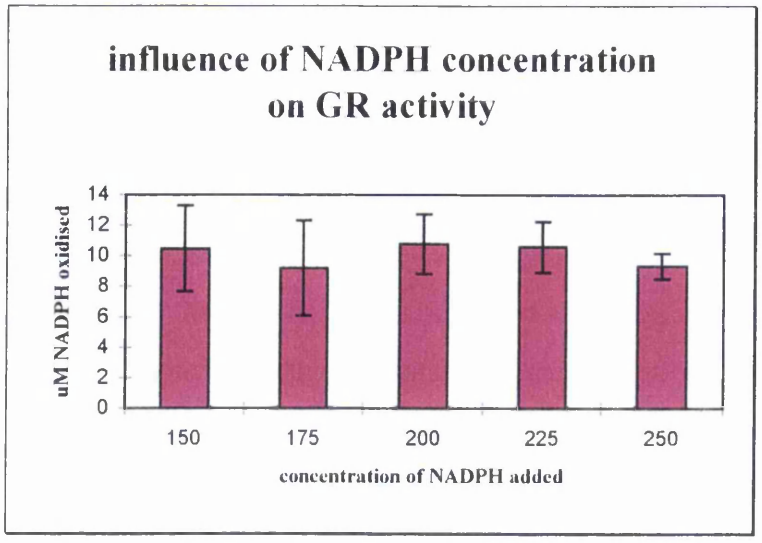

Figure 5.2. The graph represents the influence of NADPH concentration on the activity of glutathione reductase in enzyme assay. Summary statistics for this optimisation experiment can be found in Appendix 4.

5.3.4 Protein determination using Bradford's Reagent

Figure 5.3 shows a typical protein standard curve obtained for each protein assay. The OD_{595} readings of the unknown samples for *R. ponticum* were used to estimate the protein content from the curve and the amount of protein entered into the specific activity formula (Section 5.2.3).

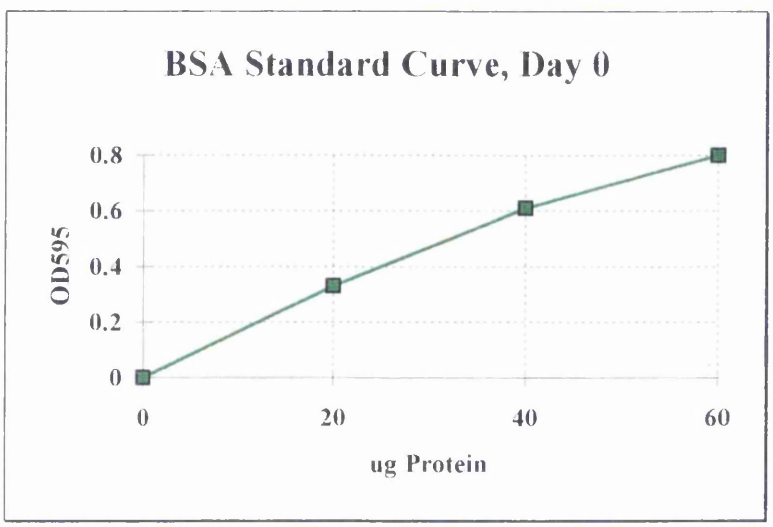

Figure 5.3. Illustration of a typical BSA Standard curve obtained from Bradford's Reagent Protein Assay. OD_{595} readings of unknown samples were used to estimate protein content.

5.3.5 RNA Extraction

Figure 5.4 illustrates one replicate of the RNA extracts from chilled and non-chilled *R. ponticum* micropropagules for each sampling day that were obtained for use in Competitive RT-PCR. As stated in chapter 5, the quality of the RNA preparation is crucial to the success of RT-PCR. Unfortunately in this case, all the extracts, with the exception of day 10, show partial degradation, with the presence of only one of the ribosomal RNA bands. The extracts from Day 10 samples illustrate the presence of two ribosomal bands, indicating a less degraded sample of total RNA. The higher quality of the sample is reflected in the result of RT-PCR for day 10, shown in Figure 5.7f.

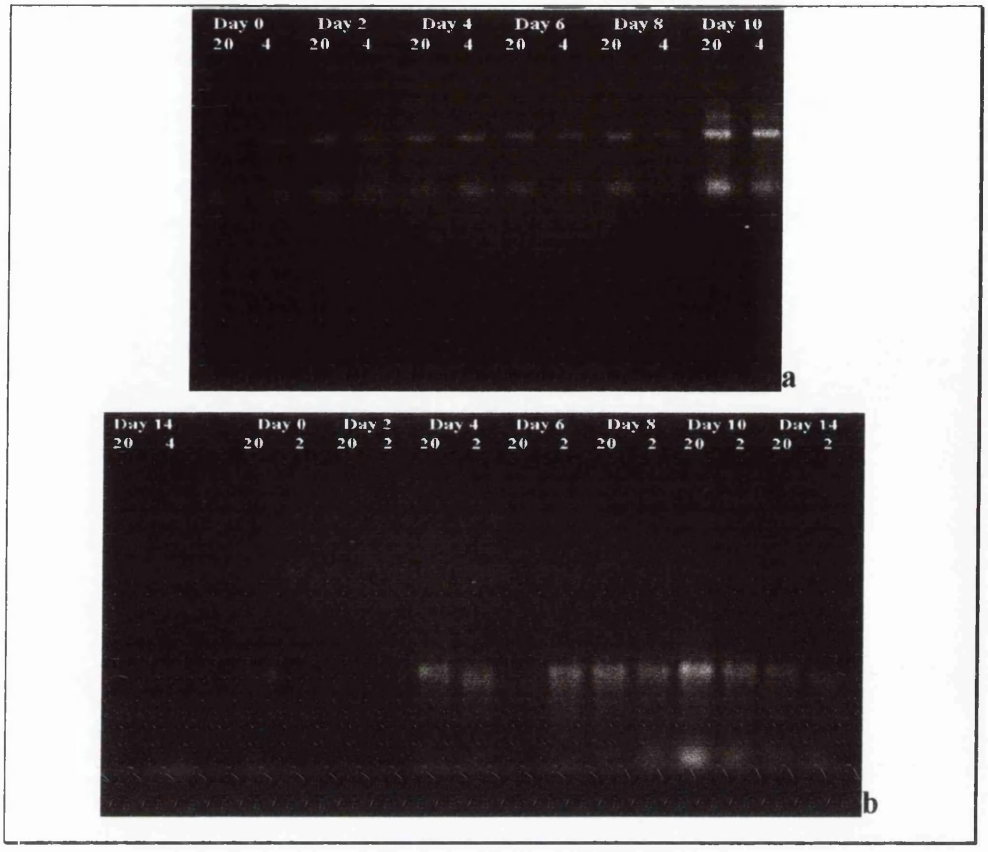

Figure 5.4. 1% (w/v agarose) TAE agarose gel electrophoresis of total RNA extractions from chilled and non-chilled *R. ponticum* micropropagules for chilling experiments 1 (5.4a) and 2(5.4b). Lanes 1 & 2 represent extracts from 20°C and 4°C/2°C day 0respectively, followed by day 2, 20°C/4°C and so on.

5.3.6 Chilling experiment 1. A comparison of plants held at 20°C and 4°C

Figure 5.5 shows that initial exposure to 4°C caused a decline in the activity of ascorbate peroxidase (APX). By day 2, there was a significant difference in the activity between the two treatments, ascorbate peroxidase in the cultures exposed to 4°C having decreased approximately two-fold. As the length of exposure of cultures at 4°C progressed, the activity of ascorbate peroxidase increased, returning to a level similar to that of the control cultures at 20°C by day 8, suggesting that the plant may have reached a level of acclimation.

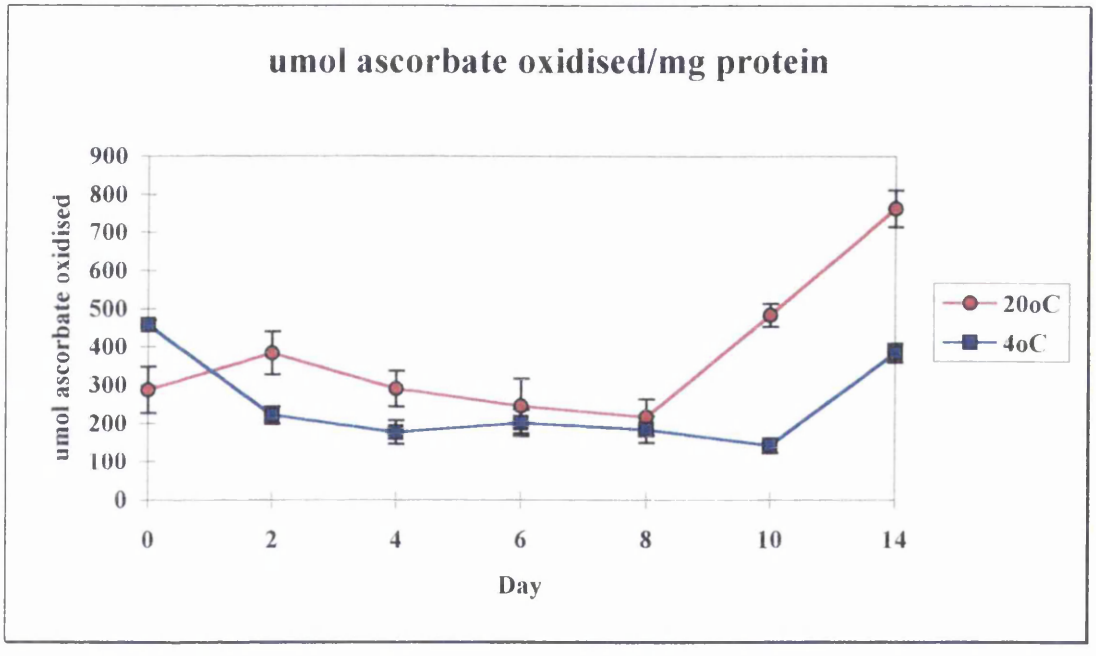

Figure 5.5. Activity of Ascorbate Peroxidase in *R. ponticum* held at 20°C and 4°C over a period of 14 days. (n=4)

However, the activity profile for cultures held at 20°C showed a sharp increase in APX activity on day 8, with the activity increasing 3-fold by day 14. This is mirrored in the 4°C cultures, where the APX activity profile increased 3-fold between days 10 and 14. It is thought that this significant increase in both treatments may be due to an additional stress

affecting the plants' responses. The cultures held at 4°C appeared to be slower to react, probably because chilling temperatures are well known to slow down all growth mechanisms (O'Kane *et al.*, 1996). The reason for the observed increases in APX activity can only be speculated upon without further experimentation, but it is thought that it may be the cultures' need for subculture onto fresh Anderson's *Rhododendron* Stage II medium that was the underlying stress. After 5-6 weeks in culture, the plants will probably have depleted the nutrients in the medium and the gaseous composition and possible humidity within the culture vessel will have altered, exposing the plant to further stress.

This phenomenon was also noted for glutathione reductase. Figure 5.6 shows that on exposure to 4°C, the plants exhibited a considerable (approximately 4-fold), but not significant increase in the activity of GR, compared to those at 20°C. By day 6, activity returned to a similar level as the control cultures held at 20°C. However, at day 10 there was a significant change in the activity of the enzyme, reflecting the possible influence of a different stress. Unlike the ascorbate peroxidase activity, the chilled plants showed a 3-fold decrease in GR activity between days 8 and 10, compared to the control plants which exhibited only a slight decrease in activity at this time. By day 14 however, the control plants showed a sharp increase in activity, the cultures exposed to 4°C mirroring this to a lesser extent, suggesting once more that the exposure to the lower temperature affected the plants' response.

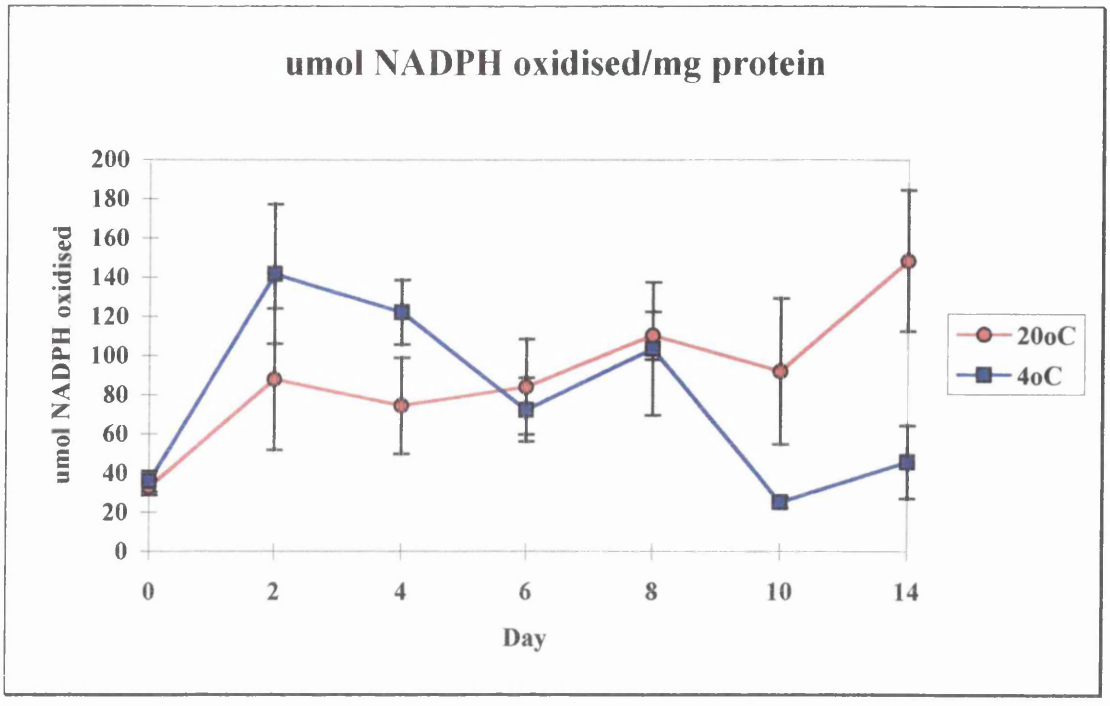

Figure 5.6. Activity of Glutathione Reductase in *R. ponticum* held at 20°C and 4°C over a period of 14 days. (n=4)

5.3.7 Ascorbate peroxidase gene expression studies - A comparison of plants held at 20°C and 4°C

As explained in Chapter 4, the use of Reverse Transcription PCR could not be used for its original intent, to quantify the changing amount of mRNA produced in stressed and unstressed *Rhododendron* tissue, because of the considerable amount of 'background' in the samples. The background interference observed in all RT-PCR samples is most likely due to the degradation found in the total RNA samples used in experimentation, arising in a loss of specificity in the amplification procedure. The assay could be used however, in a semi-quantitative manner to allow comparisons to be made between chilling treatments and determine a possible correlation between mRNA quantity and enzyme activity. Results from the RT-PCR assay are depicted in Figure 5.7 by gel electrophoresis analysis, and the corresponding graph obtained from the band densitometry readings from these gels is illustrated in Figure 5.8.

Tabulated results of the APX RNA percentages can be found in Appendix 5.

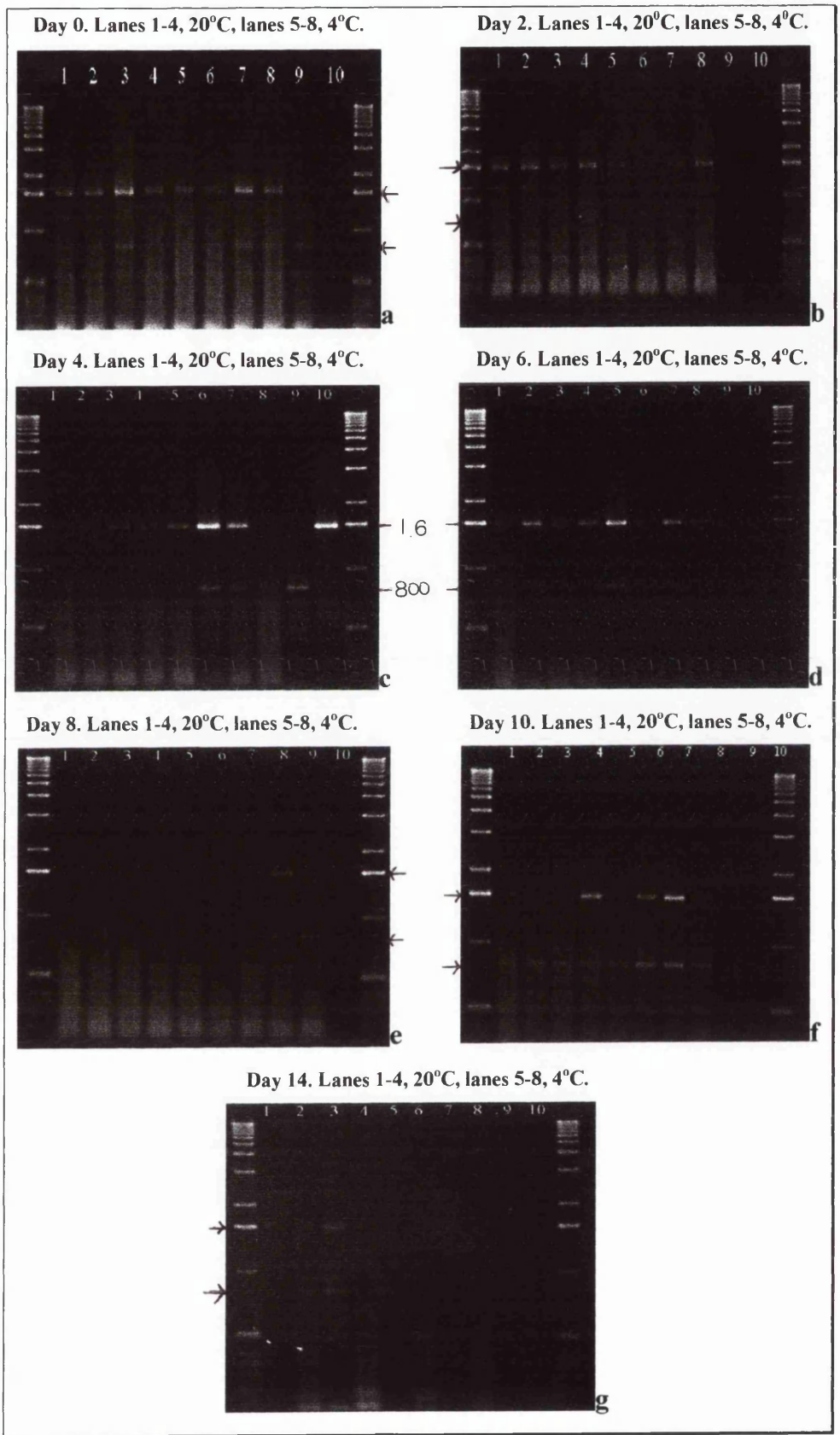

Figure 5.7. Gel Electrophoresis of Competitive RT-PCR. The competitor fragment is 1.6Kb in size, the cDNA fragment of ascorbate peroxidase, 800bp. In each figure, cDNA amplification is represented in lane 9, the competitor fragment in lane 10. (Lanes containing size markers have not been numbered).

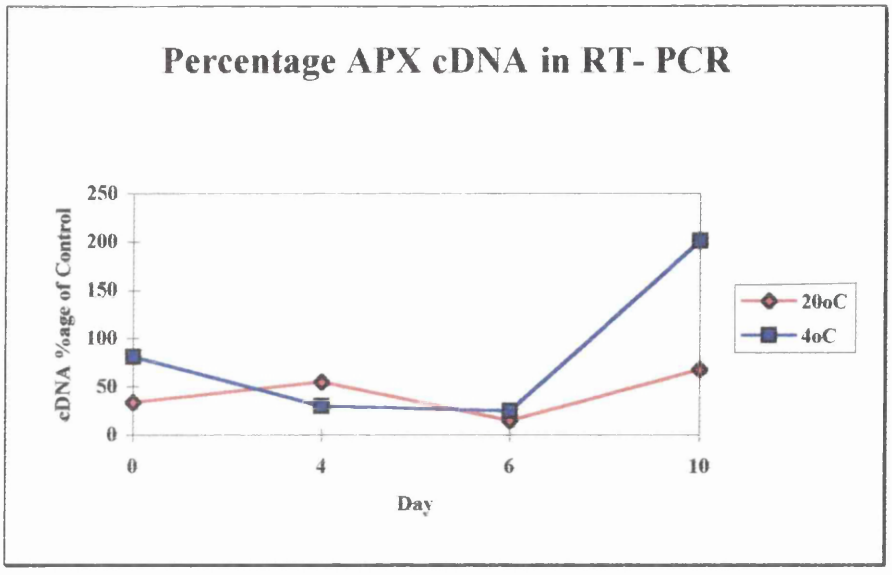

Figure 5.8. An illustration of the percentage of cDNA in the RT-PCR reaction. The calculation for the percentage of cDNA is explained in Section 5.2.5. Tabulated results can be found in Appendix 5

The results from Competitive RT-PCR for this first chilling experiment revealed an interesting pattern, illustrated in Figure 5.8. The percentage of mRNA in the reaction followed the profile of the ascorbate peroxidase activity during the first six days of the experiment. A similar initial decrease in ascorbate peroxidase was noted in the plant samples exposed to 4°C, levelling out to a similar amount as in the control plants by day 6. However, unlike the APX activity graph, it is the mRNA in the chilled plants which increased considerably in the latter stages of the study, with the control plants being slower to respond. It is interesting to note the similar patterns of the control and chilled plants for APX activity and gene expression during the early part of the study. A note of caution must be added however. Although gene expression reflected the activity of the enzyme, this was only one experiment, in which the number of replicates varied due to the delicacy of competitive RT-PCR.

5.3.8 Chilling experiment 2. A comparison of plants held at 20°C and 2°C

This study was initiated to investigate whether the response noted in experiment 1, i.e. the initial decrease in APX activity and increase in GR activity, would be more significant at a lower temperature. The results show however, that the plants' behaviour is completely different when exposed to a further 2°C drop in temperature.

Figure 5.8 displays the results of ascorbate peroxidase activity at 20°C and 2°C. The control samples at 20°C follow a similar profile to the control plants in experiment 1, the only difference being the considerable increase in activity occurring at day 6, not day 8. The behaviour of the chilled plants is somewhat different. Figure 5.8 shows that plants exposed to 2°C have similar APX activity to the control plants until day 8. As before, there appears to be the influence of another external stress taking over the plants' responses in the control samples, but this increase in activity was not observed in the chilled plants after day 8, which displayed a considerable decrease in activity, suggesting that they did not have the ability to cope with oxidative stress. At this stage in the experiment, the micropropagules were also displaying visible signs of chilling stress such as tissue dehydration , and none of recovery at this temperature.

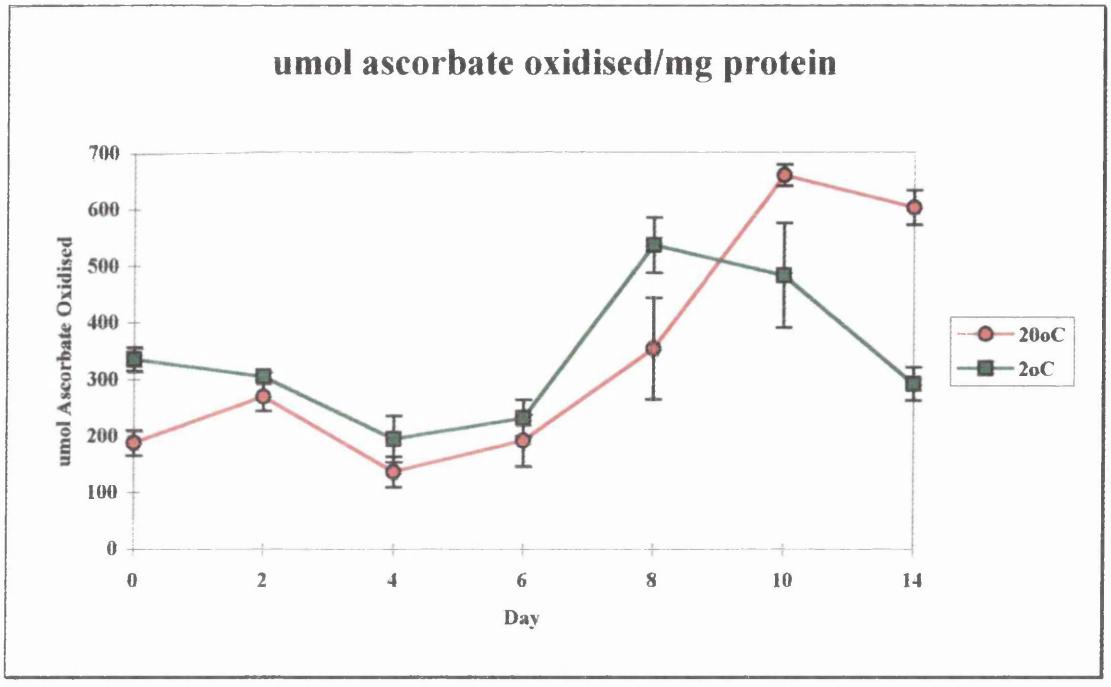

Figure 5.9. Specific Activity of Ascorbate Peroxidase in *R. ponticum* held at 20°C and 2°C over a period of 14 days. (n=4)

A similar lack of ability to cope with oxidative stress was noted with glutathione reductase activity, illustrated in Figure 5.10. Here also there was a completely different activity profile for plants exposed to 2°C, compared to 4°C. GR activity at 2°C decreased to a significantly lower level than the control plants by days 4 and 6. Day 8 revealed a rather spurious result however, which is more likely to be explained by experimental error rather than a true activity reading. It could be assumed to be highly unusual for GR activity in a control plant to decrease to almost zero on one sampling day, and then to have recovered two days later. Figure 5.11 shows the activity profile without the unusual day 8 result, and gives a far clearer explanation of what may have happened in the *Rhododendron* micropropagules. By the latter stages of the study, the activity of glutathione reductase decreased to almost negligible levels in plants exposed to 2°C. Comparing this result with the activity of APX, it could be assumed that the plants were dying rather than displaying any signs of oxidative stress resistance and cold acclimation.

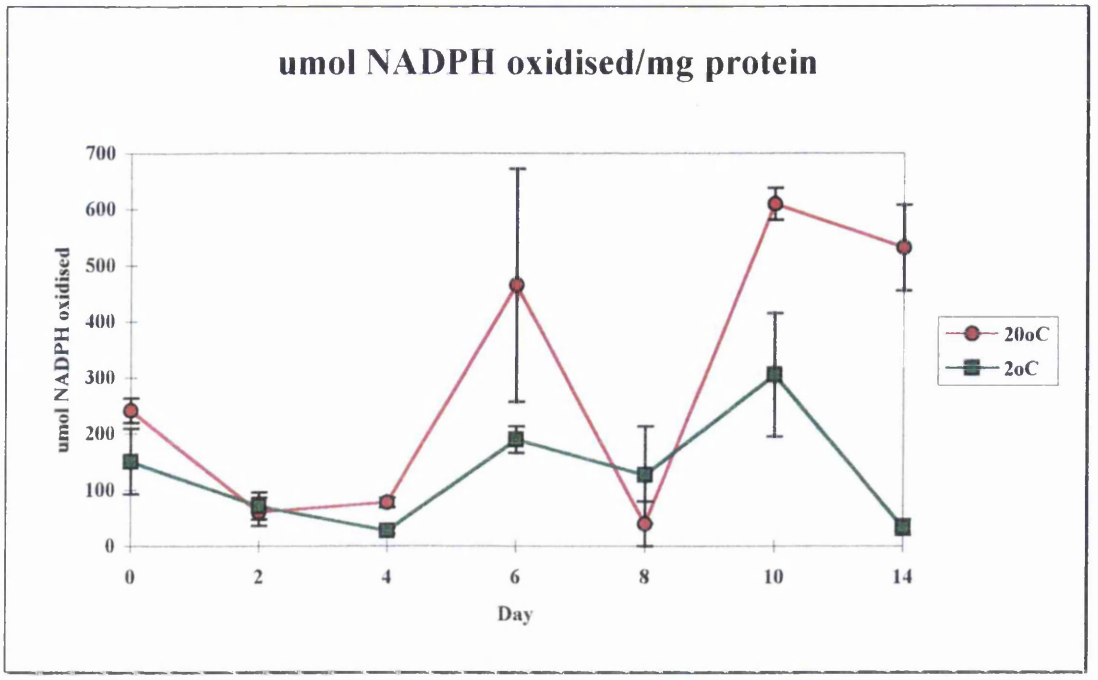

Figure 5.10. Specific Activity of Glutathione Reductase in *R. ponticum* held at 20°C and 2°C over a period of 14 days. (n=4)

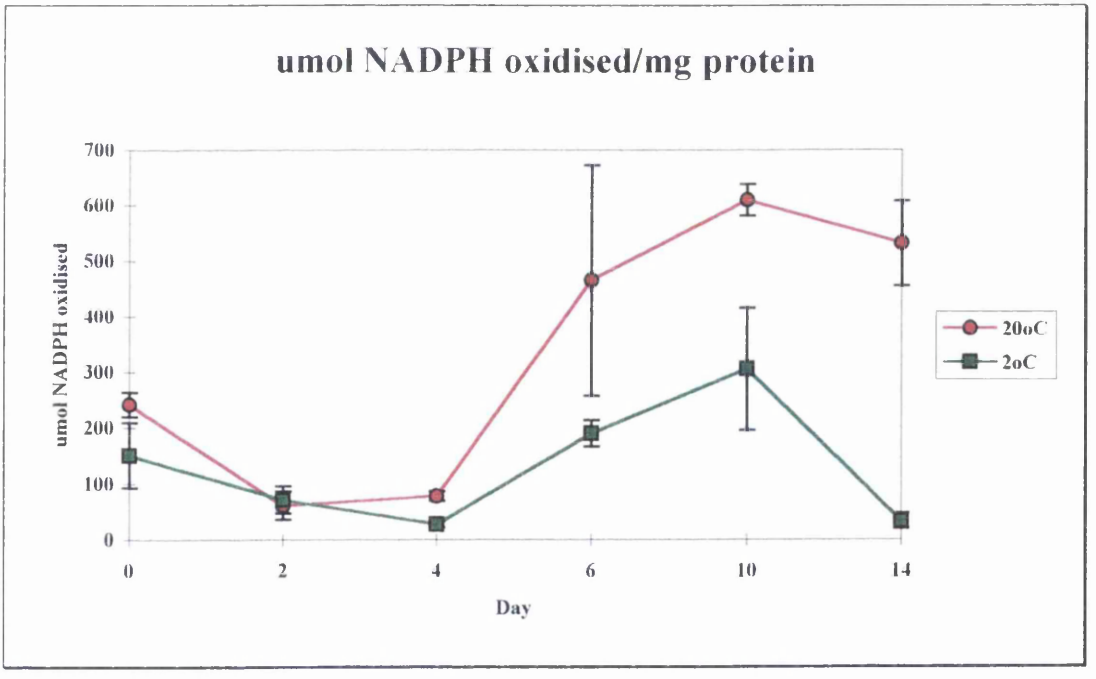

Figure 5.11. Glutathione Reductase Experiment 2 - Omission of Day 8 result.

5.3.9 Ascorbate peroxidase gene expression studies - A comparison of plants held at 20°C and 2°C

The Competitive Reverse Transcription PCR in experiment 2 was less successful in general, and so data are not available for all the sample days. However, the pattern which has been observed for the chilled plants (Figure 5.12) does reflect, to a certain extent, that of the enzyme activity of plants at 2°C. The initial decrease in percentage APX transcript is similar to the decrease in APX activity, as is the increase at day 8 followed by the substantial decrease at days 10 and 14. The partial result for the control plants is quite different however, but as mentioned previously, nothing substantial can be concluded from these results as this was only one experiment. Figure 5.11 illustrates the gel electrophoresis results of the Competitive RT-PCR. Tabulated results of the APX percentages can be found in Appendix 5.

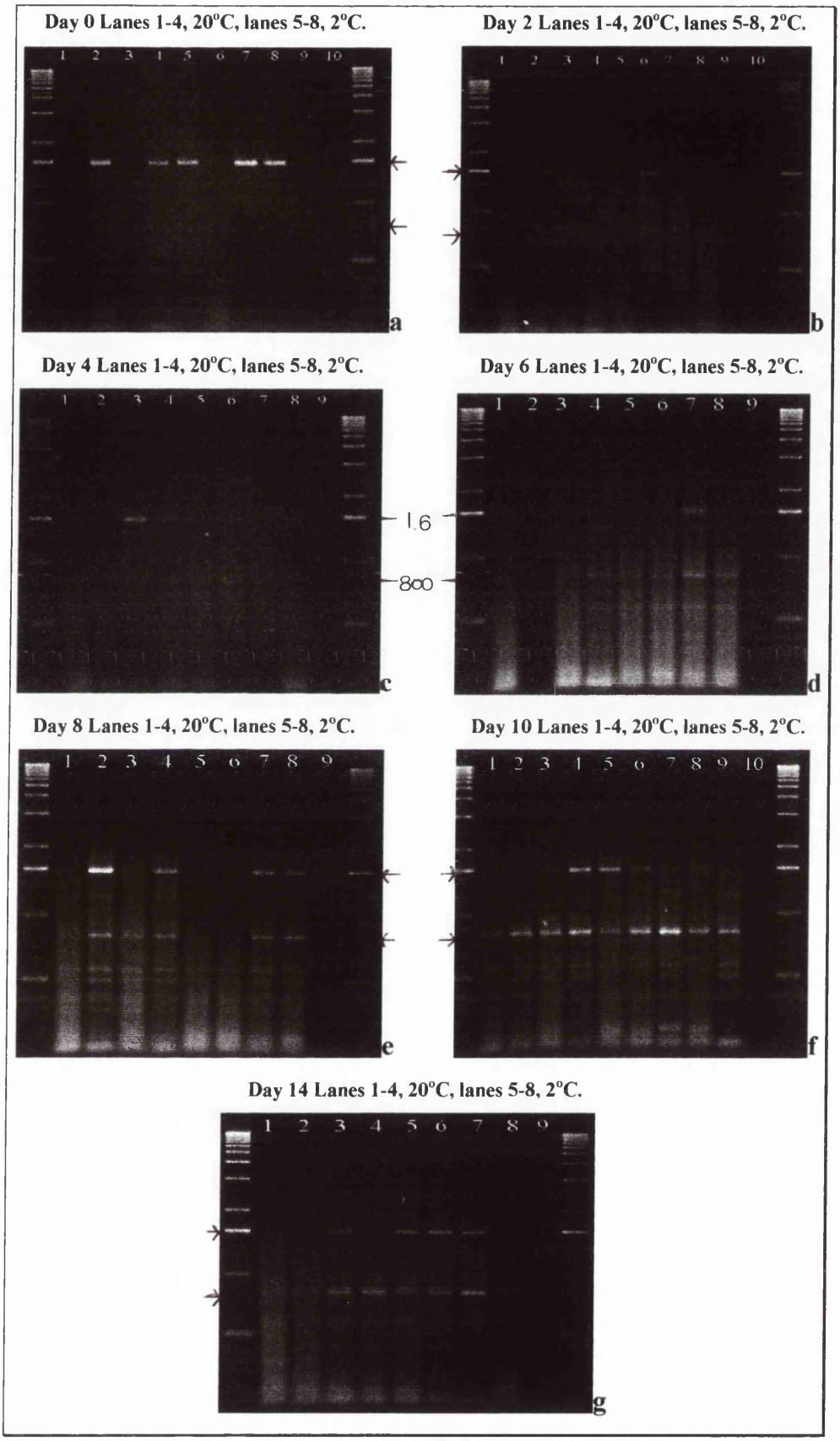

Figure 5.12 Gel Electrophoresis of Competitive RT-PCR. The competitor fragment is 1.6 kb in size, the cDNA fragment of ascorbate peroxidase, 800 bp.(Lanes with marker are not numbered)

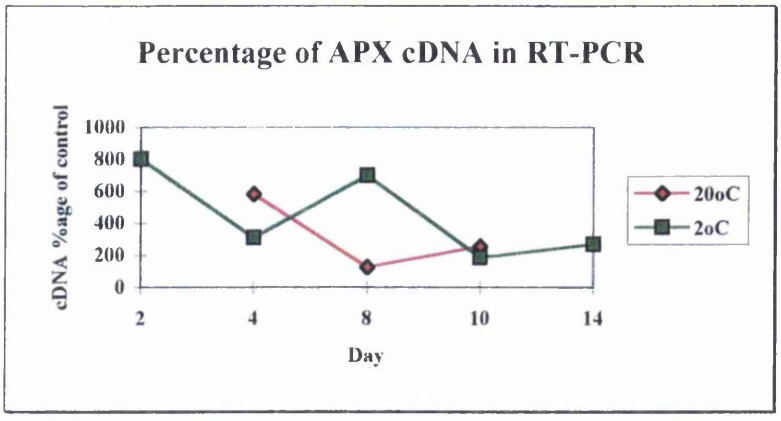

Figure 5.12. An illustration of the percentage of cDNA in the RT-PCR reaction. The calculation for the percentage of cDNA is explained in Section 5.2.5.

5.4 Discussion

It has been reported that exposure to chilling temperatures which induce oxidative stress cause an initial rise in the activity of ascorbate peroxidase (Anderson *et al.*, 1992; Kuroda *et al*, 1993; Hakam and Simon, 1996; O'Kane *et al.*, 1996). Anderson, *et al.* (1992) observed in Eastern white pine that antioxidant enzyme activities increased between 2 and 122-fold from summer to winter, and suggested the increase in activity to be linked to the cold hardening process in this particular woody plant species. Kuroda *et al.* (1993) noted in apple flower-buds that during spring dehardening, antioxidant enzyme activity decreased as the temperatures increased, again substantiating an antioxidant role in plants' response to external temperatures. Indeed, this is not only true for woody plants, but herbaceous plants also. O'Kane *et al.* (1996) found that glutathione reductase activity significantly increased in *Arabidopsis thaliana* almost immediately on exposure to 4°C. This correlates with the increase noted in *Rhododendron ponticum* micropropagules (Figure 5.5).

Concentrating on the activity of ascorbate peroxidase and glutathione reductase in *R. ponticum* micropropagules exposed to 4°C (Figures 5.4 and 5.5) over the first 8 days of the study, it could be postulated that the initial rise in GR activity may be a function of the ascorbate - glutathione pathway to produce more ascorbate, the recognised electron donor for ascorbate peroxidase (Foyer and Halliwell, 1976; Halliwell *et al.*, 1981). If more substrate were readily available, then the plant would indeed have obtained a better line of defence against oxidative stress. This theory could also account for the initial loss in APX activity, but by day 8, as the GR activity has returned to a level similar to that of the control activity, the APX activity has also recovered to a level similar to that of the non-chilled cultures.

This may not be due to acclimation however, but possibly due to changes in the isoform population of GR. Because detergents were used in the extraction of both enzymes, it is most likely a mixture of all isozymes are present in the extracts (Polle and Morawe, 1994). Guy and Carter (1987) found that glutathione reductase partially purified from cold-acclimated spinach had a greater stability against freezing and a higher affinity for substrates at low temperatures than GR purified from non-acclimated spinach. Edwards *et al.* (1994) also observed an increase in GR activity which appeared to be due to a change in isoform population. However, *Rhododendron* micropropagules do not seem to have the same extent of cold hardening ability because of the initial decrease in APX activity. Ascorbate peroxidase is well known as being one of the most important enzymes in oxidative stress, because of its ability to scavenge hydrogen peroxide (Dalton *et al.*, 1986; McKersie *et al.*, 1993; Mathews *et al*, 1997) and it could be assumed that if the increase in activity is not present on exposure to chilling, the plants will be deprived of the ability to cold-acclimate.

This lack of ability to cold-harden is further established by observations made in chilling experiment 2 (Section 5.3.8). The initial aim of the experiment was to investigate whether the response, i.e. the initial decrease in APX activity and rise in GR activity, observed in experiment 1 would be more significant at a lower temperature. The activity of ascorbate peroxidase and glutathione reductase at 2°C in *R. ponticum* micropropagules (Figures 5.8, 5.9& 5.10) is characteristic of non-acclamatory behaviour. Polle *et al.* (1996) studied the seasonal changes of ascorbate peroxidase activity in Norway spruce (*Picea abies*). When the young seedlings were exposed to temperatures of -5°C, the activity of APX was found to decline before the onset of visible cold temperature injury. Such activity was also noted in shoot cultures of rice exposed to 4°C, a species known to be chilling sensitive (Fadzillah, *et al.*, 1996). The activity of ascorbate peroxidase was observed to remain at a similar level

to cultures grown at 25°C over an 8-day period. Chilling temperatures had a marked effect on the activity of glutathione reductase however. It was noted by Fadzillah *et al* (1996) that throughout the duration of their study, the activity of GR significantly declined, and this result is reflected in the activity of GR in *R. ponticum* micropropagules. Indeed, by the end of this study, there were definite signs of visible chilling injury to the micropropagules, the cultures looking dehydrated and necrotic.

There are a variety of reasons why a species so well known to survive to temperatures as low as -25°C (Sakai *et al.*, 1981) may not have the ability to do so in micropropagation. Polle *et al.* (1996) found that seedlings and young needles were subject to cold temperature injury and death. *R. ponticum* micropropagules have very soft leaf and stem tissue, similar to young shoots in mature *R. ponticum* plants. Young tissue is often subjected to chilling and freezing injury because of their sensitivity to lipid peroxidation, which is closely linked to oxidative stress. Superoxide radicals generated in oxidative stress are thought to bind to protons in cell membrane lipids thus yielding hydroperoxy radicals, thought to initiate peroxidation (Burdon *et al.*, 1994). Peroxidised lipids interfere with normal membrane fluidity and biological function, as well as damaging membrane proteins and therefore affecting their activity also. All these mechanisms will result in cell injury and ultimately, death.

It has been thought for some time that temperature affects organisms at both a physiological and molecular level (Burdon, 1986; Ougham and Howarth, 1988). As previously mentioned in Chapter 1, oxidative stress and lipid peroxidation are closely linked, and the peroxidation of lipids is thought to be an indicator of oxidative stress (Rice-Evans *et al.*, 1991). It is still not known what triggers the change in gene expression to begin with, i.e. the upregulation of antioxidant gene expression on exposure to stress. Although the results obtained in the present study of the *R. ponticum* ascorbate peroxidase gene are far from complete, they can still

be informative. It can be said that the amount of mRNA transcribed from the APX gene reflects the activity of the enzyme during the duration of the studies (Figures 5.7 & 5.12). This is a further indication that the *in vitro* grown *Rhododendron* plant tissue did not seem to be acclimating to chilling temperatures.

5.5 Conclusions

- Optimisation of the ascorbate peroxidase enzyme activity assay allowed an accurate activity profile of enzyme activity from *R. ponticum* micropropagation tissue to be constructed

- Alteration in the amount of substrate (NADPH) added to the glutathione reductase assay optimised the assay to be specific for *R. ponticum* tissue.

- Reasonable quality total RNA extracts allowed the progression of the competitive RT-PCR assay, as an additional source of ascorbate peroxidase activity in chilled and non-chilled tissue.

- Plants exposed and held at 4°C showed an initial decrease in ascorbate peroxidase activity, indicating a poor antioxidant response, however returned to control levels by day 8 of experiment 1.

- Plants exposed and held at 4°C showed an initial increase in glutathione reductase activity, a response typical of the ascorbate-glutathione cycle to compensate for reduced ascorbate levels. Glutathione reductase activity returned to control levels by day 8 of experiment 1.

- Competitive RT-PCR results in experiment 1 indicated that the change in ascorbate peroxidase gene expression in response to chilling stress reflected the change in enzyme activity.

- Enzyme activity profiles showed that plants exposed to and held at 2°C had not the ability to cold harden. Ascorbate peroxidase and glutathione reductase activities mirrored those of control plants throughout experiment 2.

- Competitive RT-PCR results in experiment 2 reflected the change in ascorbate peroxidase enzyme activity.

- *R. ponticum* grown *in vitro* does not have the same ability to cold harden as plants grown *in vivo*.

Chapter 6 General Discussion

Molecular biological techniques can be very helpful in attempting to comprehend the causes and effects of the responses of plants to changing environments. Environmental stresses such as temperature extremes, light, anaerobiosis, water stress, xenobiotics, heavy metals and pest and pathogen attack (Gilmour *et al.*, 1988; Brown *et al.*, 1995; Iturbe-Ormaetxe, *et al.*, 1995; Sgherri & Navari-Izzo, 1995; Kirtikara & Talbot, 1996; Knörzer *et al.*, 1996; Mehdy *et al.*, 1996; de Paula *et al.*, 1996; Schwanz *et al.*, 1996; Weckx & Clijsters, 1996) all induce stress responses within plant and animal tissue (Burdon, 1993). Investigating how a plant may perceive stress at a genetic level may give an insight as to how defence mechanisms may be activated. Molecular studies in conjunction with physiological methodology may present a clearer illustration of gene regulation and expression in plants.

The production of active oxygen species (superoxide radicals, hydrogen peroxide and hydroxyl radicals) in plants is one of the most important mechanisms by which a plant incurs damage following environmental stress stimuli (Foyer and Mullineaux, 1994) and may lead to severe cell damage and death (Inzé and Van Montagu, 1995). The adaptation of plants to low temperatures may prove to be important as global climate change becomes more apparent. Milder winters may lead to earlier plant deacclimation, thus exposing them to late spring frosts, whilst plants which require a period of vernalisation may be unable to complete their natural life cycle.

Survival in such hostile habitats is enhanced by the production of antioxidant proteins and enzymes (Davies, 1995). Investigating the expression of genes encoding such antioxidant enzymes and the activity of the enzymes themselves, may lead to a better understanding of some of the mechanisms underlying cold tolerance in plant species. By

investigating the change in quantity of specific mRNA species in plant tissues or cells, it is possible to determine how much a particular gene has been expressed in response to an environmental stimulus. The employment of competitive Reverse Transcription Polymerase Chain Reaction (RT-PCR) may provide accurate quantitative changes in amounts of mRNA produced (Gilliland *et al.*, 1990). To fully develop such a molecular assay to study the expression of genes which may be up-regulated during exposure to chilling temperatures, several criteria must be met, each of which have their own specific parameters which must be adhered to;

a) the establishment of a suitable plant growth system comprising appropriate species, for the production of quality plant material (for the extraction of nucleic acids), and for ease of maintenance and environmental manipulation.

b) the development of a DNA isolation protocol producing DNA of sufficient quality to be used in PCR.

c) the generation of suitable primer sets for the amplification of ascorbate peroxidase and glutathione reductase in PCR.

d) the identification of PCR product fragments by cloning and sequencing.

e) the establishment of an RNA isolation protocol producing RNA of sufficient quality to be used in RT-PCR.

f) the synthesis of an RT-PCR competitor fragment to allow the monitoring of the changes in mRNA present in stresses and non-stressed plants, hence the establishment of a suitable competitive RT-PCR assay.

Previous acclimation and cold stress studies undertaken at SAC Auchincruive, utilised *Rhododendron* species in experimentation. A genus comprising of more than 900 species (Davidian, 1982), found throughout the globe, it was chosen as a model system suitable to represent woody plant species because of the distinct range in genotype and habitat. *Rhododendron ponticum*, *R. hatsugiri* and *R. impeditum*, distinct species within the genus, were established in a micropropagation system, chosen

because it was ideal for rapid multiplication of plants, and ease of maintenance and manipulation of the growing environment. Using *in vivo* plant material would only allow for the harvest of a single leaf for analysis, immediately introducing variation between replicate studies. The entire explant was harvested using *in vitro* material, all of which was relatively uniform in age and appearance. Explants obtained for *R. ponticum*, *R. hatsugiri* and *R. impeditum* were found to be suitable for the extraction of high quality genomic DNA (Section 2.3.4)

6.1 Development of competitor RT-PCR assay

Given that there was no sequence data available for the antioxidant enzymes ascorbate peroxidase (APX) and glutathione reductase (GR) in *Rhododendron* species, a comparison of known plant sequences was performed. Identification of conserved regions of these genes presented a starting point for the design of primers suitable for PCR amplification (Section 2.3.2). Following parameters provided by Dieffenbach *et al.* (1993) and Rychlik (1995) i.e. primer stability, primer self-compatibility and formation of a stable duplex with the target template, the sequences encoding ascorbate peroxidase in *Pisum sativum* and glutathione reductase in *Arabidopsis thaliana* were used in primer design (Section 2.3.3).

Oligo 5.0 Primer Analysis Software provided the analysis necessary for efficient primer design. Pre-programmed parameters included a high degree of homology between the 3′ end of the primer and the target sequence, the primer region being unique within the target sequence, no self-homology between the primers, and the internal stability of the primers. The latter is significant especially at the 3′ end when stability should be low to allow successful annealing, made possible accurate design (Rychlik, 1995). The melting temperature and internal stability

profiles were suitable for successful amplification according to Rychlik (1995), for several primer sets for both genes (Figure 2.5, Section 2.3.3), and using aforementioned pre-programming, Oligo 5.0 excluded unsuitable primer pairs from final analysis results. Thus, three primer sets were synthesised for each gene to be tested in PCR optimisation (Table 2.6, Section 2.3.3).

Results from PCR optimisation showed however, that although criteria for successful primer annealing and amplification were achieved, PCR met with limited success. As reported by Innes and Gelfand (1990) and observed in ascorbate peroxidase and glutathione reductase primer tests, several reaction conditions must be modified to improve the specificity, efficiency and yield of PCR. The specific temperatures of the thermal cycle, particularly primer annealing temperature, the concentration of the primers and template used and the concentration of free nucleotides and magnesium ions within the reaction mix, may need to be altered to obtain the desired PCR result (Bloch, 1990; Kidd and Ruano, 1995). Indeed, within this study all parameters comprising PCR were considered in the attainment of a discrete product for ascorbate peroxidase (2.1kb, primer set A; 1.6kb, primer set C) and glutathione reductase (~500bp primer set A; Section 2.3.6).

A reduction of primer annealing temperature was shown to decrease reaction specificity, whilst an increase in temperature resulted in an increase in specificity, correlating with results obtained by Innes and Gelfand, (1990), Saiki, (1990) and Kidd and Ruano, (1995) (Section 2.3.6). A temperature of 55°C was found to be the optimum for both genes, with 20 pmoles of each primer in the reaction mix (Section 2.3.6). Temperatures tested above the optimum resulted in reduced or no yield of PCR product. Given that the internal stability profiles were less suited to PCR amplification for APX set B and GR set B and C, none of the optimisation tests performed resulted in a desirable PCR product (Section

2.3.3). This result correlates with findings from Rychlik (1995) which showed that primer sets with high stability at the 3′ ends perform poorly in PCR.

Confirmation of PCR analysis, necessary to ensure correct annealing of the primers and amplification of the desired gene, was facilitated by gene cloning and sequencing (Chapter 3). Exploiting the T/A cloning technique derived from Clark (1988) and Mead *et al.* (1991), PCR fragments for both ascorbate peroxidase and glutathione reductase were ligated into a plasmid vector. In order to achieve this successfully, many parameters were adhered to. The PCR fragment must have the dA overhangs which *Taq* Polymerase adds to the end of the target template, irrespective of its sequence to be ligated into the dT ends of the plasmid vector. If this is not so, as was found in the first cloning attempt with ascorbate peroxidase, no ligation will occur. The PCR fragment should be fresh and unpurified from the reaction mix otherwise the fragile dA ends will be degraded (Section 3.3.1).

Indeed, it is not only the quality of the PCR reaction mix which must be considered, but the quality of the product. If the PCR product to be ligated into the plasmid vector is not discrete, then the efficiency of ligation is very much decreased. This was illustrated particularly in the second attempt at cloning the ascorbate peroxidase fragment (Section 3.3.2). Although the desired fragment of 2.1kb was indeed the most dominant band observed upon gel electrophoresis analysis, smaller bands present were possibly later responsible for the lack of success of sequencing. The chromatograms obtained from the sequencing of what was initially thought of as successfully cloned plasmids, were inconclusive - meaning that the presence of lots of smaller background chromatogram peaks made sequence difficult to determine. It could be postulated that multiple products present in the reaction mix used for ligation reactions led to different ligations within one sample, thus the chromatogram and

sequence were hard to define. This hypothesis is further substantiated by the *R. hatsugiri* ascorbate peroxidase fragment being identified as such, because it was the only reaction mix containing a discrete product, noted upon visualisation by gel electrophoresis (Figure 3.5, Section 3.3.2).

A third cloning and sequencing attempt with potential ascorbate peroxidase fragments gleaned much clearer chromatograms, but the fragments cloned lacked homology with published ascorbate peroxidase mRNA sequences, again most likely due to the quality of the PCR product. Throughout the cloning procedure, control reactions were successful, further substantiating the need to improve the quality of the experimental PCR. This necessitated the use of a different primer set, APX set C, which produced a smaller product of 1.6 kb and of significantly higher quality. DNA from all three *Rhododendron* species produced a single PCR product upon amplification with these primers, as shown by gel electrophoresis (Figure 3.12, Section 3.3.5). Sequencing provided clear chromatograms for these clones, and BLAST homology searches revealed that all species have significant homology (79%) with ascorbate peroxidase mRNA in *Pisum sativum* (Figure 3.19, Section 3.3.6).

Results for cloning and subsequent sequence analysis for glutathione reductase were however, less successful. Although the same disciplines were followed throughout the protocol as for the successful cloning and sequencing of ascorbate peroxidase fragments, the ligated PCR products shared no homology with published glutathione reductase mRNA sequences. Analysis of successfully cloned plasmids by restriction digestion revealed an insert of approximately 500 bp, the size of the initially ligated PCR product. However, the insert was not homologous with any published glutathione reductase sequence, indicating that although the primers produced a discrete PCR product, amplification and/or annealing was not specific to glutathione reductase (Section 3.4.1).

These results thus necessitated the design and synthesis of new glutathione reductase primers, from a different area of the *Arabidopsis thaliana* mRNA sequence, in an attempt to amplify a region specific to glutathione reductase. Using newly synthesised primers resulted in a single PCR product of approximately 2 kb in size using *R. ponticum* genomic DNA (Figure 3.24, Section 3.4.2). However, cloning with these PCR mixes was unsuccessful in the first instance, and due to significant time constraints, could not be repeated at this time. The delicate nature of the cloning procedure, discovered throughout the cloning of ascorbate peroxidase and outlined by Clark (1988) and Mead *et al.* (1991), most likely contributed to the lack of success with glutathione reductase.

The successful cloning of an ascorbate peroxidase fragment allowed the development of a competitive RT-PCR assay to continue. Specific information about the DNA sequence of ascorbate peroxidase in *Rhododendron* facilitated the synthesis of species-specific PCR primers, necessary for the success of mRNA-specific reverse transcription PCR (Ohan and Heikkila, 1993; Philips *et al.*, 1993). However, PCR optimisation tests using new *Rhododendron*-specific APX primers met with limited success, although primer mismatches using *Rhododendron*-specific APX upper primer and APX set C lower primer in PCR did produce a fragment of 1.6 kb, the desired product size, using *R. ponticum* and *R. impeditum* genomic DNA (Section 4.3.1). Thus established as suitable primers for the amplification of a discrete product of the expected size using genomic DNA, these primers were employed in the development of an appropriate reverse transcription protocol was developed (Section 4.3.5).

To effectively amplify RNA from plant and animal tissues, all aspects of RT-PCR must be carefully controlled due to the precise nature of the reaction, and the transient nature of RNA molecules. The specific nature of the primers had been determined previously, but the quality of the RNA

preparation and the type of reverse transcriptase used in the reaction were yet to be established. Gel electrophoretic analysis of RNA preparations using two different extraction methods showed that QIAGEN column extraction produced a preparation of higher quality, suitable for use in RT-PCR (Section 4.3.3). Indeed, initial RT-PCR tests using AMV reverse transcriptase found amplification to be successful, if unspecific, using samples prepared by this method (Section 4.3.5). However, throughout this period of experimentation, it was noted that the quality of the RNA extraction, and the degree of RNA degradation, was very important to the success of specific amplification, a phenomenon observed by Philips *et al.* (1993). Results revealed that although the quality of RNA was as high as could be obtained under laboratory conditions, undegraded preparations yielded more conclusive competitive RT-PCR results (Section 4.3.7; Figure 5.6, Section 5.3.7).

Given the relative success of initial reactions with AMV reverse transcriptase, this enzyme was used for a number of experiments aimed at increasing the specificity of RT-PCR. AMV is a DNA polymerase which utilises DNA, RNA or RNA:DNA hybrids as a template for transcription. It possesses RNase H activity, which causes cleavage of the extending strand if the enzyme pauses during transcription (Promega Corporation, 1998). This could explain the multiple banding noted during gel electrophoresis analysis (Figure 4.11). However, as RT-PCR optimisation continued, it became apparent that AMV reverse transcriptase was unsuitable for the transcription of ascorbate peroxidase mRNA. Results became consistently more inconclusive, with a smear (Figure 4.12), or no result at all being observed upon gel electrophoresis. Addition of the PCR enhancing agent, DMSO (Frackman *et al.*, 1997) did little to improve the specificity of the reaction (Figure 4.13). Indeed, the addition of the non-milk fat compound BLOTTO (De Boer *et al.*, 1995) made little impact on RT-PCR results when no amplification was noted in control samples

(Figure 4.16). It was for these reasons that the second type of reverse transcriptase was tested in RT-PCR development.

M-MLV reverse transcriptase is an RNA-dependent DNA polymerase with a weaker RNase H activity than AMV (Promega Corporation, 1998) It is better suited to the synthesis of longer cDNA molecules. Results revealed much better cDNA synthesis and amplification using this enzyme, although multiple products were still observed. RT-PCR optimisation tests using M-MLV proved to be more conclusive, with the eventual amplification of a dominant product of approximately 800bp in size, expected to be that of ascorbate peroxidase mRNA in *R. ponticum* and *R. impeditum*. Some smaller bands were still produced, but were most likely to the partial degradation of the total RNA samples that were used (Section 4.3.6).

Reverse transcription PCR made possible the study of rare, transient mRNA transcripts (Gilliland *et al.*, 1990), although a means of quantification of these molecules to glean information about gene expression was yet to be established. Results from Wang *et al.* (1989) and Gilliland *et al.* (1990) concluded that the use of exogenous standards in the reaction mix can accurately quantify target cDNA molecules. Adhering to these results, an exogenous standard was designed for RT-PCR which would compete for the same primers and reaction reagents in the same tube. This was achieved by the generation of a complementary RNA molecule, synthesised from the cloned DNA ascorbate peroxidase fragment used previously in sequencing studies, by *in vitro* transcription. By using the DNA fragment as a competitor, the same primers could be used for competitor and target molecule amplification, thus reaction efficiencies should be comparable for each (Wang *et al.*, 1989). Reverse transcription PCR of the *in vitro* transcription competitor fragment proved to be successful, generating a product of 1.6 kb, the same size as the

original DNA fragment and easily distinguishable from the dominant cDNA amplification product (Section 4.3.6).

To fully develop competitive RT-PCR, a suitable ratio of competitor and target molecules must be established. Otherwise, amplification efficiencies will differ, resulting possibly in preferential amplification of one or other molecule (Philips *et al.*, 1993). This was indeed the case when initially volumetric ratios of the two fragments were used in competitive RT-PCR. Results indicated that although a suitable volumetric ratio was found for one set of RNA preparations, when applied to another, the reaction preferentially amplified the cDNA target (Section 4.3.7). It was thus concluded that the ratios of competitor and target used in RT-PCR should be based on concentrations of molecules. Optimisation tests proved that using 2 ng total RNA extract and 6 ng control fragment in the reaction were successful in producing repeatable results (Figure 4.24, Section 4.3.7). Background amplification still proved to be a factor in the final stages of assay development, and so results were used in a semi-quantitative manner, the amount of target cDNA in the reaction expressed as a percentage of the RT-PCR reaction. Although competitor and target fragments were amplified reproducibly, the presence of smaller products, also competing in the reaction, meant that no quantitative results could be retrieved. Time and facility constraints limited further refinement of the assay, which was thus used in conjunction with physiological studies, i.e. ascorbate peroxidase and glutathione reductase activity studies, to indicate the antioxidant activity within chilled and non-chilled *Rhododendron ponticum* (Section 5.3).

6.2 Activity of ascorbate peroxidase and glutathione reductase in control and chilled *R. ponticum* micropropagules

Once appropriate activity assays had been established for ascorbate peroxidase and glutathione reductase, the former by testing different protocols (Nakano and Asada, 1980; Sen Gupta, 1993) and the latter by optimisation of reaction compounds, chilling studies were initiated. *R. ponticum* micropropagules were subjected to a 20°C control temperature or 4°C, a temperature known to induce chilling damage in plants (O'Kane *et al.*, 1996). Findings from the activity assays revealed *R. ponticum* micropropagules to have limited acclimation to chilling at 4°C (Section 5.3.6). Although there was an initial increase in the activity of glutathione reductase (Figure 5.5), possibly due to the action of the ascorbate-glutathione cycle (Foyer and Halliwell, 1976; Halliwell *et al.*, 1981), a decline was noted in the activity of ascorbate peroxidase (Figure 5.4). The cause of this decline is unknown, but could be postulated to be due to a decrease in the recognised electron donor for ascorbate peroxidase, ascorbate (vitamin C). This reasoning would account for the increase in glutathione reductase activity, to replenish the amount of ascorbate available to ascorbate peroxidase, thus increasing the plants' tolerance to chilling temperatures. This is indicated by a return of the activities of both enzymes to control levels by day 8 of the study (Section 5.3.6; Figures 5.4 and 5.5). Although the results obtained by competitive RT-PCR for ascorbate peroxidase gene expression studies could only be used for comparison between sampling days and treatments, it was shown that the changing amounts of mRNA in tissues were reflected in the activity of the enzyme (Figure 5.7; Section 5.3.7). These results suggest that gene expression may control the role of ascorbate peroxidase in chilling stress defence.

Given that the results were interpreted as *R. ponticum* micropropagule plants having a reduced ability to acclimate, a second study of plants

exposed to 20°C and 2°C was performed (Section 5.3.8). Initial interpretations were substantiated as it was noted throughout the duration of the study that the micropropagules behaved similarly to chilling sensitive plant species (Figures 5.8 and 5.9). The changes in ascorbate peroxidase (both enzymatic and molecular) and glutathione reductase in this study were characteristic of non-acclamatory behaviour. Close correlations with results from Fadzilla *et al* (1996) and Polle *et al* (1996) studying responses of chilling sensitive rice and young pine seedlings respectively, were recognised.

Rhododendron species are known to survive temperatures as low as -25°C in their natural habitats (Sakai *et al.*, 1981), but may not have the ability to do so *in vitro*. Micropropagated plantlets, like young shoots in mature plants, are comprised of soft leaf and stem tissue, and may be expected to be victim to chilling and freezing stress because of a sensitivity to lipid peroxidation, which is closely linked to oxidative stress mechanisms (Burdon *et al.*, 1994). Physical differences in mature and micropropagule *Rhododendron* samples may also contribute to such differences in acclamatory behaviour.

6.3 General conclusions

Rhododendron species maintained by micropropagation provided a useful tool for the study of responses to chilling temperatures. A growth medium which allowed straightforward multiplication and maintenance of plant material, and high quality genomic DNA and total RNA extracts, could potentially be used in controlled environment studies. However, as ultimate stress responses indicated, *Rhododendron* micropropagules cannot be used as a model to illustrate acclamatory responses in mature plants.

Rhododendron ponticum is a plant species well known to survive many habitat extremes, and indeed is often regarded as a weed due to its robust characteristics. It is perhaps surprising then that such a species should not have the ability to acclimate to chilling temperatures of 2°C when grown *in vitro*. It could be postulated that the artificial environment of micropropagation has led to some losses in acclamatory behaviour, at a physical, physiological and gene expression level. Time constraints due to the development of the molecular assay prevented further investigation into observed plant responses.

Regarding the molecular assay, the optimisation of each of the reactions involved in its development to a quality suitable for progression to the next stage were time consuming, and so refinement of competitive PCR to study ascorbate peroxidase mRNA changes in plants exposed to 20°C and 4°C or 2°C was restricted. Such a study as this would merit further development of the RT-PCR assay, a probable initial improvement being the quality of total RNA extracts used in the reaction, a factor unable to be improved upon here because of time and facility restraints. However, with the information retrieved from the assay, in conjunction with physiological data i.e. the activity of the antioxidant enzymes, it was

possible to obtain a clear indication of plant response when exposed to temperature extremes.

Future work in this area would benefit from improvements to develop a precise quantitative molecular assay for the study of antioxidative genes. The assay could be used to study a whole variety of stress responses including:

a) exposure to a lower, non-chilling temperature as a means of acclimation before exposure to known chilling temperatures,

b) responses of plants to deacclimatory temperatures,

c) responses of plants to a combination of high light/low temperature regimes

d) cross-resistance between drought and chilling stress in plant species.

Manipulation of the assay to study any number of plant species, at whatever stage of growth or development would increase current understanding of plant responses to oxidative stress.

6.4 Suggestions for further studies

1. Complete cloning and sequencing studies to yield full sequence data for both ascorbate peroxidase and glutathione reductase in *Rhododendron ponticum, R. hatsugiri and R. impeditum.*

2. Improve the quality of RNA isolation and further reduce the possibility of RNase cross-contamination by shared equipment.

3. Refine the Competitive RT-PCR assay by possible experimentation using *Tth* DNA polymerase, thus reducing the reaction to one step and possibly increasing reaction specificity.

4. Develop Competitive RT-PCR protocol for the study of glutathione reductase gene activity.

5. Compare responses of leaf tissue from mature *R. ponticum* plants and micropropagules when exposed to non-chilling and chilling conditions.

6. Expand the study to include comparisons between *in vitro*-grown and *in vivo*-grown plants of the three *Rhododendron* species initially studied.

Appendix 1

Media

LB Medium (Luria-Bertani) pH 7.0

1.0% (w/v) Bacto-tryptone	10 g
0.5% (w/v) Yeast Extract	5 g
1.0% (w/v) NaCl	10 g
1 Litre distilled water	

SOC Medium

2% (w/v) Tryptone
0.5% (w/v) Yeast Extract
10 mM NaCl
2.5 mM KCl
10 mM $MgCl_2.6H_2O$
20 mM Glucose

Anderson's Rhododendron Basal Salt Mixture(Sigma)

	mg/L
Ammonium Nitrate	400
Boric Acid	6.2
Calcium Chloride (Anhydrous)	332.2
Cobalt Chloride.$6H_2O$	0.025
Cupric Sulphate.SH_2O	0.025
Na_2-EDTA	74.5
Ferrous Sulphate.$7H_2O$	56.7
Magnesium Sulphate	180.7
Manganese Sulphate-H_2O	16.9
Molybolic Acid (Sodium Salt)-$2H_2O$	0.25
Potassium Iodide	0.3
Potassium Nitrate	480
Sodium Phosphate (Monobasic)	330.6

Anderson's Rhododendron Stage I Medium

Anderson's Rhododendron Basal Salt Mixture	1.89 g
Myo-Inositol	0.05 g
Adenine Hemisulphate	.0.4 g
6-γ-γ-Dimethylallylaminopurine	0.01 g
Indole-3-Acetic Acid	0.0025g
Sucrose	15 g
Agar	10 g
Distilled Water	1 litre

Anderson's Rhododendron Stage II Medium

Anderson's Rhododendron Basal Salt Mixture	1.89 g
Myo-Inositol	0.05 g
Adenine Hemisulphate	0.04 g
6-γ-γ-Dimethylallylaminopurine	0.08 g
Indole-3-Acetic Acid	0.001g
Sucrose	15 g
Agar	10 g
Distilled Water	1 litre

Appendix 2

Ascorbate Peroxidase and Glutathione Reductase DNA Sequences

i) DNA Sequence for *Pisum sativum* Ascorbate Peroxidase. Sequence regions highlighted in green represent exons coding for mRNA. Red areas within these represent APX primer set C used in PCR and subsequent experiments.

```
   1 atgataatat aggtcaagcc ttagccaaat tcaaaacaaa ttataaaata tttttattta
  61 ttttaaaata tttaatatga ataaatattt ttatgtaatg aacataatta taatttacgt
 121 tatcactcat tatttaaaaa ataaaataat aatttataag gttctacgaa aattgaatac
 181 attgtactaa aacaaattga atagattatc tttaagaata atattttatc atttaaacat
 241 ctaaaaaaaa taaaaataaa gttggcattt atcaataatt aattaatact aatattaatt
 301 ttaaaataaa aaaactaaga ataatttatt atcaatataa ataatttttac atcattattt
 361 aatcaaatta aaatatttcg tcacgtcata ttaatatttt aaaatcaata tgctagtata
 421 acttaataga atatatattc gtaattaatt tataatataa aaatatttta tattatcaca
 481 tttttcttat ctaaataaga ataaaaatat tgtatgcacg actttctttt ccgaaataat
 541 acaaatacaa tattactata gtcaacaata gggctctgtc ataactcatc acgcaacgaa
 601 acccgttttc acgcattcaa aaactcacga cctttcgaat ttatatttct taattattcc
 661 attattactc catttctctc acttctctca ttcgtcgttc acgaaaccta cctcacctac
 721 ccaataatcc acacactgtg aatttagtca tttacacgtg tagattcatc ttaacattca
 781 actctccgaa tcaacaataa ccgtacgatt ccaaccatcc aacgtctcac agtaaagcta
 841 ctagaacttt ctcctcttcc ttcctcttta tattacgtgt tcttcatttc taacacacgc
 901 tcttcactct tggcttgtgc tctcctcgtg tcactagggt ttaacttctt cgtttttgct
 961 tcttagattt cgaggttcga acgtttcgct tttgattcag ctttttttctt cgtcgtaggg
1021 attgttaata ctcgaattgc agttgattat tttctagaat catttttttt agattagatc
1081 catgttttga tcttgttttt gcttcgattt attggtatac gcgatgatct gattctgttt
1141 gtgatgtatg gatttttgtt tgattttgct gtagagtcgt ttgctatggg aaaatcatac
1201 ccaactgtta gtcccgatta ccagaaggcc attgaaaagg ctaagaggaa gctcagaggt
1261 tttatcgctg agaagaaatg cgctcctcta attctccgtt tggcgtaagt tttgttatct
1321 gcaatttttt tatagaatcg ttgatttgtg agattttgat tttttgaatt gtctgtgttt
1381 aagtttttga gtgatttact ttgttgatga tttgtttgtg atattttatt tgcgatggtt
1441 ttgttttgtg tagatggcac tctgctggta cttttgattc caagacaaag actggtggtc
1501 ctttcggaac aattaagcac caagccgagc ttgctcatgg tgctaacaac ggtcttgata
1561 tcgcggttag gctgttggag cctattaagg agcaattccc tattgtgagc tatgctgatt
1621 tctaccaggt tggtaatttt tgtgtgtgtt tagtttttag atttgaattt atgtggttgt
1681 tcaatttttg tgatcatgtg gttgatggtt tattttaata cgtaacgcag ttggctggtg
1741 ttgttgctgt tgagattacc ggtggacctg aagttccttt ccaccctggt agggaggtat
1801 gtttgaccac aactatcgct tttgtcttca aatctaattt acatgattag taaatcaatt
1861 attgggtatc acttttttcg gttatataat gattggattc atgttggtgg gtacctttt
1921 ttttaaagaa tagtgtatgt ttaattttta tatcatgttc ggacattagt ttgtaagctt
1981 tgatatttgt cacttttttgg tgccttctgg ttttcaagaa tttccattgg ttacataatt
2041 gcggtcagaa tcacaacaat taatctaatg tgatggaata attggaaatg cttttcttac
2101 atgttttact aaaatgtatg taaagtgtgg ttatattatt ttacacagtt gttgatgatg
2161 tattctttat ctttttttac tcagtttttc agatagttga agctataaca gtccttttgt
2221 tttgttttca tatcaggaca agcctgagcc accacctgag ggtcgcttgc ctgatgccac
2281 taagggtcag tgatctgatt tgtgatgtga attaatctat atgattgatg tatttatttg
2341 tctaaggatt tgattcttga ttattattgc aggttctgac catttgaggg atgtgtttgg
2401 aaaggctatg gggcttagtg atcaggacat tgttgctcta tctggtggtc acaccattgt
2461 atgtcataac tttaagctcg ctcctacttt tattgtagta ttaataaaac cattaatatt
2521 gagatttgca taatcatctg cattatgatt gtgcaattca ggatgtggat gttaatatta
2581 ctactactaa tggggctgga atatcaaaag cttgatactt ctaactgtat aattcagctg
2641 acttttgtgt aaatgttata tatgataatg tatttttgat gctctgggct taactctttc
2701 ggaatagctt aatgtaattc ttagggagct gcacacaagg agcgttctgg atttgaggga
2761 ccatggactt ctaatcctct catttttgac aactcatatt tcacgtaagt cttctaaaac
2821 attatctttc aaccatgcca ccttttatct attttataaa tctcttcatt gacaacatta
2881 ttaataaatg tatagtgagt tgttgactgg tgagaaggat ggccttcttc agttgccaag
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2941 tgataaggct cttttgactg actctgtatt ccgccctctt gttgagaaat atgctgcggt
3001 aagtatattt ctatatttct attctatgtg aaattataat gtctactgta aaggtaataa
3061 aaataactgt catctgtcaa tgtttacagg atgaagatgt tttctttgct gattatgctg
3121 aagcacatct taagctctct gagcttgggt aggtcatttt atcaatttaa ctttggagct
3181 tatgaagaat atacttgttt atagagagtt gattcatttt tatgtatttt ttaccttgca
3241 gatttgctga agcctaagtc acagttgttt ggtgtttaga gaggagcact gtcctgaatc
3301 ttacataaat ttcatagacg ttgcttttat tttcaatgtg gattcatctt agttgggtag
3361 cattttggat gtattttgga agtttgattg ttttctctat tgttgatcct tggttaaata
3421 acattgttaa gtggtaatgc ccagctattg cattttcctg ataattactg gttttattct
3481 tattttgcgg gtgaaatttc atctaaagtg agccatgtat ttagtttaga ttaaaatttt
3541 aaacatcatc cgttgataaa cttcaaatgt atgatatttc atttcttatc tcccatgctt
3601 cagaaatggt gaaaactcta ttatt

ii) DNA sequence for *Arabidopsis thaliana* glutathione reductase. Sequence regions highlighted in green represent exons coding for mRNA. Red areas within these represent GR primer set A and GRa and GRb used in PCR and subsequent experiments.

```
   1 tggccattta aaacatgaac taaacgttga ctaccattta agacataaaa atatcgttga
  61 ccagaccaaa atagacatgt ctttatcagt agaacctaga aacccgttaa taactgttaa
 121 tctgtccaaa acaacgtcgt ttgagaataa tcgaaagaac ataaagattt ttcccatatg
 181 aaatctccat atcaaaaagt tttaagtaga acccaagaac atagtttgat aaagagataa
 241 tcgcagtgtc agtaattgga agcaaataaa ggtattttcg tttatatatt tgttttaaat
 301 ttgaaattga atcatggaag caaattgaag actcattata agattgttcg tcttgtttga
 361 gcggttgtag aaatagattt ggggaactgg ggtttcattt agaaaacaat cctgattttc
 421 ttttgattat tctcaaaaca aagtcgtttt agggatacta atagagtcgt taacaagtga
 481 taatgaatgt tataagttct tttaatgaat gatgacaaca tgtttatttt ggcttggtca
 541 acgaatttct catgttttaa atggtagtca acgcttagtt catgtttaaa attgccacat
 601 ataaagtcga tgtttaaatt gaccacaatt taaaacttga ctaataaagt cgttaacgga
 661 tgataacgga cgttagaagt tccgttaatg actgataacg acatgtctat tttggcatga
 721 tcaacgaata atcatgtttt aaaagttagt caacatttaa ttcatgtttt aaacagccgc
 781 atacgaaatt gatgtataaa atgaccattt tttgaaagtt aggagattaa ttgatatttt
 841 tccctttata gtttacaggt acatacgttg ataacacaaa tcgtataact ttctaacggt
 901 acagaaaata gtaaaccgta caaaaataat aaaaaagggt aagatgtaga agtataaaac
 961 taatagtaca aattaaaaga ttttggttcc acttgtatgt ataagtgatt acatcaaaat
1021 ggtattacta agtcatcatt tagatttgat gaagtttgta ttcgtgtcat aaaccgggcg
1081 taggtggagt tattgtcagt ttggtagcgt tttacgacca cacataaaaa catgtttata
1141 aattgttaaa aaacaataag acatgtcata aatctaaatg ccacaagtag aattatcgaa
1201 ataaagcttt tacataaata cacacaccca aactaaaaaa gaaaaaaaaa agaaagagag
1261 agagagaaga agaatcgtgg agatggcttc gaccccgaag cttaccagta caatttcatc
1321 atcttctcca tctcttcaat tcctctgcaa aaaactccca atcgcaattc atctaccatc
1381 atcttcttcc tctagctttc tctcgcttcc taaaacccta acctctctct attctctccg
1441 tccccgtatc gccctactct caaaccaccg ctattaccac tctcgccggt tttctgtttg
1501 tgccagtacc gataatggag ctgaatcaga ccgccactac gattttgatc tcttcactat
1561 cggtgccgga agcggcggcg tccgcgcctc tcgcttcgcc actagcttcg gtgcatccgc
1621 cgccgtttgc gagcttcctt tttccactat ctcttccgat actgctggag gcgttggagg
1681 aacgtaagat ttcttccttc ttgtggctta tatcgatagc tagtgaattt gactttagta
1741 gtagctatac cagaatttga agcatttgct tgatgattta tggtttgatt gtgttgtcta
1801 ctcatttttg gttattttgc aggtgtgtat tgagaggatg tgtaccaaag aagttacttg
1861 tgtatgcatc caaatacagt catgagtttg aagacagtca tggatttggt tggaagtatg
1921 agactgagcc ttctcatgat tggactactt tgattgctaa caagaatgct gagttacagc
1981 ggttgactgg tatttataag aatatactga gcaaagctaa tgtcaagttg attgaaggtc
2041 gtggaaaggt atgccgaggc tcggtgttta tatagttcat gagttaacct ctatgtattg
2101 atttgagctt gagtatgtat tttatatgtg taggttatag acccacacac tgttgatgta
2161 gatgggaaaa tctatactac gaggaatatt ctgattgcag ttggtggacg tcctttcatt
2221 cctgacattc caggaaaaga gtttgctatt gattctgatg ccgcgcttga tttgccttcc
2281 aagcctaaga aaattgcaat agttggtggt ggctacatag ccctggagtt tgcggggatc
2341 ttcaatggtc ttaactgtga agttcatgta tttataaggc aaaagaaggt gctgagggga
2401 tttgatgaag atgtaagtaa aaaattgaga atgacttttg ctgctttcat ttatccgtct
2461 ggatattttc taattgtaat tgttattctt gtttcaggtc agggatttcg ttggagagca
2521 gatgtcttta agaggtattg agtttcacac tgaagaatcc cctgaagcca tcatcaaagc
2581 tggagatggc tcgttctctc tgaagaccag caagggaact gttgagggat tttcgcatgt
2641 tatgtttgca actggtcgca agcccaacac aaaggtatag tgtgattttt attattttcc
2701 aaaggatgtt tataaaatga aaactggtag caaagttcat cctttaataa tttgtctaag
2761 ataaactgac cactgctgcc ccttgtttat gactttaatg gaatatgatt gttcattgat
2821 gtaatgttgg ctgttataac agaacttagg gttggagaat gttggcgtta aaatggcgaa
2881 aaatggagca atagaggtat gatccagtgt tttacctagt ttttttgtca cttaggattt
```

2941 ctattattgc attgtgcaca gttctcttgt tatacctggt tacttcttgt gattgtgctc
3001 catgtttcca ctttttttt gtaaggttga cgaatattca cagacatctg ttccatccat
3061 ctgggctgtt ggggatgtta ctgaccgaat caatttgact ccagttgctt tgatggaggg
3121 aggtgcattg gctaaaactt tgtttcaaaa tgagccaaca aagcctgatt ataggtaaca
3181 taacaatctt aacttttgat gtttaaacct ttgggatttt ttggtgattg atgagagttt
3241 ttctctgttt agtcttacat gctgcatcag tgatctataa cattttataa ctgttgtctc
3301 tgatggcttt tatcctttct gtgttctata atcaactaga gctgttccct gcgccgtttt
3361 ctcccagcca cctattggaa cagttggtct aactgaagag caggtaagta ggatgatttg
3421 tcaaaaatat ggtttatcat gcatgccact acatagaaat attaactacg tgtttgcagg
3481 ccatagaaca atatggtgat gtggatgttt acacatcgaa ctttaggcca ttaaaggcta
3541 ccctttcagg acttccagac cgagtattta tgaaactcat tgtctgtgca aacaccaata
3601 aagttctcgg tgttcacatg tgtggagaag attcaccaga aatcatccag gtttcatttt
3661 ttaacatgat tggtattctc tgaaaagtaa tcacggaaaa catatatgat tgaatatgaa
3721 ttttatttta caatttttca gggatttggg gttgcagtta aagctggttt aactaaggcc
3781 gactttgatg ctacagtggg tgttcacccc acagcagctg aggagtttgt cactatgagg
3841 gctccaacca ggaaattccg caaagactcc tctgaggttt gtttgtgaac agtcaaaact
3901 tatccatcta gttatttctc gtggttttgc aacatgggaa aatgattgga actgagtgag
3961 atgtgtatgt ccaatttcat ttcagggaaa ggcaagtcct gaagctaaaa cagctgctgg
4021 ggtgtagaga aggttgcaaa aaagattgta tttacggcat tggagccccc tgataaggta
4081 ttcattcacc agtttccgaa gtattggtcc taattcctaa aattagaaaa gacatgcaat
4141 gcattagctc ataccatgct taagtaagat gtcaattagg gccaaagctt tgtttctgcc
4201 caccccggaa aaatagggta gactagaatt ctcctccata acggtgtttg atatttgact
4261 tgcatgtttg caggagaaca ttgtgtctgt gaagaagaag cttttctttt ggcagagaat
4321 atttttttcat aatgtcgtgt gaagatatga tcgctgtcct gcaatacttt tgatcgtttg
4381 acacacgacc aaattcctcg agagagagag gttgtaacta ttccgaaaga gctctaaaac
4441 tgaagctttc agttcttgtg acatcataat gaaggctgta tagatataac agtttcgagt
4501 tttatttgga tttgtagtat ttttgttttc tattgtgttg aacttgtctc tttgaagtca
4561 ccacgggaga cctcatatca gcatcgttgc gaatcaaatg aggttttgag ttttgacaag
4621 aaattcaaca cgatcaccaa atccttgagt cattatcata ttcagatttc agagtctcct
4681 accataatca tatttatggt tcgactgtac attattactg tttaaaattg atttgatttt
4741 tgaaaagtta aaaactaata gttgactaaa aaattcataa acaaatatat acttttttaaa
4801 gagatacgtt ttgattaata acaaaaagga aatatataca ttaagtatta taattaatat
4861 acttatataa ctaaaaatca ctattataag taacaatatc tctaaaactt tgaatggcca

Appendix 3

BLAST Search Results of Unsuccessful Cloning Attempts

i)APX 1 forward sequence BLAST homology - first ten results.

Sequences Producing Significant Alignments	*Score (bits)*	*E Value*
E.coli plasmid pMM234 DNA	*4.4e-206*	*6*
Artificial DNA. Bicistronic eukaryot..	*6.0e-205*	*5*
Artificial DNA; bicistronic eukaryot...	*9.0e-205*	*5*
Cloning vector pCI-neo, mammalian ex...	*1.1e-204*	*5*
Cloning vector pTet-Off, complete se...	*5.0e-204*	*5*
Cloning vector pTet-On, complete seq...	*5.0e-204*	*5*
Cloning vector pSV2neo aminoglycosid...	*6.6e-204*	*5*
pRSVNeo cloning vector for high effi...	*7.7e-204*	*5*
Cloning vector pSV2neo aminoglycosid...	*8.6e-204*	*5*
Cloning vector cosmid pTCF DNA seque...	*4.0e-203*	*5*

ii)APX 1 reverse sequence BLAST homology - first ten results.

Sequences producing significant alignments:	*Score (bits)*	*E Value*
Vibrio cholerae beta-galactosidase (lacZ)	*2e-25*	
Uncultured gamma proteobacterium SUR-ATT-2...	*2e-25*	
Mus musculus Ampd3 gene, exon 1, partial s...	*2e-25*	
Homo sapiens DNA for p58 NK receptor gene	*2e-25*	
Mus musculus proapoptotic protein (Siva) g...	*2e-25*	
Uncultured bacterium OS9F 16S ribosomal RN...	*2e-25*	
Dengue virus type 3 DOH 33 nonstructural pro...	*2e-23*	
Dengue virus type 3 SLMC 54 nonstructural pr...	*2e-23*	
Uncultured bacterium OS9C 16S ribosomal RN...	*2e-23*	
Hevea brasiliensis mRNA for chitinase	*2e-23*	

iii) APX 2 forward sequence BLAST homology - first ten results

Sequences producing significant alignments:	*Score (bits)*	*E Value*
Anopheles gambiae mRNA for infection respo...	*2e-22*	
Mus musculus proteinase-3 and neutrophil...	*2e-22*	
Picea jezoensis internal transcribed sequence 1, 5...	*4e-21*	
Sorghum bicolor var. White Martin gene enco...	*4e-21*	
Babesia bovis strain Ur rhoptry associated...	*4e-21*	
Uncultured bacterium OS9F 16S ribosomal RN...	*2e-20*	
Haemophilus influenzae 16S ribosomal RNA g...	*2e-20*	
Homo sapiens D15S1506 ca repeat region, co...	*6e-20*	
Mus musculus Clone pad69, Complete Sequenc...	*6e-20*	
Uncultured bacterium 1A 16S ribosomal RNA ...	*6e-20*	

iv) APX 2 reverse sequence BLAST homology - first ten results

Sequences producing significant alignments:	*Score (bits)*	*E Value*
Mus musculus Ampd3 gene, exon 1, partial s...		*2e-29*
Homo sapiens DNA for p58 NK receptor gene		*2e-29*
Vibrio cholerae beta-galactosidase (lacZ) ...		*2e-29*
Uncultured bacterium OS9F 16S ribosomal RN...		*2e-29*
Uncultured gamma proteobacterium SUR-ATT-2...		*2e-29*
Mus musculus proapoptotic protein (Siva) g...		*2e-29*
Uncultured bacterium OS9C 16S ribosomal RN...		*3e-28*
Dengue virus type 3 DOH 33 nonstructural pro...		*3e-28*
Dengue virus type 3 SLMC 54 nonstructural pr...		*3e-28*
Hevea brasiliensis mRNA for chitinase		*1e-27*

v)APX 3 forward sequence BLAST homology - first ten results

Sequences producing significant alignments:	*Score (bits)*	*E Value*
Uncultured bacterium OS9E 16S ribosomal RN...		*4e-18*
Rattus norvegicus melanocortin-4 receptor mR...		*4e-18*
Sorghum bicolor var. White Martin gene enco...		*4e-18*
Haemophilus influenzae 16S ribosomal RNA g...		*4e-18*
Cloning vector pcDNA3ZEO DNA		*4e-18*
Uncultured bacterium OS9C 16S ribosomal RN...		*4e-18*
Rattus norvegicus clone for microsatellite...		*4e-18*
Uncultured bacterium OS9F 16S ribosomal RN...		*4e-18*
Alu 2 region-T-cell receptor J delta 1 fusion ...		*4e-18*
Papio hamadryas plasminogen (BABPEPSG) mRN...		*4e-18*

vi) APX 3 reverse sequence BLAST homology - first ten results

Sequences producing significant alignments:	*Score (bits)*	*E Value*
Uncultured bacterium OS9F 16S ribosomal RN...		*2e-26*
Mus musculus Ampd3 gene, exon 1, partial s...		*2e-26*
Uncultured gamma proteobacterium SUR-ATT-2...		*2e-26*
Homo sapiens DNA for p58 NK receptor gene		*2e-26*
Vibrio cholerae beta-galactosidase (lacZ) ...		*2e-26*
Mus musculus proapoptotic protein (Siva) g...		*6e-26*
Sphenostylis stenocarpa class III chitin...		*4e-24*
Hevea brasiliensis mRNA for chitinase		*4e-24*
Dengue virus type 3 SLMC 54 nonstructural pr...		*2e-23*
Dengue virus type 3 DOH 33 nonstructural pro...		*2e-23*

1)GR 1 forward sequence BLAST homology - first ten results

Sequences producing significant alignments:	*Score (bits)*	*E Value*
Sorghum bicolor var. White Martin gene enco...		*1e-27*
Uncultured bacterium OS9E 16S ribosomal RN...		*1e-27*
Uncultured bacterium 1A 16S ribosomal RNA ...		*1e-27*
Anopheles gambiae mRNA for infection respo...		*1e-27*
Homo sapiens D15S1506 ca repeat region, co...		*1e-27*
Homo sapiens asthmatic clone 1 mRNA, 3' UTR		*1e-27*
Papio hamadryas plasminogen (BABPEPSG) mRN...		*1e-27*
Amoeba proteus symbiotic bacterium macro...		*1e-27*
Mus musculus proteinase-3 and neutrophil...		*1e-27*
Haemophilus influenzae 16S ribosomal RNA g...		*2e-26*

ii) GR 1 reverse sequence BLAST homology - first ten results

Sequences producing significant alignments:	*Score (bits)*	*E Value*
Cloning vector pSG930, HIS4-based plasmid, c...		*1e-27*
Mus musculus proteinase-3 and neutrophil...		*1e-27*
P.capsici gene for cutinase		*7e-26*
Expression vector pCOR116aN		*1e-18*
Cloning vector pSG928, HIS4-based plasmid,		*1e-18*
Cloning vector pSG929, HIS4-based plasmid,		*1e-18*
Calonectris diomedea random amplified polymorph		*1e-18*
Cloning vector pZeRO-2T		*4e-18*
Cloning vector pKIL PCR-2		*4e-18*
Expression vector pB5T-MRz, complete cds		*7e-17*

iii) GR 4 forward sequence BLAST homology - first ten results

Sequences producing significant alignments:	*Score (bits)*	*E Value*
Vibrio cholerae beta-galactosidase (lacZ) ...		*6e-39*
Mus musculus Ampd3 gene, exon 1, partial s...		*6e-39*
Uncultured gamma proteobacterium SUR-ATT-2...		*2e-38*
Homo sapiens DNA for p58 NK receptor gene		*3e-28*
Uncultured bacterium 1A 16S ribosomal RNA ...		*1e-27*
Papio hamadryas plasminogen (BABPEPSG) mRN...		*1e-27*
Uncultured bacterium OS9E 16S ribosomal RN...		*1e-27*
Anopheles gambiae mRNA for infection respo...		*1e-27*
Amoeba proteus symbiotic bacterium macro...		*1e-27*
Homo sapiens asthmatic clone 1 mRNA, 3' UTR		*1e-27*

iv) GR 4 reverse sequence BLAST homology - first ten results

Sequences producing significant alignments:	*Score (bits)*	*E Value*
Mus musculus proteinase-3 and neutrophil..		*6e-45*
Cloning vector pSG930, HIS4-based plasmid, c...		*9e-44*
P.capsici gene for cutinase		*9e-44*
Cloning vector pZeRO-2T		*1e-36*
Cloning vector pKIL PCR-2		*5e-36*
Calonectris diomedea random amplified polym...		*1e-24*
Cloning vector pSG929, HIS4-based plasmid, c...		*1e-24*
Cloning vector pSG928, HIS4-based plasmid, c...		*1e-24*
Expression vector pCOR116aN (modified from...		*1e-24*
Cloning vector pKMZB containing zero-backgro...		*4e-24*

Appendix 4

Glutathione Reductase Enzyme Assay
NADPH Concentration Calculations

150uM NADPH	
Mean	10.48
Standard Error	2.8068161
Median	9.425
Standard Deviation	5.6136322
Sample Variance	31.512867
Kurtosis	1.9440727
Skewness	1.057619
Range	13.37
Minimum	4.85
Maximum	18.22
Sum	41.92
Count	4
Confidence Level (95.0%)	8.9325499

175uM NADPH	
Mean	9.2075
Standard Error	3.1125241
Median	8.79
Standard Deviation	6.225048
Sample Variance	38.75122
Kurtosis	0.020185
Skewness	0.355132
Range	14.77
Minimum	2.24
Maximum	17.01
Sum	36.83
Count	4
Confidence Level (95.0%)	9.9054501

200uM NADPH	
Mean	10.7775
Standard Error	1.9731801
Median	10.015
Standard Deviation	3.9463601
Sample Variance	15.573758
Kurtosis	0.5857404
Skewness	0.9622181
Range	9.12
Minimum	6.98
Maximum	16.1
Sum	43.11
Count	4
Confidence Level (95.0%)	6.2795455

225um NADPH	
Mean	10.59
Standard Error	1.660441
Median	9.85
Standard Deviation	3.320883
Sample Variance	11.02826
Kurtosis	1.5
Skewness	1.159960
Range	7.72
Minimum	7.47
Maximum	15.19
Sum	42.36
Count	4
Confidence Level (95.0%)	5.2842715

250uM NADPH	
Mean	9.3325
Standard Error	0.8352283
Median	9.71
Standard Deviation	1.6704565
Sample Variance	2.790425
Kurtosis	2.4170699
Skewness	-1.2635884
Range	3.95
Minimum	6.98
Maximum	10.93
Sum	37.33
Count	4
Confidence Level (95.0%)	2.6580716

Appendix 5

Experiment 1 and 2

Enzyme Statistical Data &

RT-PCR Percentage Calculations

i)Raw data for ascorbate peroxidase enzyme activity experiment 1: a comparison of plants held at 20°C and 4°C. Values were derived from the formula stated in section 5.2

20oC		4oC	
0	169	0	478.8
0	367	0	434.8
0	327	0	459.2
0	*	0	*
2	425.2	2	178.5
2	526.3	2	219.8
2	288.2	2	284.3
2	297.6	2	202.4
4	235.9	4	191.3
4	312.5	4	252.9
4	200	4	107.1
4	412.1	4	154.8
6	125.7	6	248.2
6	444.5	6	175.9
6	246.7	6	265.5
6	163.7	6	114.7
8	92.8	8	139.9
8	200	8	107.7
8	255	8	252.7
8	315.6	8	232.1
10	457.8	10	167
10	572.9	10	175.4
10	446.4	10	134.7
10	454.5	10	91.2
14	717.2	14	386.7
14	907.9	14	451.5
14	731.3	14	361.9
14	698.8	14	336.9

ii) Descriptional statistics for ascorbate peroxidase enzyme activity, experiment 1: a comparison of plants held at 20°C and 4°C.

Day0 20 °C	
Mean	287.66667
Standard Error	60.446487
Median	327
Standard Deviation	104.69639
Sample Variance	10961.333
Skewness	-1.451986
Range	198
Minimum	169
Maximum	367
Sum	863
Count	3
Confidence Level (95.0%)	260.08042

Day0 4°C	
Mean	457.6
Standard Error	12.726874
Median	459.2
Standard Deviation	22.043593
Sample Variance	485.92
Skewness	-0.324904
Range	44
Minimum	434.8
Maximum	478.8
Sum	1372.8
Count	3
Confidence Level (95.0%)	54.759359

Day 2 20°C	
Mean	384.325
Standard Error	56.707515
Median	361.4
Standard Deviation	113.41503
Sample Variance	12862.969
Skewness	0.6350161
Range	238.1
Minimum	288.2
Maximum	526.3
Sum	1537.3
Count	4
Confidence Level (95.0%)	180.46879

Day 2 4°C	
Mean	221.25
Standard Error	22.657394
Median	211.1
Standard Deviation	45.314788
Sample Variance	2053.43
Skewness	1.1879801
Range	105.8
Minimum	178.5
Maximum	284.3
Sum	885
Count	4
Confidence Level (95.0%)	72.106007

Day 4 20°C	
Mean	290.125
Standard Error	46.940927
Median	274.2
Standard Deviation	93.881854
Sample Variance	8813.8025
Kurtosis	-0.789237
Skewness	0.752871
Range	212.1
Minimum	200
Maximum	412.1
Sum	1160.5
Count	4
Confidence Level (95.0%)	149.38712

Day 4 4°C	
Mean	176.525
Standard Error	30.745254
Median	173.05
Standard Deviation	61.490507
Sample Variance	3781.0825
Kurtosis	-0.087306
Skewness	0.2978087
Range	145.8
Minimum	107.1
Maximum	252.9
Sum	706.1
Count	4
Confidence Level (95.0%)	97.845211

Day 6 20°C	
Mean	245.15
Standard Error	71.089867
Median	205.2
Standard Deviation	142.17973
Sample Variance	20215.077
Kurtosis	1.401912
Skewness	1.316914
Range	318.8
Minimum	125.7
Maximum	444.5
Sum	980.6
Count	4
Confidence Level (95.0%)	226.2399

Day 6 4°C	
Mean	201.075
Standard Error	34.720032
Median	212.05
Standard Deviation	69.440064
Sample Variance	4821.9225
Kurtosis	-2.285834
Skewness	-0.574041
Range	150.8
Minimum	114.7
Maximum	265.5
Sum	804.3
Count	4
Confidence Level (95.0%)	110.49474

Day 8 20°C	
Mean	215.85
Standard Error	47.324509
Median	227.5
Standard Deviation	94.649018
Sample Variance	8958.4367
Kurtosis	0.2345942
Skewness	-0.640474
Range	222.8
Minimum	92.8
Maximum	315.6
Sum	863.4
Count	4
Confidence Level (95.0%)	150.60785

Day 8 4°C	
Mean	183.1
Standard Error	35.114764
Median	186
Standard Deviation	70.229528
Sample Variance	4932.1867
Kurtosis	-4.588637
Skewness	-0.104854
Range	145
Minimum	107.7
Maximum	252.7
Sum	732.4
Count	4
Confidence Level (95.0%)	111.75096

Day 10 20°C	
Mean	482.9
Standard Error	30.095432
Median	456.15
Mode	#N/A
Standard Deviation	60.190863
Sample Variance	3622.94
Kurtosis	3.8787231
Skewness	1.9616317
Range	126.5
Minimum	446.4
Maximum	572.9
Sum	1931.6
Count	4
Confidence Level (95.0%)	95.777185

Day 10 4°C	
Mean	142.075
Standard Error	19.092903
Median	150.85
Mode	#N/A
Standard Deviation	38.185807
Sample Variance	1458.1558
Kurtosis	-0.454351
Skewness	-0.952871
Range	84.2
Minimum	91.2
Maximum	175.4
Sum	568.3
Count	4
Confidence Level (95.0%)	60.762197

Day 14 20°C	
Mean	763.8
Standard Error	48.491941
Median	724.25
Standard Deviation	96.983882
Sample Variance	9405.8733
Kurtosis	3.6378986
Skewness	1.8870204
Range	209.1
Minimum	698.8
Maximum	907.9
Sum	3055.2
Count	4
Confidence Level (95.0%)	154.32314

Day 14 4°C	
Mean	384.25
Standard Error	24.613868
Median	374.3
Standard Deviation	49.227736
Sample Variance	2423.37
Kurtosis	1.1041277
Skewness	1.0440772
Range	114.6
Minimum	336.9
Maximum	451.5
Sum	1537
Count	4
Confidence Level (95.0%)	78.332387

iii)Raw data for glutathione reductase enzyme activity experiment 1: a comparison of plants held at 20°C and 4°C. Values were derived from the formula stated in section 5.2

20oC		4oC	
0	32.3	0	48.4
0	36.9	0	23.9
0	21.5	0	30.5
0	40.3	0	40.3
2	23.3	2	105.9
2	148.4	2	105.9
2	92.2	2	212.9
2	*	2	*
4	115.7	4	136.9
4	30.9	4	131.6
4	76.8	4	73.7
4	*	4	146.6
6	106.4	6	109.9
6	31.5	6	32.6
6	140.1	6	83.2
6	58.3	6	63.9
8	134.4	8	43.6
8	103	8	161.3
8	93.6	8	105.9
8	*	8	*
10	48.2	10	27.8
10	63.1	10	25.8
10	166.8	10	30.2
10	*	10	16.7
14	209.7	14	90.8
14	161.2	14	61.2
14	179.2	14	11.6
14	44.4	14	19.1

iv) Descriptional statistics for glutathione reductase enzyme activity, experiment 1: a comparison of plants held at 20°C and 4°C.

Day 0 20°C	
Mean	32.75
Standard Error	4.092575
Median	34.6
Standard Deviation	8.185149
Sample Variance	66.99667
Kurtosis	1.028814
Skewness	-1.12098
Range	18.8
Minimum	21.5
Maximum	40.3
Sum	131
Count	4
Confidence Level (95.0%)	13.02441

Day 0 4°C	
Mean	35.775
Standard Error	5.390636
Median	35.4
Standard Deviation	10.78127
Sample Variance	116.2358
Kurtosis	-2.03154
Skewness	0.15088
Range	24.5
Minimum	23.9
Maximum	48.4
Sum	143.1
Count	4
Confidence Level (95.0%)	17.15543

Day 2 20°C	
Mean	87.96667
Standard Error	36.17524
Median	92.2
Standard Deviation	62.65735
Sample Variance	3925.943
Skewness	-0.30265
Range	125.1
Minimum	23.3
Maximum	148.4
Sum	263.9
Count	3
Confidence Level (95.0%)	155.6496

Day 2 4°C	
Mean	141.5667
Standard Error	35.66667
Median	105.9
Standard Deviation	61.77648
Sample Variance	3816.333
Skewness	1.732051
Range	107
Minimum	105.9
Maximum	212.9
Sum	424.7
Count	3
Confidence Level (95.0%)	153.4614

Day 4 20°C	
Mean	74.46667
Standard Error	24.50744
Median	76.8
Standard Deviation	42.44813
Sample Variance	1801.843
Skewness	-0.24661
Range	84.8
Minimum	30.9
Maximum	115.7
Sum	223.4
Count	3
Confidence Level (95.0%)	105.4471

Day 4 4°C	
Mean	122.2
Standard Error	16.46223
Median	134.25
Standard Deviation	32.92446
Sample Variance	1084.02
Skewness	-1.78478
Range	72.9
Minimum	73.7
Maximum	146.6
Sum	488.8
Count	4
Confidence Level (95.0%)	52.39021

Day6 20°C	
Mean	84.075
Standard Error	24.26535
Median	82.35
Standard Deviation	48.5307
Sample Variance	2355.229
Kurtosis	-2.57402
Skewness	0.143075
Range	108.6
Minimum	31.5
Maximum	140.1
Sum	336.3
Count	4
Confidence Level (95.0%)	77.22325

Day 6 4°C	
Mean	72.4
Standard Error	16.27672
Median	73.55
Standard Deviation	32.55344
Sample Variance	1059.727
Kurtosis	-0.1267
Skewness	-0.18677
Range	77.3
Minimum	32.6
Maximum	109.9
Sum	289.6
Count	4
Confidence Level (95.0%)	51.79984

Day 8 20°C	
Mean	110.3333
Standard Error	12.3355
Median	103
Standard Deviation	21.3657
Sample Variance	456.4933
Skewness	1.362576
Range	40.8
Minimum	93.6
Maximum	134.4
Sum	331
Count	3
Confidence Level (95.0%)	53.07539

Day 8 4°C	
Mean	103.6
Standard Error	33.99652
Median	105.9
Standard Deviation	58.8837
Sample Variance	3467.29
Skewness	-0.1755
Range	117.7
Minimum	43.6
Maximum	161.3
Sum	310.8
Count	3
Confidence Level (95.0%)	146.2753

Day 10 20°C	
Mean	92.7
Standard Error	37.29884
Median	63.1
Standard Deviation	64.60348
Sample Variance	4173.61
Skewness	1.628976
Range	118.6
Minimum	48.2
Maximum	166.8
Sum	278.1
Count	3
Confidence Level (95.0%)	160.4841

Day 10 4°C	
Mean	25.125
Standard Error	2.948835
Median	26.8
Standard Deviation	5.897669
Sample Variance	34.7825
Skewness	-1.45547
Range	13.5
Minimum	16.7
Maximum	30.2
Sum	100.5
Count	4
Confidence Level (95.0%)	9.384516

Day 14 20°C	
Mean	148.625
Standard Error	36.15472
Median	170.2
Standard Deviation	72.30944
Sample Variance	5228.656
Kurtosis	2.693627
Skewness	-1.54075
Range	165.3
Minimum	44.4
Maximum	209.7
Sum	594.5
Count	4
Confidence Level (95.0%)	115.0606

Day 14 4°C	
Mean	45.675
Standard Error	18.58455
Median	40.15
Standard Deviation	37.16911
Sample Variance	1381.543
Kurtosis	-2.93168
Skewness	0.484195
Range	79.2
Minimum	11.6
Maximum	90.8
Sum	182.7
Count	4
Confidence Level (95.0%)	59.1444

v) Data used to determine the percentage of cDNA amplified during RT-PCR to determine the expression of the Rhododendron gene encoding ascorbate peroxidase. Experiment 1: A comparison of plants held at 20°C and 4°C.

Day/Temp.	Control (C)	cDNA (D)	Background (B)	C-B	D-B	cDNA %age of control
0, 20oC	54.29	43.65	38.04	16.25	5.61	34%
0, 4oC	49.35	47.37	38.52	10.83	8.85	81%
2, no result						
4, 20oC	19.93	15.65	10.22	9.71	5.43	55%
4, 4oC	56.63	26.27	16.91	39.72	9.36	23%
4, 4oC	34.67	22.26	15.11	19.56	7.15	36%
6, 20oC	19.03	6.78	4.66	14.37	2.12	15%
6, 4oC	29.3	9.12	2.25	27.05	6.87	25%
8, 20oC	6.71	17.56	8.52	-1.81	9.04	499%
8 4oC	19.11	19.55	13.33	5.78	6.22	107%
10, 20oC	33.36	30.11	23.3	10.06	6.81	68%
10, 4oC	25.96	34.26	22.11	3.85	12.15	315%
10, 4oC	34.29	32.03	17.96	16.33	14.07	86%
14, 20oC	44.84	39.54	40.07	4.77	-0.53	-11%

vi) Raw data for ascorbate peroxidase enzyme activity experiment 2: a comparison of plants held at 20°C and 2°C. Values were derived from the formula stated in section 5.2

20oC		4oC	
0	158.1	0	297.6
0	228.2	0	334.3
0	178	0	374.5
2	260.6	2	255.1
2	263.9	2	339.3
2	285.7	2	322.2
4	133.2	4	188.4
4	67.8	4	151.8
4	209.5	4	244.8
6	189	6	154.2
6	137.2	6	230.4
6	249.4	6	312.6
8	262.9	8	417.7
8	375	8	714.2
8	428.6	8	484.8
10	757.5	10	452.9
10	753.3	10	483.3
10	477.3	10	519.3
14	546.6	14	306.8
14	634.9	14	234.7
14	636.1	14	336.5

vii) Descriptional statistics for ascorbate peroxidase enzyme activity, experiment 2: a comparison of plants held at 20°C and 2°C.

Day 0 20°C	
Mean	188.1
Standard Error	20.856734
Median	178
Standard Deviation	36.124922
Sample Variance	1305.01
Skewness	1.1597884
Range	70.1
Minimum	158.1
Maximum	228.2
Sum	564.3
Count	3
Confidence Level (95.0%)	89.739344

Day 0 2°C	
Mean	335.46667
Standard Error	22.206781
Median	334.3
Standard Deviation	38.463273
Sample Variance	1479.4233
Skewness	0.1363683
Range	76.9
Minimum	297.6
Maximum	374.5
Sum	1006.4
Count	3
Confidence Level (95.0%)	95.548132

Day 2 20°C	
Mean	270.06667
Standard Error	7.8745017
Median	263.9
Standard Deviation	13.639037
Sample Variance	186.02333
Skewness	1.6186782
Range	25.1
Minimum	260.6
Maximum	285.7
Sum	810.2
Count	3
Confidence Level (95.0%)	33.88127

Day 2 2°C	
Mean	305.53333
Standard Error	25.695287
Median	322.2
Standard Deviation	44.505543
Sample Variance	1980.7433
Skewness	-1.448854
Range	84.2
Minimum	255.1
Maximum	339.3
Sum	916.6
Count	3
Confidence Level (95.0%)	110.55797

Day 4 20°C	
Mean	136.83333
Standard Error	40.945587
Median	133.2
Standard Deviation	70.919837
Sample Variance	5029.6233
Skewness	0.2299369
Range	141.7
Minimum	67.8
Maximum	209.5
Sum	410.5
Count	3
Confidence Level (95.0%)	176.17477

Day 4 2°C	
Mean	195
Standard Error	27.048845
Median	188.4
Standard Deviation	46.849973
Sample Variance	2194.92
Skewness	0.6213574
Range	93
Minimum	151.8
Maximum	244.8
Sum	585
Count	3
Confidence Level (95.0%)	116.38187

Day 6 20°C	
Mean	191.86667
Standard Error	32.421049
Median	189
Standard Deviation	56.154905
Sample Variance	3153.3733
Skewness	0.229123
Range	112.2
Minimum	137.2
Maximum	249.4
Sum	575.6
Count	3
Confidence Level (95.0%)	139.49661

Day 6 2°C	
Mean	232.4
Standard Error	45.737075
Median	230.4
Standard Deviation	79.218937
Sample Variance	6275.64
Skewness	0.1135368
Range	158.4
Minimum	154.2
Maximum	312.6
Sum	697.2
Count	3
Confidence Level (95.0%)	196.79089

Day 8 20°C	
Mean	355.5
Standard Error	48.817039
Median	375
Standard Deviation	84.553592
Sample Variance	7149.31
Skewness	-0.982605
Range	165.7
Minimum	262.9
Maximum	428.6
Sum	1066.5
Count	3
Confidence Level (95.0%)	210.04291

Day 8 2°C	
Mean	538.9
Standard Error	89.764822
Median	484.8
Standard Deviation	155.47723
Sample Variance	24173.17
Skewness	1.3762391
Range	296.5
Minimum	417.7
Maximum	714.2
Sum	1616.7
Count	3
Confidence Level (95.0%)	386.22713

Day 10 20°C	
Mean	662.7
Standard Error	92.707928
Median	753.3
Standard Deviation	160.57484
Sample Variance	25784.28
Skewness	-1.730717
Range	280.2
Minimum	477.3
Maximum	757.5
Sum	1988.1
Count	3
Confidence Level (95.0%)	398.8903

Day 10 2°C	
Mean	485.16667
Standard Error	19.190739
Median	483.3
Standard Deviation	33.239334
Sample Variance	1104.8533
Skewness	0.2519156
Range	66.4
Minimum	452.9
Maximum	519.3
Sum	1455.5
Count	3
Confidence Level (95.0%)	82.571141

Day 14 20°C	
Mean	605.86667
Standard Error	29.635358
Median	634.9
Standard Deviation	51.329946
Sample Variance	2634.7633
Skewness	-1.730985
Range	89.5
Minimum	546.6
Maximum	636.1
Sum	1817.6
Count	3
Confidence Level (95.0%)	127.51074

Day 14 2°C	
Mean	292.66667
Standard Error	30.224843
Median	306.8
Standard Deviation	52.350963
Sample Variance	2740.6233
Skewness	-1.1263307
Range	101.8
Minimum	234.7
Maximum	336.5
Sum	878
Count	3
Confidence Level (95.0%)	130.04709

viii) Raw data for glutathione reductase enzyme activity experiment 2: a comparison of plants held at 20°C and 2°C. Values were derived from the formula stated in section 5.2.

20oC		2oC	
0	220.7	0	209.7
0	264.1	0	92.8
0	*	0	*
2	111.2	2	49.8
2	39.1	2	119.4
2	32.6	2	45.8
4	77.4	4	31.9
4	62.8	4	33.5
4	92.2	4	16.6
6	436.3	6	151.2
6	838.7	6	232.9
6	120.1	6	186.1
8	50.9	8	47.9
8	34.7	8	33.5
8	161.3	8	299.5
10	630.5	10	93.2
10	645.2	10	460.8
10	552.9	10	362.9
14	399.4	14	45.2
14	665.3	14	50.4
14	529.9	14	5.62

ix) Descriptional statistics for glutathione reductase enzyme activity, experiment 2: a comparison of plants held at 20°C and 2°C.

Day 0 20°C	
Mean	242.4
Standard Error	21.7
Median	242.4
Standard Deviation	30.688434
Sample Variance	941.78
Range	43.4
Minimum	220.7
Maximum	264.1
Sum	484.8
Count	2
Confidence Level (95.0%)	275.72346

Day 0 2°C	
Mean	151.25
Standard Error	58.45
Median	151.25
Standard Deviation	82.660783
Sample Variance	6832.805
Range	116.9
Minimum	92.8
Maximum	209.7
Sum	302.5
Count	2
Confidence Level (95.0%)	742.67449

Day 2 20°C	
Mean	60.966667
Standard Error	25.186659
Median	39.1
Standard Deviation	43.624573
Sample Variance	1903.1033
Skewness	1.6888919
Range	78.6
Minimum	32.6
Maximum	111.2
Sum	182.9
Count	3
Confidence Level (95.0%)	108.36952

Day 2 2°C	
Mean	71.666667
Standard Error	23.894583
Median	49.8
Standard Deviation	41.386632
Sample Variance	1712.8533
Skewness	1.7138668
Range	73.6
Minimum	45.8
Maximum	119.4
Sum	215
Count	3
Confidence Level (95.0%)	102.81017

Day 4 20°C	
Mean	77.466667
Standard Error	8.4871144
Median	77.4
Standard Deviation	14.700113
Sample Variance	216.09333
Skewness	0.0204076
Range	29.4
Minimum	62.8
Maximum	92.2
Sum	232.4
Count	3
Confidence Level (95.0%)	36.517131

Day 4 2°C	
Mean	27.333333
Standard Error	5.3865058
Median	31.9
Standard Deviation	9.3297017
Sample Variance	87.043333
Skewness	-1.674918
Range	16.9
Minimum	16.6
Maximum	33.5
Sum	82
Count	3
Confidence Level (95.0%)	23.17628

Day 6 20°C	
Mean	465.03333
Standard Error	207.93885
Median	436.3
Standard Deviation	360.16065
Sample Variance	129715.69
Skewness	0.3567215
Range	718.6
Minimum	120.1
Maximum	838.7
Sum	1395.1
Count	3
Confidence Level (95.0%)	894.68927

Day 6 2°C	
Mean	190.06667
Standard Error	23.668005
Median	186.1
Standard Deviation	40.994187
Sample Variance	1680.5233
Skewness	0.4313508
Range	81.7
Minimum	151.2
Maximum	232.9
Sum	570.2
Count	3
Confidence Level (95.0%)	101.83528

Day 8 20°C	
Mean	82.3
Standard Error	39.775872
Median	50.9
Standard Deviation	68.893831
Sample Variance	4746.36
Skewness	1.624932
Range	126.6
Minimum	34.7
Maximum	161.3
Sum	246.9
Count	3
Confidence Level (95.0%)	171.14188

Day 8 2°C	
Mean	126.96667
Standard Error	86.366763
Median	47.9
Standard Deviation	149.59162
Sample Variance	22377.653
Skewness	1.7140122
Range	266
Minimum	33.5
Maximum	299.5
Sum	380.9
Count	3
Confidence Level (95.0%)	371.60645

Day 10 20°C	
Mean	609.53333
Standard Error	28.632868
Median	630.5
Standard Deviation	49.593582
Sample Variance	2459.5233
Skewness	-1.562428
Range	92.3
Minimum	552.9
Maximum	645.2
Sum	1828.6
Count	3
Confidence Level (95.0%)	123.19737

Day 10 2°C	
Mean	305.63333
Standard Error	109.91215
Median	362.9
Standard Deviation	190.37343
Sample Variance	36242.043
Skewness	-1.231165
Range	367.6
Minimum	93.2
Maximum	460.8
Sum	916.9
Count	3
Confidence Level (95.0%)	472.91415

Day 14 20°C	
Mean	531.53333
Standard Error	76.763063
Median	529.9
Standard Deviation	132.95752
Sample Variance	17677.703
Skewness	0.0552725
Range	265.9
Minimum	399.4
Maximum	665.3
Sum	1594.6
Count	3
Confidence Level (95.0%)	330.28503

Day 14 2°C	
Mean	33.74
Standard Error	14.139906
Median	45.2
Standard Deviation	24.491035
Sample Variance	599.8108
Skewness	-1.644621
Range	44.78
Minimum	5.62
Maximum	50.4
Sum	101.22
Count	3
Confidence Level (95.0%)	60.839146

x) Data used to determine the percentage of cDNA amplified during RT-PCR to determine the expression of the Rhododendron gene encoding ascorbate peroxidase. Experiment 2: A comparison of plants held at 20°C and 2°C.

Day	Control (C)	cDNA (D)	Background (B)	C-B	D-B	cDNA %age of control
0, no result						
2, 20oC	22.66	24.28	22.43	0.23	1.85	804%
4, 20oC	41.99	37.95	35.66	6.33	2.29	36%
4, 20oC	39.42	41.17	39.25	0.17	1.92	1129%
4, 20oC	36.67	39.36	36.07	0.6	3.29	548%
4, 20oC	27.99	27.38	25.84	2.15	1.54	71%
6, 20oC	37.89	48.42	40.12	-2.23	8.3	-372%
6, 2oC	36.13	56.27	36.69	-0.56	19.58	-3492%
8, 20oC	64.87	53.19	39.35	25.52	13.84	54%
8, 20oC	39.95	44.69	35.15	4.8	9.54	198%
8, 20C	30.51	40.6	24.28	6.23	16.32	1009%
8, 2oC	23.05	35.3	18.85	4.2	16.45	391%
10, 20oC	41.53	59.49	30.2	11.33	29.29	258%
10, 2oC	38.8	45.26	31.28	7.52	13.98	186%
14, 2oC	25.31	29.13	220.4	3.27	7.09	217%
14, 2oC	23.35	28.7	18.73	4.62	9.97	216%
14, 2oC	17.24	28.3	13.18	4.06	15.12	372%

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