STUDIES ON TRANSGENIC TOBACCO PLANTS CONTAINING ESCHERICHIA COLI GLUTATHIONE REDUCTASE.

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PREFACE

The experimental work described in this thesis was carried out in the Department of Biological Sciences, University of Natal, Durban, from January 1993 to April 1995, under the supervision of Professor Alan M. Amory.

These studies represent the original work by the author and have not been submitted in any form to another university. Where use was made of the work of others it has been duly acknowledged in the text.

Zodwa Lawrentia Dlamini

this 12th day of November 1996

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ABSTRACT

Glutathione reductase (GR) and superoxide dismutase (SOD) enzymes are thought to play an important role in the plant chloroplast antioxidant system. Tobacco plants transformed with *E. coli* glutathione reductase and superoxide dismutase genes were used to investigate the role of these gene products (enzymes) in the chloroplast antioxidant system. These plants were T131B (transformants with increased levels of cytoplasmic glutathione reductase activity) and GOR10T (transformants with increased levels of cytoplasmic glutathione reductase activity and chloroplastic superoxide dismutase).

In addition, 10μM methyl violegen (paraquat), was used to perturb the system experimentally under high light, low light and in darkness. During these experiments GRA (glutathione reductase activity) was assayed and the results expressed as mg^{-1}protein, mg^{-1} chlorophyll and g^{-1} tissue, using different types of transgenic plants.

T131B-cytosolic GOR transformants had a higher GRA under high light intensity. Under low light intensity T131B had a small increase in GRA compared to controls (T131Bs in 1mM $CaSO_4$). Also leaf discs in the dark showed similar GRA as did controls. The three treatments had no effect on the GRA of untransformed plants. GOR10T (cytoplamic GOR and chloroplastic SOD transformants) had a slight increase in GRA under high light intensity and in darkness . At low light intensity GOR10T showed similar results to controls.

The results indicate the overall absolute increase in GRA in transgenic plants after methyl viologen treatment. The higher activity than that of nontransgenic controls indicate that bacterial GRA must have also increased following exposure to methyl viologen.

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ABBREVIATIONS

bp	base pairs
°C	degree Celsius (centigrade)
CaMV	cauliflower mosaic virus
CAT	chloramphenicol acetyltransferase
cm	centimeter
DNA	deoxyribonucleic acid
DHA	dehydroascorbate
DHAR	dehydroascorbate reductase
g	gram
GRA	glutathione reductase activity
GSH	reduced glutathione
GSSG	oxidised glutathione
MDA	monodehydroascorbate
MDAR	monodehydroascorbate reductase

mg	milligram
min	minute (time)
μ	micro-(10^{-6})
μg	microgram
μl	microlitre
n	nano-(10^{-9})
NADP	nicotinamide adenine dinucleotide (oxidized)
NADPH	nicotinamide adenine dinucleotide (reduced)
neo	neomycin phosphotransferase gene
nos	nopaline synthase gene
pH	hydrogen ion concentration, negative logarithm
Ri	root inducing (plasmid)
SOD	superoxide dismutase
T-DNA	transferred DNA
TEMED	tetramethylethylenediamine
Ti	tumour inducing(plasmid)
u.v.	ultra violet
%	percent

CHAPTER 1: GENERAL INTRODUCTION

Life in the presence of oxygen is energetically favorable, because in an aerobic environment oxygen acts as a terminal electron acceptor of respiration and is reduced to water with concomitant increased energy yields. It is obvious that the combination of photoautotrophic and aerobic lifestyles offers advantages of relative energy dependence and efficiency, two factors that can largely explain the success of modern plants. On the other hand, oxygen is potentially hazardous. Oxygen has been reported to undergo single electron reductions, precipitating the formation of toxic oxygen species (Salin, 1988). These reactive oxygen species include the hydrogen peroxide, the hydroxyl radical, singlet oxygen and superoxide anion. These active species are produced in most tissues, but are more likely to form in leaves; here the oxygen concentration is slightly raised due to the light reactions of photosynthesis (Halliwell and Gutteridge, 1989). Hydrogen peroxide is toxic because it inhibits carbon dioxide fixation at concentrations as low as 50^{-5}M (Kaiser, 1976). The site of inhibition seems to be the fructose and sedoheptulose bisphosphatase enzymes, which are oxidized by hydrogen peroxide to forms that cannot participate in the Calvin cycle (Halliwell, 1985). The formation of singlet oxygen by oxygen is toxic because it leads to lipid peroxidation. The hydroxyl radical is the most reactive species

known to chemists, it attacks and damages almost every molecule found in living cells. Superoxide has been implicated as an agent in a number of oxygen-mediated toxic reactions which include membrane damage, cellular toxicity, single strand breaks in DNA, and lipid peroxidation.

Levels of the toxic oxygen species should be controlled, to minimize unwanted oxidation and destruction of cell components. Higher plants chloroplast contain an antioxidant system that has evolved to combat photosynthetically generated free radicals (Salin, 1987; Halliwell, 1987). This system comprises a series of enzyme catalyzed redox reactions that reduce toxic oxygen species to water. The rate limiting step in this pathway is believed to be the reduction of glutathione by NADPH using glutathione reductase (Jablonski and Anderson, 1981).

In an effort to increase knowledge of the plant antioxidant system, tobacco, containing enhanced levels of GR (suggested rate-limiting step), has been engineered. The GR gene, under the control of the Cauliflower Mosaic virus 35S promotor, was inserted into the T-DNA region of the binary vector pBin19. The use of such transgenic plants could provide insight into the role of cytoplasmic GR during oxidative stress.

The present study was developed in an effort to investigate the response of glutathione reductase in transgenic tobacco plants to environmental and chemical stresses. Firstly, background on the transformation of plant tissue and the relationship between the antioxidant system and environmental stress will be discussed. Materials and methods used are described. Thereafter the results will be discussed.

CHAPTER 2: TRANSFORMATION AND THE CHARACTERISTICS OF THE TRANSFORMING VECTOR

2.1 INTRODUCTION

In this section theories behind genetic engineering of plants will be discussed. Also, the transforming vector will be analyzed.

2.1.1 Plant transformation

Plant transformation is one of the technologies of plant genetic engineering i.e. the manipulation of plant genomes via the introduction of a DNA segment. The novel genetic information of the introduced DNA will either specify a new protein or alter expression level (overexpressing or underexpressing) of an endogenous gene. This powerful approach can improve agronomic and quality traits such as nutritional value, composition, flavour and storage ability. By this process DNA is introduced into the genome of an organism (Comai, 1993).

2.1.1.1 Agrobacterium and plant transformation

Agrobacterium tumefaciens is a gram negative soil bacterium and causes crown gall. This bacterium is often employed in the transformation of tobacco plants, and carries a large Ti- plasmid, the most commonly studied of which are the Octopine and the Nopaline types. (Figure 1)

A segment of this plasmid, designated T-DNA (tumour DNA) is introduced by this bacterium into the plant nuclear genome. The wild type *Agrobacterium* T-DNA is a plant pathogenic element since it carries genes for plant hormone production. The T-DNA segment is transmitted by this organism into individual plant cells, usually within wounded tissue. This segment penetrates the plant cell nucleus and integrates randomly within the genome where it is stably incorporated and inherited like any other plant gene in a predictable dominant Mendelian fashion (Fisk and Dandekar, 1993). Expression of these genes induces proliferation of transformed cells and results in tumour growth (Gruber and Crosby, 1993).

The oncogenic genes responsible for this phenotype are often experimentally removed, leaving only regions of DNA needed in *cis* for transforming and called borders. The borders define the start and the end of the T-DNA. The T-DNA

used to transform plants is usually 5 to 10 kb, but may be up to 50 kb in size, with the capacity to encode 2 to 20 genes.

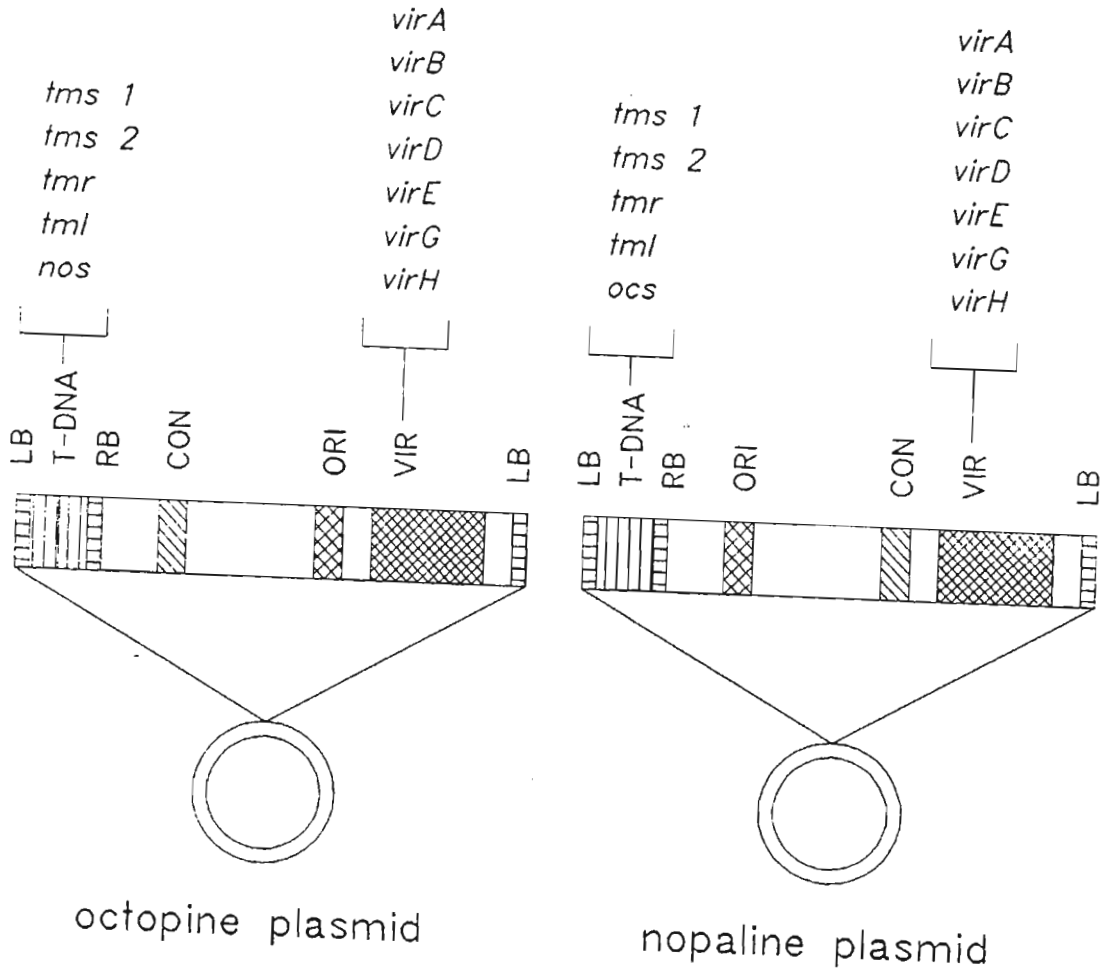


Figure 1. The general organization of octopine and nopaline-type Ti plasmids (adapted from Draper *et al.*, 1988).

The Ti-plasmid carries an antibiotic resistance gene for the selection of transformed cells, called a selectable marker gene. The most common selectable marker confers resistance to the aminoglycoside antibiotic kanamycin and encodes the bacterial enzyme neomycin phosphotransferase. The T-DNA also carries one or rarely two additional genes which vary according to the objective of the experiment (Fisk et al., 1993; Gruber and Crosby, 1993).

2.1.1.2 Characteristics of the tumour-inducing plasmid

Two regions of the Ti-plasmid (tumour-inducing) and Ri-plasmid (root-inducing) of *A. tumefaciens* and *A. rhizogenes* respectively (are essential for virulence) have been identified. These regions are the T-DNA (transferred DNA) and the *vir* region.

The *vir* region (50 kb) of a Ti- plasmid is responsible for virulence and confers upon the host *Agrobacterium* the ability to transmit a natural or genetically modified T-DNA. Mutational and DNA sequence analysis have shown that this region can be divided into six complementation groups, to which have been designated as *vir* A, B, C, D, E and G (Klee *et al.*, 1982, 1983). The genes *vir* A and G are the regulatory genes responsible for the induction of transcription of the other genes. Their gene products operate as a two component signal

transduction system responding to a variety of environmental signals (Stachel and Zambryski, 1986). Mutations in any of the *vir* regions genes abolishes T-DNA transfer and hence virulence. It has also been shown that some of these genes are not expressed unless the bacterial cells are either mixed with plant tissue or separated from growing plant tissue by a dialysis membrane, meaning that the plant tissue provides the inducer factor (Gruber & Crosby, 1993).

The T-DNA is a specific region of the Ti-plasmid or Ri-plasmid that is integrated into the plant nuclear genome. The integrated T-DNA is transcribed; for the octopine type Ti-plasmid, eight polyadenylated transcripts have been identified representing 0,001% of the total polyadenylated mRNA in the plant tissue and this has been found by northern blotting experiments. T-DNA transcription is inhibited by α-amanitin indicating that it is RNA polymerase 11 dependent. Either polymerase 1 or 111 is also amanitin-sensitive, although at different concentration. DNA sequence analysis show reading frames that correlate with these transcripts which are preceded by TATA boxes and in some cases CAAT boxes and succeeded by AATAAA boxes. The former two sequence boxes are thought to be involved in initiation of transcription and the latter in polyadenylation of transcripts in a wide variety of eukaryotes. Four genetic loci in the T- DNA that map within open reading frames have been identified using transposon and deletion mutagenesis genetic analysis. These loci are the *ocs* locus encoding the octopine synthase enzyme, the *tmr* locus encoding an enzyme involved in

cytokine biosynthesis. Mutations in the latter locus (rooty mutants) results in massive root proliferation. The third and fourth loci are the *tms*1 and *tms*2 loci encoding functions involved in auxin biosynthesis and mutations in either of these loci result in shoot proliferation. The origin (ORI) and conjugation site (CON) are involved with conjugative transfer and replication of the plasmid within *Agrobacterium* (Draper *et al.,* 1988). The Ti-plasmid based vectors have been developed that have deletions of the *onc* region (the genes *tms1, tms2* and *tmr*) yet retain the boarder sequences that allow T-DNA transfer. T-DNA is also flanked by 25 base-pair near perfect reapeats and these sequences are thought to be involved in transfer of the T-DNA to the plant genome since the end points of the integrated T-DNA are close to these sequences. Removal of the right border by deletion mutagenesis of the Ti-plasmid abolishes the transfer of T-DNA.

2.1.2 *Agrobacterium* based transformation systems.

There are two mechanisms involving *Agrobacterium* transformation, the co-intergrating system and the binary vector system. The co-integrating system features two independent plasmids: a Ti-plasmid in *Agrobacterium* and an intermediate vector in *Escherichia coli (E. coli)*. Both plasmids have a region of homology which undergoes recombination to form a large, co-integrated plasmid after conjugation between *Agrobacterium* and *E.coli* (Fig. 2)

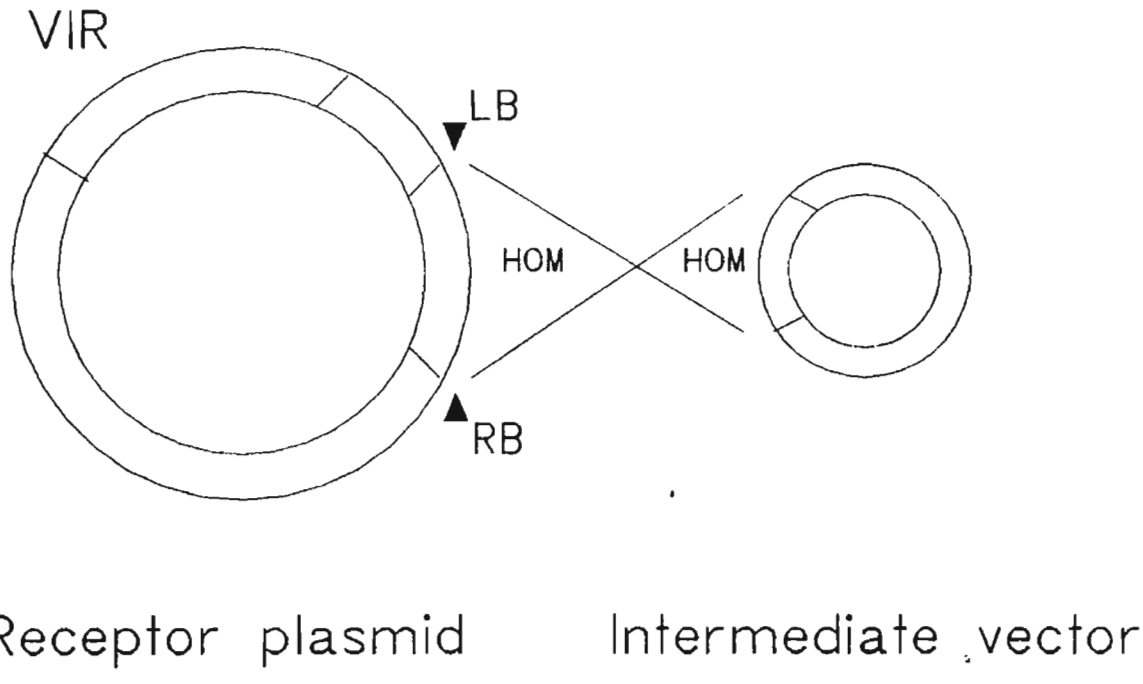


Figure 2. Schematic diagram of a cointergrative vector system (adapted from Draper *et al,* 1988).

Genes of interest are cloned and manipulated in *E. coli* and, after recombination with the Ti-plasmid in *Agrobacterium*, are situated between two T-DNA border repeats. The *E. coli* plasmid does not have an origin of replication for maintenance in *Agrobacterium* and is not retained without the recombination step. The example of a co-integrating system is the split-end vector (SEV) system in which the right and left border sequences reside each on one of the independent plasmids (Gruber & Crosby, 1993). The two plasmids form a cointergrate following a single recombination event (Fig. 3). Binary vectors exploit the observation that when the vir region and the T-DNA region of the Ti-plasmid are on two separate plasmid replicons the *vir* region on one plasmid can complement in *trans* to effect transfer of DNA to plants (Gruber & Crosby, 1993). In such

systems the *Agrobacterium* host strain contains a wild-type Ti-plasmid or disarmed (tumor genes deleted) Ti-plasmid that carries the *vir* functions and

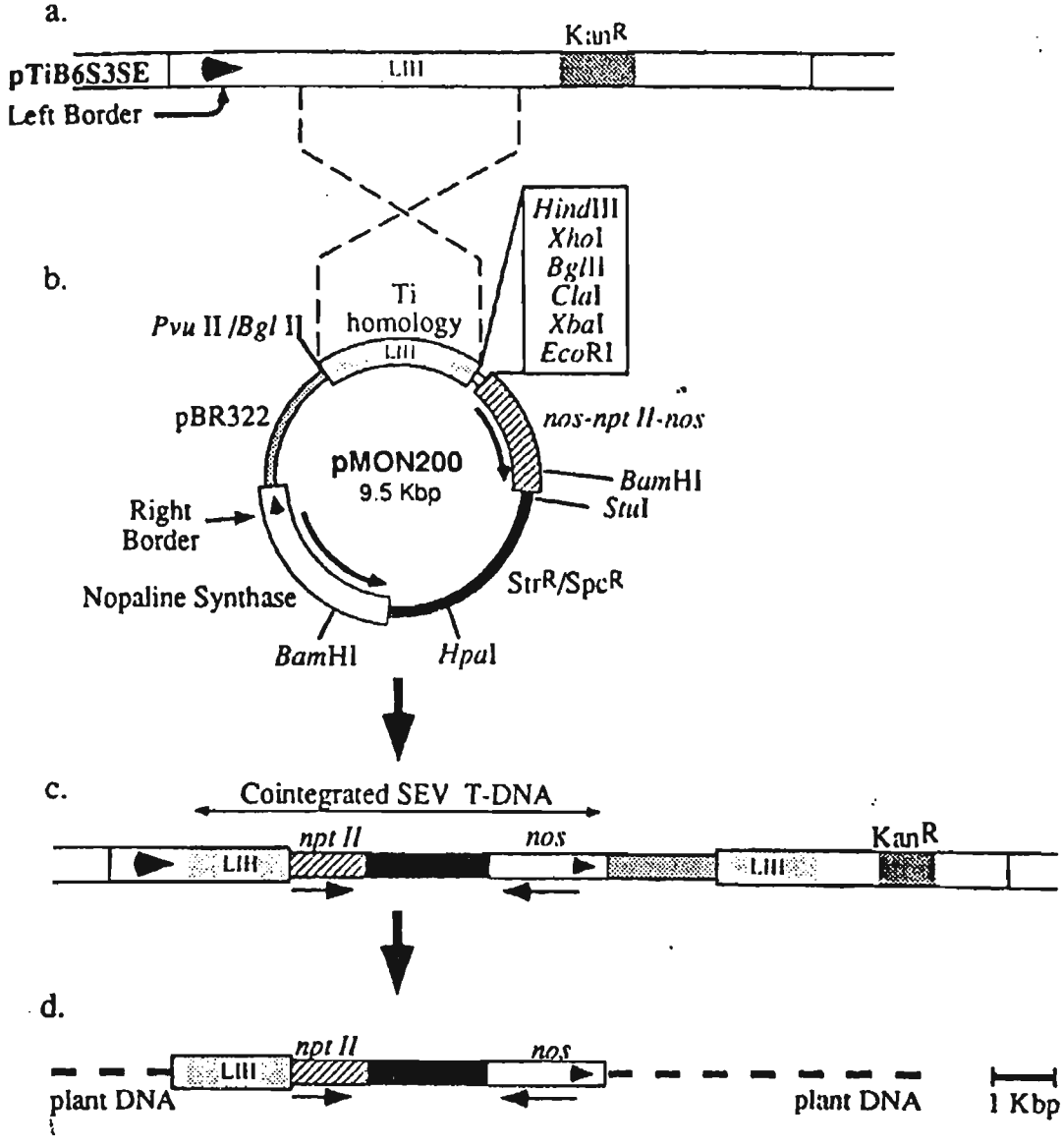


Figure 3. Cointergrate formation and plant intergration of plasmid pMON200 using the SEV system: (a) pTiBS3SE, an engineered Ti plasmid residing in *Agrabacterium*; (b) restriction map of intemediate vector pNMO200 residing in *E.coli*; (c) cointergrated plasmid residing in *Agrobacterium* after conjugation; (d) T-DNA intergrated into plant chromosome after transformation; other elements are represented by boxes. (From Rogers *et al.*, Gene transfer in plants : production of transformed plants using Ti plasmid vectors, in *Methods for Plant Molecular Biology,*Weissbach , A. & Weissbach, H., Eds., Academic Press, 1988, pp. 423)

serves as a helper. The T-DNA borders are located on a compatible replicon that will function both in *E coli* and *Agrobacterium* (An *et al.*, 1988). The example is shown in Fig. 4.

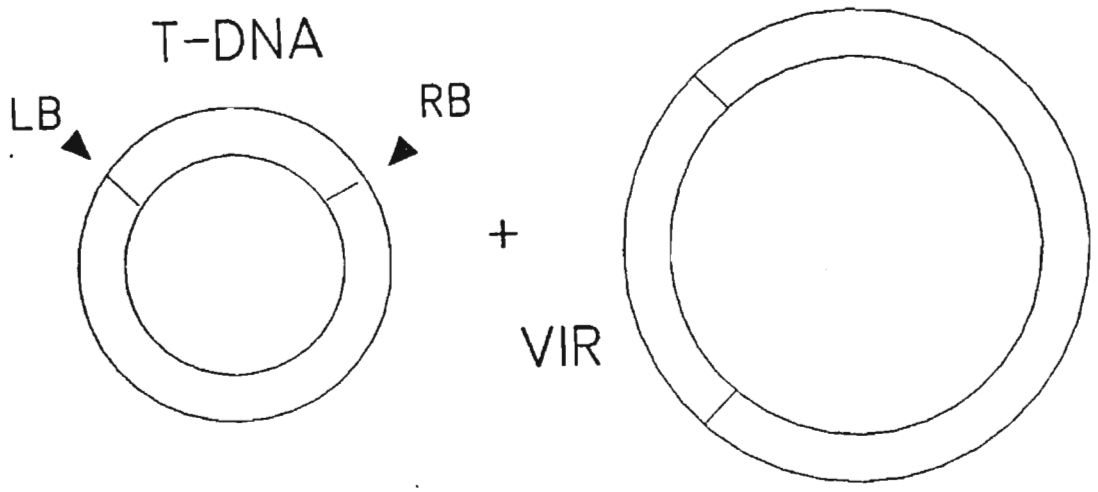


Figure 4. Schematic diagram of a binary vector system (adapted from Draper *et al.*, 1988).

The binary plasmid which has been used in this investigation is the expression vector pBIN 19. This was constructed to contain only border repeats and a selectable *nos-npt* 11 sequence, thereby providing multiple cloning sites for the insertion of foreign genes. The construction of pBIN 19. (Fig. 4), used in this study to introduce a bacterial GR gene into tobacco will be briefly discussed. The selectable marker gene *npt*11 was excised from the bacterial transposon 5, modified, then ligated between *nos* promotor and *nos* polyadenylation site on a PUC 9 plasmid (Bevan, 1984). This plasmid provided a suitable skeleton on

which to construct pBIN 19. The right border from the nopaline plasmid, TiT37, was ligated to the chimeric marker gene sequence, and the left border made flush with DNA polymerase 1 (Fig. 5).

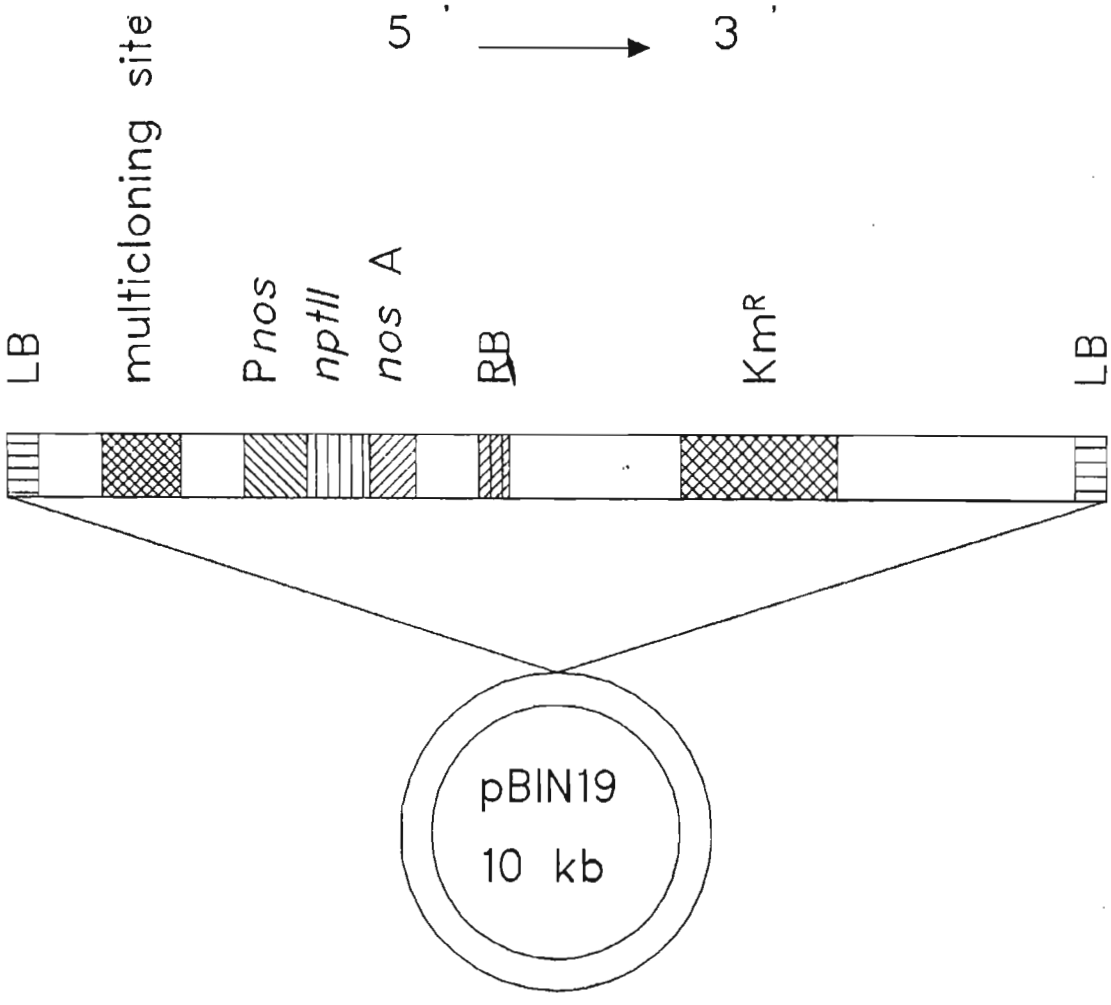


Figure 5. The expression vector pBIN19 (adapted from Bevan, 1984).

Both border elements of TiT37, including the selectable marker, were then ligated into the wide host range plasmid pRK 252. The latter contain a type 111 aminoglycoside phosphotransferase gene from *Streptococcus* for selection in *Agrobacterium*. This prototype, called pBIN 6, was modified by replacing a portion of T-DNA with a fragment of DNA from vector M13mp 19 containing an array of restriction sites for the cloning of foreign genes (Fig. 5) (Bevan, 1984). The *npt*11 gene confers resistance to G418 and the less toxic analogue, kanamycin (Horsch *et al.*,1985). The *nos* promotor has been used extensively to direct the expression of foreign genes contained on both oncogenic cointegrate and binary plasmids (Bevan, 1984).

2.1.2.1 The insertion of GR into pBIN 19

The bacterial glutathione reductase gene has been mapped, isolated and sequenced in earlier studies (Greer & Perham 1986). The GR gene was mapped and excised as a 2.8 kb *Dde*1 fragment from the pGR plasmid. The *Dde*1 fragment was treated with DNA polymerase 1 to create blunt ends and cloned into the DNA polymerase 1 treated Hind 111 site of a vector M13mp 19. *Hind* 11 sites were recreated at both ends of the GR gene. Deletions were subsequently created at the 5' end of the GOR gene with *Bal* 31 exonuclease digestion and the GOR DNA cut with *HIND* 111. The resulting GOR fragment was inserted into *Sma*1/*HIND* 111 sites of an M13mp 9 vector. After sequencing analysis, the GOR gene (without the promotor region), was inserted as a *Hpa*1/*HIND* 111 fragment into the *Hinc*11 site of the vector pUC 19 (Kunert *et al.*, 1990). This

al., 1990). This vector was cloned into the vector pJIT62 designed by Guerinean and co- workers (1990) (Fig. 6).

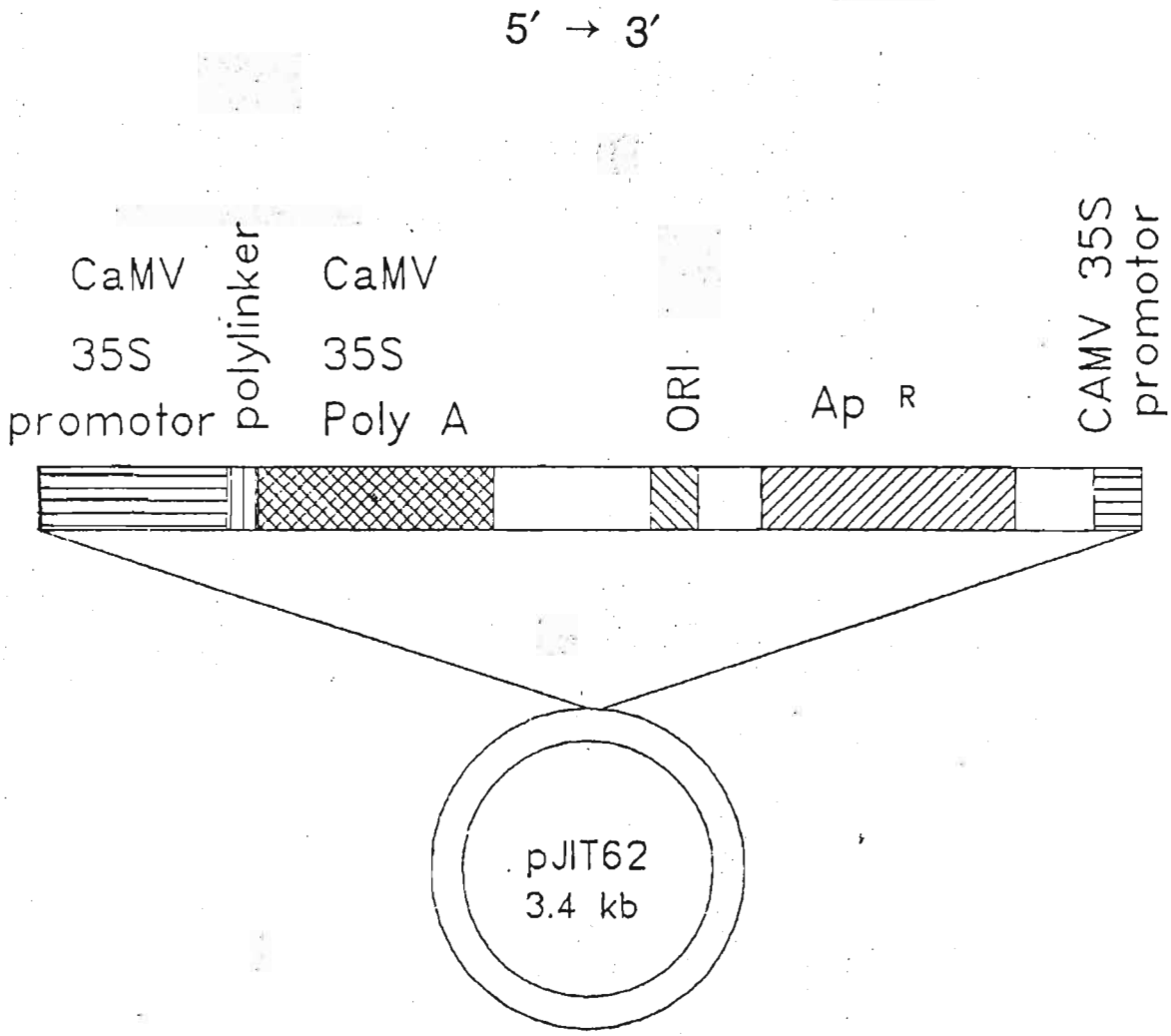


Figure 6. The expression vector pJIT62 (adapted from Guerinean *et al.*, 1990)

Plasmid pJIT62, a derivative of pJIT30 is an expression cassette made of a pUC-derived vector carrying the 35S promotor, corresponding to the co-ordinates 7040-7432 and the CaMV polyadenylation signal, corresponding to the co-ordinates 7435-126 (Guerinean *et al.*, 1990). GOR was cloned from the transconjugant as *Sst1/Xho*1 fragment into the *Sst*1-Sa*l*1 site of the poly linker of pBIN 19 (Bevan 1984). The transconjugant (fig. 7) was mobilised by the helper plasmid, pRK2013, containig the Ti-plasmid derivative pAL4404.

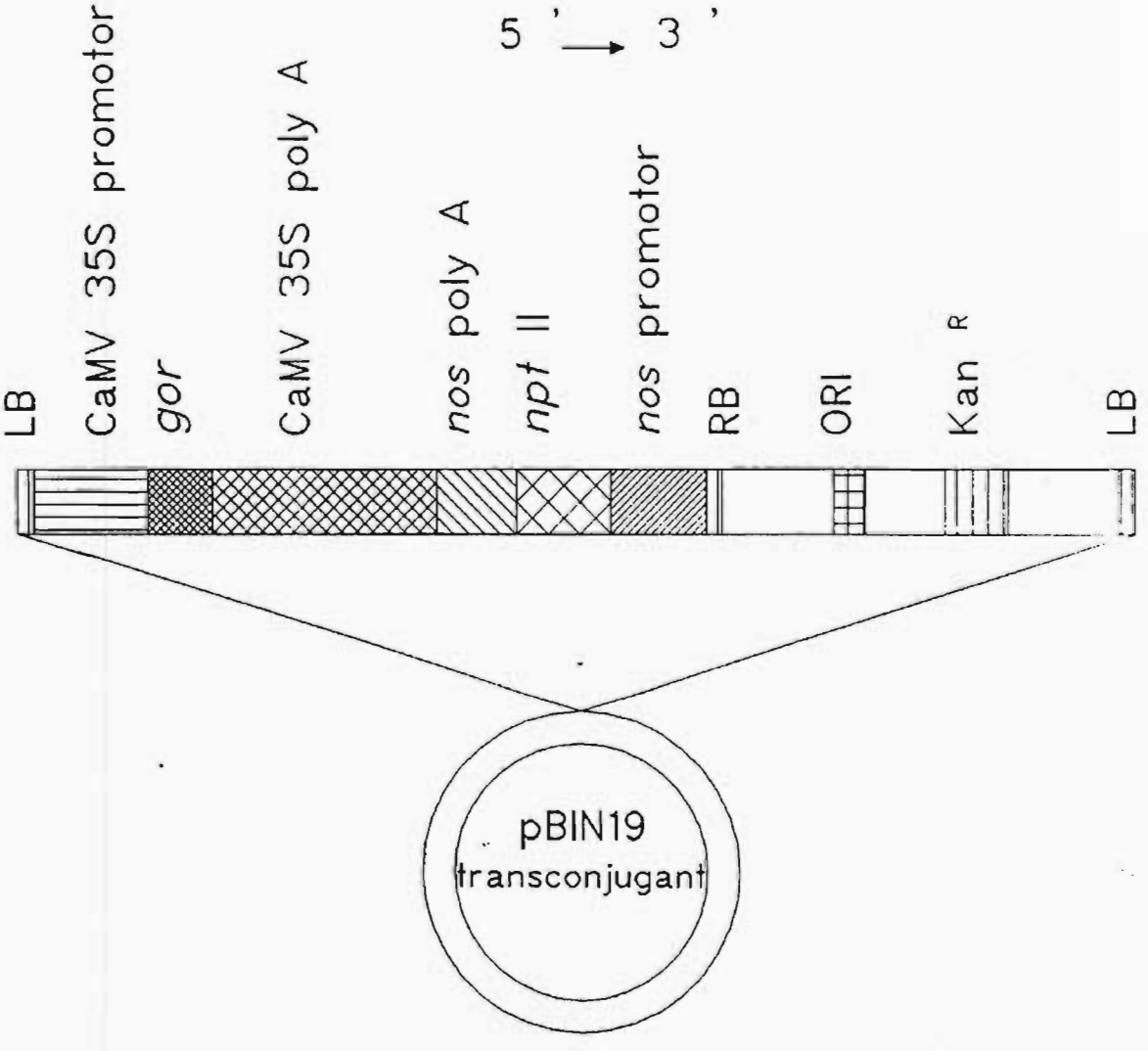


Figure 7. The pBIN19 transconjugant vector (adapted from Bevan, 1984).

CHAPTER 3: RESPONSE OF GLUTATHIONE REDUCTASE TO ENVIRONMENTAL AND CHEMICAL STRESS

3.1 INTRODUCTION

Aerobic organisms are exposed to an atmosphere that contains at least 20% oxygen. A life with oxygen, while highly efficient, carries with itself a potential danger. Molecular oxygen inhibits cellular enzymes for instance, it directly inhibits nitrogenase in *Clostridium pasteurinum*. The nitrogenase enzyme depends on maintaining some of its cofactors in a highly reduced state and on exposure to oxygen they are irreversibly oxidised and the enzyme is inactivated (Halliwell, 1981). The best example of the direct effect of oxygen on aerobic organisms comes from green plants. During photosynthesis, illuminated green plants fix carbon dioxide into sugars by a complex metabolic pathway known as the Calvin Cycle. The first enzyme in this pathway, ribulose bisphosphate carboxylase (rubisco) combines CO_2 with a five-carbon sugar (ribulose 1,5-bisphosphate) to produce two molecules of phosphoglyceric acid. O_2 is an inhibitor of this reaction, competing with CO_2 (oxygenase activity).

While the energetic advantages of life in the presence of oxygen should be evident, it induces destruction of cell components. The methods of containing it have attracted considerable research, and were the primary reason for generating the transformants used in this study. To understand the motivation for this

transformation, a discussion of oxygen and oxygen scavenging systems is thus advisable. Such analysis is even more pertinent considering the apparent effects of oxygen stress on the expression of GR in these transgenic plants (Whittaker, 1990).

3.1.1 THE CHEMISTRY OF OXYGEN AND ITS DERIVATIVES

In general the rates of enzyme inactivation by O_2 in aerobic cells are too slow and too limited in extent to account for the rate at which toxic effects develop. Also many enzymes are totally unaffected by oxygen. The most damaging effects of O_2 could be attributed to the formation of oxygen free radicals (Gutteridge and Halliwell, 1989). A free radical can be defined as any species capable of independent existence that contains one or more unpaired electrons, which cause the species to be attracted slightly to a magnetic field, and sometimes makes the species highly reactive. Radicals can be formed by the loss of a single electron from a non-radical, or by the gain of a single electron by a non-radical. This can easily happen when a covalent bond is broken and one of the electrons from each of the pair shared remain with each atom, a process known as *homolytic fission.*

When the reduction of oxygen proceeds in univalent steps, reactive intermediates are produced. Among these are superoxide (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH^-). Figure 8 depicts the pathways and interrelations between substrates and oxy-intermediates.

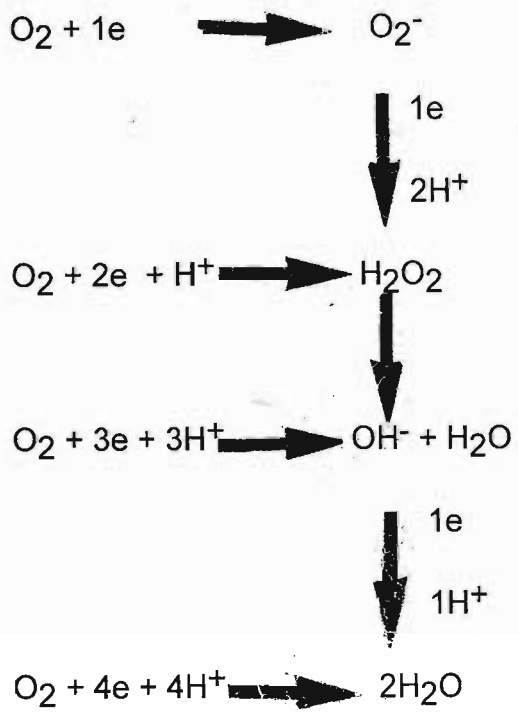


Figure 8. Pathways in O_2 reduction: formation of oxygen intermediates (from Salin, 1988)

It is through these species that oxygen exerts its effects, and thus an understanding of this group is required before an accurate assessment of the dangers of oxygen can be made.

3.1.1.1 Superoxide

Superoxide is produced either through the univalent reduction of oxygen or the univalent oxidation of H_2O_2. It can also be enzymatically produced by some

flavoprotein dehydrogenases or non-enzymatically through the autoxidation of substrates such as ferredoxins hydroquinones, thiols and reduced hemoproteins (Fridovich, 1974). Superoxide is not as toxic as other oxy-radical species. It has been implicated as an agent in a number of oxygen-mediated toxic reactions. These include lipid peroxidation, membrane damage, cellular toxicity and single strand breaks in DNA leading to mutations.

Among a few effects directly attributed to the superoxide anion are the inactivation of catalase, glutatione peroxidase and NADP(H) (Fridovich, 1986). It appears that superoxide anion exerts its effect through the formation of other species, notably hydrogen peroxide.

3.1.1.2 Hydrogen peroxide.

Hydrogen peroxide while toxic, it is not an free radical. It is a weak oxidising agent, capable of oxidising thiol groups of proteins. Several Calvin cycle enzymes within the chloroplast are extremely sensitive to hydrogen peroxide and high levels of it directly inhibit carbon dioxide fixation (Kaiser, 1979). The site of inhibition seems to be the fructose and sedoheptulose bisphosphatase enzymes, which are oxidised by hydrogen peroxide to forms that cannot participate in the Calvin cycle (Halliwell, 1985).

Like superoxide, it can act as both an oxidant and mild reductant. It does not exhibit radical properties. In general, hydrogen peroxide has a low reactivity and

toxic effects are frequently seen only at non-physiological concentrations. In the presence of metal catalysts the toxicity is enhanced, perhaps, due to a metal-catalyzed hydroxyl radical formation.

Hydrogen peroxide and the superoxide anion can react in a "Haber-Weiss" reaction to generate the hydroxyl radical (OH^-), which is the most potent oxidant known (Scandalios, 1990; Bowler et. al, 1992).

$H_2O_2 + 0_2^- \rightarrow OH^- + O_2^- + OH\cdot$ (Haber-Weiss reaction)

3.1.1.3 Hydoxyl radical

The product of a univalent reduction of hydrogen peroxide is the highly reactive hydroxyl radical (OH·) This is a weak acid and has a pK similar to that of hydrogen peroxide (11,85). It is one of the strongest oxidising agents and reacts at almost diffusion-controlled rates ($k>19^9 M^{-1}s^{-1}$) with most organic compounds (Gutteridge and Halliwell, 1989).

This is the most reactive species known to chemistry. It will attack and damage almost every molecule found in living cells. It can hydroxylate the purine and pyrimidine bases present in DNA so giving rise to mutations and can abstract hydrogen radicals from membrane lipids and, as a result, trigger peroxidation.

3.1.1.4 Singlet oxygen

Molecular oxygen or dioxygen (O_2) in the ground state is a triplet molecule containing two unpaired electrons with parallel spins. Electronically excited species of oxygen are formed when one of the outer shell electrons is elevated to a higher orbital and the spin is inverted, the resulting antiparallel spin is referred to as the singlet state. The orbital depiction of ground state triplet and the two excited singlet states as well as the energy levels above ground state are as follows:

State	ΔG, kJ	Spin
$^1\Sigma g$	155	↑↓
$^1\Delta g$	92	↑ ↓
$^3\Sigma g$		↑ ↑

Fig.8 Pathways in oxygen reduction.

Because $^1\Delta g$ is stable as compared to $^1\Sigma g$ state because of its half-life, the singlet oxygen reactions involve the former species. Although singlet oxygen is not a free radical it can be formed in some radical reactions and can trigger off

others. The higher electronic excitation states formed on illumination of the chlorophyll molecule are capable of transferring energy into oxygen and this leads to the singlet state of oxygen. It can interact with other molecules in essentially two ways: it can either combine chemically with them, or transfer its excitation energy to them returning to the ground state while the molecule enters an excited state. Singlet oxygen species are extremely reactive and cytotoxic in all organisms. They can react with unsaturated fatty acids to cause peroxidation of essential membrane lipids in the plasmalemma or intracellular organelles. This leads to the leakage of cellular contents, rapid desiccation and cell death. Intracellular damage can affect respiration in mitocondria, cause pigment breakdown, and cause loss of carbon-fixing ability in chloroplasts (Halliwell, 1985; Scandalios, 1993).

In addition to normal metabolic activity, cellular exposure to various environmental conditions (Fig. 9) such as UV light factor and other forms of radiation, herbicides such as methyl violegen (paraquat) and diquat, temperature fluctuations and various other stresses are known to induce free radical formation in most aerobic organisms (Scandalios, 1993).

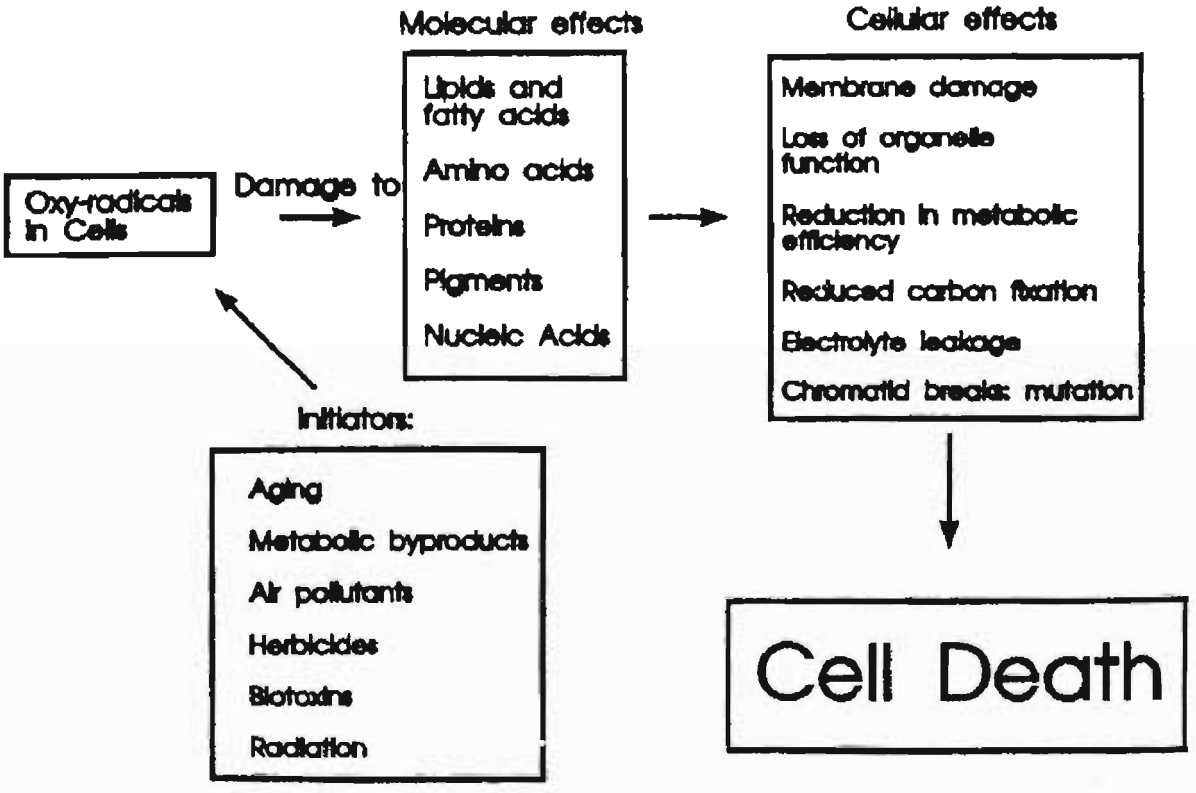


Figure 9. Scheme showing some initiators of oxyradicals and the biological consequences leading to biological dysfunctions and cell death.

3.1.2 THE DEFENCE MECHANISM AGAINST OXIDATIVE STRESS IN CHLOROPLASTS.

The fact that hydroxyl radicals are far too reactive to be controlled easily, aerobic organisms eliminate the less reactive forms as efficiently as possible, the species such as hydrogen peroxide and superoxide are removed before they have the opportunity to produce the hydroxyl radical.

In view of the susceptibility of chloroplasts to oxidative damage, one would expect to find antioxidant protective mechanisms in these organelles. Two antioxidant systems are found in the chloroplasts: the thylakoid membrane based (non enzymatic) system (Halliwell, 1987) and the stromal based antioxidant system (Asada-Halliwell pathway). These two systems will be discussed separately.

3.1.2.1 The thylakoid membrane based antioxidant system

The thylakoid membrane is very rich in α-tocopherol (vitamin E) and chloroplasts appear to be the site of synthesis of this substance in plants. Vitamin E interrupts the chain reaction of lipid peroxidation by scavenging the peroxy and alkoxy radicals. It also can quench and scavenge singlet oxygen. The free radical that is formed from chain-breaking antioxidation of vitamin E can be reduced back to vitamin E by ascorbic acid. This is likely to happen in chloroplasts since the stroma contains vitamin C at millimolar concentrations.

Carotenoids are another component of the thylakoid membrane. There are two main types (β-carotene and xanthophylls), which are oxygen-containing derivatives of carotenes. Carotenoids can quench singlet oxygen extremely rapidly (Knox and Dodge, 1985; Asada *et.al.*, 1987). As integral components of the light harvesting complex they function mostly to block free radical chain initiation, and act as energy traps to deactivate species such as triplet chlorophyll and singlet oxygen (Fig. 10).

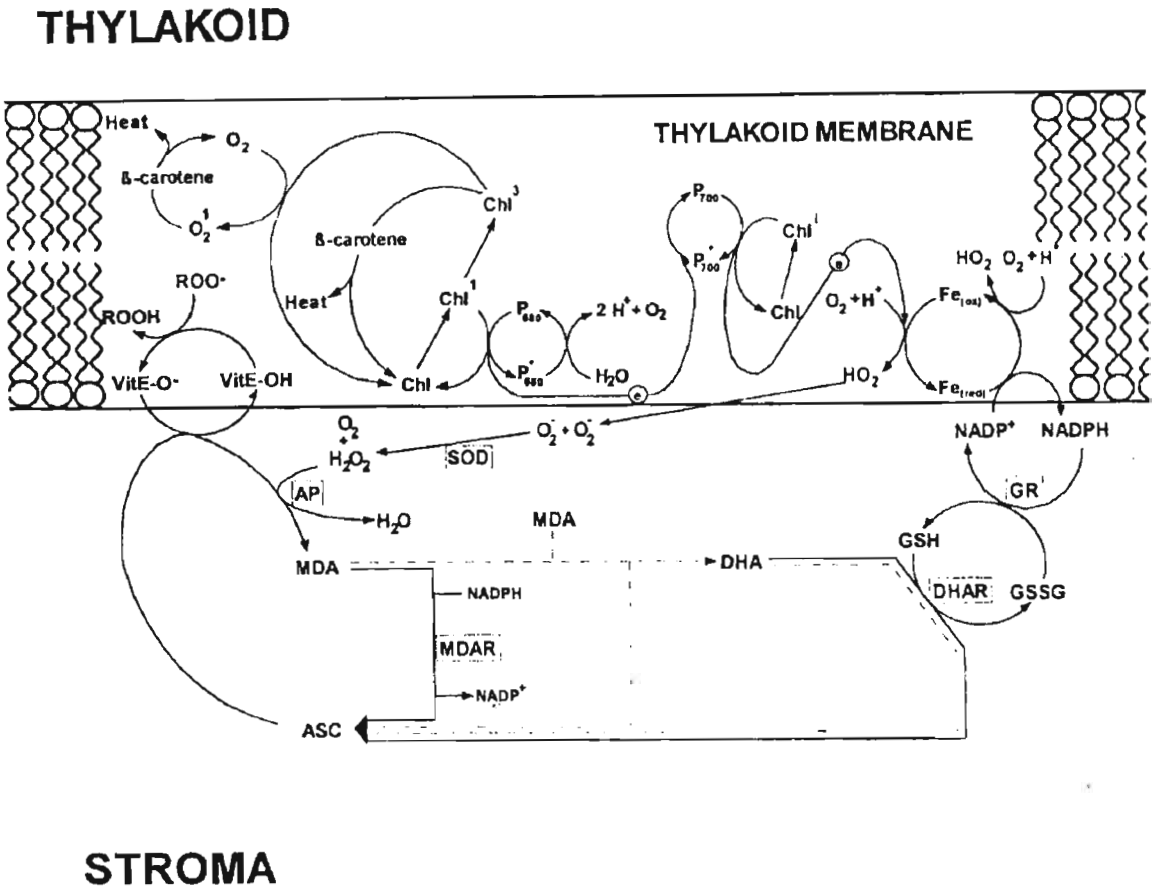


Figure 10. The chloroplast antioxidant system. Diagram indicates the interaction between the membrane and stromal free radical scavenging pathways. In the stroma enzyme catalyzed reactions are indicated by solid lines, while broken lines indicate non enzymatic reactions. Enzyme components are surrounded by boxes. Abbreviations are as in the text (adapted from Badenhorst, 1993).

3.1.2.2 Stromal based antioxidant system.

The stromal antioxidant system comprises a myriad of radical-quenching reactions. The crux of the cycle is the Asada-Halliwell pathway which, through a series of enzyme catalysed-reactions, uses photosynthetically-produced reductants to maintain the antioxidants, ascorbate and glutathione, in the reduced state. Peripheral to this engine are the interactions of ascorbate and glutathione with free radicals, and the activities of other enzymes such as superoxide dismutase.

Superoxide dismutase.

Superoxide produced in the chloroplast is dismutated by a superoxide dismutase enzyme. This reaction is called the disproportionation of superoxide:

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

This enzyme is present in all subcellular compartments where oxidative stress is likely to arise, especially in the chloroplast stroma (Bowler *et al.*,1992). The action of superoxide dismutase results in the formation of a higher concentration of hydrogen peroxide, which have a negative feedback on itself. The increased concentrations of hydrogen peroxide may lead to reaction with the superoxide and the formation of the hydroxyl free radical. Thus the superoxide dismutase activity will be meaningless without a system that functions at least as effectively

to remove the resultant hydrogen peroxide which also blocks the Calvin cycle. In plants this is achieved by the Asada-Halliwell cycle.

The Asada-Halliwell cycle.

Of more importance in the context of oxidative stress is a chloroplast localized ascorbate specific peroxidase activity. Together with glutathione reductase and dehydroascorbate reductase it is thought to remove hydrogen peroxide through a mechanism termed the Asada-Halliwell pathway (Fig. 11).

The action of superoxide dismutase results in the formation of hydrogen peroxide and is intimately linked with this pathway. Glutathione reductase, which is another key component of this pathway, has a regulatory function because of the dependence of its activity on the availability of NADPH. Glutathione reductase is found in chloroplasts, mitocondria and the cytoplasm, where it cooperates with SOD to remove superoxide radicals. Besides dehydroascorbate, ascorbate peroxidase activity also generates monodehydroascorbate.

The ascorbate radical is converted back to ascorbate by monodehydroascorbate reductase which uses either NADPH or NADH as reductant.

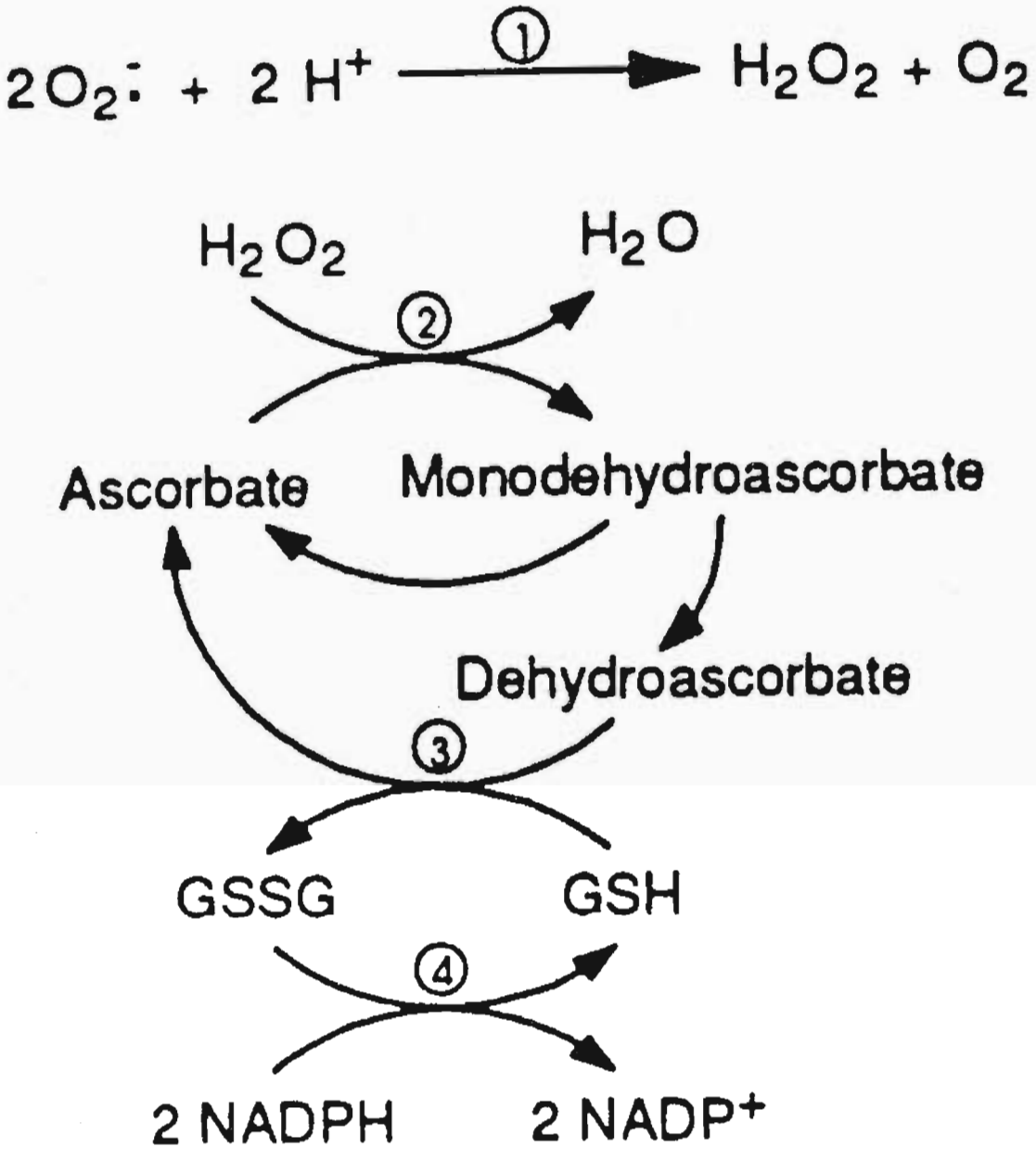


Figure. 11 Inducible reactions of the Halliwell-Asada Pathway. Abbreviations: GSH, reduced glutathione; GSSG, oxidised glutathione. Enzymes catalysing the reactions are indicated by the following numbers: 1, superoxide dismutase; 2, ascorbate peroxidase; 3, dehydroascorbate reductase; 4, glutathione reductase.

3.1.3 THE EFFECT OF PARAQUAT ON GR.

Paraquat is a bipyridal herbicide, which means that the structure contains two pyridine rings. There are aromatic rings in which one carbon atom is replaced by a nitrogen. A methyl group is attached to each nitrogen giving a full chemical name as 1,1′-dimethyl-4,4′-dipyridiniumchloride (Fig. 12).

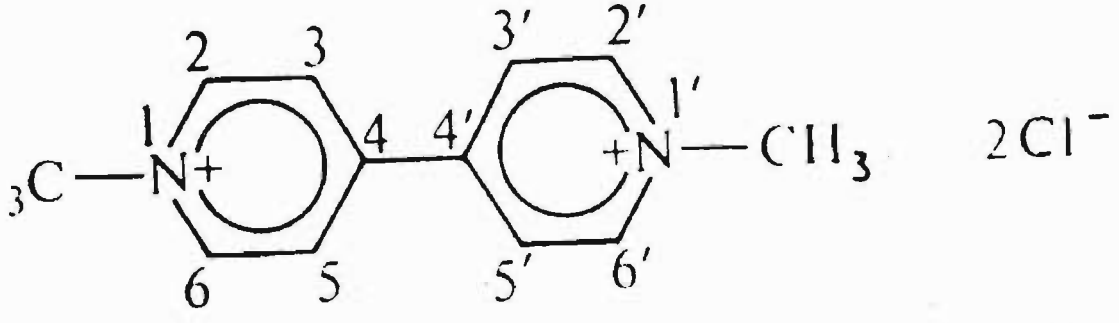


Figure 12. The chemical structure of Paraquat dichloride.

As is shown in the structure, paraquat is usually manufactured as a salt with chloride (Cl^-) ion. It seems feasible that methyl violegen should influence the Asada-Halliwell pathway by the normal production of superoxide from photosystem 1. This compound increases the oxidative stress directly by generating oxygen radicals. Also known as methyl violegen (1,1′-dimethyl-4,4′-bipyridinium chloride), paraquat is a redox-active compound that is photoreduced by photosystem 1 and subsequently reoxidised by transfer of its electrons to oxygen, forming the superoxide anion (Halliwell & Gutteridge, 1989). Highly reactive hydroxyl radicals and related species produced from this superoxide are

presumably the agents that cause cellular death. In fact, paraquat can cross the chloroplast envelope easily and can accept electrons from the non-haem-iron proteins associated with photosystem 1 and also from the flavin at the active site of ferredoxin-NADP reductase, in both cases becoming reduced to its radical form. On reaction with oxygen, the radicals disappear because they react with oxygen extremely rapidly (Fig. 13).

$$BP^{2+} \xrightarrow{\text{electron-transport chain (1 electron)}} BP^{\cdot+}$$

$$BP^{\cdot+} + O_2 \rightarrow BP^{2+} + O_2^{\cdot-} \quad (K_2 = 7.7 \times 10^8\ M^{-1}\ S^{-1})$$

Figure 13. Reactions involving paraquat and its radicals. BP stands for paraquat.

The treatment of illuminated chloroplasts *in vitro* with paraquat leads to a rapid uptake of oxygen as methyl violegen is continuously reduced and reoxidised (Gutteridge & Halliwell, 1989). The oxygen is converted into hydrogen peroxide by chloroplast superoxide dismutase. For the reason that chloroplasts contain no catalase, hydrogen peroxide is dealt with by the Asada-Halliwell pathway, but glutathione (GSH) and ascorbate are quickly oxidised and this leads to the inactivation of the Calvin cycle enzymes such as fructose bisphosphatase, and as a result of this carbon dioxide fixation comes to a halt. The other reason for the inhibition of CO_2-fixation is that diversion of electrons from photosystem 1 onto

paraquat will decrease the supply of NADPH both for the Calvin cycle and for glutathione reductase activity (Fig. 14).

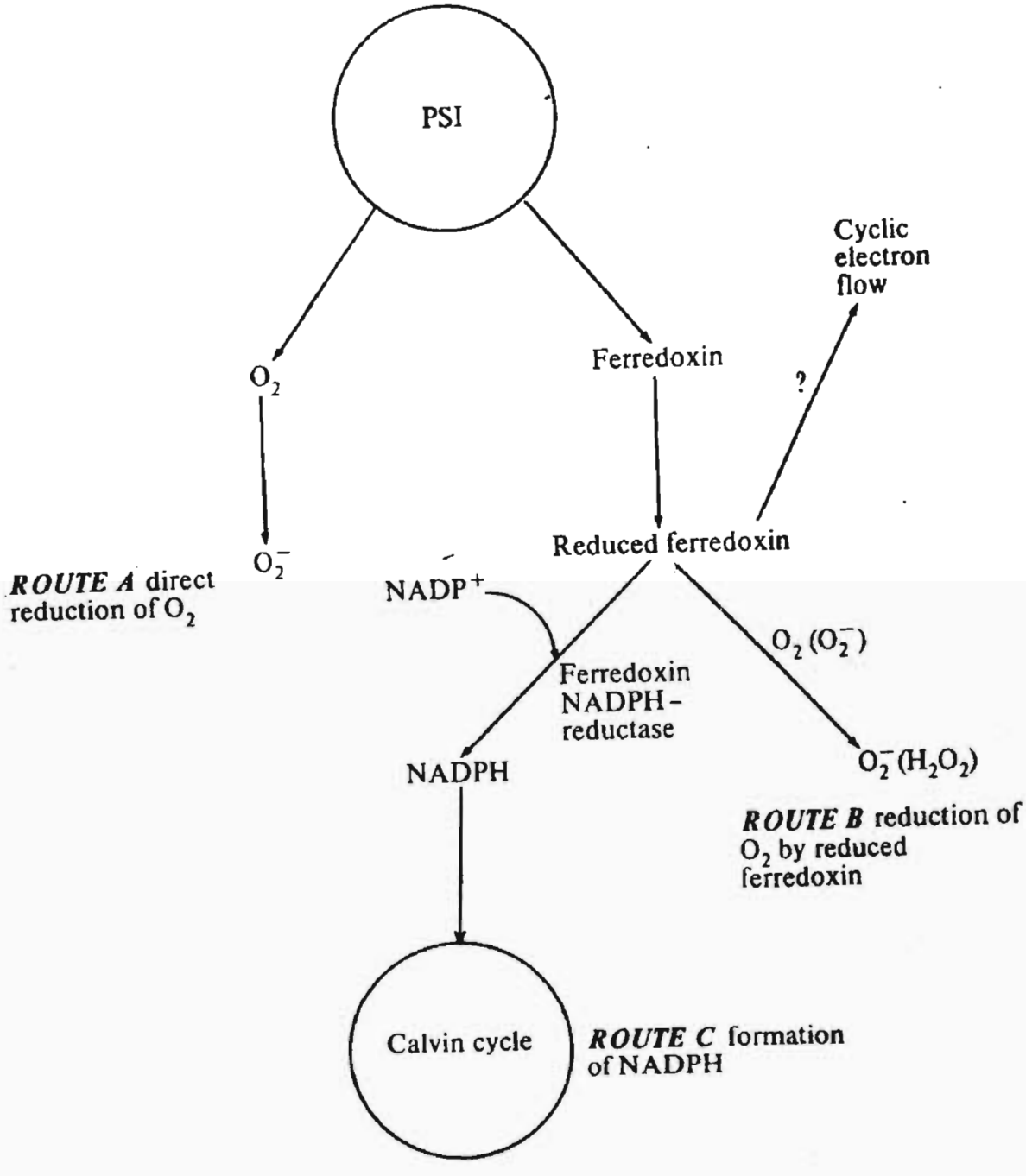


Figure 14. The effect of paraquat on the Calvin cycle (from Halliwell & Gutteridge, 1989).

Lipid peroxidation and other cellular toxic effects such as leaf death follow. A number of flavoprotein enzymes, including glutathione reductase, have been experimentally proved or shown to be capable of reducing methyl violegen. If it can penetrate into the active sites of the enzymes, 1,1′-dimethyl-4,4′-dipyridinium dichloride seem able to take electrons from the flavin ring and then, in the presence of oxygen, to generate the superoxide anion.

3.1.4 AIMS OF THE PRESENT INVESTIGATION.

The objective of the present investigation was the *Agrobacterium tumefaciens*-mediated transformation of *Nicotiana tabacum* (tobacco) using pBIN19 constructs carrying the bacterial gene for chloroplastic glutathione reductase (GR). Essentially GR provides the connection between the antioxidant system and photochemically -produced reductant (NADPH) in the chloroplasts and this is vital to the continued operation of the Asada-Haliwell pathway. In addition it would seem that the GR is the rate limiting step in dehydroascorbate reduction (Jablonski & Anderson, 1981). This suggests that the effectiveness of the enzyme antioxidant cycle may be limited by the degree of GR activity. The second objective was to test the resultant transgenic plants for the level of this enzyme and to select and clone high expressing individuals. The third objective was to study the response of glutathione reductase to environmental and chemical stresses using transgenic plants as a research tool. The fourth objective was to indirectly measure toxic oxygen species formation by measuring the chlorophyll content.

CHAPTER 4: MATERIALS AND METHODS.

4.1 TRANSFORMATION STUDIES

4.1.1 Bacterial culture and plasmid preparation.

Agrobacterium tumefaciens (strain LBA4404 pBIN19/gor T_2), containing the plasmid pBIN19/gor, which has a glutathione reductase gene with a chloropastic leader sequence (transit peptide), was maintained at 28°C, on GT medium, supplemented with 100μg ml^{-1} Kanamycin, 100μg mL^{-1} Rifampicin and 200μg mL^{-1} Streptomycin.

Plasmid pBIN19/gor T_2 was isolated using a modified method of Birnboim and Doly (1979). *A. tumefaciens* was grown by innoculating 5 mL of GT broth containing the appropriate antibiotics, with a 48 hour culture of the *Agrobacterium* strain. The broth was then incubated at 28°C in a shaking environmental incubator for 28 hours and 1.5 mL was centrifuged for 1 minute in an Biofuge B microfuge (Heraeus Sepatech, Germany) at 11000 rpm. The remainder of the overnight culture was stored at 4°C. The medium was removed by aspiration, leaving the bacterial pellet as dry as possible. The bacterial pellet was resuspended by vortexing in 100 μL of an ice-cold solution of 50 mM glucose, 10 mM EDTA and 25 mM Tris (pH 8.0), stored for five minutes at room temperature with the top of an eppendorf tube open. 200μL of a freshly prepared solution of

0.2 N NaOH and 1% SDS was added. The top of the tube was closed and contents mixed by inverting the tube rapidly two or three times. This was then stored for 5 minutes on ice. Aliquot of 150 μL of an ice-cold solution of 5M potassium acetate (~pH 4.8) was added. The cap of the tube was closed and tube gently vortexed in an inverted position for 10 seconds and then stored on ice for 5 minutes. The tubes were then centrifuged at 4°C in an eppendorf centrifuge and supernatant transferred to a fresh tube. An equal volume of phenol / chloroform was added and the solution mixed by vortexing. After centrifuging for 2 minutes in an eppendorf centrifuge, the supernatant was transferred to a fresh tube. Two volumes of absolute ethanol was added at room temperature to precipitate DNA. This was then mixed by vortexing and allowed to settle at room temperature for 2 minutes. Thereafter the tubes were again centrifuged for 5 minutes in an eppendorf centrifuge at room temperature. The supernatant was removed and the tube was placed in an inverted position on a paper towel to allow fluid to drain away. About 1 mL of 70% ethanol was added, the tube was briefly vortexed and then centrifuged for one minute to wash out salts (this was done two or three times). The supernatant was again removed and the pellet briefly dried in a vacuum dessicator . Exactly 50μL of TE (pH 8.0) containing DNAse-free pancreatic RNAse (20μg /mL) was added and the tube briefly vortexed. The quality of DNA was assessed by determining the absorption at 260 nm and 280 nm using a Beckman DU 7500 spectrophotometer. The plasmid was stored at -20°C until required.

4.1.2 Restriction digest and DNA agarose gel electrophoresis.

All restriction digests and DNA agarose electrophoresis were performed using enzyme supplied by Boehringer Mannheim, Germany. DNA was digested with one unit of restriction enzyme per μg target DNA for two hours at 37°C. After 2 hours enzymes were inactivated by heating at 65°C in a water bath for 10 minutes followed by 5 minutes chilling on ice. Gel electrophoresis was performed on horizontal (100 x 75 mm) 1% (w/v) agarose gel using a Hoefer Scientific Mini instrument. Agarose used was analytical grade agarose, supplied by Biorad, USA. The electrophoresis buffer used was 1 x TBE (89 mM Tris, 89 mM boric acid and 2 mM EDTA, pH 7.0) . Exactly 16μL of DNA per well in one third loading buffer (1.46 M sucrose, 4 M urea, 1.5 mM bromophenol blue (Merck, Germany), 50 mM EDTA, pH 7.5-8.0) was loaded onto wells. Electrophoresis was performed at 70V until the dye front had migrated to within two cm of the end of the gel. The gels were stained in 0.5 μg mL^{-1} ethidium bromide for 20 minutes, and subsequently destained in distilled water for 30 minutes prior to viewing with a Hoefer ultraviolet (300 nm) transilluminator. Results were recorded photographically with a Nikon SLR camera equipped with a 1 x Red filter and Kodak Tmax 400 ASA film.

4.1.3 Leaf disc transformation.

Green house-grown plants of tobacco (*Nicotiana tabacum* var *Samsun*) were used for transformation. Plant transformation was carried out using a modified

method described by Horsch *et al.* (1985) using the *Agrobacterium tumefaciens* strain LBA4404. Mature tobacco leaves were removed from 10 week old plants and surface-sterilized for 15 minutes with 1% sodium hypochlorite. After sterile rinsing, leaf discs of diameter 10 mm were cut and innoculated for 20-40 minutes with an *A. tumefaciens* plasmid, a plasmid construct containing a glutathione reductase gene with a chloroplastic leader sequence (LBA 4404 pBIN 19/gor T_2) that had been grown for 24 hours at 28°C in GT medium (Yeast extract, 3g L^{-1}, Oxoid, England; Tryptone, 10g L^{-1}, Difco, USA; Glucose, 2g L^{-1}, BDH, England; Sodium glycerophosphate, 10g L^{-1}, Merck, Germany; Tris, BDH, England; $CaCl_2$, 0.04 gL^{-1}, BDH, England, pH 7.4) supplemented with 100μg mL^{-1} kanamycin, 100 μg mL^{-1} Rifampicin and 200 μg mL^{-1} Streptomycin. The *Agrobacterium* was pelleted by centrifugation and resuspended in 1x MS (Murashige and Skoog, 1962,) containing 3% sucrose. After an incubation of an hour, the leaf discs were placed on agar plates of 1x MS without antibiotcs for 24 hours at 25°C with a 16:8 hr day:night schedule. Leaf discs were then transferred to selection medium of 1x MS supplemented with antibiotics (500μg mL^{-1} Cefotaxime and 50μg mL^{-1} kanamycin). Callus was initiated in the dark for 7 weeks after which the culture tubes were transferred to a 16 hour day / 8 hour night schedule at 25°C to initiate shoot formation. Subculturing onto fresh medium was performed weekly if there was an *Agrobacterium* growth.

4.1.4 Plant tissue culture and growth conditions.

Transformed tobacco (*Nicotiana tabacum* var Samsun) seeds T131B containing a high cytoplasmic glutathione reductase (GR) activity, and GOR10T, containing high cytoplasmic glutathione reductase and chloroplastic superoxide dismutase were used in this study. The T131B transformants were generated by Mr. M. Roberts (Biology Department, University of Natal, Durban), while the GOR10T plants were produced by Prof. Dvora Aviv (Israel Institute). Seeds were germinated in aseptic conditions and screened for kanamycin resistance according to the method of Horsch *et al.* (1984). Resistant plants were transferred to the greenhouse. Control seeds [SAMX (Samsun x Xanthi) provided by Prof. Dvora Aviv (Israel Institute)] were sown directly onto potting soil and mantained under greenhouse conditions.

Two to three weeks before enzyme analysis, plants were transferred to growth chambers (Conviron Model EF7, controlled environments, USA), and grown under a 16 hour light 8 hour dark regime at 250 μmol m^{-2} s^{-1} and 25°C. This provided ample time for the plants to acclimatize to the photoperiod. Plants were watered as above, when required.

4.1.5 Screening for the highest expressors and selection of transgenic plants

Leaf discs (10 mm in diameter) were obtained from greenhouse plants using a cork borer. Thereafter the discs were surface sterilized in 1% (v/v) commercial bleach (Jik) and 0.1% (v/v) tween 20 (Merck, Germany) for 15 minutes. Discs were then rinsed two or three times with sterile water before being placed on 1 x MS (Murashige and Skoog, 1962) induction medium [(consisting of 30 g L^{-1} sucrose, 1 mg L^{-1} naphthyl acetic acid (NAA), 10 g L^{-1} agar, pH 5.6) and kanamycin (100 µg ml^{-1})] in 65 mm Petri dishes (Carbi, South Africa). The petri dishes were placed in the light under a 16 hour light, 8 hour dark photoperiod at 200 µmol m^{-2} s^{-1} and 25°C, for three weeks to allow embryos to form.

After three weeks, embryos were excised and placed on germination medium consisting of half strength MS and 30 g L^{-1} sucrose, 10 g L^{-1} agar, pH 5.6) in tubes (100 x 25 mm), in the light, under the same conditions specified above. Embryos were allowed to germinate and when the shoots were about 50 mm high, they were transferred to pots containing moistened potting soil. The plants were enclosed in plastic bags and hardened off under greenhouse conditions. The bags were kept closed for a week but, subsequently, were opened slowly, allowing plants to acclimatize to the moisture differential. Once plants were fully established the bags were removed. Plants were watered with Long Ashton solution (Hewitt, 1952) (consisting of 0.208 g L^{-1} NaH_2PO_4. $2H_2$, 0.369 g L^{-1} $MgSO_4$. $7H_2O$, 0.00223 g L^{-1} $MnSO_4$. H_2O, 0.00024 g L^{-1} $CuSO_4$. $5H_2O$,

0.00029 g L^{-1} $ZnSO_4$. $7H_2O$, 0.00186 g L^{-1} H_3B_3, 0.00003 g $L^{-1}(NH_4)_6Mo_7O_{24}$. $4H_2O$, 0.00002 g L^{-1} $CoSO_4$. $7H_2O$, 0.00585 g L^{-1} NaCl, 0.505 g L^{-1} KNO_3, 0.820 g L^{-1} $Ca(NO_3)_2$. $4H_2O$, and 0,03 g L^{-1} FeEDTA).

4.2 BIOCHEMICAL STUDIES.

4.2.1 Tissue harvesting and processing

Leaf discs (10 mm in diameter), were cut from the uppermost fully expanded leaves of the three clones, randomized and placed in glass petri dishes containing 1mM $CaSO_4$. Disks were either kept in the dark or exposed to low (200 μmoles m^{-2} s^{-1}) or high (1200 μmoles m^{-2} s^{-1}) light levels in the absence or presence of 5 μM methyl violegen for 5 hours. Leaf discs were also taken for immediate enzyme analysis.

4.2.2 Glutathione reductase assay.

Glutathione reductase was extracted from leaf tissue according to the procedure of Smith *et al.* (1988). Previously-collected leaf samples were ground in ten volumes of extraction buffer (0.1 M potassium phosphate buffer (pH 7.5) containing 0.5 mM EDTA) using a mortar and pestle on ice. The extract was then centrifuged for 5 minutes at 11000 rpm and 4°C in a Biofuge B microfuge (Heraeus Sepatech, Germany). The supernatant was promptly removed and used to assay enzyme activity.

The GR assay was performed according to the procedure of Carlberg and Mannervik (1985). Extract (100 µL) was added to 500 µL of the assay buffer (0.4 M potassium phosphate buffer (pH 7.5) containing 1mM EDTA), 100µL 2 mM NADPH (Boehringer Mannheim, Germany), 100µl 16 mM GSSG (Boehringer Mannheim, Germany). The decline in absorbance of NADPH at 340 nm was followed spectrophotometrically using a Beckman (USA) DU7500 spectrophotometer. Rates of oxidation of NADPH were calculated using the DU7500 kinetics package and the molar extinction coefficient of 6.22 x 10^6 cm^2 for NADPH (Merck index). GR activity was expressed on a protein basis after extract protein levels were determined by the method of Bradford (1976), using bovine serum albumin (Boehringer Mannheim, Germany) as protein standard.

4.2.3 Superoxide dismutase assay.

Sample preparation

Leaf tissue was harvested in the same manner as in the GR assay. Leaf material was ground in two volumes of 0.05 M phosphate buffer (pH 7.8) containing 0.5 mM EDTA plus 1.4 gram isoascorbate per 100 mL of buffer just before use. Homogenate was centrifuged at 11000 rpm for 30 minutes in a Biofuge B microfuge (Heraeus Sepatech, Germany) and the clear supernatant assayed for protein content according to the method of Bradford (1976).

Native- polyacrylamide gel electrophoresis.

Non-denaturing PAGE was performed according to the method of Laemmli (1970). Aliquots (90-110µg) with 1/10 volume 0.1% bromophenol blue and 50% sucrose tracker dye (Merck, Germany) were loaded onto 1.5 mm thick non-denaturing 10% polyacrylamide gels containing 2.7% bis-acrylamide (BDH, England), 0.375 M tris (pH, 8.8) and 0.3 µL L^{-1} TEMED (Sigma, USA), 0.5 g L^{-1} ammonium persulphate (Biorad, USA)), with a 4% polyacrylamide stacking gel containing 2.7% bis-acrylamide (BDH, England), 0.125 M tris containing(pH 6.6), 0.3 µL L^{-1} TEMED (Sgma, USA) and 0.5 g L-ammonium persulphate (Biorad, USA). Gels were assembled in a SE 600 Vertical Slab Electrophoresis Unit. The gel solution was degassed (KF Neuberger vacuum pump) for 5 minutes, before adding TEMED and ammonium persulphate. The solution was poured into the assembled glass plates (16 cm x 18 cm) to a height of about 14 cm and allowed to polymerise at room temperature. Separating gel was overlayed with a deaerated stacking gel solution .

Running buffer containing Tris (0.025 M, pH 8.3) and glycine (0.192 M) was added to the chambers and gels subjected to electrophoresis at 4mA for 10 hours using PS 500X power supply (Hoefer Scientific Instruments, USA). The first gel was stained for protein in 0.25% Coomassie Blue G-250 for 15 minutes and destained in 7% acetic acid and 5% methanol for 25 minutes. For the quantification of gels, the protein separations were scanned with a GS 300 Densiometer at a speed of 13.5 cm / min.

Superoxide dismutase staining

Solution A [(19 mL Wing buffer x 4 (0.2 M Na phosphate buffer pH 7.8, 4 mM EDTA), 57 mL H_2O, 16 mg Nitroblue Tetrazolium)] was mixed with 40 mL of solution B [(40 mL Wing x 1 (10 mL of Wing x 4 + 30 mL of H_2O), 10 mg Riboflavin)] and 0.2 mL of TEMED (to start the reaction) and this solution was immediately poured onto the gels which were transferred into small plastic boxes. The boxes were then covered with aluminium foil and gently shaken for 40 minutes. The gels were then transferred onto a light box, light was turned on for 5-10 minutes. The yellow background colour turned purple. Where SOD was present bands remained colourless. The gels were kept in 7% acetic acid until they were scanned with a GS 300 Densiometer.

4.2.4 Chlorophyll assay.

For chorophyll determination 200μL extract (same extract used for GR assay) was mixed with 2.8 μL 80% acetone and centrifuged for five minutes at 11000 rpm and 4°C in a Biofuge B microfuge (Heraeus Sepatech, Germany). The supernatant was promptly removed and the absorbance was read at 652 nm using a Beckman (USA) DU 7500 spectrophotometer. The chlorophyll concentration was calculated using the DU7500 kinetics package.

4.3 STATISTICAL ANALYSIS

Estimates of sample variability are provided in terms of the standard deviation(SD) of the mean. For each plant the assay was repeated three times.

CHAPTER 5 : RESULTS

5.1 INTRODUCTION

Glutathione reductase (GR) is believed to be a rate-limiting enzyme in the plant chloroplast antioxidant system (Jablonski & Anderson, 1981). To test this hypothesis, transformed tobacco plants containing either enhanced chloroplastic or cytoplasmic GR activities and enhanced cytoplasmic GR plus chloroplastic SOD activity were subjected to paraquat treatment at two light intensities and in the dark. Plants with high cytoplasmic *gor* expression with and without SOD were grown from seed and propagated by tissue culture. Plants with high levels of chloroplastic *gor* expression were produced by *Agrobacterium* transformation. The results section will first highlight the problems associated with this transformation, discuss plant selection and finally report on the effects of paraquat in the light and dark.

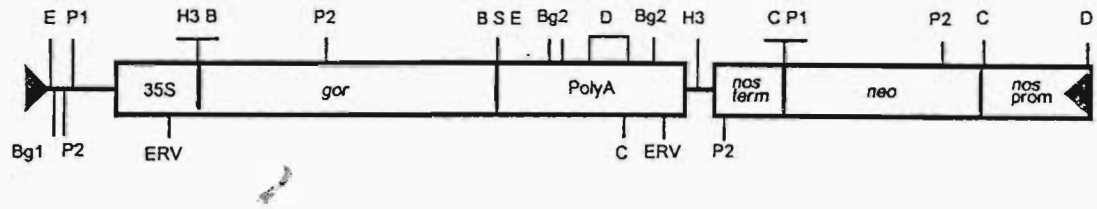
5.2 TRANSFORMATION STUDIES

5.2.1 Restriction analysis of the T-DNA of pKG2

The restriction map derived from the theoretical sequence compilation (Paul Badenhorst, 1993 Msc reference) is shown in Fig. 15A. This was found from the information of the pBIN19 binary vector construction (Bevan, 1984).

It was suggested that *Eco* R1 and *Hind* 111 should be used for restriction analysis of the T-DNA of pKG2 since the theoretical restriction indicated that *Hind* 111 and *Eco*R1 sites occurred in the T-DNA of pKG2 (Fig. 15B).

A



B

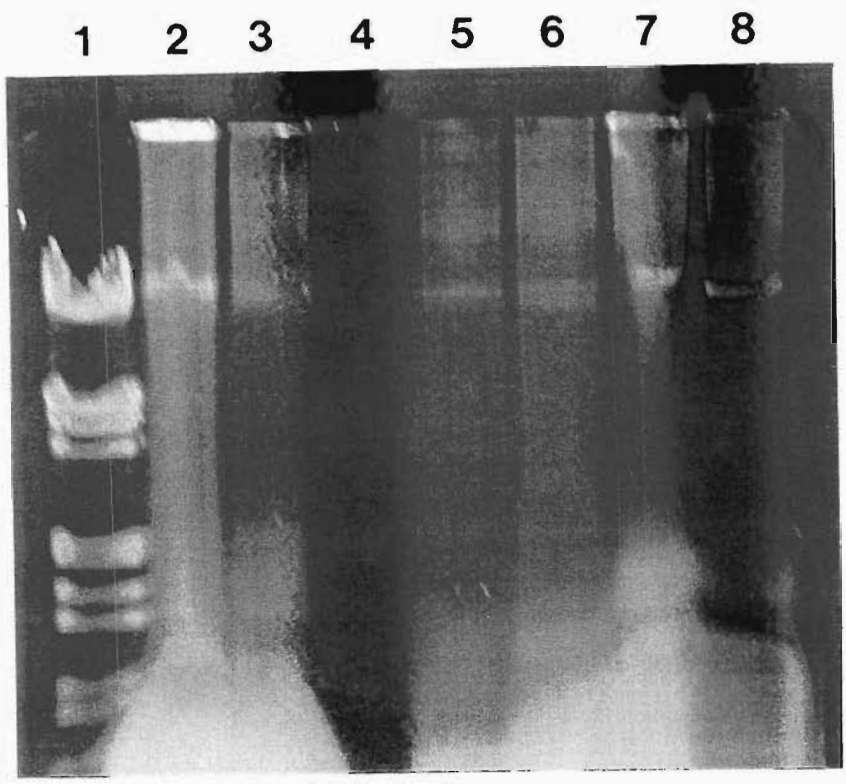


Figure 15. (A) The restriction map derived from the theoretical sequence compilation (Badennhorst, 1995). (B) Lane 1 is the molecular weight marker of *Eco*R1/*Hind*111 digestion of λ-DNA. Lanes 2 and 3 is pKG2 digested with *Eco*R1. Lanes 5 and 6 is pKG2 undigested. Lanes 7 and 8 is the digestion of pKG2 with *Hind*111. 12μl was loaded on each lane and the molecular weight marker loaded on lane 1 was 3.5μl.

As there was only one band in each lane the DNA sequence could not be successfully cut with these two enzymes.

5.2.2 Plant selection.

Seeds of transgenic plants were germinated in vermiculite under greenhouse conditions to obtain plants which would be screened for the highest expressors of *gor* and *sod* (Fig 16A and B). Transformants used in this study were the T131Bs (cytoplasmic *gor* transformationts), GOR10Ts (cytoplasmic *gor* and chloroplactic *sod* transformants).

Germination on kanamycin was used as a primary screen for transgenic plants. Plants that germinated on kanamycin containing medium were tested for the ability to root on kanamycin-containing medium. This was done because the T-DNA carries an antibiotic resistance gene for the selection of transformed cells, called a selectable marker gene, and this confers resistance to the aminoglycoside antibiotic kanamycin, because it encodes the bacterial enzyme neomycin phosphotransferase.

Fig. 16A shows the germination of the seeds of transformed plants in vermiculite and in Fig. 16B plants from vermiculite have been transferred to pots before plant selection is done.

As a first step in plant selection, GOR10T transformants which had been hardened off under greenhouse conditions, were assayed for glutathione reductase activity (GRA). Plants number 1 and 10 are control plants (SamX) and plants number 2-9 are transformants (Table 1).

A

B

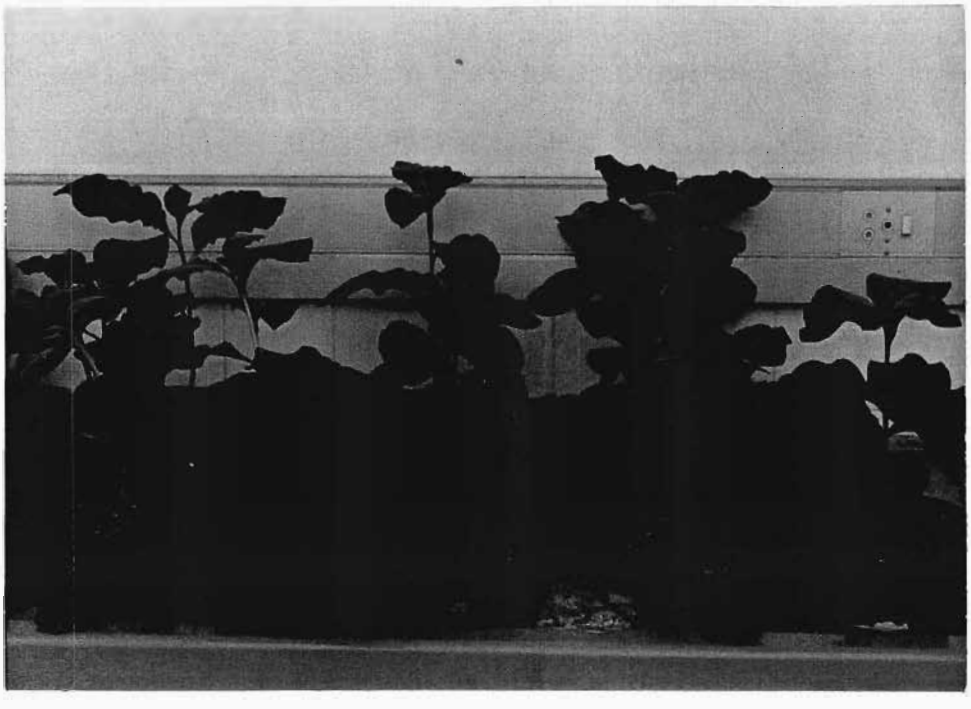


Figure16. Plant selection. (A) Germination of seeds in vermiculite. (B) Plants transferred to pots with potting soil.

Table 1. GRA of 10 GOR10T plants expressed as per mg protein. Plants number 1 and 10 are the controls (untransformed plants) and n is the number of separate GRA determinations on each plant.

Plant number	**Mean GRA (nmoles NADPH/min/mg protein)**	**n**
1	50±5	3
2	139±16	3
3	167±12	3
4	143±15	3
5	187±16	3
6	190±18	3
7	181±12	3
8	179±17	3
9	180±23	3
10	49±5	3

As is seen in this table, plant designated 6 had the highest GRA

To determine the highest expressor of the *sod* enzyme, 8 GOR10T transformants, as in Table 1, were assayed for *sod* using Native PAGE (Fig. 17).

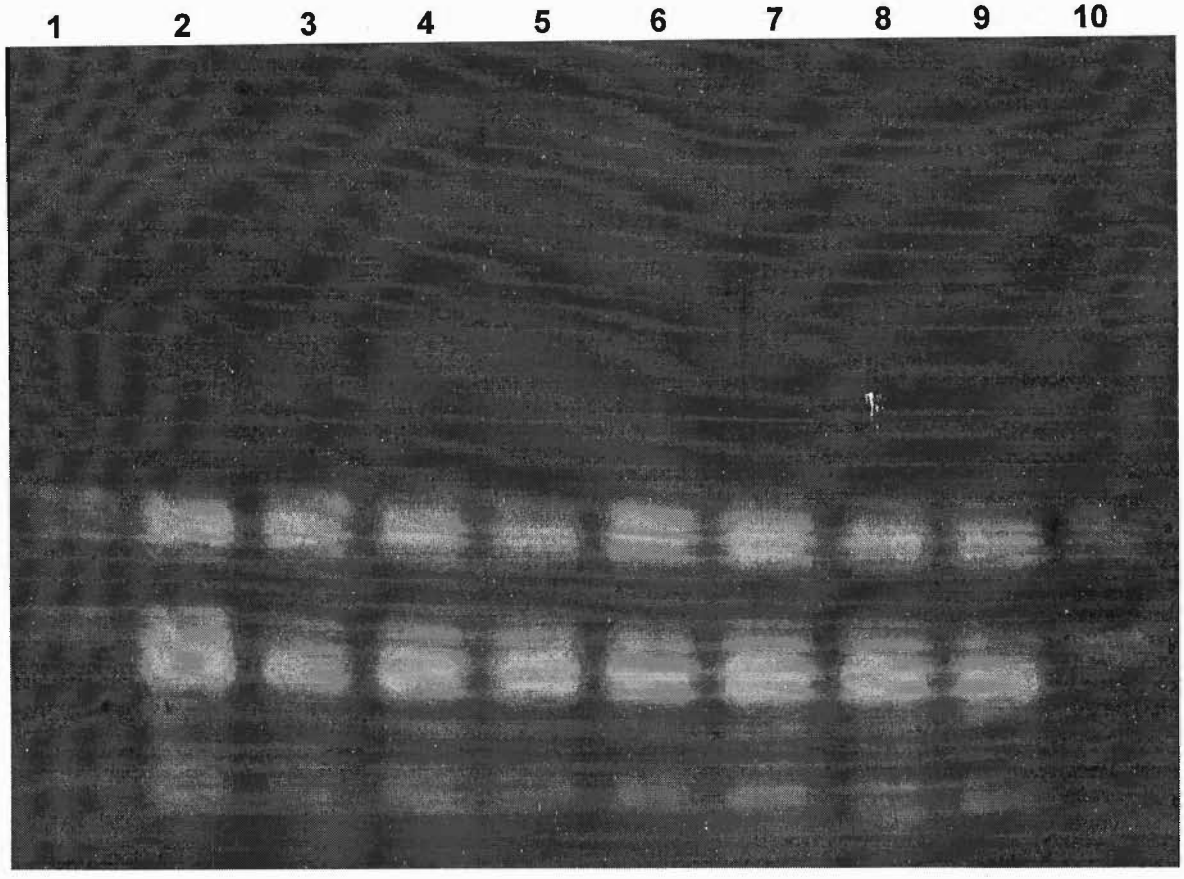


Figure 17. Native PAGE showing the SOD assay of the 8 GOR10T and two control plants selected. Control plants (lanes 1 and 10) and transformants expressed SOD activity. The levels of the cytoplasmic isoform (a) was similar in all plants. Transformants had higher chloroplastic activity due to additional expression of bacterial SOD (b). The third isoform (c), situated in the cytoplasm, was equally expressed in all transformants but expressed at a lower level in the controls.

GOR10T and T131B plants with the highest activity of SOD and GR, which were cloned and screened for kanamycin resistance and later hardened off in

GOR10T and T131B plants with the highest activity of SOD and GR were cloned and screened for kanamycin resistance and later hardened off in greenhouse conditions for further experimental usage. Fig. 18 demonstrates germination and rooting in kanamycin containing medium that confirms the presence of the neomycin phosphotransferase gene in the selected clones.

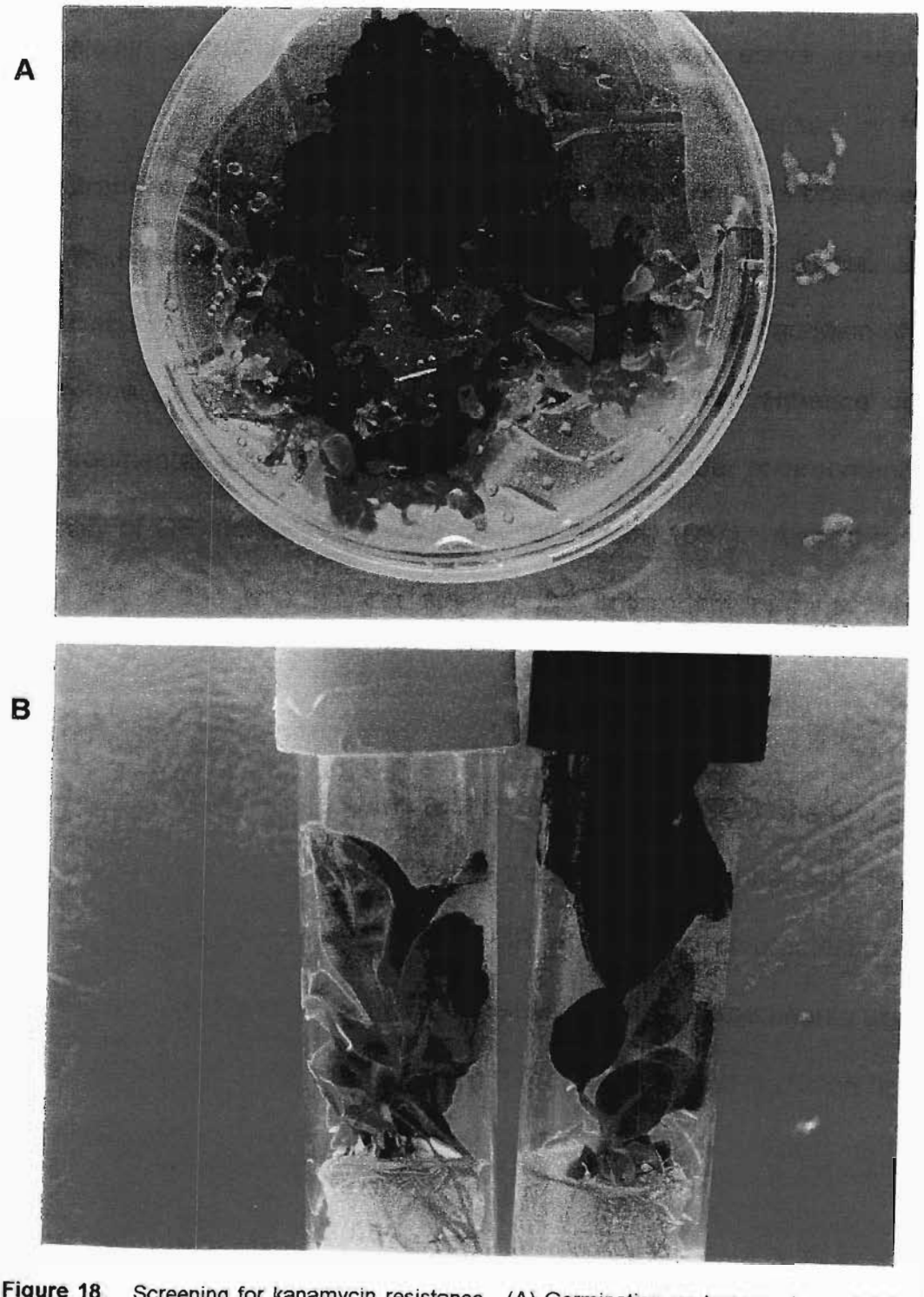


Figure 18. Screening for kanamycin resistance. (A) Germination on kanamycin containing medium. (B) Rooting on kanamycin containing medium.

5.3 BIOCHEMICAL STUDIES

5.3.1 Effect of paraquat on GRA of transgenic plants exposed to different enviromental conditions.

Paraquat increases the oxidative stress directly by generating oxygen radicals (Halliwell & Gutteridge, 1989). It is a redox-active compound that is photoreduced by photosystem I and subsequently reoxidised by transfer of its electrons to oxygen, forming the superoxide anion which is presumed to produce highly reactive hydroxyl radicals and related species. These active oxygen species are involved in the process of cellular death. The addition of paraquat will therefore increase oxidative damage that will further enhance damage under environmental stress (e.g. high light intensity). Under environmental stress the activity of GR increases (Asada & Takahashi, 1987). Also, it is hypothesized that GR is the rate limiting step in the antioxidant pathway (Jablonski & Anderson, 1981).

To test this hypothesis and the relationship between SOD, the first enzyme in the pathway, and GR, the two different transformants (enhanced cytoplasmic GR activity, and enhanced cytoplasmic GR activity and chloroplastic SOD activity) were subjected to paraquat (10 μM methylviolegen). Experiments were conducted in the dark (no or little effect), low light (paraquat stress) and high light (light and paraquat stress).

As a first step the response of GR to darkness in the presence and absence of methyl violegen, was investigated. Leaf discs were soaked in 10 μM methyl violegen and kept in the dark for five hours and assayed for GR activity (Fig. 20). Transgenic plants showed higher GRA than did controls. Also, methyl violegen had no effect on GRA in the dark.

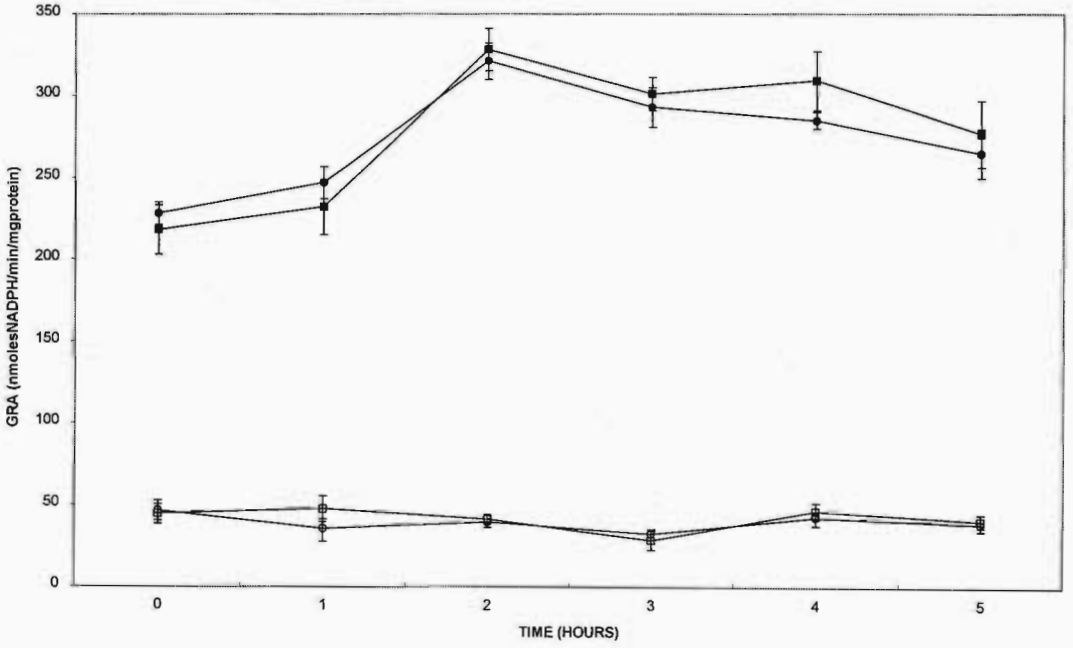


Figure 20. The effect of methyl violegen on GRA of T131B transformants put in darkness for 5 hours. [Untransformed plant without Methyl violegen (O), untransformed plant with methyl violegen (□), a high expressing transformant T131B without Methyl violegen (●) and a high expressing transformant T131B with Methyl violegen (■)]. Each point represent the mean, n=3 and bar represents standard deviation.

To examine GR response in T131B transformants treated with methyl viologen and kept under low light intensity, GRA was assayed in leaf discs incubated in the presence and absence of 10 μM Methyl viologen under 200 μmole m^{-2} s^{-1} over a five hour period (Fig. 21). The presence of methyl viologen had little effect on GRA in the light and dark in either control or transformed plants. After five hours there appeared to be a small increase in GR activity in transformed tissue.

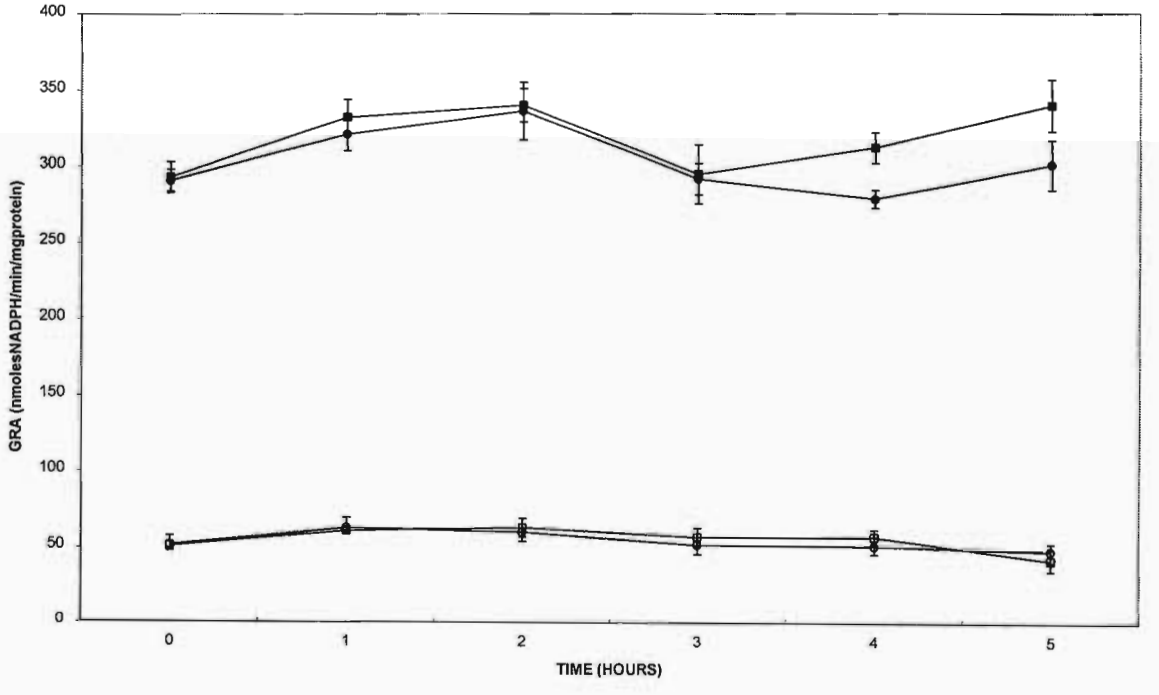


Figure 21. Effect of methyl viologen on T131B transformants placed under low light intensity for five hours. [Untransformed plant untreated with Methyl viologen (O), untransformed plant with Methyl viologen ( ), a high expressing transformant T131B untreated with Methyl viologen (●) and a high expressing itransformant T131B with Methyl viologen (■)]. Each data point represents the mean, n=3, bar represents standard deviation.

GRA was also assayed in leaf discs of T131B transformants and control plants treated with paraquat and subjected to high light intensity (1000 μmole m^{-2} s^{-1})(Fig. 22).

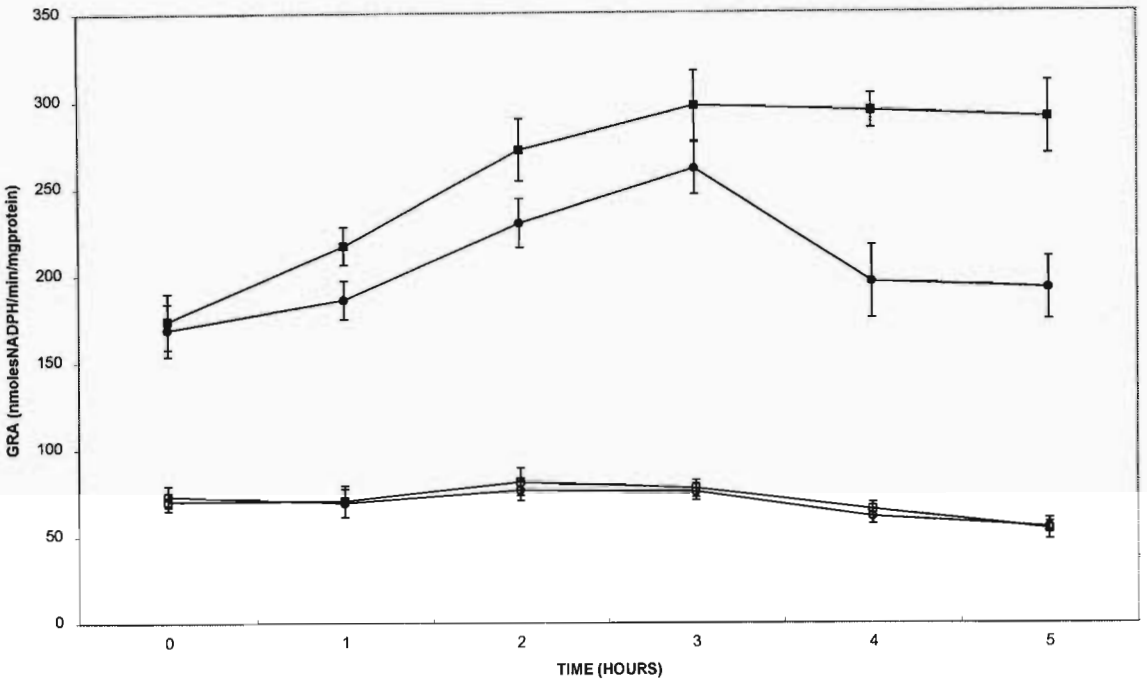


Figure 22. Effect of methyl violegen on GRA of T131B transformants exposed to high light intensity for five hours. Untransformed plant not soaked in paraquat (O), untransformed plant soaked in paraquat ( ) a high expressing transformant T131B not soaked in paraquat (●) and a high expressing transformant T131B soaked in paraquat (■). Each data point represents the mean, n=3, bar represents standard deviation.

There was a sharp increase in GRA in T131B transformants treated with paraquat under high light intensity. There was also a significant rise in GRA of T131B transformants not treated with paraquat. Interestingly GR activity increased up to the third hour and thereafter remained constant in paraquat treated T131B transformants. With the paraquat untreated transformants GRA also increased

during the first three hours then declined. There was a slight rise in GR activity in untransformed plants either soaked or not soaked in paraquat but this declines after the third hour. The level of GR activity was higher in control plants subjected in high light (Fig. 22) compared to plants at low light (Fig. 21).

GR response was also examined in the clones of high expressing *sod* and *gor* (GOR10T) transformants. Leaf discs of GOR10Ts were treated with 10 μM methy violegen and kept in the dark for five hours.

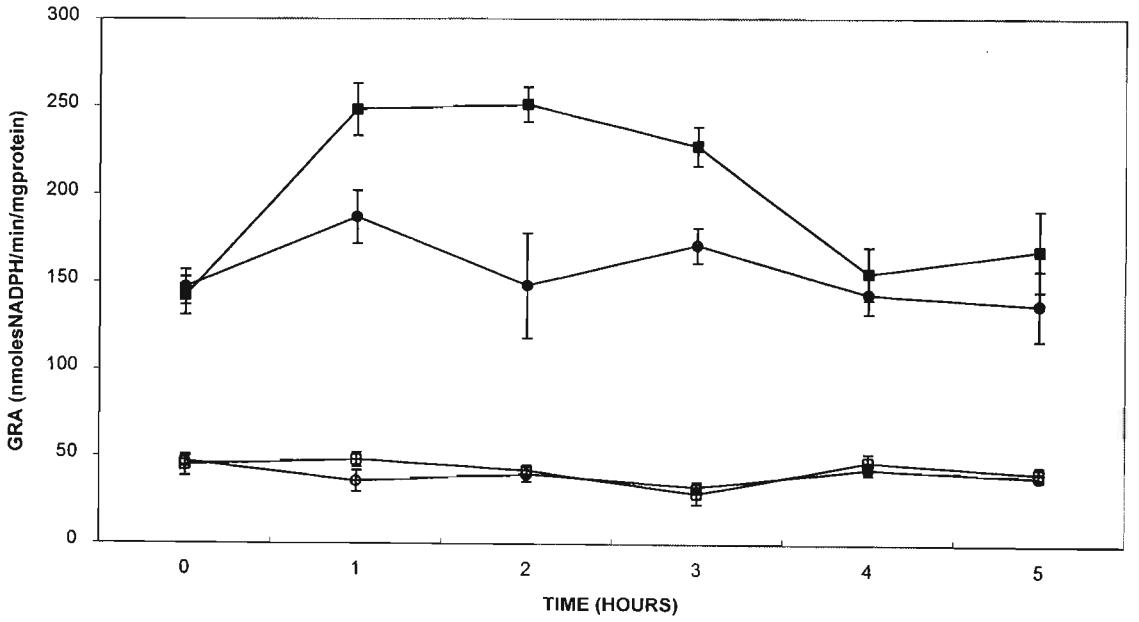


Figure 23. An effect of methyl violegen on GRA of GOR10T transformants kept in darkness for five hours. [Untransformed plant untreated with paraquat (O), untransformed plant treated with paraquat ( ), a high expressing transformant GOR10T untreated with paraquat (●) and a high expressing transformant treated with paraquat (■)]. Each data point represents the mean, n=3, bar represents standard deviation of three separate determinations.

As can be seen in Figure 23 there was no increase in GR activity in untransformed plants treated or not treated with paraquat. There was a significant rise of GRA in GOR10T transformants treated with methyl violegen that declined after the second hour back to the baseline levels. The activity of GR fluctuated over the five hour experiment in transformants not exposed to methyl violegen (Fig. 23).

GRA was again assayed in leaf disc of GOR10T transformants treated with paraquat and maintained under low light intensity.

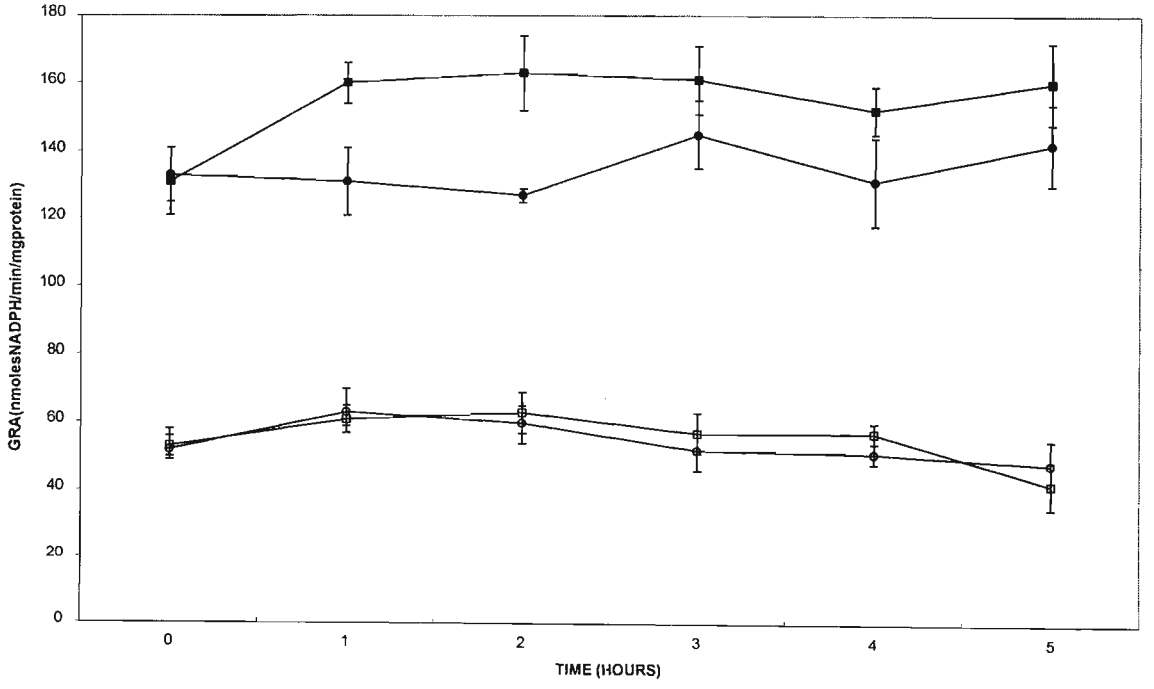


Figure 24. An effect of methyl violegen on GRA of GOR10T transformants placed under low light intensity for five hours. [Untransformed plant untreated with paraquat (O), untransformed plant treated with 10μM paraquat ( ), a high expressing transformant GOR10T untreated with paraquat (●) and a high expressing transformant GOR10T treated with 10 μM paraquat (■)]. Each data point represents the mean, n=3, bar represents standard deviation of three separate determinations.

There was an increase in GR activity of GOR10T treated with methyl violegen, but in untreated transformants GRA increased slightly. Under low light condition transformants showed a slightly elevated level of GR activity in the presence of methyl violegen. In the control plants methyl violegen had little effect in GR activity (Fig. 24).

GR activity was also determined in GOR10T transformants subjected under high light intensity in the presence or absence of methyl violegen for five hours.

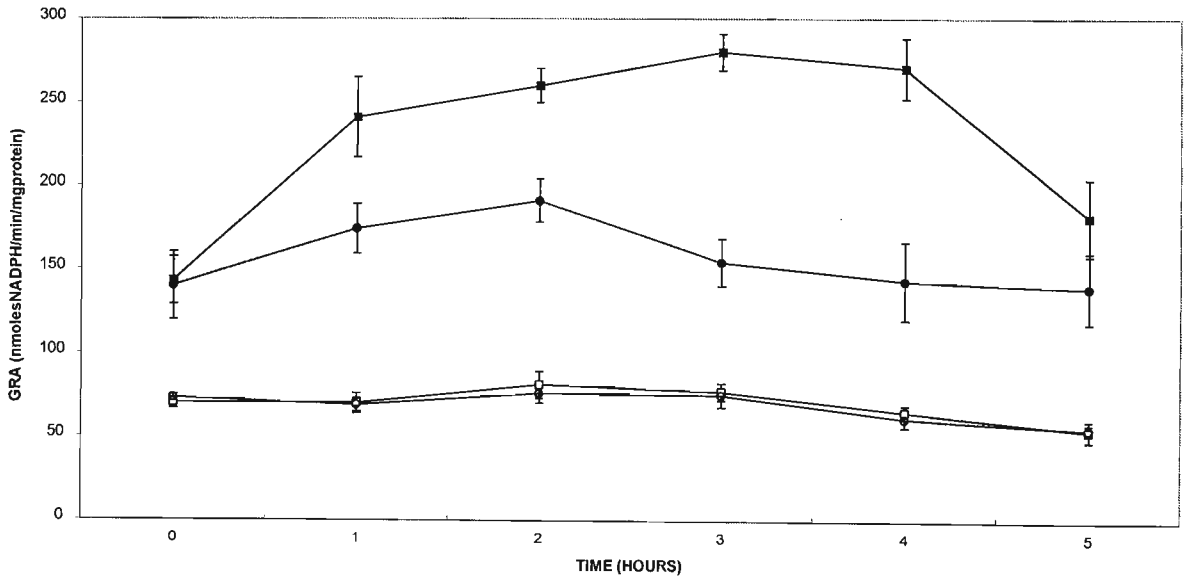


Figure 25. An effect of methyl violegen on GRA of GOR10T transformants exposed to high light intensity. [Untransformed plant not soaked in paraquat (O), untransformed plant soaked in 10 μM paraquat ( ), A high expressing transformant GOR10T not soaked in paraquat (●) and a high expressing transformant GOR10T soaked in 10 μM paraquat (■)]. Each data point represents the mean, n=3, bar represents standard deviation of three separate determinations.

There was a significant increase in GRA of GOR10T transformants treated with paraquat although an increase in GR activity is also seen in transformants in the absence of paraquat (Fig. 25). There was no change in GR activity in untransformed plants treated or not treated with paraquat. The significant rise of GR activity in paraquat treated transformants, compared to that of T131B transformants might be due to the increased levels of SOD activity in these transformants.

5.3.2 Chlorophyll bleaching

Metabolic reactions in the chloroplasts of higher plants have the potential to generate many forms of toxic oxygen species. Interaction between triplet chlorophyll and oxygen results in the generation of singlet oxygen. Oxygen may accept electrons from the terminal electron carriers of photosystem I and thus become reduced to the superoxide anion. The dismutation of the latter by superoxide dismutase forms hydrogen peroxide, while reaction of hydrogen peroxide with superoxide generates the highly reactive hydroxyl radical.

Toxic oxygen species can lead to extensive damage to the photosynthetic apparatus especially the chlorophyll molecules (Gillham & Dodge, 1985). The carotenoids are very important in protecting chlorophyll from photosensitized reactions and play a role in preventing chlorophyll bleaching. They deactivate triplet chlorophylls and transform singlet oxygen to its triplet ground state. Chlorophyll content may therefore be used as an indirect measure of toxic oxygen species formation.

Chlorophyll content was therefore determined under identical experimental conditions as the paraquat experiments. Chlorophyll content was measured in leaf dics of high expressing GR transformants (T131Bs) that were soaked in 10 μM methyl violegen and subjected to darkness, low and high light intensities over a period of five hours.

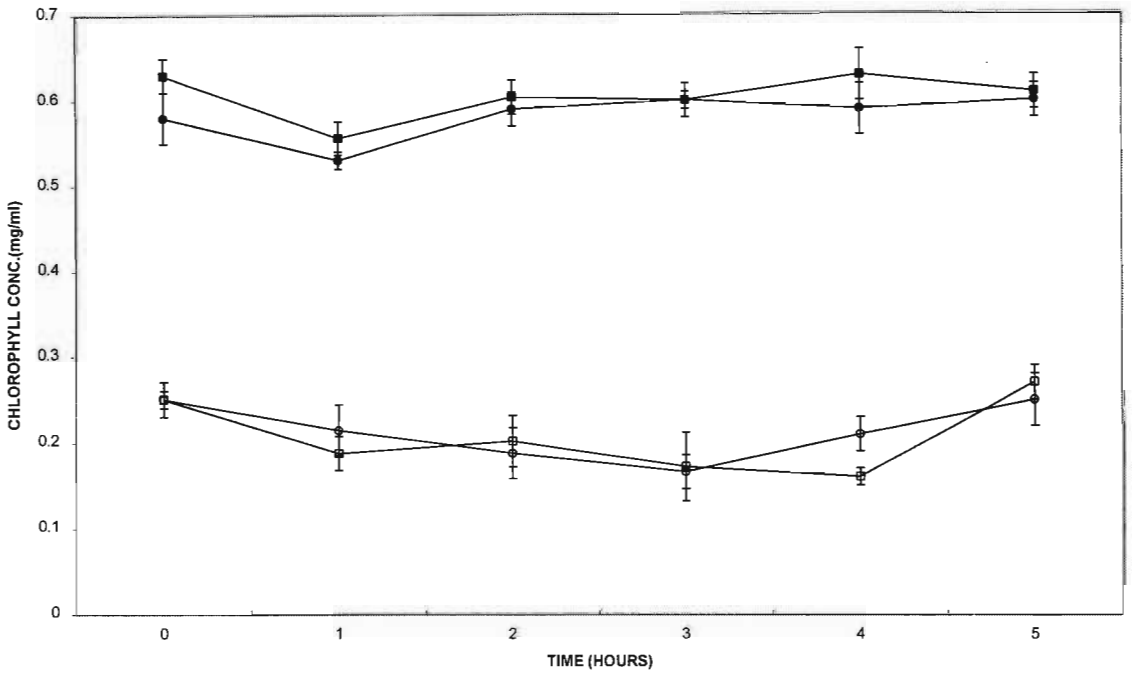


Figure 26. Chlorophyll assay of T131B transformants in darkness over a period of five hours. [(O) Untransformed plant not treated with methyl violegen, ( ) untransformed plant treated with 10 μM methyl violegen, (●) a high expressing transformant T131B without methyl violegen and (■) a high expressing transformant T131B with 10 μM methyl violegen]. Each data point represents the mean, bar represents standard deviation of three separate determinations.

In the dark there was not much change in the chlorophyll content in transformants in the presence or absence of methyl violegen (Fig. 26). Interestingly the chlorophyll content of the transformants was higher than that of the control plants treated or untreated with paraquat.

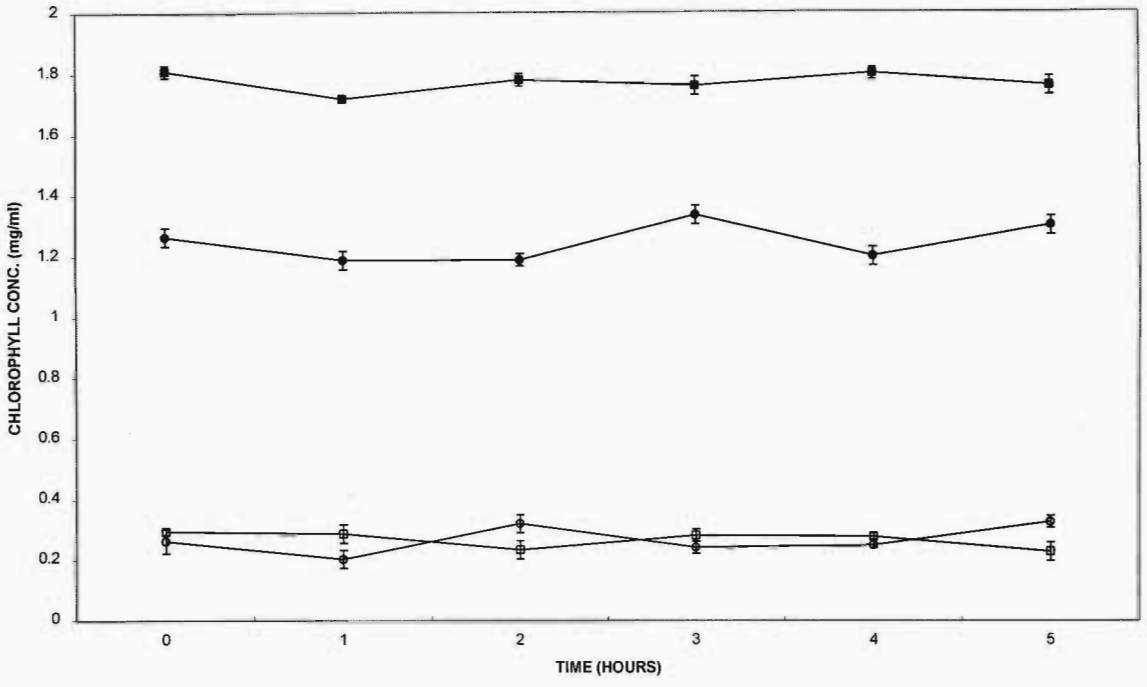


Figure 27. Chlorophyll Assay of T131B transformants put under low light intensity over a period of five hours. [(0) Untransformed plant with no methyl viologen, ( ) untransformed plant treated with 10 μM methyl viologen, (●) a high expressing transformant T131B without methyl viologen and (■) a high expressing transformant T131B with methyl viologen]. Each data point represents the mean, bar represents standard deviation of three separate determinations.

Under low light intensity there was no change in the chlorophyll content in both controls and transformants in the presence or absence of methyl viologen (Fig. 27). The chlorophyll content of transformants was much higher than that of untransformed plants treated or not treated with methyl viologen.

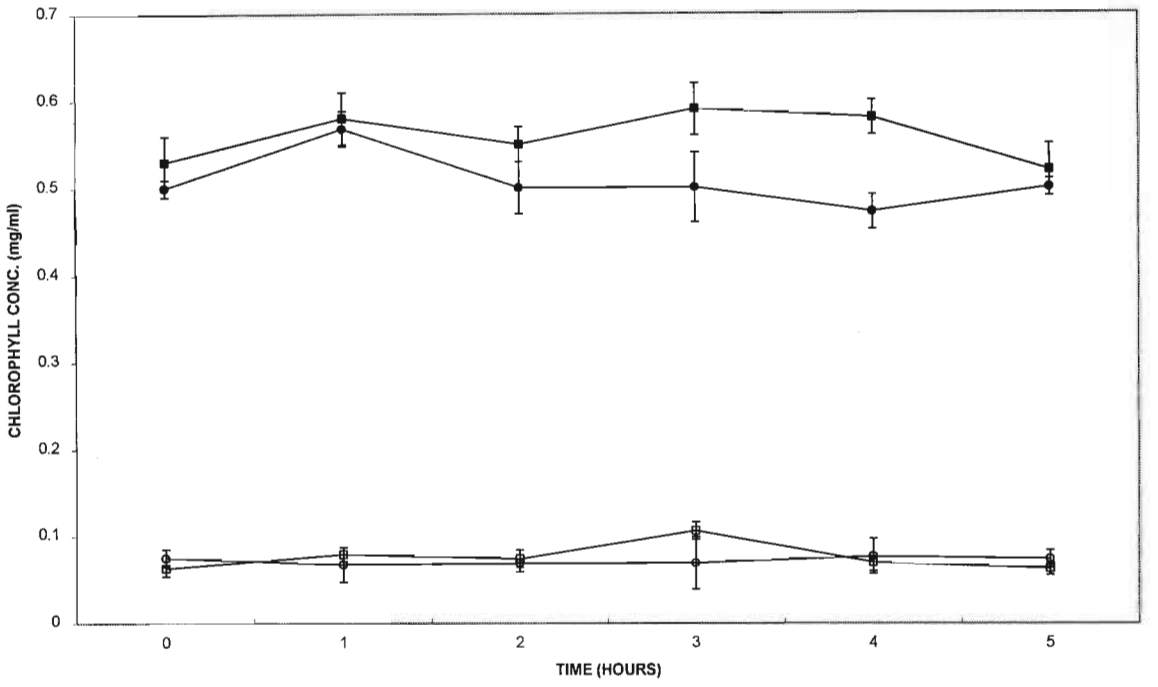


Figure 28. Chlorophyll assay of T131B transformants put under high light intensity over a period of five hours. (0) Untransformed plant not treated with methyl violegen, ( ) untransformed plant treated with methyl violegen, (●) a high expressing transformant T131B without 10 μM methyl violegen and (■) a high expressing transformant T131B with 10 μM methyl violegen. Each data point represent the mean, bar represents standard deviation of three separate determinations.

There was no noticeable change in the chlorophyll content of both controls and transformants put under high light intensity in the presence or absence of paraquat. The chlorophyll content of transformed plants was seen to be higher than that of the untransformed plants.

Chlorophyll content was also measured in leaf discs of GOR10T transformants that were soaked in 10 μM paraquat and subjected to darkness, low light and high light intensities over a period of five hours.

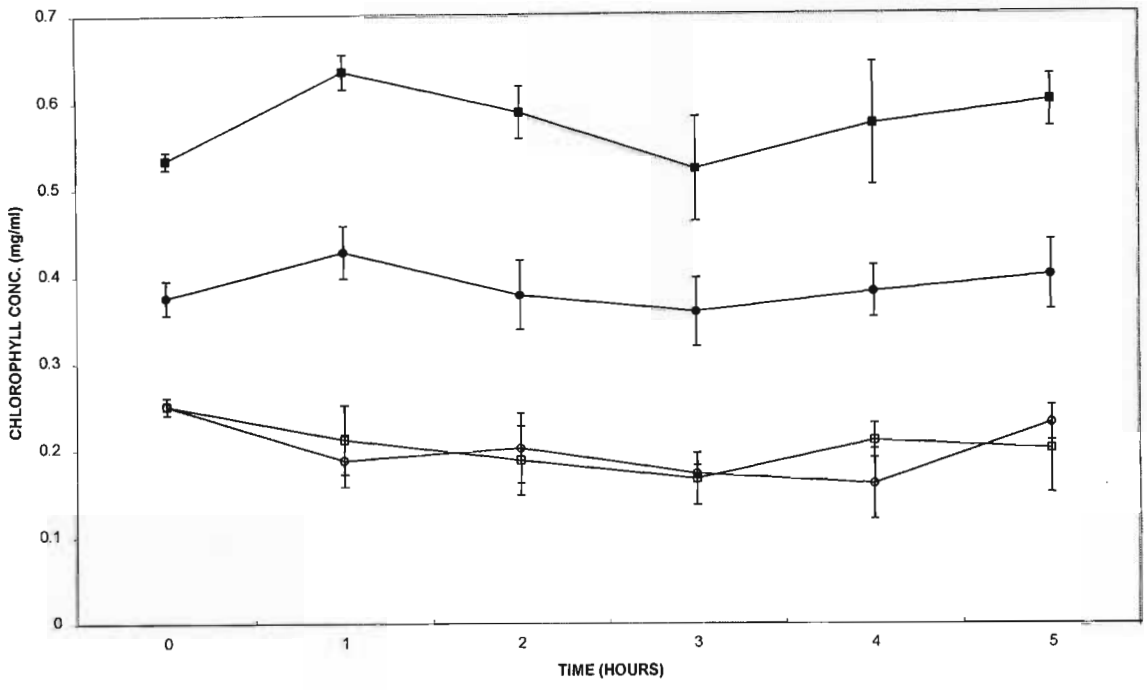


Figure 29. Chlorophyll assay of GOR10T transformants placed in darkness over a period of five hours. (0) Untransformed plant not treated with methyl viologen, ( ) untransformed plant treated with methyl viologen, (●) a high expressing transformant GOR10T without paraquat and (■) a high expressing transformant GOR10T with 10 μM paraquat. Each data point represent mean, bar represents standard deviation of three separate determinations.

In the dark there was no change in the chlorophyll content in both controls and transformants in the presence and absence of paraquat (Fig. 29). A high chlorophyll content was again seen in the transformants as compared to the untranformed plants

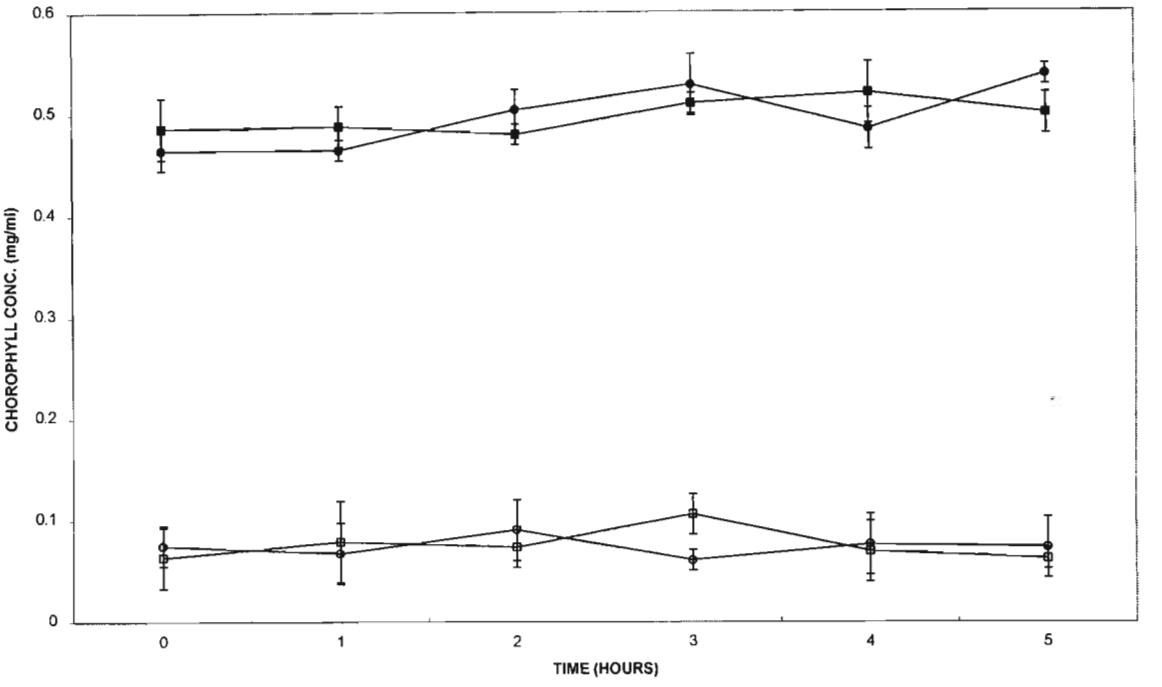


Figure 30. Chlorophyll assay of GOR10T transformants put under low light intensity over a period of five hours. (0) Untransformed plant not treated with methyl viologen, ( ) untransformed plant treated with methyl viologen, (●) a high expressing transformant GOR10T without methyl viologen and (■) a high expressing transformant GOR10T with 10 μM methyl viologen. Each data point represent the mean, bar represents standard deviation of three separate determinations.

Under low light intensity there was again no change in the chlorophyll content in both controls and transformants in the presence or absence of paraquat (Fig. 30). The chlorophyll content of tranformants was seen to be higher than that of untransformed plants.

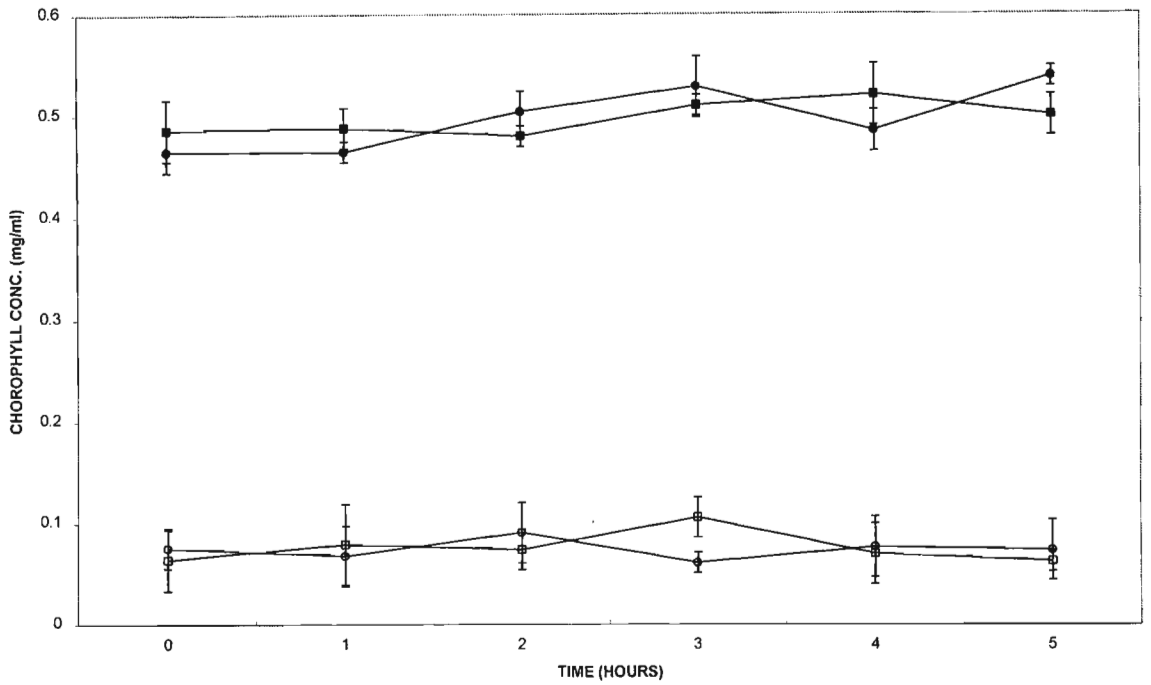


Figure 31. Chlorophyll assay of GOR10T transformants put under high light intensity over a period of five hours. (0) Untransformed plant not treated with methyl violegen, ( ) untransformed plant treated with methyl violegen, (●) a high expressing transformant GOR10T without methyl violegen and (■) a high expressing transformant GOR10T with 10 μM methyl violegen. Each data point represent the mean, bar represents standard deviation of three separate determinations.

Under high light intensity the chlorophyll content remained unchanged in both controls and transformants in the presence or absence of paraquat, but the chlorophyll content of transformants was higher than that of untransformed plants (Fig.31).

CHAPTER 6 : DISCUSSION

6.1 INTRODUCTION

The aims of the present study were to produce tobacco plants expressing high levels of *Escherichia coli* glutathione reductase in the chloroplast, and to investigate the effect of oxidative stress on tobacco with (1) increased levels of cytoplasmic GRA, (2) increased levels of chloroplastic GRA and (3) increased levels of cytoplasmic GRA and chloroplastic SOD. This project was undertaken to investigate the role of GR in plants subjected to environmental stress using transformed plants as a research tool.

6.2 TRANSFORMATION STUDIES

6.2.1 Transformation of tobacco using *E. coli* gene for GR.

Leaf disc transformation was done according to the method of Horsch *et al.,* 1985. Young actively-growing leaves were used in the transformation. The experiment was done three times but without success. Either calli did not form completely and the leaf discs became brown after some weeks in the dark or calli formed in the dark and when leaf discs were transferred to root inducing media in a 16 hour day/8 hour night regime they did not form roots and became yellowish in colour. The transformation method was then modified following the method of David *et al.*

(1993) by virulence induction of *Agrobacterium.* Leaf discs were placed in the dark for 21 days with the selective antibiotics and then transferred to induction medium without antibiotic in the dark for 21 days. This was unsuccessful as very little calli formed. Calli that was produced also failed to root and subsequently died.

In the final attempt, leaf discs were pre-treated with 0.5 μM NAA (naphthalene acetic acid) (Gui'varch', 1993) to improve transformation efficiency. This was also unsuccessful.

Many factors may have contributed to the failure of these experiments. It has been reported that a temperature of about 25°C is required for transformation to take place (Fisk *et al., 1993*). The temperature in the growth room where leaf discs were placed in the dark was 28°C or higher. The second factor that led to the failure of this experiment could be DNA methylation of the transgenes which were present on the integrated T-DNA and subsequent inactivation of the *nos-npt*11 marker gene and this might have been the reason for the problems in shoot regeneration (Mandal *et al., 1992*). The selective antibiotic concentration was also reduced to 25 mgL^{-1} so that transformation might not be inhibited. Problems encountered might have been mainly caused by the inhibition of the expression of the *nos-npt*11 gene in the integrated T-DNA rather than T-DNA loss or mutation. It was very unfortunate that it was not easy to obtain a DNA methylation inhibitor such as 5-azacytidine to remove the block in regeneration of shoots from calli.

6.2.2 Restriction analysis of the pKG2 T-DNA

It was suggested that Eco R1 and Hind 111 should be used for restriction analysis of T-DNA of PKG2 since the theoretical restriction indicated that Hind 111 and Eco R1 sites occurred in the T-DNA of PKG2. The rectriction mapping procedure was commenced by using restriction enzymes that were specific to the polycloning site of pBIN19 and these enzymes were expected to cleave only within the T-DNA, and not generate additional bands. The preliminary restriction map (Fig. 15A) indicated that two *Hind*111 sites occurred in the T-DNA of pKG2 . Analysis showed that the two sites were absent (Fig. 15B, lanes 7 and 8). This could mean that there was a problem in cutting the DNA with *Hind* 111. The positions of the *Eco* R1 sites on pKG2 were analysed and also here the expected 1.9 kb fragment was obtained. The reasons which might have contributed to the failure of the two enzymes to cut the T-DNA might have been that one unit of enzyme to was not enough to cut the T-DNA. The time of the digestion was about 2 hours at 37°C and this was long enough because usually 1 hour restriction is enough. The plasmid DNA was checked for purity and the value was 1.8, which means that it was pure enough to be cut with enzymes. The bands might have been to small to be seen for the concentration of DNA seemingly was very small. Only uncut bands were seen.

6.2.3 Screening for the highest expressor.

Plants exhibiting highest expression of SOD and GR were to be cloned and used in the experiments. Seeds of transgenic plants were provided by Prof. Dvora Aviv (Israel Institute). These were germinated in vermiculite under greenhouse conditions (Fig. 16A and 16B) to be screened for the highest expressors of GOR and SOD. Screening was done in 8 GOR10T (cytoplasmic GR and chloroplastic SOD transformants) plants by assaying the SOD and the GR activity. Table 1 shows results of gor activity for the 8 GOR10T plants expressed as per mg protein. Plant number 6 was found to be the highest expressor of GOR activity i.e 190±18 nmoles NADPH/min/mg protein. Before the SOD assay was performed using native polyacrylamide gel electrophoresis, a native PAGE was done to see whether the gel could show the distribution of protein bands (Fig. 17). The SOD assay was then performed on the 8 GOR10T plants (Fig.18). 100μg protein was loaded onto each lane. The SOD activity was the same because bands of the 8 plants were all equal. This experiment was repeated three times and the banding pattern was similar at all instances. This led to the conclusion that the highest expressor plant was plant number 6 because it had the highest GRA as compared to all other plants including the controls.

6.2.4 Cloning the highest expressor.

Explants derived from the highest GR and SOD expressor which had also been screened for kanamycin resistance were placed on induction medium after being

sterilised (Fig. 19A). After two to three weeks embryos were excised and placed on germination medium (Fig. 19B). Embryos were allowed to germinate and grow to 50 mm, after which shoots were removed and hardened off in greenhouse conditions, by transferring to pots containing moistened potting soil (Fig. 16B). Cloning was done in order to mass produce experimental material.

6.3 BIOCHEMICAL STUDIES

6.3.1 An effect of methyl violegen on glutathione reductase activity.

In this study tobacco plants, transformed with bacterial reductase genes, were used to elucidate the role of glutathione reductase during oxidative stress. Oxygen stress was induced by soaking leaf discs in 10 μM methyl violegen and placed under high light intensity, low light intensity and in darkness for five hours. Soaking in methyl violegen would increase the oxidative stress because this compound increases the oxidative stress directly by generating oxygen free radicals (Halliwell and Gutteridge, 1989).

In the present investigation there was a significant increase in the levels of GRA in the leaves of transformants with enhanced cytoplasmic GR (T131Bs) and control plants following exposure to methyl violegen at high light intensity (Fig. 22). These results support those of Foyer *et al.* (1991) who showed that both light and the oxidative stress mediator, methyl violegen, caused an increase in the amount of extractable GRA in transgenic and nontransgenic plants. Transgenic

plants with enhanced cytoplasmic GR showed higher GRA than did the controls. Under low light the enhanced cytoplasmic GR transformants showed higher GRA than did controls (Fig. 20). The rise in GRA seen could be as a result of paraquat stress because this is consistent with findings of Asada and Takahashi (1987) that light should influence the Asada-Halliwell pathway, by the normal production of superoxide from photosystem 1 or the Mehler reaction. However, this will be enhanced under high light, as this condition deplete stromal NADP levels, resulting in an increase in the reduction state of ferredoxin and photosystem 1 (Bowler *et al.* 1982). Given the effect of such superoxide generation on the antioxidant pathway, it seems likely that light should influence GR. Also paraquat had no effect on enhanced cytoplasmic GR transformants GRA placed in the dark. This was expected since paraquat is photoreduced by photosystem 1 and subsequently reoxidised by transfer of its electrons to oxygen forming the superoxide anion (Halliwel an Gutteridge, 1989) which leads to highly reactive hydroxyl radicals and related species being formed. In darkness oxidative stress by this compound is not possible since it cannot be photoreduced by photosystem 1.

In enhanced cytoplasmic GR and chloroplastic SOD (GOR10T) transformants the overall glutathione reductase activity was further enhanced during oxidative stress, but with time GRA declined (Fig. 23, 24 & 25). With enhanced levels of SOD the concentration of hydrogen peroxide increases. Although GR is the rate limiting enzyme, the supply of NADPH is the most crucial factor responsible for antioxidant functioning (Robinson, 1988). The decline seen in GRA with time

could reflect a possible decline in reductant and / or an increase in the rate of hydrogen peroxide production (Law *et al.,* 1983; Smith *et al,* 1985). Investigations have shown that hydrogen peroxide is responsible for the oxidation of GR (Law *et al* 1983). Excessive hydrogen peroxide production and the loss of metabolite pools are both suggested to inactivate the antioxidant enzymes (Hossain and Asada, 1984a; Nakano and Asada, 1981). Oxidation of GR due to higher hydrogen peroxide concentration led to the inactivation of the Calvin cycle enzymes and this led to cell death. There was no noticeable change in the chlorophyll content during the experiment and this might be due to a short period within which the experiment was conducted, which is only five hours. If time had been extended chlorophyll beaching might have resulted. The chlorophyll content of transformants was higher than that of untransformed plants and that might be due to the fact that transformants were purely samsun plants whereas untranformed plants were Samx (Samsun X Xanthi) plants. It is not good to increase levels of SOD in plants because it leads to more oxidative damage.

Protection against oxygen free radicals damage appears to be vital in times of stress, such stress conditions are frequently found to induce increases in the extractable activities of free metabolising enzymes such as GR and other protective enzymes, this is also shown in this study because at high level of stress, GR increased, leading to protection against oxygen free radicals damage. Also when GRA was expressed (g fresh weight)$^{-1}$ results followed a similar pattern (data not shown).

6.4 CONCLUSION

The main aim of the present investigation was to investigate the effect of oxidative stress on tobacco with enhanced levels of cytoplasmic GR and / or chloroplastic SOD. Preliminary results have indicated that the use of genetically engineered plants can provide a powerful tool with which to examine the relationship between gene expression and antioxidant functioning. Paraquat, the oxidative stress mediator, was shown to induce an increase in the levels of glutathione reductase in both control and transformed plants. The increase in GRA was more marked in transformants with enhanced cytoplasmic GR and chloroplastic SOD (GOR 10T) when they were subjected to high light intensity. This is consistent with the findings that the glutathione reductase levels in transgenic tobacco had been shown to be affected by oxygen stress (Whittaker, 1990) and light (Foyer *et al.*, 1991).

CHAPTER 7

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