CHARACTERISATION OF *CIS*-ELEMENTS IN THE *GST-27* PROMOTER OF *ZEA MAYS*

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by

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ABSTRACT

The ability to control the expression of selected genes has many applications both in industry and academic research. The 27 kDa subunit of the glutathione S-transferase II (*GST-27*) gene in maize is not transcribed in the aerial organs of maize during normal growth and development. However, it can be induced by treatment with herbicide safeners (*e.g.* dichlormid) resulting in high levels of expression. Since the promoter of this gene may be useful as a tool to control the expression of other genes, we have investigated the molecular basis of its induction by the identification of the *cis*-acting promoter elements involved. Safeners also induce the production of enzymes and substrates which are involved in a plant's defense against pathogen attack and in combating oxidative stress. Thus, information relating to *GST-27* induction may also add to our knowledge relating to these other responses.

Deletion analysis of the *GST-27* promoter was carried out by transient expression of *GST-27*::GUS transcriptional fusions in Black Mexican Sweet (BMS) maize cells. These experiments indicated that a 378 base pair region of the promoter can confer safener-inducibility on GUS expression. This region of the promoter was *in vivo* footprinted and four putative safener-responsive elements were thus identified (regions in which G residues are protected when the gene is expressed). These elements are similar to each other and to an ethylene responsive element in the *GST1* gene of carnation, Electrophoresis mobility-shift assays indicated that one or more nuclear proteins from maize leaves specifically interact with all of the elements. These elements were mutated in *GST-27*::GUS constructs, which were tested in transient assays in BMS cells in order to obtain direct evidence for their involvement in safener-dependent transcription. The transient assay technique was, however, found to be insufficiently sensitive for this purpose. The *GST-27*::GUS constructs containing mutations of the putative elements were therefore tested in transgenic tobacco.

The 570 base pairs of the wild type *GST-27* promoter upstream of the transcription start point was found to retain inducibility in transgenic tobacco plants, but a 378 base pair *GST-27*::GUS transcriptional fusion (which was inducible in BMS maize cell lines) was not inducible when tested in tobacco. In order to test for loss of function, mutations of the putative safener-responsive elements were introduced into the 378 base pair truncated promoter; in order to test for a gain of function the putative elements were fused to the -60 and -90 35S cauliflower mosaic virus minimal promoters and tested in stable tobacco transformants. These experiments did not, however, prove that these elements play a role in safener-dependent transcription. Surprisingly, the -90 minimal promoter itself was found to be responsive to safener. A previously characterised *cis*-element, activation sequence-1 (*as-1*), is contained within the -90 minimal promoter and may account for its inducibility; a similar element is present in the *GST-27* promoter but its role in safener-dependent transcription, if any, is unclear.

ABBREVIATIONS

The following abbreviations are used in the text:

AEBSF	4-(2-aminoethyl)-benzenesulfonylflouride,HCl
ATP	adenosine 5'-triphosphate
BAP	6-benzylaminopurine
bp	base pairs
BSA	bovine serum albumin
CaMV	cauliflower mosaic virus
cfu	colour forming units
CIP	calf intestinal alkaline phosphatase
cpm	counts per minute
dH_2O	deionised water
DMS	dimethyl sulphate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
EDTA	ethylenediamininetetraacetic acid
EMSA	electophoretic mobility shift assay
g	gramme
GUS	β-glucoronidase
GST	glutathione *S*-transferase
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
IPTG	isoproyl-β-D-thiogalactopyranoside
kb	kilobase pairs
MOPS	3-(N-morpholino)propanesulphonic acid
mRNA	messinger ribonucleic acid
MS	Murashige and Skoog slats
MU	methylumbelliferone
MUG	4-methylumbelliferyl β-D-glucoronide
NAA	α-naphtaleneacetic acid
NP-40	nonidet P-40
OD	optical density (absorbance)
PCR	polymerase chain reaction
PEG	polyethylene glycol
PIC	preinitiation complex
PolII	RNA polymeraase II
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulphate

SDS-PAGE	sodium dodecyl sulphate polacrylamide gel electrophoresis
TAF	TBP-associated factor
TBP	TATA-binding protein
Tm	melting temperature
Tris	tris(hydroxymethyl)aminomethane
U	units
UV	ultraviolet
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
X-gluc	5-bromo-4-chloro-3-indolyl-β-D-glucoronide

CONTENTS

Chapter One

GENERAL INTRODUCTION

1.1 TRANSCRIPTION

This study is concerned with analysing the molecular basis of the transcriptional regulation of the *GST-27* gene from *Zea mays*. A great amount of work has led us to our current understanding of transcriptional regulation in eukaryotes. It has been discovered that, with up to 100,000 genes to regulate in the average eukaryotic organism, RNA polymerase II (Pol II) requires the involvement of many transcription factors in order to achieve correct temporal and spatial levels of transcription. These transcription factors mediate their effects through interaction with different DNA sequence elements found within a gene's promoter, which is defined as the regulatory region of untranscribed DNA normally situated upstream of the 5' end of a gene. The regulatory sequences (*cis* elements) include the TATA box, which is situated about 30 base pairs upstream of the transcription start point (TSP), and the initiator element, which spans bases -2 to +4 relative to the TSP, which together determine where transcription is initiated. In addition, gene-specific upstream promoter elements proximal to the TSP and distal elements (enhancers and silencers) determine the rate of transcript elongation and reinitiation.

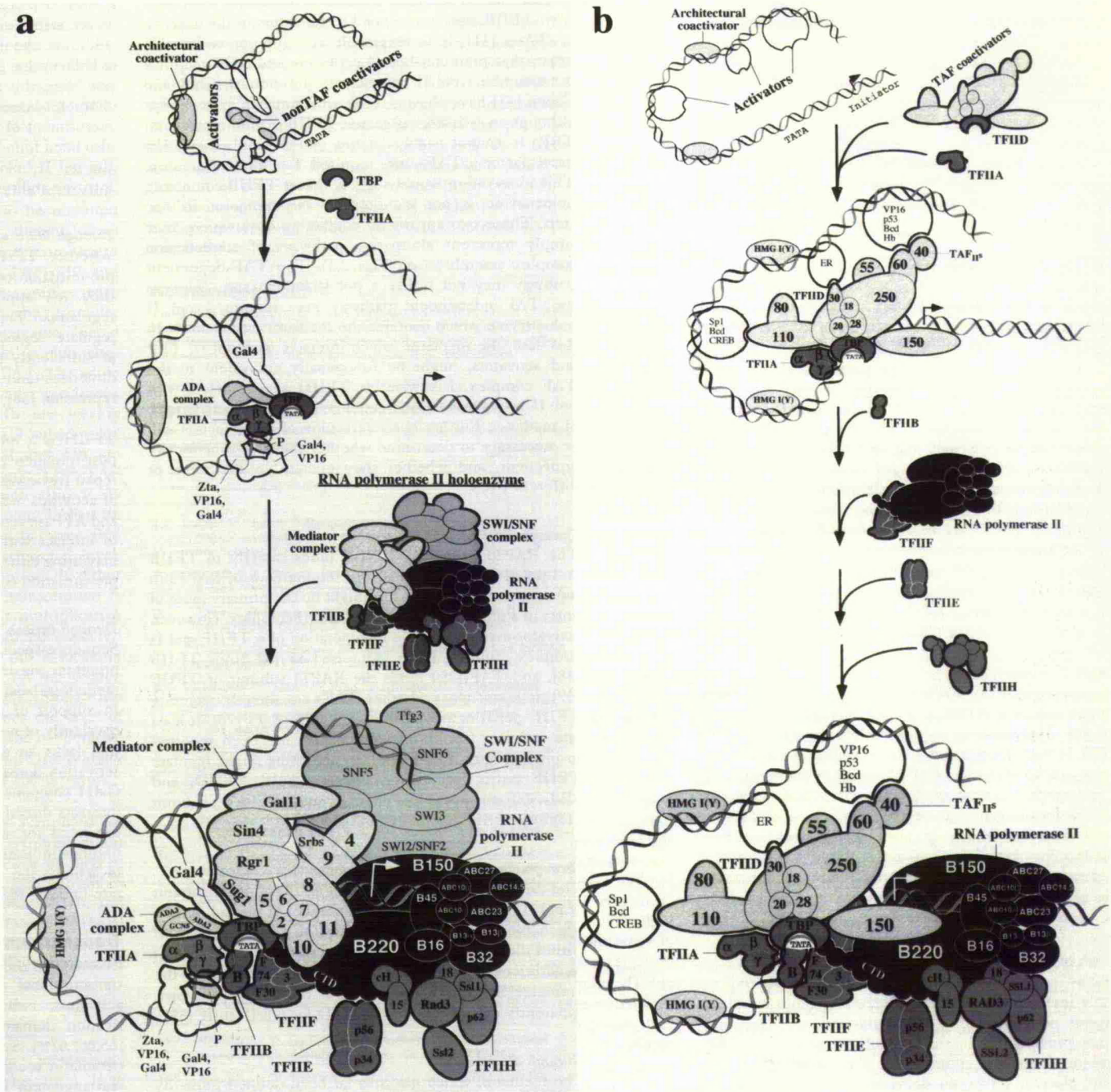
1.1.1 Formation of the pre-initiation complex

RNA polymerase II requires the assembly of a multiprotein complex, containing several general initiation factors, in order for transcription to occur (for reviews see Conaway and Conaway, 1993 and Buratowski, 1994). Although much of the research into transcription machinery is performed in non-plant systems, the general characteristics of transcription have been found to be conserved in species ranging from yeast to mammals and so are also likely to be applicable, to a large extent, to plant species (Zawel and Reinberg, 1992). It was originally thought that this pre-initiation complex (PIC) involved the stepwise assembly of general transcription factors on to the promoter but recent experimental evidence suggests that a 'holo PIC', containing all the transcription factors necessary for basal transcription, is assembled prior to binding to the promoter (Ossipow *et al.,* 1995) (see figure 1). The minimal components necessary for basal-level initiation from a core promoter has been defined *in vitro* using purified factors from yeast or rat and consist of TATA-binding protein (TBP), transcription factor IIB (TFIIB), TFIIE, TFIIF and TFIIH. *In vivo*, other factors which are involved in mediating the effect of upstream promoter elements are also present in the PIC, namely TFIIA, TFIID, TFIIG, TFIIH and THIIJ. TFIID, which binds to the TATA box, is a complex of the TBP and TBP associated factors (TAFs). Some promoters lack a TATA box but still require TBP for transcription which indicates that the TBP may interact with other sequence-specific DNA-binding proteins. TFIID, via $TAF_{II}150$ and $TAF_{II}250$, also interacts with the initiator and with downstream bases within the gene up to approximately 35 base pairs away from the TSP which provides additional promoter specificity (Verrijzer *et al.,* 1995). TAFs, in addition to their contribution to promoter specificity, also function as coactivators by forming a bridge between the activation domains of some transcription factors and the transcription machinery on the promoter, although non-TAF

Figure 1.1

Schematic diagrams illustrating the two potential pathways of transcription complex assembly (a) A TAF-independent two-step process involving a pre-assembled pol II holoenzyme (as described predominantly in yeast) and (b) A TAF-dependent stepwise assembly of basal factors and Pol II (as described predominantly in higher eukaryotic systems). The two pathways represent a composite of many different observations and are not mutually exclusive or inclusive of each other. Reproduced from: Pugh (1996) Curr. Opin. in Cell Biol., **8**:303-311.

This figure has been included to illustrate the complexity of interactions, and the diversity of factors, in a regulated transcription complex. Each oval represents a cloned gene product which is thought to be a component of a regulated transcription complex. Activators are shown in white, coactivators in light grey, basal factors in dark grey and pol II subunits in black. Where feasible, interacting factors are shown to interact. Factors whose interactions have not been fully mapped are positioned arbitrarily. Different activators with the same target are listed in the same oval and activators with multiple target sites are listed in more than one oval.


a
Architectural coactivator
Activators
nonTAF coactivators
TATA
TBP
TFIIA
Gal4
ADA complex
TFIIA
P
Gal4, VP16
Zta, VP16, Gal4
RNA polymerase II holoenzyme
Mediator complex
SWI/SNF complex
RNA polymerase II
TFIIB
TFIIF
TFIIE
TFIIH
Tfg3
SNF6
SNF5
SWI/SNF Complex
Gal11
SWI3
Sin4
Srbs
4
SWI2/SNF2
Rgr1
9
B150
Gal4
Swg1
8
5
6
7
2
11
10
B220
B16
B32
HMG I(Y)
ADA complex
TFIIA
TFIIF
TFIIB
TFIIE
TFIIH
b
Architectural coactivator
Activators
TATA
Initiator
TAF coactivators
TFIID
TFIIA
VP16
p53
Bcd
Hb
HMG I(Y)
ER
40
TAF$_{II}$s
55
60
TFIID
30
250
80
18
20
28
Sp1
Bcd
CREB
110
150
TFIIA
TFIIB
RNA polymerase II
TFIIF
TFIIE
TFIIH
B150
B45
B220
B16
B32
CTD
Rad3
p62
p56
p34
Ssl2

coactivators are also used in this role (reviewed by Goodrich and Tijan,1994). TFIIA binds to TBP and its role may be to enhance the interaction between TBP and the TATA box (Pugh, 1996). TFIIB and TFIIF are involved in the recruitment of RNA Pol II to the TBP-core promoter complex, while TFIIE and TFIIH are involved in converting the pre-initiation complex into an elongation complex, which leaves the promoter and proceeds downstream to transcribe the gene. TFIIH is a multisubunit complex with ATPase and DNA helicase activities, which together may function to provide energy for unwinding a short stretch of DNA near to the TSP (for review see Drapkin and Reinberg,1994).

1.1.2 Regulatory transcription factors

Regulatory transcription factors, which bind to specific *cis* elements within the promoter, contribute to determining the rate of transcription by affecting the rate of the formation of the pre-initiation complex (PIC) (for review see Travers, 1993). The factors can function to increase transcription by stabilising or promoting the production of the PIC via contact with one or several of the general transcription factors, by freeing a promoter from histone-mediated interference (see 1.1.3) or by antagonising the repressors which inhibit basal transcription *in vivo* (Zawel and Reinberg, 1992). Examples of factors which function to stabilize the PIC, by promoting the formation of the three-dimensional nucleoprotein complexes, are factors which contain a high mobility group (HMG) domain. These function by bending the promoter DNA and thereby bring into proximity proteins which may have been separated by a region of DNA (Grosschedl *et al.,* 1994). The interactions between the proteins within the PIC require that the regulatory proteins are precisely positioned relative to each other and the promoter. Therefore, the position of the *cis* elements relative to each other and to the TSP, which determine the position of the protein in the transcription complex, are very important.

Many transcription factors show activity in heterologous systems indicating that mechanisms of activation are conserved in eukaryotes. For example, the yeast GAL4 activator is active in *Drosophila* (Fischer *et al.,* 1988), mammalian cells (Kakidani and Ptashne, 1988) and plant cells (Ma *et al.,* 1988) and plant transcription activators can function in mammalian cell extracts and in yeast (Sablowski *et al.,* 1994). Transcription factors from all systems are thought to have a modular structure, consisting of a DNA-binding domain and, in positive regulators, an activation domain (Frankel and Kim, 1991). The DNA binding domains can be grouped into a diverse but limited range of protein structures as follows: basic-leucine zippers (bZIP) *i.e.g. jun* and *fos* (Kerppola and Curran, 1991); zinc fingers *i.e.g.* TFIIA (Schmeideskamp and Klevit, 1994); basic helix-loop-helix *i.e.g.* MyoD from muscle cells (Ellenberger, 1994); helix-turn-helix *i.e.g.* bacterial repressors and activators (Luescher and Eisenman, 1990); MADS-box *i.e.g.* *agamous* from *Arabidopsis* (Yanofsky *et al.*, 1991); and the homeodomain family *i.e.g. knotted* from maize (Duboule, 1994). Most of the well characterised binding domains make use of the α-helix protein conformation to make contact with bases in the major groove of DNA, although

surrounding regions of the protein are also involved in DNA interactions (Pabo and Sauer, 1992). The α-helix's shape and dimension allows it to fit into the major groove of DNA. While it is thought that this groove is more important than the minor groove for site-specific recognition some base contacts do occur in the minor groove, such as those made with the N-terminal arm of the homeodomain (Travers, 1993). Some groups of binding domain are present in proteins which control target genes with diverse functions. For example, zinc fingers occur in proteins induced by growth signals, in proto-oncogenes, in general transcription factors and in the regulatory genes of eukaryotic organisms (Pabo and Sauer, 1992). However, others are involved in the control of a more specific set of genes. For example, the homeodomain proteins act as master regulatory genes during development in most eukaryotes (Duboule, 1994).

Activation domains can also be classified into a number of groups and include acidic (Ma and Ptashne, 1987), glutamine-rich, (Courey and Tjian, 1988) and proline-rich domains (Mermod *et al.,* 1989). The yeast factor GAL4, involved in galactose metabolism, contains a typical acidic activation domain as it has a high proportion of acidic residues. The human factor CTF/NF1 is an example of a proline-rich activation domain. However, the presence of one of these activation domains does not necessarily mean that the protein will function as an activator. The repression or activation properties of a protein are influenced by which promoter it binds to and by the presence of other transcription factors on that promoter. In yeast, for example, RAP1 is one transcription factor involved in the activation of genes encoding ribosomal proteins and glycolytic enzymes, but it can also function as a repressor, silencing mating type genes in the HML and HMR loci (Shore, 1994). In addition, the concentration of a transcription factor can affect its properties to activate or repress transcription. This is true of the Krueppel factor from *Drosophila melonogaster* which can activate transcription when present at low concentrations, when it functions as a monomer, while at higher concentrations it forms homodimers which act on the same DNA binding site to repress transcription (Sauer and Jaeckle, 1993).

1.1.3 Chromatin structure

In order for transcription to occur, transcription factors must be able to gain access to the promoter. This involves the disruption of the local chromatin domain and nucleosome arrays (for reviews see Paranjape *et al.*, 1994; Wolffe, 1994 and Wallrath *et al.*, 1994). Nucleosomes thus have the potential to act as transcriptional repressors. For example, an *in vivo* study in the yeast *Saccharomyces cerevisiae* found that a reduction in histone H4 synthesis (a component of the nucleosome) resulted in a reduction of gene repression, and normally inactive genes were expressed (Wallrath *et al.,* 1994). Nucleosomes have also been shown to be involved in gene activation. This is illustrated by the heat shock protein *hsp26* gene of *Drosophila melanogaster* where HSF and GAGA factors bind to elements approximately 200 base pairs apart and are thought to be brought into proximity with each other by the intervening DNA wrapping around

a specifically positioned histone octamer (Lu *et al.,* 1993). The primary function of some proteins, such as the yeast SWI-SNF and NURF (Nucleosome-Remodelling Factor) protein complexes, is to reorganise the nucleosome structure in order to allow transcription factors (for a recent review see Peterson and Tamkun,1995). Each of these protein complexes contains an ATPase and they are thought to use the energy from the hydrolysis of ATP to alter the structure of the nucleosome. Certain repressors, such as the yeast α2 repressor, can act in an opposite way, by dictating the position of nucleosomes on the promoter and so blocking access of the transcription machinery to the DNA (Shimizu *et al.,* 1991).

Preset genes contain binding sites for *trans*-acting factors which are accessible prior to activation of the gene, and the promoter does not require remodelling of nucleosomes in order for transcription to occur. The *Drosophila hsp26* referred to above (Lu *et al.,* 1993) is a good example of an inducible preset gene. This gene, which is induced in almost all the cells in the organism within minutes after heat shock, has been shown to have the GAGA factor bound to its *cis* element, and RNA pol II bound but paused at position +25 of the gene prior to heat shock. After heat shock, HSF binds and transcription can commence. No major changes in chromatin structure occur at any stage (Thomas and Elgin, 1988). An example of an inducible gene with a promoter which does require remodelling is found in the mouse mammary tumour virus long terminal repeat (MMTV LTR) promoter region. Nucleosomes are positioned so as to obscure the TATA box and some upstream elements, while two binding sites for the glucocorticoid receptor remain accessible (Pina *et al.,* 1990). When the glucocorticoid receptors bind steroids they move from the cytoplasm to the nucleus, bind to these sites and act in concert with additional proteins to disrupt the nucleosomes, thereby exposing the other *cis* elements which are required for transcription to occur.

1.2 INDUCIBLE GENE EXPRESSION SYSTEMS

The reason why we are interested in studying the molecular basis of the transcription of the *GST-27* gene from *Zea mays* is because this gene is not normally expressed in the aerial organs of the plant during normal growth. However, it can be induced to express at high levels following treatment with herbicide safeners *e.g.* dichlormid (see section 1.4 for definition of safeners). Therefore this promoter may be useful for use in an inducible gene expression system. The importance of inducible gene expression systems for regulating the production of specific proteins in transgenic organisms has long been recognised. Such systems allow external control over the expression of specific genes, and are useful for both academic and commercial purposes. Such systems have been developed for use in bacteria, yeast, mammals and plants and are described below.

1.2.1 Inducible gene expression systems in bacteria

Inducible gene expression systems were first developed in bacteria. In 1977 Itakura *et al.* succeeded in achieving inducible production of the hormone somatostatin in *Escherichia coli* by placing the gene under the control of the lac operon. The somatostatin gene was chosen as it encodes a short peptide of 14 amino acids. The lac operon, responsible for lactose utilization, was chosen because it had been well characterised by Jacob and Monod in 1961. The operon's expression is induced by lactose. The natural inducer of this promoter is allolactose (an analog of lactose), but a gratuitous inducer, isopropyl-ß-D-1-thiogalactoside (IPTG), can be used to turn on transcription (Lewis *et al.,* 1996). In the absence of lactose a repressor molecule prevents transcription by tightly binding to the promoter DNA, but when lactose is present it loses its ability to bind to the DNA. Monod *et al.* (1963) proposed that the binding affinity of the repressor for the promoter was affected by the inducing molecule (allolactose or IPTG) causing a change in the repressor's conformation due to allosteric effects. Over 30 years later, using X-ray crystallography, this theory has been proved (Lewis *et al.,* 1996). This inducible promoter system is now widely used as a tool to control transgene expression in bacteria.

1.2.2 Inducible gene expression systems in yeast

In yeast the *GAL* operon, responsible for galactose utilization, is effective for use in an inducible gene expression system because the genes controlled by the operon are barely transcribed in the absence of galactose but in its presence transcription can increase 1,000 fold (St. John and Davis, 1979). *GAL4* encodes a protein necessary for the expression of *GAL1, GAL7* and *GAL10. GAL80* encodes a protein that directly interacts with GAL4 thereby preventing transcription of *GAL1, GAL7* and *GAL10* (Laughon and Gesteland, 1982). In the presence of galactose, or one of its metabolites, the conformation of GAL80 changes so that it can no longer bind to GAL4 which results in the induction of the *GAL* genes (Oshima, 1982). The operon was first used in an inducible expression system by Johnston and Davis in 1984 when the yeast *HIS3* gene was placed under the control of the *GAL* operon DNA situated between the genes *GAL1* and *GAL10* . The *GAL-HIS3* fusions were used to study *GAL* gene regulation and to determine which region of the operon was responsible for the regulation of which gene.

1.2.3 Inducible gene expression systems in higher organisms

In mammalian cells steroid hormones are able to modulate gene expression by binding hormone-specific intracellular receptor proteins which, after binding the hormone, move to the nucleus and can associate with specific DNA *cis* elements (Anderson, 1983). The glucocorticoid receptor and its corresponding *cis* element comprise a steroid system which has been used as an effective gene switch in mammalian cell lines (Hirt *et al.,* 1994). One such system utilises the long terminal repeat (LTR) of the mouse mammary tumour virus (MMTV)

promoter which contains steroid-responsive enhancer elements. In 1983 Chandler *et al.* pioneered the use of the MMTV LTR in an inducible expression system by placing the herpes simplex virus thymidine kinase (*tk*) gene under its transcriptional control. By adding the synthetic glucocorticoid, dexamethasone, to the growth medium, a 50-fold induction in expression of *tk* was observed thereby transforming a *tk*- rat cell line to *tk*+. Problems can occur using this system in some cell types as, for example, glucocorticoids can induce programmed cell death in lymphocytes (Migliorati *et al.,* 1992) which illustrates the need for tight specificity of the inducing agent.

The metallothionein-I gene from mouse has a promoter which can be used in mammalian cells and in whole mammalian organisms as part of an inducible gene expression system, as it responds to glucocorticoids and several heavy metals (Durnham *et al.,* 1980). Palmiter (1982) fused this promoter to the rat growth hormone gene and when he used this construct to create transgenic mice he generated a 'supermouse'. Palmiter's transgenic mice, when fed on a diet supplemented with low concentrations of zinc sulphate, showed growth rates 1.8-fold greater than littermates which did not carry the transgene, although accelerated growth was observed before the supplement was added, indicating that the promoter may have responded to endogenous hormones. This particular transgenic mouse model is important academically, since it allows the study of the biological effects of growth hormone. It also has potential applications in farming as a means of generating larger livestock.

1.2.4 Inducible gene expression systems in plants

The external control of gene expression is more difficult in plants than in micro-organisms or cell cultures. In an agricultural setting, the transfer of crops to special growth conditions for the purpose of gene induction cannot easily be achieved. However, a variety of systems have been developed to control gene expression, with varying degrees of success and usefulness to industry or academia. They can be divided into two classes: endogenous and exogenous systems.

There are many endogenous inducible genes in plants. Transcription, translation or post-translational modification of these genes can be activated by a variety of factors including dehydration (Yamaguchi-Shinozaki *et al.,* 1995; Kiyosue *et al.,* 1993), ethylene (Itzhaki and Woodson, 1993; Montgomery *et al.,* 1993; Ohme-Takagi and Shinshi, 1995; Shinshi *et al.,* 1995), low temperature (Jiang *et al.,* 1996), hypoxia (de Vetten and Ferl, 1995), auxins (Hong *et al.,* 1995; Boot *et al.,* 1993; Droog *et al.,* 1995; Takahashi and Nagata, 1992), senescence (Itzhaki *et al.,* 1994), pathogen attack (Mauch and Dudler, 1993; Ohshima *et al.,* 1990; van der Rhee *et al.,* 1990) safeners (Gaillard *et al.*, 1994) and wounding (Shufflebottom *et al.,* 1993). An inherent problem with an endogenous system is that the inducing agent may turn on the transcription of native genes. For example, pathogen treatment can induce as least nine sets of

genes in tobacco making pathogen-induced promoters unsuitable candidates for a gene switch system which will be used in tobacco (Ward *et al.,* 1991). However, the promoter of the tobacco gene pathogenesis-related (PR) protein 1a, which is induced by salicylic acid, has been used to drive GUS expression in transgenic tobacco plants to which salicylic acid was applied (Ohshima *et al.,* 1990). The same promoter has also been used to control expression of the insecticidal CryIA(b) protein from *Bacillus thuringiensis* (Williams *et al.,* 1992), a result which has potential agricultural importance. If transgenic crops were produced which can produce the CryIA(b) insecticide, when induced to, at times of insect infestations, development of toxin resistance among the insect population may be reduced compared to transgenic crops which produced the insecticide constitutively.

Genes involved in phenylpropanoid metabolism are induced by a variety of biotic and abiotic factors and so have the potential to be used in gene switch systems. Doerner *et al.* (1990) created transgenic tobacco which contained the promoter from the bean chalcone synthase gene fused to the reporter gene GUS. On addition of inducing agents, such as mercury chloride, or following pathogen infection, transcription of the gene increased 18-fold. The chalcone synthase gene catalyses the key regulatory step in the synthesis of a class of plant natural products that function as antibiotics (phytoalexins) and so this system can be used as an assay to screen for potential enhancers of natural resistance to disease. Although this system is useful in this respect, its use is limited since the inducing agents are not specific and by inducing the phenylpropanoid pathway toxic metabolites may accumulate in the plant.

Inducible gene systems for use in plants have been developed using exogenous promoters. As the inducible promoter used does not occur naturally in the plant there is a higher probability that only the transgene will be activated by the inducing agent. However, problems may be encountered with such systems, which include achieving the uptake of the inducing compound by the plant and ensuring that once the compound is in the plant it is systemically transported, in an active form, to the site of action (Ward *et al.,* 1993). The signal transduction pathways for many inducing compounds are unclear as yet but the site of action may be in the cytoplasm, such as a steroid receptor protein, or a receptor on the surface of the cell.

A mammalian steroid inducible system has been developed for use in plants (Schena *et al.,* 1991; Lloyd *et al.,* 1994). Schena *et al.*(1991) created a hybrid cauliflower mosaic virus (CaMV) 35S promoter, which contained several tandem copies of the rat glucocorticoid response element, fused to a reporter gene. The CaMV 35S promoter was used at it is a strongly expressing constitutive promoter active in many plant species. Tobacco protoplast cultures containing the hybrid promoter were co-transfected with the MMTV glucocorticoid receptor. When dexamathasone was added to the culture, the glucocorticoid receptor bound the hormone ligand and this receptor/hormone complex could then bind to the response elements in

the hybrid promoter, which led to a 150-fold increase in expression of the reporter gene. The system was further developed for use in *Arabidopsis* (Lloyd *et al.,* 1994). The *Arabidopsis* mutant *ttg* lacks trichomes and anthocyanin pigments, but these are restored by the constitutive expression of the maize transcriptional regulator (R). By fusing the coding region of the rat glucocorticoid receptor to R a chimeric protein was formed which was responsive to hormone treatment. When *ttg* mutants were transformed with the chimeric gene WT phenotype was restored by the addition of dexamethasone, and by using this system the pattern and timing of trichome formation on the developing leaf epidermis were defined.

The prokaryotic Tn*10* tet repressor(R)/operator(O) has also been used in plants as an inducible switch, but in order to reduce expression of a transgene (Gatz *et al.,* 1991). Transgenic tobacco plants constitutively expressing *tetR* were transformed with a 35S-GUS construct which included three *tetO* sequences. The repressor molecules produced from *tetR* bound to the *tetO* operator sequences and interfered with the normal transcription of the gene. The double transformant showed a 50-fold decrease of GUS expression. However, in the presence of tetracycline the repressor is prevented from binding to the operator and it was shown that the application of 0.1 mg/l tetracycline could relieve repression in 30 minutes. This system has been used in tobacco to study the function of a family of transcription factors *in vivo* (Rieping *et al.,* 1994). The dominant-negative mutant of the transcription factor PG13, which leads to reduced amounts of DNA-binding complex ASF-1 in transgenic plants, was placed under control of the tetracycline-inducible promoter. It was shown that the low activity of a truncated CaMV 35S promoter was due to low levels of ASF-1 which were reduced by the presence of the mutant PG13, as when tetracycline was removed and the mutant form of PG13 was not expressed, the activity of the CaMV 35S promoter increased. This system has also been successfully used in potato and tomato plants, although the high levels of the bacterial Tet repressor present in the transgenic plants appears to reduce growth rates (Gatz, 1996). A similar system using the *Escherichia coli lac* operator-repressor system has also been used to control gene expression in tobacco (Wilde *et al.,* 1992). Double tobacco transformants carrying the *lacI* gene driven by the CaMV 35S promoter and the reporter gene GUS driven by the chlorophyll a/b binding protein (CAB) promoter, were generated. The *lac* operator element was introduced into the CAB promoter in order that the *lac* repressor could bind to its cognate operator site and block transcription of the reporter gene. Levels of GUS were reduced in these plants compared to those transformants which contained the same constructs, but without the addition of the *lac* operator elements. However, repression was relieved 15-fold when protoplasts made from the double-transformants were treated with IPTG.

1.2.5 Applications of inducible gene systems

Inducible gene systems have the potential to be used as gene 'switches' in both academic and applied systems. A gene switch can be defined as a system which enables the rigid external

control of transcription levels of a specific gene. To create an inducible gene switch an organism is transformed with a gene of interest fused to an inducible promoter which responds to a specific stimulus. When this specific stimulus is applied to the organism, the gene of interest is transcribed (for review see Ward *et al.* 1993). To create the optimum switch transcription must be tightly regulated by the gene's promoter, there must be low uninduced expression of the gene which can rapidly increase to high levels of expression upon application of the inducer and, in addition, it is desirable that the inducer does not activate transcription of endogenous genes. Dependent on the required function of the inducible gene system, it may be desirable that the gene, when induced to transcribe, is active for either a short or a long period of time. If it is desired that the gene is active for a short period of time, the inducing agent can be applied briefly and then removed, and, assuming that the promoter becomes inactive when the inducing agent is removed, the gene will be transcribed for the short period of time when the inducing agent is present. However, the long term presence of the inducing agent which would be required for long term gene activation may be deleterious to the plant. Systems are being developed to overcome this problem, including the use of site-specific recombinases capable of inserting, inverting or eliminating DNA sequences involved in gene regulation. One such system makes use of a heat shock inducible FLP/FRT site-specific recombination system from yeast, in which the soybean heat-shock promoter *Gmhsp 17.5 E*, which is active at 42°C but inactive at 20°C, is fused to the recombinase FLP (Lyznik *et al.,* 1995). The recombinase functions at specific FRT target sites, and it was demonstrated in a transformed maize cell line that FLP, when activated by a two hour incubation at 42°C, could function to delete a fragment of DNA flanked by FRT sites present in a co-transformed test vector, which led to the expression of the reporter gene GUS. Such a system could be used in order that an inducing signal was amplified by recombination-induced activation of a strong constitutive promoter driving expression of the gene of interest. Once achieved, the rearrangement of DNA would be stable and the gene of interest would be transcribed until the FLP gene was reactivated by the inducing agent.

There are numerous applications for gene switches, one example being the production of the thermoplastic polyhydroxybutyrate. This is synthesised in three steps from acetyl-CoA by the bacterium *Alcaligenes eutrophus*. The first enzyme in the pathway is 3-ketothiolase which is an enzyme endogenous to plants. By expressing the bacterial genes for the remaining two steps in *Arabidopsis* Poirer *et al.* (1992) produced plants that could synthesize the plastic. However, expression of the genes was harmful to the plant and led to reduced growth rates. In this situation it would be useful to allow the plant to grow to full size, without it expressing the genes necessary for the synthesis of the plastic. If these genes could then be induced to transcribe shortly before harvesting of the plants, an increased yield of plastic could be obtained. In addition to their use in the production of specific compounds gene switches could be used to control the development of an organism; for example, if genes known to control

flowering, such as *CO*, could be externally regulated, the timing of flowering could be manipulated to the advantage of the producer (George Coupland, pers. comm.). Gene switches also have use in academic situations, for example, when trying to elucidate the function of a gene. A gene switch utilising the inducible promoters of genes responsive to safener has potential for large scale agricultural use and this topic is covered more fully in section 1.3.4 and 1.4.

1.3 GLUTATHIONE *S*-TRANSFERASES

This study is concerned with the transcriptional regulation of the *GST-27* gene from *Zea mays*. The glutathione *S*-transferase (GST) family of enzymes catalyze the nucleophilic attack of the sulphur atom of the tripeptide glutathione on the electrophilic centre of a variety of toxic chemical compounds (for general reviews see Daniel, 1993; Mannervick and Danielson, 1988; Marrs, 1996; Timmerman, 1989). The reaction forms water-soluble products which are less toxic to the organism. This ubiquitous enzyme family can be found in most organisms including mammals (Daniel, 1993), plants (Lamoureux and Rusness, 1989), insects (Lamoureux and Rusness, 1989), parasites, yeast, fungi and bacteria (Lamoureux and Bakke, 1984). The first GST was identified in rat liver in 1961 (Booth *et al.,* 1961) and in 1973 the first GST purified to homogeneity was isolated from rat liver (Fjellstedt *et al.,* 1973). The GST enzyme family has been the subject of considerable research efforts, as in animal species GSTs are thought to play a role in the detoxification of carcinogens, mutagens and heavy metals (Daniel, 1993; Rushmore and Pickett, 1993). Agronomically, GSTs are of importance for the role they play in herbicide, fungicide and insecticide detoxification (Hatzios, 1991).

1.3.1 Classification of the GST family of enzymes

In animals the most extensive research has been carried out using rat liver where more than ten isozymes have been identified (Mannervik, 1985). Generally, in mammals, the numerous isozymes are differentially expressed in various tissues; some are constitutively expressed while others are inducible. The enzymes, many of which have been cloned, have been shown to normally consist of homodimers or heterodimers of subunits which, in mammals, have been classified into four groups: alpha, mu, pi and theta. This classification is based on sequence homology, substrate specificity and immunoreactivity (Daniel, 1993; Mannervik *et al.,* 1992). A fifth GST activity is membrane bound and referred to as the microsomal GST (Mannervik and Danielson, 1988; Mannervik, 1985). The cDNAs encoding the rat and human microsomal GSTs have been sequenced (Jong *et al.,* 1988). Although they share 95% amino acid similarity to each other they show low homology to the cytosolic GSTs and are thought to be the result of convergent, rather than divergent, evolution. It is not known if they have a specific function which differs from the cytosolic GSTs.

The GSTs identified in animals are similar to those identified in plants in that they normally exist as homodimers or heterodimers (Jepson *et al.,* 1994; Reinemer *et al.,* 1996; Singhal *et al.,* 1991) but there are exceptions including a putative monomer isolated from maize (Dean *et al.,* 1995). Although microsomal forms of plant GSTs have been found (Guddewar and Dautermann, 1979; Wu *et al.*, 1996) most of the literature relates to the cytosolic forms of GSTs. All known plant GSTs are most similar to the theta class, but attempts have been made to further classify them according to their sequence homology and, by constructing a phylogenetic tree, Droog *et al.* (1995) have identified three major classes. Class I includes GSTs from maize (Grove *et al.,* 1988), wheat (Dudler *et al.,* 1991), tobacco (Takahashi and Nagata, 1992), and *Arabidopsis thaliana* (Bartling *et al.,* 1993; Zhou and Goldsbrough, 1993) and, where the gene structure is known, the genes encoding the GSTs contain three exons and two introns. The genes which encode class II GSTs contain ten exons and nine introns and have only been identified in carnation (Itzhaki and Woodson, 1993; Meyer *et al.,* 1991). The genes which encode class III GSTs, where the structure is known, contain two exons and one intron, and were originally identified as a group of auxin-related proteins, and include GSTs from soybean (Ulmasov *et al.,* 1995), tobacco (Droog *et al.,* 1993; Takahashi *et al.,* 1991), potato (Taylor *et al.,* 1990) and *Nicotiana plumbaginifolia* (Dominov *et al.,* 1992). However, this classification is speculative and as yet there is no unified nomenclature for plant GSTs as there is for animal GSTs. The sequence homology between plant GSTs is variable. For example, the predicted sequence of *GST-27* shares 57 % identity with the 29 kDa subunit of GST I and GST II, 43.7 % identity with the 26 kDa subunit of GST III, 46.4 % identity with the wheat *GstA1*, 39.1 % identity with a *Silene* GST and 11 % identity with tobacco *Nt103* (Jepson *et al.*, 1994). The homology between plant GSTs is particularly noticeable in the N-terminal region which may have functional significance as the glutathione-binding domain (G-site) is thought to occur in this region (Rushmore and Pickett, 1993).

1.3.2 GST structure

The three-dimensional structures of several animal GSTs have been solved, including two class theta GSTs (Rienemer *et al.,* 1991; Reinemer *et al.,* 1992), the rat 3-3 isozyme (Liu *et al.,* 1992), the squid 3-3 isozyme (Ji *et al.,* 1992) and a GST from *Schistosoma japonicum* (Lim *et al.,* 1994). Recently the first plant GST had its structure solved (Reinemer *et al.,* 1996). This isozyme from *Arabidopsis thaliana* shares about 60% sequence homology with the maize isozymes, and so may provide a model to unravel the mechanisms underlying herbicide resistance in plants. The *Arabidopsis* GST structure indicates that each subunit in the dimer consists of two spatially distinct domains with an active site in a cleft between the two domains (Reinemer *et al.,* 1996). The active site consists of a highly specific glutathione binding region and a less specific hydrophobic binding region. Significant structural homology was observed between mammalian isoforms of the enzyme and the*Arabidopsis* enzyme, although in mammals a conserved tyrosine residue which is thought to play a key role in the catalysis reaction was

absent in the *Arabidopsis* GST (Dirr *et al.,* 1994). However, a hydroxyl group of a serine residue from the plant GST superimposes close to the position of the important tyrosine residue from animal GSTs and as this serine is conserved in most of the known plant GSTs it may play a key role in plant GSTs.

1.3.3 Regulation of expression of GSTs

In animals the GST enzymes are differentially induced by many agents including drugs, xenobiotics (foreign compounds), food additives, natural dietary compounds (Daniel, 1993), glucocorticoids (Fan *et al.,* 1992) and insulin (Hatayama *et al.,* 1991). It has been suggested that the common chemical feature between the diverse array of compounds that induce GSTs is a carbon-carbon double bond adjacent to an electron-withdrawing group, termed a 'Michael acceptor' (Talalay *et al.,* 1988). The inducing compound may naturally contain this feature or it may be acquired by phase I metabolism; phase I enzymes introduce functional groups into compounds by oxidation or reduction while phase II enzymes (*i.e.* GSTs) conjugate functionalised compounds with endogenous ligands. Specific cis elements present in the promoters of GST genes confer specific compound inducibility to the gene. The regulation of expression of the GST Ya subunit from mouse and rat are particularly well studied. To study the mechanism of induction of the mouse GST Ya subunit Friling *et al.* (1987) carried out promoter deletion studies using chloramphenicol acetyltransferase (CAT) as a reporter gene. They discovered that a single *cis* element, located between -754 and -714 nucleotides upstream of the TSP, named the electrophile-response element (EpRE), was sufficient to drive inducible expression by electrophiles. This element was later discovered to consist of two adjacent nine base pair motifs which are related to the Activator Protein -1 (AP-1) binding site (Friling *et al.,* 1992) (see table 1 for sequence of EpRE; the regions similar to AP-1 are underlined.) The transcription factors *c-jun* and *c-fos*, which bind to AP-1, were shown to bind and activate transcription from the element.

Five elements have been identified in the rat GST Ya subunit promoter. One is the antioxidant-responsive element (ARE), located between -722 and -682 nucleotides upstream of the TSP (Rushmore and Pickett, 1990) (see table 1 for sequence of the element). Extensive deletion and mutational studies indicate that the ARE is necessary for basal expression of the gene and induction by phenolic antioxidants or planar aromatics. The element is significantly similar to the EpRE in the mouse gene, however, it is interesting to note that in electrophoretic mobility shift assays the ARE did not bind *c-jun* or *c-fos* (Rushmore and Pickett, 1993). A xenobiotic-responsive element (XRE) found in this promoter lies between nucleotides -908 and -899 and was found to respond to electrophilic compounds or metabolised phenolic antioxidants (Rushmore *et al.,* 1990). This element is similar to an XRE found in multiple copies in the 5' region of the cytochrome p1-450 gene (see table 1 for sequence of the XRE; the region underlined indicates the similarity to the XRE in P1-450). To achieve induction by phenolic

compounds via the XRE in the Ya gene promoter, it is thought that there is a requirement for functional aryl hydrocarbon (*Ah*) receptors and cytochrome P1-450 . The *Ah* receptors are required to transport the phenolic antioxidants to the nucleus and once there cytochrome P1-450 activity, another gene involved in xenobiotic metabolism, is required to metabolise the compound. Experiments conducted with murine hepatoma cells which lacked either *Ah* receptors or the P1-450 gene indicated that, in this situation, the XRE could not be activated by phenolic compounds, only by electrophilic compounds. The rat GST Ya subunit is expressed preferentially in the liver and two *cis* elements are thought to be responsible for this. One is thought to be recognised *in vitro* by HNF1 (hepatocyte nuclear factor 1), is located between nucleotides -860 to -850 (Rushmore *et al.,* 1990)(see table 1 for sequence) and in promoter deletion studies this was found to be required for basal expression. The other element, which is located between nucleotides -775 and -755, is thought to be recognised *in vitro* by HNF4 (Paulson *et al.,* 1990)(see table 1 for sequence). These binding studies were carried out with crude protein extracts and so the identity of the regulatory factors which bound to these elements cannot be conclusive. The final *cis* element in the rat Ya promoter, identified by computer-aided sequence examination, was a glucocorticoid-responsive element (GRE)(Rushmore and Pickett, 1993). Human homologues of the rat GST Ya subunit have been characterised (Klone *et al.,* 1992) and it is interesting to note that although the coding sequences share homology, the 5' flanking sequences are quite different, which suggests that the human homologues may be regulated in a different way to the rat or mouse genes.

A number of reports have indicated that the Pi family of GST genes in animals show increased expression in tumours and drug-resistant cell lines, and so there is considerable interest in identifying the factors which control the transcription of these genes (Cowell *et al.,* 1988; Masaharu *et al.,* 1988). Deletion analysis of the rat GST P gene (grouped in the Pi family) revealed two enhancing elements, located 2.5 and 2.0 kb upstream of the TSP, designated GPEI and GPEII (Masaharu *et al.,* 1988). Detailed analysis of GPE1 revealed that it was composed of two imperfect inverted repeats of the phorbol 12-*O*-tetradecanoate 13-acetate-responsive elements (TREs) (Okuda *et al.,* 1990) (for sequence of the TREs see table 1- the inverted repeats are underlined). The sequences, which share some homology with the AP-1 element (TGA$^{G}/_{C}$T$^{C}/_{A}$A), on their own showed no activity, but together acted synergistically to form a strong enhancer. A silencing element was also found in this promoter consisting of several *cis* elements capable of binding at least three proteins (Imagawa *et al.,* 1991), one of which may be silencing factor A (SF-A) which has been partially purified (Imagawa *et al.,* 1991). Similar to the comparison of rat and human forms of the Ya GST subunit, analysis of the human Pi GST gene has revealed some different controlling elements to the rat Pi gene (Cowell *et al.,* 1988). Two Sp1 recognition sequences were identified (G+C boxes), located at nucleotide -43 to -38 and at -53 to -48. In addition, an AP-1 recognition sequence was found between nucleotides -69 and -63 (see table 1 for sequences).

Table One A list of some of the elements which may be involved in the transcriptional regulation of GST sub-units in mammals. Underlined sequences - * = similar to AP1 element
+ = similar to element in pi-450 promoter

Name of Element	GST subunit it is present in	Sequence and reference
EpRE	Ya (mouse)	-754TAGCTTGCTAAATGACATTGCTAATGGTGACAAAGCAACTTT-714 Friling *et al.* (1992)
ARE	Ya (rat)	-722GAGCTTGGAAATGGCATTGCATTGCTAATGGTGACAAGCAACTTT-689 Rushmore *et al.* (1990)
XRE	Ya (rat)	-908TCAGGCATGTTGCGTGCATCTGAGGCCAGCC-899 Rushmore *et al.* (1990)
HNF1	Ya (rat)	-872GGTAATGATTAATAACCAAGACCCATG-845 Rushmore *et al.* (1990)
HNF4	Ya (rat)	-770TGAACCTTG-761 Paulson *et al.* (1990)
TRE	Pi (rat)	GTCAGTCACTATGATTCAG Okuda *et al.* (1990)
SF-A	Pi (rat)	GGAGCAGGA Imagawa *et al.* (1991)
TRE	Pi (human)	-66TGACTCAG-59 Cowell *et al.* (1988)
SP1	Pi (human)	-43GGGCGG-38 -53GGGCGG-48 Cowell *et al.* (1988)

Several Mu class GST genes have been characterised (Rushmore and Pickett, 1993) and one isolated from hamster was found to be transcriptionally up-regulated by glucocorticoids (Fan *et al.,* 1992). The element responsible for this response was isolated using a 5' deletion strategy and found to be situated between nucleotides -353 and -239. Although no classic glucocorticoid responsive element (TGTTCT) could be found in this section of DNA, four potential sites for helix-loop-helix binding domains were identified. Another glucocorticoid

regulatory element was also identified between nucleotides -239 and -136.

In plants, as in animals, constitutively expressed and inducible GSTs have been identified. Constitutive GSTs have been found in maize, including GST III (Moore *et al.,* 1986) and GST I (Mozer *et al.,* 1983), in sugarcane (Singhal *et al.,* 1991) and in *Silene cucubalus* (Kutchan and Hochberger, 1992). Inducible GSTs, or proteins that share homology with GSTs, have been identified in many plant species and have been shown to be responsive to a variety of inducing agents. Many, for example, appear to be induced by auxins. Takahashi *et al.* (1991) characterised the auxin-regulated *parB* gene isolated from tobacco mesophyll protoplasts. When the cDNA was cloned into an expression vector in *E. coli* active GST was produced, as determined by its ability to conjugate the substrate 1-chloro-2,4-dinitrobenzene (CDNB) to glutathione. Other auxin-induced tobacco proteins have been characterised and these were also found to encode active GSTs (Droog *et al.,* 1993). A *cis* element responsible for auxin induction in these genes has been identified, with a consensus sequence TGACG(N7)TGACG, which is similar to the *as*-1 element and octopine synthase (*ocs*) element from CaMV 35S (van der Zaal *et al.,* 1996). This element, and the abberations of it found in some tobacco auxin-inducible genes, when fused to a heterologous promoter in stable tobacco transformants, were found to be sufficient to drive auxin-inducible expression (van der Zaal *et al.,* 1996). No essential difference was found between these slightly differing *cis* elements and a consensus *as*-1 element, apart from their relative strength in driving transcription in yeast and plant cells. *In vitro* experiments with the cloned transcription factor TGA1a, which was used as it had previously been shown to promote increased expression from the CaMV 35S promoter in yeast, indicated that the protein binds to the tobacco auxin-inducible elements, and that the strength of binding *in vitro* was related to the strength of the elements to drive transcription *in vivo* (van der Zaal *et al.,* 1996). Therefore, this factor may be involved in the regulation of these genes. *Ocs*-like elements have also been identified in the promoters of the soybean *GH2/4* gene, the wheat *GstA1* gene, the tobacco *parA* and *parB* gene and in the *Silene* GST (Marrs, 1996). This element shares some similarity with the EpRE as both of the elements contain tandem duplication of binding sites (tandem AP-1 sites in the EpRE and a tandem core sequence of ACGT in the *ocs* element) and both are the binding sites of bZip transcription factors (Marrs, 1996). It has been suggested that the *ocs* element in plants plays a similar role to the AP-1 element in animals by acting as a stress-inducible element, responsive to a variety of electrophilic agents which may generate conditions of oxidative stress (Marrs, 1996).

In soybean a protein, GH2/4 (Gmhsp26-A), which has GST activity when expressed in *E. coli,* has been isolated and is responsive to elevated temperature and to a wide range of chemical agents including auxins, non-auxin analogues and heavy metals (Ulmasov *et al.,* 1995). Within the 780 base pairs of sequenced promoter, putative elements have been identified using computer homology searches, including an *ocs*-like element between nucleotides -286 and -305

with the sequence 5'TTACGTAATCTCTTACATCA3'. Two putative salicylic acid-inducible elements lie further downstream and between these and the *ocs*-like element a G-box element has been identified, with the sequence 5'CCACGTT3'. By fusing the promoter to the reporter gene β-glucoronidase (GUS) it was shown, in transgenic tobacco, that the application of inducing chemicals affect transcription in a tissue-specific and concentration-dependant manner. In general, low concentrations of an agent were found to induce expression of the gene in root tips, while increased concentrations led to GUS expression in the upper parts of the root and in the hypocotyl. This suggests that different organs have different sensitivities to the applied chemicals and illustrates the complexity of the regulation of expression of GSTs in plants. The *ocs*-element alone, when excised from this promoter, has been shown to confer responsiveness to auxins, inactive auxins, salicylic acid and salicylic acid analogs to a minimal promoter (Ulmasov *et al.,* 1994).

Ethylene-induced GSTs have been identified in *Arabidopsis* (Meyer *et al.,* 1991) and carnation (Itzhaki and Woodson, 1993). Promoter deletion analysis of the carnation *GSTI* gene was carried out using the particle bombardment technique in petal tissue (Itzhaki and Woodson, 1993). This indicated that 126 base pairs of the promoter, located between -596 and -470 relative to the TSP, fused to a CaMV 35S minimal promoter, could drive ethylene-regulated transcription, in an orientation-independent manner (Itzhaki *et al.,* 1994). Gel electrophoresis mobility-shift assays (EMSAs) revealed that this element could bind protein *in vitro* from extracts prepared from flower petals at anthesis (low ethylene levels), petals treated with ethylene and petals producing ethylene as they senesced. As the nuclear protein can interact with the *cis* element *in vitro* in an ethylene-independent manner, it was hypothesised that post translational modifications or interactions with other proteins were necessary for ethylene-regulated expression. DNase I footprinting was utilised to further characterise this *cis* element (Itzhaki *et al.,* 1994). Nucleotides between -510 and -488 were shown to be protected from DNase I digestion after incubation with a nuclear protein extract isolated from carnation petals in the ethylene climacteric (high ethylene levels). Therefore, these bases may be protected by an interacting protein. The top strand of sequence containing regions of protected nucleotides is -510GTGATTTACCACCTATTTCAAAG-488. Eight base pairs of this sequence, ATTTCAAA, are very similar to sequences in an ethylene-responsive region of the tomato E4 gene. Thirty-two nucleotides in the E4 gene's promoter were shown to be protected from DNAse I digestion after incubation with nuclear protein extract, and the sequence similar to that from the carnation gene is AATTCAAA. A putative transcription factor for the GST1 element, designated carnation ethylene-responsive element-binding protein-1 (CEBP-1), has now been cloned by Southwestern screening (Maxson and Woodson, 1996). The factor is a predicted 32 kDa protein which homology searches indicate contains a conserved nucleic acid binding domain, an acidic region, a putative nuclear localisation signal and two highly conserved RNA-binding motifs. It is proposed that CEBP-1 is involved in the activation of *GST1* transcription and in the

processing, stability and transport of the RNA (Maxson and Woodson, 1996). By conducting computer homology searches an AP-1 like site has also been identified in the promoter of the GST1 gene from carnation between nucleotides -567 and -561, with the sequence TGAAATT (Itzhaki and Woodson, 1993) but the functional significance of this site, if any, has not been determined.

Pathogens have been shown to regulate GST levels in wheat (Dudler *et al.,* 1991). It was observed that winter wheat showed local, induced resistance against the pathogen *Erysiphe graminis* following exposure to the fungi's non-pathogenic form. Differential screening isolated a cDNA that was 50% homologous to the GSTI gene from maize and had GST activity when expressed in *E.coli.* (Mauch and Dudler, 1993). This protein is estimated to be about 29 kD in size. Two other GSTs of 25 kD and 26 kD were also identified. These had high basal levels of expression and were induced by xenobiotics such as cadmium and the herbicides atrazine, paraquat and alachlor. However, pathogen attack did not induce the smaller GSTs which indicates that at least two classes of GST exist in wheat. It is clear from the above that GSTs in plants and animals respond to a variety of stimuli, both environmental and chemical, and that promoter-responsiveness is conferred via a number of distinct *cis* elements.

1.3.4 Maize GSTs

Much attention on plant GSTs has focused on herbicide metabolism in crop and weed species. The role of GSTs in herbicide detoxification was discovered in maize, which is naturally tolerant to the herbicide atrazine. However, in 1960, all the maize seedlings of the Mississippi selection of the inbred line GT112 were killed by atrazine which had been applied at the recommended rate (Grogan *et al.,* 1963). Five other GT112 lines responded normally and were not susceptible to atrazine. Therefore, it was possible to study atrazine tolerance in sibling strains, atrazine-susceptible GT112 and atrazine-tolerant GT112 RfRf (Eastin, 1971). The first report of GST activity in plants was published in 1970 (Frear and Swanson). When GST was purified from the GT112 plants the specific activity of the GST from GT112 RfRf was found to be 50-fold higher than from the atrazine-susceptible line (Shimabukuro *et al.,* 1971). In the 1980's glutathione conjugation was shown to be the primary mechanism used to detoxify the herbicide in maize and in the related species sorghum (Lamoureux and Rusness, 1986) and so, in this way, the existence and importance of GSTs in plants was established.

Maize GSTs comprise 1-2% of total soluble protein (Holt *et al.,* 1995) and, in terms of biochemistry and molecular biology, they are the best characterised plant GSTs. Maize can be selectively protected from herbicide injury by compounds known as herbicide safeners or antidotes (described in more detail in section 1.4) which have been shown to elevate levels of GSTs (Hatzios, 1991). These safener induced GSTs have been particularly well characterised (for review see Timmerman, 1989). As some plant GSTs are specifically induced by herbicides

they have been considered to be suitable candidates for the development of effective gene switches (Jepson, 1994). There are numerous GSTs in plant systems and by using anion exchange chromatography up to 15 GST isoforms have been shown to exist in maize which have different substrate specificities (Dean *et al.,* 1991). The substrates used include *trans*-cinnamic acid, the herbicide atrazine, 1-chloro-2,4-dinitrobenzene, metalochlor and the sulfoxide derivative of *S*-ethyl dipropylcarbamothioate; it is important to use a variety of substrates when characterising GSTs as, for example, the atrazine-specific GSTs have no activity against 1-chloro-2,4-dinitrobenzene (Timmerman, 1989). The isoforms are classified on the basis of molecular weight, substrate specificity and responsiveness to safener. Isoforms GST I, II, III and IV have been particularly well characterised and all appear to have a role to play in herbicide detoxification. cDNA clones have been isolated for all four isoforms (Jepson *et al.,* 1994; Moore *et al.,* 1986; Weigand *et al.,* 1986). More recently, two novel GST isoforms from maize have been characterised (Dean *et al.*,1995; Marrs *et al.*,1995).

GST I is a homodimer of a 29 kDa protein which, by protein sequencing, has been shown to be identical to the 29 kDa subunit of GST II (Mozer *et al.,* 1983). Western blotting analysis revealed that *GST-29* is constitutively expressed throughout the plant (Holt *et al.,* 1995) but it can also be induced 3 to 4-fold by herbicide safener treatment (Weigand *et al.,* 1986). It is active against alachlor and CDNB and recently has been found to be active against a range of substrates including ethacrynic acid, 4-nitrobenzyl chloride (NBC) and 1,2-dichloro-4-nitrobenzene (DCNB) (Dixon *et al.*, 1996). GST II is induced when the plant is treated with the safener dichlormid, also referred to as R-25788 (*N,N*-diallyl-2,2-dichoroacetimide) and R-29148 (3-(dichloro-acetyl)-2,2,5-trimethyl-1,3-oxazolindone) (Jepson *et al.,* 1994). It has been purified to homogeneity and is a heterodimer composed of a 29 kDa (*GST-29*) and a 27 kDa (*GST-27*) subunit. Both of the subunits have been shown to be active against alachlor and CDNB (Dixon *et al.*, 1996; Holt *et al.,* 1995; Mozer *et al.,* 1983). Recently GST II has also been found to be active against a range of substrates including ethacrynic acid and NBC (Dixon *et al.*, 1996). *GST-27* is normally only present in the roots of untreated plants but, following application of safener, is expressed in all aerial parts of the plant (Holt *et al.,* 1995). A range of hormonal, environmental and physiological stimuli were tested for the ability to induce expression of this subunit (Jepson *et al.,* 1994) but an increase in *GST-27* mRNA was only observed in the late stages of leaf senescence and after treatments resulting in phytotoxic effects. Such specific inducibility means that the promoter of this gene is a good candidate for the development of a gene switch. In addition, the inducing agent, dichlormid, is a registered chemical already in agricultural use. In addition to its activity as a GST the 27 kDa sub-unit has also been found to have activity as a glutathione peroxidase (Dixon *et al.*, 1996). GST III, a homodimer made up of 26 kDa subunits, is constitutively expressed (Timmerman, 1989) and is active against CDNB and alachlor (Moore *et al.,* 1986). It is about 45% similar at the amino acid level to GST I (Grove *et al.,* 1988). Safener-inducible GST IV consists of a homodimer of

a 27 kDa subunit (Irzyk and Fuerst, 1993). Protein sequence analysis indicates that the 27 kDa subunit present in GST IV is identical to 27 kDa subunit of GST II (Holt *et al.,* 1995). It is active against chloroacetamide herbicides but has no activity against CDNB. Recent research has indicated that a fifth isoform, containing the 29 kDa subunit from GST I and the 26 kDa subunit from GST III exists (Dixon *et al.*, 1996). This isoform was found to be active against alachlor, metalochlor, CDNB, NBC and ethacrynic acid. Although some of the maize GSTs have been shown to be inducible, the exact mode of regulation for this remains unclear. It has not been shown that the induction is due to an increase in the transcription of the gene as nuclear run-on experiments or promoter analysis experiments have not been performed. Experiments which indicate that higher levels of mRNA are achieved after the application of a herbicide safener (Jepson *et al.*, 1994; Weigand *et al.,* 1986) do not necessarily indicate that the gene's transcription rate is increased; they may indicate that the mRNA has increased stability. An experiment has shown that the *de novo* synthesis of protein is required for both the basal and safener induced levels of GST I and GST III achieved by maize cell suspension cultures (Miller *et al.*, 1994). The authors of this paper imply that the response of these genes to safener is controlled at the transcriptional level. However, they also point out that genes involved in the signal transduction pathway between safener treatment and its effect may be the genes which are transcriptionally regulated by safener.

The two GSTs from maize which have been characterised more recently include the product of the gene *Bronze-2* (Marrs *et al.*, 1995) and a GST (GST (4-CA)) which is active with p-coumaric acid and other unsaturated phenylpropanoids (Dean *et al.*, 1995). The GST encoded by the *Bronze-2* gene has not been characterised in terms of its form or subunit composition, but it is known to be induced by the products of the maize regulatory genes *R* and *C1* which together transactivate the structural genes for anthocyanin biosynthesis. GST (4-CA) unusually appears to exist as a monomer, with a molecular weight of 30 kDa. It is induced by *p*-coumaric acid and 7-hydroxycoumarin (Dean *et al.*, 1995). The biological function of these GSTs are described in section 1.3.5. A summary of the characterised maize GSTs is given in table 2.

1.3.5 The biological function of GSTs

As previously described, one of the biological functions of GSTs involves herbicide detoxification (Timmerman, 1989). It has been shown that maize lines tolerant to herbicide have higher levels of GST and glutathione compared to susceptible lines (Sari-Gorla *et al.,* 1993) which allows faster detoxification of the herbicide by conversion to a less toxic water-soluble conjugate. However, plants did not evolve this system to cope with man-made chemicals. It appears that GSTs play a general role in the detoxification of numerous potentially toxic or damaging exogenous and endogenous compounds. Evidence that GSTs may have evolved in plants to cope with the products of their own defense systems is seen in a GST from maize

Table 2 Summary of the characterised maize GSTs

Isozyme	Form	Subunit	Expression	Reference
GST I	Homodimer	29 kDa	Constitutive and inducible	(Mozer *et al.*, 1983)
GST II	Heterodimer	29 kDa 27 kDa	Inducible	(Jepson *et al.*, 1994; Mozer *et al.*, 1983)
GST III	Homodimer	26 kDa	Constitutive	(Moore *et al.*, 1986)
GST IV	Homodimer	27 kDa	Inducible	(Fuerst *et al.*, 1993; Irzyk and Fuerst, 1993)
GST I/III	Heterodimer	29 kDa 26 kDa	Constitutive	(Dixon *et al.*, 1996)
Bronze-2	?	?	Inducible	(Marrs *et al.*, 1995)
GST (4-CA)	Monomer	30 kDa	Inducible	(Dean *et al.*, 1995)

(which, unusually, appears to function as a monomer), which is active against the endogenous substances coumaric acid and other phenylpropanoids (Dean *et al.*, 1995). This GST is associated with defense responses and was found to be induced by fungal elicitors in bean suspension-cultured cells (Edwards and Dixon, 1991). Other GSTs have been found to be induced by hydrogen peroxide, which is a compound generated transiently by plants within two to three minutes after pathogen attack, in a process known as the oxidative burst (Levine *et al.*, 1994; Tenhaken *et al.*, 1995). The hydrogen peroxide functions to reinforce cell walls, restrict fungal growth and to limit the pathogen spreading. By inducing GSTs the toxic by-products of these processes are detoxified by conjugation with glutathione. In addition, the GSTs can function as glutathione peroxidases which catalyse the glutathione-dependent reduction/inactivation of hydrogen peroxide and increase glutathione synthesis by feedback

induction (Bartling *et al.,* 1993; Dixon *et al.*, 1996; Smith *et al.,* 1985; Tenhaken *et al.,* 1995). The elevated glutathione levels induce the transcription of other genes involved in defense, including in some cases, GSTs (Levine *et al.,* 1994; Wingate *et al.,* 1988). The dehydration-induced putative GST from *Arabidopsis* may be involved in the detoxification of stress compounds which accumulate in drought conditions (Kiyosue *et al.,* 1993). Similar to the effects of stress caused by pathogen attack or tissue damage, active oxygen species are generated when a plant is subjected to the stress of low water availability, and these cause DNA damage and lipid peroxidation; the GST may serve to metabolise the resulting toxic products. Auxins are involved in a number of cellular processes including cell division and elongation and it has been suggested that the auxin-induced GSTs are required to detoxify the oxidising by-products of these processes (Droog *et al.,* 1993). The *ocs*-like element, which is present in the promoters of many auxin responsive promoters, is proposed to be similar in structure to tandem AP-1 elements (Ulmasov *et al.,* 1995) and as AP-1 elements have been shown to be responsive to oxidative stress in animal GST promoters (see section 1.3.3) it would follow that the *ocs*-like elements are involved in dealing with oxidative stress in plants. Therefore, evidence is accumulating which links GSTs to a role in protecting tissues from endogenous compounds which are produced when a plant is subjected to stress.

In addition to their enzymatic properties GSTs also possess ligand binding properties. In animals they are involved in the intracellular transport of hydrophobic and amphiphatic compounds (Ketley *et al.,* 1975; Listowski *et al.,* 1988; Litwack *et al.,* 1971). This may be true in plant systems as a GST from *Hyoscyamus muticus* is isolated by virtue of its ability to bind to indolacetic acid affinity columns (MacDonald *et al.,* 1991), and other researches have identified active GSTs that bind to the natural auxin indole-3-acetic acid (IAA) without detecting the formation of IAA-glutathione conjugates (Bilang and Sturm, 1995; Jones, 1994). The binding may allow temporary storage of IAA or serve to traffic the molecule from membranes to receptors. A proposed function for the ligand binding activity of GSTs is to prevent cytotoxic events which could result from an excess of certain molecules within cells (Jones, 1994). Another example involves the *Bronze-2* gene in maize, which performs the last genetically defined step in anthocyanin biosynthesis, and results in the deposition of red and purple pigments into the vacuoles of maize tissue (Coe *et al.,* 1993), and which has been shown to encode a GST with activity in *E. coli*, *Arabidopsis* and maize (Marrs *et al.,* 1995). As an ATP driven glutathione *S*-conjugate pump has been identified which is thought to transport glutathione conjugates across the vacuolar membrane (Gaillard *et al.,* 1994; Martinoia *et al.,* 1993) it has been suggested that *Bronze-2* catalyses the conjugation of cyanidin-3-glucoside (bronze) to glutathione, so they can then be transferred to the vacuole. This is supported by the experimental evidence that vanadate, a known inhibitor of the pump, decreases the accumulation of pigments in protoplasts (Marrs *et al.,* 1995). This suggests that a common mechanism exists in plants to sequester structurally similar but functionally diverse molecules in the vacuole. Once

the anthocyanin-glutathione compound is in the vacuole, it has been suggested that a carboxypeptidase is responsible for the removal of the glutathione tag (Wolf *et al.,* 1996). This enzyme has been shown to be responsible for the first step in the degradation of the herbicide alachlor-glutathione conjugate once it has been excreted into the vacuole, and indicates that glutathione conjugation is used as a transport mechanism and not as a storage mechanism (Wolf *et al.,* 1996).

1.4 SAFENERS

The cost of weed control in Australia is estimated to be similar to the gross value of the Australian wheat crop (Sindel, 1995) and so it clear that weed control is a large scale problem in agriculture. Herbicides are often used as part of an integrated weed management system but as perfectly selective herbicides have not been discovered safeners, or herbicide antidotes, are used to increase selectivity by elevating detoxification of the herbicide in the crop plant but not in the weed (Stephenson and Yaacoby, 1991). The first published reports on the use of safeners appeared in the early 1960's (Hoffman, 1962) and were inspired by the observation in 1947 that tomato plants injured accidently on a hot summers day in a greenhouse by vapours of 2,4-D survived if they had been pre-treated with 2,4,6-T, an analogue of 2,4-D. Detoxification of xenobiotics consists of a three phase reaction; in phase one the compound is chemically modified, in phase two it is conjugated to an endogenous ligand and in phase three the detoxified compound is excreted (Daniel, 1993). Safeners work by increasing levels of the detoxifying enzymes and ligands which are involved in these three steps, including GSTs, cytochrome P450 oxidases, UDP-glucosyl transferases and glutathione (Hatzios, 1991; Hatton *et al.,* 1996). In addition, safeners have been shown to induce the activity of the vacuolar transporters necessary for excreting the metabolised herbicide into the plant vacuole (Gaillard *et al.,* 1994; Martinoia *et al.,* 1993; Wolf *et al.,* 1996). The enzymes and ligands which are induced by the application of safeners also play important roles in other aspects of a plant's biochemistry. For example, glutathione plays an important role as an antioxidant which prevents cellular damage by maintaining essential thiols in their reduced state, scavenging hydrogen peroxide and hydroxyl radicals (Field, 1996). GSTs and glutathione are also induced when a plant is the subject of pathogen attack and they are thought to be involved in reducing the effects of oxidative stress caused by the hypersensitive response (El-Zahaby *et al.*, 1995; Levine *et al.*, 1994; Wingate *et al.,* 1988).

Commercial safeners are members of diverse chemical groups including naphthopyranones (eg. naphthalic anhydride), dichloroacetamides (eg. dichlormid and benoxacor), substituted thiazoles (eg. flurazole) and phenylpyrimidines (eg. fenclorim) (Hatzios, 1991). The different classes of safener protect against different classes of herbicide. For example, dichlormid is effective against carbamothioate, chloroacetanilide and sulfonylurea herbicides while flurazole is

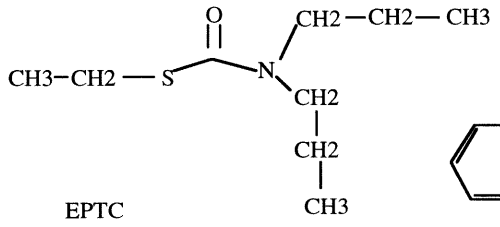
effective against chloroacetanilide herbicides only (see figure 2 for the chemical structures of some herbicides and figure 3 for the chemical structures of some herbicide safeners). The safeners are applied either directly to the seed crop before planting or applied with the herbicide in the field (Hatzios, 1991). Safener induced effects are only observed in grass crops including maize, rice, wheat and sorghum, but the reason for the botanical specificity is unknown (Hatzios, 1991).

The signal transduction mechanism between safener application and effect is unclear. There is some debate as to whether safeners act as competitive antagonists of the herbicide or if they act solely by enhancing herbicide detoxification (Hatzios, 1991). In figures 2 and 3 it can be seen that dichlormid is structurally similar to the herbicide it protects against, EPTC. This example, therefore, argues towards safeners acting as antagonists of herbicides. Other safener/herbicide combinations which do not show such obvious structural similarities have been found, using computer-assisted molecular modelling, to have similarities such as degrees of bonds, charge distribution and molecular volumes (Hatzios, 1991). A protein which binds dichloroacetamide safeners (*i.e.* dichlormid) has been observed in etiolated maize seedlings and this may be involved in the signal transduction pathway (Walton and Casida, 1995). It is suggested that binding of safeners to this cytosolic protein receptor leads to increased levels of glutathione and GSTs but if a herbicide binds the result is phytotoxicity. This hypothesis, therefore, combines both schools of thought as to the action of safeners. Interestingly, in some species which are protected to an extent by dichloroacetamide safeners *i.e.* barley, bean and wheat, the protein receptor was not detected (Walton and Casida, 1995). Therefore, either the method of detection was not efficient in these species or safening action occurs through a different pathway in these plants.

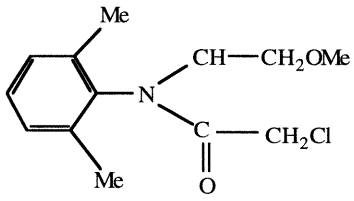
It should be remembered that there is a certain risk associated with the use of herbicides and herbicide safeners on crop plants, both to the consumer and the environment. Before commercial use, however, products are thoroughly tested in terms of toxicity to plants, animals, and the environment. It has been shown that even stable chemical compounds are rarely found in quantities exceeding 1 mg/kg of fresh plant material in parts of the plant other than that which was directly sprayed (British Crop Protection Council, 1982). As the lethal dose of dichlormid, for example, required to kill half the rats in a test population is approximately 2000 mg/kg (data from Stauffer Chemical Company), the levels of this compound left in plants should not prove to cause any toxicological problems to mammals. In addition, during a 13 week study rats had to ingest over 40 mg/kg/day of dichlormid before the toxic effects of the chemical were observed in liver cells, and so the long term effects of exposure to very low levels of the chemical may be minimal. Current legislation reduces risks of toxicity to low levels. However, not every eventuality can be tested in the laboratory before a product is available on the market and these issues should be kept in mind when developing further uses for herbicides and their

Figure 2

The chemical structure of the herbicides S-ethyl dipropylthiocaranate (EPTC), a thiocarbamate compound, and metalochlor, a chloroacetanilide compound.



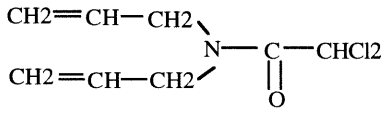
EPTC



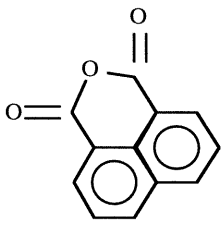
Metalochlor

Figure 3

The chemical structures of two herbicide safeners, dichlormid and naphthalic anhydride.



Dichlormid



Naphthalic anhydride

safeners.

1.5 AIM OF WORK

Although safeners are widely used in agriculture as herbicide antidotes, and industry continues in its attempt to develop new safener compounds, little is known about their mechanism of action. Safeners are capable of inducing enzymes and compounds which are involved in herbicide detoxification (Jepson *et al.*, 1994; Mozer *et al.*, 1983; Timmerman, 1989), defense against pathogen attack and oxidative stress (El-Zahaby *et al.*, 1995; Lamb *et al.*, 1994). In animal systems common features have been identified between enzymes involved in oxidative stress and xenobiotic detoxification and it may be that such commonality exists in the related plant systems. Therefore, by increasing the understanding of how safeners induce GSTs more may be learnt about the regulation of a plant's response to pathogen attack or oxidative stress; to date the promoters of the genes involved in these processes have not been studied in great detail. The aim of increasing our knowledge of these systems will be achieved by defining the *cis* element which confers safener inducible expression to the *GST-27* gene. Similar elements may be in the present in the promoters of the other genes which are induced by safeners; a precedent for this is set by the XRE element in the rat GST Ya subunit promoter which is also present in multiple copies in the cytochrome p1-450 gene (Rushmore *et al.*, 1990). In addition, because GSTs are involved in the detoxification of xenobiotics they are increasingly being investigated as a source of naturally occurring bioremediation enzymes (Field, 1996; Plumachar and Schroder, 1994). Therefore, by increasing the knowledge of how GSTs are regulated information may be gained on how to effectively use these enzymes in the fight against pollution.

An effective inducible gene switch has not yet been developed for wide-scale use in agriculture. The promoter of the GST-27 gene from maize is considered to be an ideal candidate for such a system as it combines specific inducibility with an inducing agent, dichlormid, which is suitable for agricultural use. Another aim of this work was to define *cis* acting elements which confer inducibility to this promoter so that they may be used in heterologous systems to drive inducible expression of specific genes by the application of dichlormid. Similar experiments have proved successful with ethylene responsive *cis* elements from the *GST1* gene of carnation (Itzhaki *et al.*, 1994) and auxin responsive *cis* elements from tobacco genes which exhibit *in vitro* GST activity (van der Zaal *et al.*, 1996). However, promoters are complex and the response of a promoter to a specific stimulus may be the result of the interplay of a combination of elements and factors (Benfey *et al.*, 1990; Puente *et al.*, 1996). Therefore, the addition of a single responsive element to an heterologous promoter may not necessarily be successful in conferring responsiveness to that promoter. Nevertheless, once the *cis* element is identified it can be used in Southwestern screening experiments to identify its cognate *trans* acting factor. When the

mechanisms responsible for inducibility are defined, a gene switch of improved efficiency could be developed *i.e.* the *cis* element could be concatemerised or a mutant *trans* acting factor could be engineered. *In vivo* footprinting will be used in order to locate the *cis* element which confers inducibility to the *GST-27* promoter and electrophoresis mobility shift assays will be employed in order to determine if maize nuclear protein extracts can interact with the putative elements *in vitro*. Assuming that these experiments are successful a series of wild type and mutant 5' deleted promoter constructs will be engineered so that the involvement of the putative elements in safener-dependent transcription can be determined. The promoter studies will also add to the information available in the literature on the mechanism by which safeners increase levels of GSTs in plants and will provide information on the mechanism by which the *GST-27* promoter confers constitutive expression to the roots of maize seedlings. In addition, the information gained from this work could be used in the development of an effective screen for the identification of new and more effective safener compounds which are capable of inducing a gene via the safener responsive *cis* element.

Chapter Two

MATERIALS AND METHODS

2.1 REAGENTS

Unless otherwise stated reagents were obtained from BDH, Sigma Chemical Company or Aldrich Chemical Company Ltd.. Suppliers of enzymes and unusual reagents are given in the text. Deionised water was used, unless otherwise stated. Phenol was equilibrated with 0.1 M Tris-HCl pH 8.0 and contained 1 g/l 8-hydroxyquinoline. Phenol/chloroform consisted of equal volumes of phenol and chloroform with 1:50 addition of isoamyl alcohol. Autoradiography was performed using X-Omat AR5 film (Kodak Company) at -70 °C. Unless otherwise stated DNA was precipitated by the addition of 1 volume of isopropanol (IPA) and 0.1 volume of 3 M NaAc pH 5.2.

2.2 PLASMID CONSTRUCTION AND CLONING TECHNIQUES

2.2.1 DNA Plasmid minipreps

Plasmid minipreps were prepared using an alkaline lysis method modified from that described by Birboim and Doly (1979). A 10 ml culture of LB (1 % (w/v) Bacto tryptone [Difco], 0.5 % (w/v) yeast extract [Difco], 0.5 % NaCl, 0.1 % D-glucose) was set up overnight containing selecting antibiotic, generally at a concentration of 50 µg/ml. The bacteria were pelleted by centrifugation at 3,000 rpm for 10 minutes (Sorvall RC3C centrifuge, H6000A rotor), resuspended in 300 µl solution I (50 mM Tris-HCl, pH 8.0; 10 mM EDTA) and transferred to an eppendorf. Three hundred microlitres of solution II (200 mM NaOH, 1 % SDS) were added and the eppendorf was hand-mixed vigorously. 300 µl solution III (3.0 M KAc, pH 5.5) were then added and the eppendorf was mixed again. After 5 minutes centrifugation at 13,000 rpm (MSE Microcentaur benchtop microfuge) the supernatant was removed and DNA was precipitated by the addition of 1 volume isopropanol followed by 10 minutes centrifugation at 13,000 rpm (MSE Microcentaur benchtop microfuge). The DNA pellet was washed with 70 % (v/v) ethanol to remove salt and then resuspended in 15 µl water. Fifteen microlitres of this DNA was sufficient for use in sequencing reactions and 1 µl was sufficient for use in restriction enzyme digests.

2.2.2 DNA restriction enzyme digests

Restriction digestion of DNA was performed as described in Sambrook *et al.* (1989) with commercial enzymes, buffers and conditions recommended by the manufacturers. In general the digests was carried out in a 10 µl volume with 1 µl miniprep DNA, 1 µl 10X buffer, 1 µl enzyme and 7 µl water. This mix was incubated at 37 °C for 1 hour and then run on an agarose electrophoresis gel.

2.2.3 Agarose gel electrophoresis of DNA

Agarose gel electrophoresis and ethidium bromide staining were carried out as described by Sambrook *et al.* (1989). The gels were made with 0.5X TBE (0.045 M Tris-borate, 0.0001 M EDTA), contained 0.5 μg/ml ethidium bromide and were run in a 1X TBE buffer.

2.2.4 DNA fragment purification

DNA fragments were purified using the QUIquick gel extraction kit (QIAGEN). Manufacturer's methods were followed. For fragments of less than 100 bp in size the phenol-squeeze method was used, based on that described by Hammond-Kosack (1990). The band requiring purification was cut from the gel and squeezed through a 2 ml syringe (no needle attached). An equal volume of phenol was added and the solution vortexed, resulting in the formation of a milky white suspension. This was frozen in liquid nitrogen for 60 seconds and immediately centrifuged for 10 minutes at 13,000 rpm (MSE Microcentaur benchtop microfuge). The supernatant was extracted twice with equal volumes of phenol/chloroform and IPA precipitated with the addition of 0.01 volumes of 1 M $MgCl_2$.

2.2.5 Quantification of DNA

High concentrations of DNA were determined spectrophotometrically according to Sambrook *et al.* (1989). Five microlitres of the DNA solution were added to 995 μl water and measured in a 1 ml quartz cuvette in a spectrophotometer, set at 260 nm wavelength. For double-stranded DNA A_{260} = 1 for 50 μg/ml DNA while for single-stranded DNA A_{260} = 1 for 33 μg/ml DNA. Low concentrations of DNA were quantified using the Hoescht method (Labarca and Paigen, 1980). Two microlitres of DNA at >10 ng/μl were added to 2 mls of a 0.1 μg/ml solution of Hoescht 33258 in TEN buffer (10 mM Tris, pH 7.4: 1 mM EDTA; 50 mM NaCl). Using a fluorometer, with excitation filters set at 365 nm wavelength and emission filters set at 458 nm wavelength, fluorometric readings were taken. These readings were compared to a standard curve prepared using DNA of known concentration. An alternative method also used involved quantification of the fragments in 5 μl drops containing 0.5 μg/ml ethidium bromide. The fluorescence of the drops on a UV-transilluminator was compared with DNA standards, usually 1 Kb ladder (Gibco) at 0, 20, 50 and 100 ng/drop.

2.2.6 Large scale DNA plasmid preps

Large scale plasmid preps were performed using the QIAGEN plasmid maxi kit. Minor modifications were made to the manufacturer's instructions as follows; 400 ml overnight culture was used. After the centrifugation step, following the addition of solutions P1, P2 and P3, only half of the supernatant was passed over the QIAGEN tip, which was then washed and the DNA eluted. The tip was re-equilibrated and the remaining half of the supernatant was passed over the tip. In this way higher yields of DNA could be recovered. Yield was also increased if the

final precipitation step was performed in a 50 ml falcon tube spun at 3,500 rpm for 30 minutes (Sorvall RC3C centrifuge, H6000A rotor).

2.2.7 Cloning

Ten microgrammes of vector plasmid were digested with appropriate enzymes for an hour (see section 2.2.2). One unit of calf intestinal alkaline phosphatase (Boehringer Mannheim) was added direct to the tube of digesting DNA to dephosphorylate the ends of the vector. Incubation was continued for 30 minutes. The vector was run on an electrophoresis gel and purified with the QUIquick gel extraction kit (see section 2.2.4). Vector and insert were mixed at appropriate concentrations and ligated using bacteriophage T4 DNA ligase (Promega). The reaction was carried out for 3 hours at room temperature or overnight at 4°C.

2.2.8 Preparation of competent cells and their transformation

DNA was transformed into *Escherichia coli* (DH5α cells :*supE*44, Δ*lac*U169, *hsdR*17, *recA*1, *gyrA*96, *thi*-1, *relA*-1). The method used was described by Nishimura *et al.* (1990). To prepare competent cells 50 mls medium A (LB supplemented with 10 mM $MgSO_4$ and 0.2 % glucose) was inoculated with 0.5 mls overnight culture. This was grown at 37°C until A_{600} = 0.48 (approximately 3 hours). The cells were kept on ice for 10 minutes and then pelleted by centrifugation for 10 minutes at 3,500 rpm at 4°C (Sorvall RC3C centrifuge, H6000A rotor). The cells were gently resuspended in 0.5 mls precooled medium A and then 2.5 mls cold medium B (LB supplemented with 36 % glycerin, 12 % PEG [MW 7,500], 12 mM $MgSO_4$, filter sterilised) were added. One hundred microlitre aliquots of the competent cells were transferred into eppendorf tubes and stored at minus 80°C until required. To transform, the cells were thawed on ice, plasmid DNA in a volume less than 20 µls was added and the cells were incubated on ice for 15 minutes. The cells were then subjected to a heat shock for 60 seconds at 42°C which was followed by 2 minutes incubation on ice. Eight hundred microlitres LB were added and the samples were incubated at 37°C for 1 hour. The cells were then plated on LB agar (as broth, with the addition of 8 g agar/l) containing suitable antibiotics for selection of the plasmid, and grown overnight at 37°C. If the plasmid contained the *lacZ* gene for blue/white selection IPTG (4 µl of a 200 mg/ml solution) and X-Gal (40 µl of a 20 mg/ml dimethylformamide solution) were added to the plate.

2.2.9 Synthesis and cloning of oligonucleotides

Oligonucleotides (oligos) were synthesised using a Pharmacia LKB Gene Assembler Plus according to the manufacturer's instructions. Oligos were recovered from the cassette by overnight incubation in 1 ml concentrated ammonia at 50°C. The DNA was IPA precipitated and quantified. Ten microgrammes of each oligo were phosphorylated using 10 units bacteriophage T4 polynucleotide kinase (Pharmacia Biotech) in a volume of 45 µl, with 4.5 µl one-phor-all

10X buffer and 2 µl 2 mM ATP. This mix was incubated at 37°C for 60 minutes. Complementary phosphorylated oligonucleotides (with non-complimentary protruding ends for cloning into suitable restriction sites) were then combined and 10 µl 10X annealing buffer was added (50 mM $MgCl_2$, 100 mM NaCl, 10 mM Tris pH 8.0) giving a final volume of 100 µl. The oligo pair mixes were overlaid with mineral oil and floated on 2 l water at 95°C for 5 minutes. The oligos were left on the water, which was allowed to cool to room temperature overnight. Three microlitres of each oligo pair were ligated into Blueskript KS- (Stratagene). The cloned oligos were always sequenced.

2.3 DNA SEQUENCING

Fifteen microlitres miniprep DNA or 10 µg QUIGEN DNA was used for sequencing reactions. The DNA was incubated with 10 µl 1 M NaOH for 15 minutes at 37°C and then spin-dialysed through a BioGel P-30 column (BioRad) to desalt the sample. The column was prepared by piercing the bottom of a 0.5 ml eppendorf with the tip of a 0.6 mm diameter needle, and filling it with BioGel P-30 equilibrated with Tris-HCl 10 mM, EDTA 1 mM, pH 8.0. This was placed in a 1.5 ml eppendorf tube which had been modified, by the removal of the bottom few mm's of the tube with a razor blade, to form a hollow tube. After centrifugation for 2 minutes at 2,500 rpm (IEC centra-3C centrifuge) the BioGel column was placed in an intact 1.5 ml eppendorf tube, the DNA sample was placed on the surface of the column and the whole was centrifuged for 2 minutes at 2,500 rpm (IEC centra-3C centrifuge). Seven microlitres of the collected sample were used for sequencing which was performed using the Sequenase Sequencing Kit (United States Biochemical) according to the manufacturer's instructions. The method is based on that described by Sanger *et al.* (1977). Appropriate primers were used at a concentration of 10 µg/ml. One microlitre of primer, 7 µl of the denatured DNA sample and 2 µl of 5X sequencing buffer (200 mM Tris-HCl, pH 7.5; 100 mM $MgCl_2$; 250 mM NaCl) were mixed together and the primer annealed by incubation for 2 minutes at 65°C followed by 30 minutes at 37°C, 5 minutes at room temperature and 5 minutes on ice. Five and a half microlitres labelling mix were then added (1 µl 100 mM DTT, 2 µl labelling mixture [7.5 µM each of dGTP, dCTP and dTTP], 0.5 µl α-35S-dATP [5 µCi] and 4U (2 µl) T7 DNA polymerase (diluted in 10 mM Tris-HCl, pH 7.5; 5 mM DTT, 0.5 mg/ml BSA)). After 2 minutes incubation at room temperature 3.5 µl aliquots of the labelling mix were removed and added to 2.5 µl of 1 of 4 termination mixes (each containing one ddNTP at 8 µM in 50 mM NaCl with 80 µM each of dGTP, dCTP, dATP and dTTP). After 5 minutes at 37°C 4.5 µl stop solution were added (95 % (v/v) formamide, 20 mM EDTA, 0.05 % (w/v) of bromophenol blue and xylene cyanol FF). The reactions were subjected to electrophoresis in a 6 % polyacrylamide/urea sequencing gel as described by Sambrook *et el.* (1989).

2.4 *IN VIVO* FOOTPRINTING OF MAIZE LEAVES

2.4.1 Preparation of chromosomal DNA

This technique was based on that described by Hammond-Kosack and Bevan (1993). All solutions used were exactly as described in the above reference. The first step involved an *in-vivo* DMS (dimethyl sulphate) treatment of the maize DNA. A 1 % solution of R-29148 safener was applied to the upper and lower surfaces of 3 week old, greenhouse grown, maize leaves. Five grammes of leaf material were harvested and placed in a 50 ml falcon tube. MS medium [Flow Laboratories] containing 0.1 % DMS was added until the material was submerged. Transferring to a vacuum desiccator in a fume hood a vacuum was applied for 1 minute and then slowly released. After 5 minutes the media was removed and the tissue was washed several times in fresh MS media. The tissue was blotted dry and stored at -70°C until all time points (0, 6, 24 and 48 hours) were collected. Chromosomal DNA was then prepared from the tissues using 'protocol 1' described by Hammond-Kosack and Bevan (1993). The DMS treated tissue was crushed to a fine powder using a pestle and mortar and liquid nitrogen. Thirty mls hot (65°C) extraction buffer (100 mM Tris HCl pH 8.0, 50 mM EDTA pH 8.0, 500 mM NaCl, 1.25 % SDS, 8.3 mM NaOH, 0.38 g/100 ml Na bisulphite) was added and the resulting slurry was incubated for 15 minutes at 65°C. Prior to 20 minutes incubation on ice, 6.16 ml 5 M KAc were added. After centrifugation at 3,500 rpm for 5 minutes (Sorvall RC3C centrifuge, H6000A rotor) the supernatant was filtered through Miracloth and 0.7 volumes IPA were added. This was spun at 4,000 rpm for 10 minutes (Sorvall RC3C centrifuge, H6000A rotor), the pellet was washed twice in 70 % ethanol and then resuspended in 0.84 ml T5E and 0.36 ml 10 M NH4Ac. This solution was centrifuged for 5 minutes at 13,000 rpm (MSE Microcentaur benchtop centrifuge). and the supernatant was retained. The pellet was resuspended and precipitated once more, the supernatants were combined and DNA was precipitated by the addition of 0.73 mls IPA. The DNA was dissolved in 100 µl T10E1 and then digested with HindIII. The DNA samples were then amplified using ligation mediated PCR (LMPCR).

2.4.2 Ligation mediated PCR (LMPCR)

Two sets of three nested primers were designed to amplify both strands of the GST-27 promoter between 0 and 350 bp upstream of the transcription start point. Primers FOR1, FOR2 and FOR3 were used to amplify the bottom strand of DNA. Primers REV1, REV2 and REV3 were designed to amplify the top strand. Sequence and melting temperatures (Tm) of primers are shown below; see the results section of chapter three for their position on the promoter. Tm was calculated according to the following formula: Tm = 69.3 + 0.41 X (G+C)%-650/L, where G+C = number of G and C residues and L = length of the oligonucleotide (Sambrook *et al.*, 1989). The two primer sets were designed to be nested *i.e.* to share a degree of common overlapping sequence, and such that the annealing temperature increased between primers. In this way, specificity in amplification is achieved. Two annealed linker primers were used in the

ligation step; their sequence is shown below.

FOR1	5'TAACAAAAGATACTAGCCTCTCTAC3'	Tm=58.1°C
FOR2	5'TCTCTACTATTTGAGTATATTCGGTGC3'	Tm=60.4°C
FOR3	5'GTATATTCGGTGCACCGAATAGACCG3'	Tm=64.8°C
REV1	5'TGCAGGATCAAGATCAGCAGCTG3'	Tm=62.3°C
REV2	5'TCAGCAGCTGGAATTGGATGGGTG3'	Tm=64.4°C
REV3	5'GGATGGGTGTGTGCAGCTCCTGCC3'	Tm=69.6°C

Linker primer Ib	5'GCAATCATTTGAGAGATCTGAATTC3'
Linker primer II	5'GAATTCAGATC3'

Before use the primers were subjected to electrophoresis on a 15 % polyacrylamide gel (15 % (w/v) acrylamide/M,N-methylene-bisacrylamide (19:1), 42 % (w/v) urea in 1 X TBE) using sequencing gel apparatus. This was in order to remove any incomplete contaminating products. Oligonucleotides were synthesised as in section 2.2.9; 30 µg oligonucleotide were mixed with an equal volume of sequencing dye and heated to 55°C for 5 minutes prior to loading into large 8 cm long wells in the gel. The gel was run for 3.5 hours at 40 W after which the apparatus was taken apart and cling film lain over the gel which was retained on one glass plate. The oligonucleotide was visualised by placing the glass with the gel on over a thin layer chromatography plate coated with a 250 µm thick layer of 254 nm fluorescent indicator (Sigma) and illuminating with longwave UV light from a hand-held UV lamp (Model UVLS.25, Mineralight). The oligonucleotide appeared as a ladder with the uppermost thickest band being the full length product. This was cut out of the gel and the oligonucleotide was eluted by placing the strip of gel into an eppendorf with 1 ml 1 X TBE, incubating at 37°C whilst shaking overnight. The buffer was twice extracted with phenol/chloroform, then once with chloroform before precipitating with isopropanol. After dissolving in 50 µl water their concentration was measured according to section 2.2.5. PCR reactions, overlain with mineral oil, were performed in a microtitre plate with a Techne PHC-3 PCR machine. The first primer for each strand (FOR1 or REV1) was annealed to the chromosomal DNA using the program 95°C 2 minutes, 55°C 30 minutes, 20°C hold. For strand extension 0.5 µl T7 DNA polymerase (10 U/µl, Pharmacia) and 8.5 µl extension buffer (20 mM $MgCl_2$, 20 mM DTT, 200 mM dNTPS) were added and incubation proceeded at 40°C 10 minutes, 65°C 10 minutes, 15°C hold. T7 DNA polymerase is used instead of Taq polymerase as the latter adds an extra single A residue to the end of approximately 95 % of extension products, which are therefore not suitable for use in the subsequent blunt ended ligation. T7 DNA polymerase produces approximately 50 - 70 %

suitable products for blunt ended ligations (Garrity and Wold, 1992). The annealed linker primers were annealed to the end of each extended molecule. 51 µl annealing mix, consisting of 6 µl 310 mM Tris-HCl pH 7.5, 20 µl ligation buffer A (17.5 mM $MgCl_2$, 42 mM DTT, 125 µg/ml BSA), 17 µl ligation buffer B (14.7 mM $MgCl_2$, 29 mM DTT, 4.4 mM ATP, 74 µg/ml BSA), 5 µl annealed primer (15 µM in 250 mM Tris-HCl pH 7.5) and 3 µl T4 DNA ligase (1 U/µl) were added, and the ligation carried out overnight at 15°C, followed by heat inactivation of 70°C for 10 minutes. The samples were IPA precipitated adding 10 µg glycogen to ensure efficient precipitation. After a 70 % ethanol wash the pellets were resuspended in 20 µl water. In a fresh microtitre plate the samples were mixed with 27 µl PCR mix, containing 5 µl 10X buffer (100mM Tris-HCl pH 8.9, 500 mM KCl, 0.01 % gelatin, 30 mM $MgCl_2$), 5 µl 2 mM dNTPs and 0.5 µl Amplitaq (Perkin Elmer). No primers were added at this stage. Samples were denatured at 94°C for 2 minutes, after which primers FOR2 or REV2 were added with primer Ib while pausing the first cycle at 85°C. The cycling was then continued, using the program 30 X (94°C 1 minute, 60°C 2 minute, 72°C 3 minutes). 25 µl of the PCR product were used for the final extension cycling; the rest was stored at -20°C. In the final extension the product was mixed with 5 µl 2 mM dNTPs, 2.5 µl 10X PCR buffer, 0.5 µl Amplitaq, 16 µl water and 1 µl end-labelled primer FOR3 or REV3 (labelled as in section 2.5.1). The samples were denatured by incubation at 94°C 2 minutes and then cycling was performed, using the program 9 X (94°C 1 minute, 65°C 3 minutes, 76°C 5 minutes). The samples were phenol-chloroform extracted, IPA precipitated and resuspended in 10 µl sequencing loading buffer. To visualise the footprint the samples were run on a 6 % sequencing gel, which was then dried and exposed to film.

2.5 RADIOLABELLING OF PROBES

2.5.1 End-labelling of primer for LMPCR

This method of labelling was adapted from Rigaud *et al.*(1991). Ten picomoles of primer were labelled for each PCR reaction (10 pm = primer length x 3.3 ng). Three point three picomoles of γ-32P dATP (1 µl Easytides, DuPont) were required to label 10 pm DNA. Appropriate quantities of primer and label were mixed in a 30 µl volume with 10X kinase buffer and T4 polynucleotide kinase (Pharmacia Biotech) and incubated at 37°C for 45 minutes. The enzyme was inactivated by a 5 minute incubation at 65°C. The sample was purified over a BioGel P30 column (see section 2.3). One microlitre of labelled primer was used in each PCR reaction.

2.5.2 Labelling of probes for electrophoretic mobility shift assay (EMSA)

Probes were labelled by filling in 3' recessed termini with labelled dNTPs using the Klenow DNA polymerase fragment. Ten probes were prepared; the sequences are shown below.

Probe name	Sequence
290WTN	5'AGCTT GC TATTTCAGAAT GC A3'
	3'A CG ATAAAGTCTTC CG TTCGA 5'
290MUTN5	5'AGCTT GC TATGGCCTAAT GC A3'
	3'A CG ATACCTGATTA CG TTCGA 5'
284WTN	5'AGCTT GC GAATCCGAAAT GC A3'
	3'A CG CTTAGGCTTTA CG TTCGA 5'
284MUTN6	5'AGCTT GC GACGAATCAAT GC A3'
	3'A CG CTGCTTAGTTA CG TTCGA 5'
275WTN	5'AGCTT GC AATTTCATAAA GC A3'
	3'A CG TTAAAGTATTT CG TTCGA 5'
275MUTN5	5'AGCTT GC AATGGACGAAA GC A3'
	3'A CG TTACCTGCTTT CG A 5'
326WTN	5'AGCTT GC GGTTCCTAAAA GC A3'
	3'A CG CCAAGGATTTT CG TTCGA 5'
326MUTN6	5'AGCTT GC GGCGGAAGCAAAGC A3'
	3'A CG CCGCTTCGTTT CG TTCGA 5'
ERE-WT	5'AGCTT GC TATTTCAAAAT GC A3'
	3'A CG ATAAAGTTTTA CG TTCGA 5'
ERE-MUT	5'AGCTT GC TATGGTCCAAT GC A3'
	3'A CG ATACCAGGTTA CG TTCGA 5'

Twenty-five nanogrammes of probe were mixed in a volume of 25 µl with 1 µl (10 µCi) of each of the 4 α–^{32}P-labelled dNTPs (DuPont Easytides), 2.5 µl 10 X one-phor-all buffer, and 2 units of Klenow. After a 10 minute incubation at room temperature 1 µl of a mix of 2 mM of each dNTP was added and the reaction allowed to continue for 15 minutes at room temperature. The sample was then phenol/chloroform extracted and spin dialysed through a BioGel P30 column (see section 2.3). Specific activities were typically 2 X 10^4 cpm/ng as measured using a benchtop Probe Counter (Oncor).

2.5.3 Labelling of probe for Northern

Using *Eco*RI an 800 bp fragment of DNA was from the pIJ21 construct (supplied by Ian Jepson, Zeneca). This fragment spans the first exon and intron of the *GST-27* gene. Approximately 100 ng of probe DNA in a 33 µl volume were incubated at 100°C for 5 minutes and then incubated on ice. Ten microlitres random hexamer oligonucleotide mix (50 units

random hexamer oligos, 20 µM dATP, dTTP, dGTP, 5 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$, 200 mM HEPES), 1 µl 20 mg/ml BSA (bovine serum albumin), 1 µl Klenow fragment (2 units, Pharmacia Biotech) and 5 µl α–^{32}P dCTP (50 µCi, DuPont) were added. This mix was incubated at 37°C for 90 minutes, then passed through a Sephadex G-50 column (prepared in the same way as a BioGel p30 column, described in section 2.3). Before use the probe was incubated at 100°C for 5 minutes.

2.6 PREPARATION OF MAIZE RNA

The RNA preparation method was as described by Jepson *et al.* (1991). RNase contamination was avoided by the use of autoclaved, disposable plastic ware and the treatment of solutions with a 0.1 % DEPC solution. A 1 % solution of R-29148 safener was applied to the upper and lower surfaces of 5 week old, greenhouse grown, maize leaves. 200 mg leaf material were harvested at time points 0, 4, 6, 8, 12, 18, 24, 36 and 48 hours after the application of safener. The samples were stored at -70°C until all the time points had been collected. The material was ground to a fine powder using a pestle and mortar and liquid nitrogen. One ml homogenising buffer (400 mM NaCl, 50 mM Tris-HCl pH 9.0, 1 % SDS, 5 mM EDTA, 4u/ml heparin, 1 mM ATT and 10 mM DTT) and 0.5 mls phenol cresol (phenol with 10 % (v/v) *m*-cresol) were added. The resulting slurry was transferred to 2 eppendorf tubes. The pestle was rinsed with 0.5 mls homogenising buffer and 0.25 mls phenol/cresol and the rinse was added to the eppendorf tubes. The samples were centrifuged at 4°C for 15 minutes at 13,000 rpm (MSE Microcentaur benchtop centrifuge). The pink coloured supernatant was extracted twice with an equal volume of phenol/chloroform. The RNA present in the aqueous phase was precipitated by the addition of 12 M LiCl to a final concentration of 2 M and incubated overnight at 4°C. The solution was centrifuged at 4°C for 15 minutes at 13,000 rpm (MSE Microcentaur benchtop microfuge). The resulting pellet was resuspended in 100 µl 5 mM TrisHCl, pH 7.5 and precipitated again, overnight, with LiCl. The solution was centrifuged at 4°C for 15 minutes at 13,000 rpm (MSE Microcentaur benchtop centrifuge), the pellet was washed with 70 % EtOH and resuspended in 50 µl water. RNA was quantified using a spectrophotometer (see section 2.2.5). Readings were taken at wavelengths of 260 nm and 280 nm. These values, for pure RNA samples, have a ratio of 2:1.

2.7 AGAROSE GEL ELECTROPHORESIS OF RNA

Ten microgrammes total RNA (see section 2.6) were run on a 1.3 % agarose/formaldehyde gel. The gel was prepared with 1.95 g agarose, 15 mls 10 X MOPS (0.2 M MOPS, 50 mM Na Ac, 10 mM EDTA) and 130.5 mls diethylpyrocarbonate (DEPC)-treated water. It was cooled to 50°C before 7.65 mls 37 % formaldehyde were added. The RNA samples, in a 5 µl volume,

were mixed with 2 µl 10 X MOPS, 10 µl deionised formamide, 3.5 µl 37 % formaldehyde, 1 µl water and 1 µl 1 mg/ml ethidium bromide and then denatured by incubation at 65°C for 20 minutes. Two microlitres RNA loading buffer were added to each sample (50 % glycerol, 1 mM EDTA, 0.25 % bromophenol blue, 0.25 % xylene cyanol) prior to electrophoresis. Electrophoresis was carried out in a 1 X MOPS/7 % formaldehyde buffer for 90 minutes at 160 volts. The gel was visualised on a UV-transilluminator.

2.8 NORTHERN BLOTTING

The RNA gel was soaked for 2 X 15 minutes in 20 X SSPE (3.6 M NaCl, 0.2 M NaH_2PO_4, 0.02 M EDTA, pH 7.7) and then blotted overnight onto Hybond-N nylon membrane (Amersham). Capillary blotting apparatus were set up as described by Sambrook *et al.*(1989)using 20 X SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.4). After blotting the membrane was briefly washed twice in 2 X SSC and then the RNA was bound to the membrane by UV irradiation using a Stratalinker (Stratagene).

2.9 HYBRIDISATION

The membrane, with bound RNA, was pre-hybridised in 25 mls pre-hybridisation solution (6 X SSPE, 1 X milk buffer [2.5 % skimmed milk, 3 % SDS, 0.1 % DEPC], 100 µg/ml salmon sperm DNA) for 3 hours at 65°C in an hybridisation oven (Bachofer). 20 mls of the solution were then removed and the denatured radio-labelled probe was added. Hybridisation was continued overnight. Two low stringency washes were performed (2 X SSC, 0.1 % SDS) for 5 minutes at room temperature. This was followed by 2 moderate stringency washes (1 X SSC, 0.1 % SDS) for 30 minutes at 60°C and then 2 high stringency washes (0.2 X SSC, 0.1 % SDS) for 30 minutes at 60°C. The membrane was sealed in a clear plastic bag and radioactive bands were visualised by autoradiography.

2.11 PREPARATION OF NUCLEAR PROTEIN EXTRACTS

Nuclear protein extracts were prepared from 3 week old, greenhouse grown, maize plants. A 1 % solution of R-29148 safener was applied to the upper and lower surfaces of each leaf on 40 plants. After 24 hours the plants were harvested and washed thoroughly in water. The nuclear protein extracts from these plants were referred to as the '24 hour' preparation. 40 plants were not treated with safener and were referred to as the '0 hour' preparation. The samples were frozen in liquid nitrogen and ground to a fine powder in a pestle and mortar. From this time on the samples were kept at 4°C. The powder was transferred to a 125 ml falcon tube and 60 mls freshly prepared extraction buffer were added (0.25 M sucrose, 10 mM NaCl, 10 mM Mes-NaOH, 5 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 0.2 mM AEBSF, 10 mM NaF,

20 mM β-mercaptoethanol, 0.1 % BSA and 0.6 % Non-idet P40). The resulting slurry was filtered through 3 layers of Miracloth (Calbiochem) into 4 corex tubes. A 25 % Percoll solution was prepared (10 mls Percoll (Sigma), 1.88 mls 20X salts [200 mM NaCl, 200 mM Mes-NaOH pH 6.0, 100 mM EDTA], 4.7 mls 2 M sucrose, 23.4 mls water, 250 µl Non-idet P40, 40 µl antipain (5 mg/ml) and 40 µl leupeptin (5 mg/ml)). Five mls of the Percoll solution were carefully pipetted into the bottom of the corex tubes, so forming a cushion between the nuclear prep solutions and the base of the corex tube. The samples were centrifuged at 4°C for 40 minutes at 3,000 rpm (Sorvall RC-5B centrifuge, HB4 rotor). The nuclei were collected at the bottom of the tube and resuspended in 10 mls extraction buffer (with no BSA added). They were centrifuged again at 4°C for 5 minutes at 5,000 rpm (Sorvall RC-5B centrifuge, HB4 rotor). After removing as much of the supernatant as possible the nuclei were carefully resuspended in 200 µl dialysis buffer (40 mM KCl, 24.7 mM HEPES-NaOH pH 7.9, 5 mM EDTA, 25 % glycerol, 1 mM DTT, 10 mM NaF, 1 µg/ml leupeptin and 1 µg/ml antipain) using a blunt ended pipette tip. Proteins were precipitated by the addition of 1/10 volume of 4 M $(NH_4)_2SO4$ followed by 30 minutes incubation on ice. The solution was centrifuged at 4°C for 20 minutes at 13,000 rpm (MSE Microcentaur benchtop microfuge). The supernatant was retained and nuclear proteins were precipitated by the addition of 1.5 volumes 4 M $(NH_4)_2SO4$ followed by 90 minutes incubation on ice. The solution was then centrifuged at 4°C for 20 minutes at 13,000 rpm (MSE Microcentaur benchtop microfuge). The resulting pellet was resuspended in 100 µl dialysis buffer and twice dialysed against 500 mls dialysis buffer. The protein concentration was estimated using the Bradford assay (see section 2.12).

2.12 BRADFORD ASSAY

Protein concentration of extracts was estimated using the Bradford assay (1976). Commercial Bradford reagent (Biorad) was used, diluted 1/5 with water. Standards ranging in concentration from 0 to 5 µg BSA/10 µl water were prepared and mixed with 190 µl Bradfords reagent in a clear 96-well microtitre plate. Two aliquots of each sample were assayed with 190 µl Bradfords reagent. Generally, 2 µl of nuclear protein extract were used and 10 µl of a 1/5 dilution of the leaf extracts used in GUS assays. Absorbance of samples was measured at 600 nm, and protein concentration was calculated using a Titertek Multiscan spectrophotometer (Flow Laboratories) and software provided by the manufacturer.

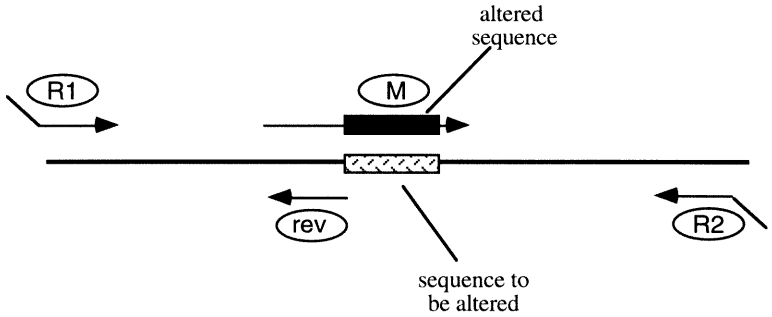
2.13 ELECTROPHORETIC MOBILITY SHIFT ASSAYS (EMSA)

EMSA was performed essentially as described by Holdsworth and Laties (1989). 1 µg protein (see section 2.11) was mixed in a volume of 10 µl with 5 mM DTT, 50 mM KCl, 1 µg poly-

dIdC (Pharmacia), 100 mM $MgCl_2$, 1 µl gel retard buffer [250 mM HEPES pH 7.6, 10 mM EDTA, 50 % glycerol] and approximately 10^4 cpm of radiolabelled probe (see section 2.5.2). This mix was incubated at room temperature for 20 minutes. The samples were loaded onto a 6 % polyacrylamide 0.5 X TBE gel, which had been pre-run for 20 minutes at 100 Volts. Electrophoresis of the samples was carried out for 90 minutes at 150 Volts. The gel was transferred onto 3 mm Whatman paper, dried and autoradiographed at -70°C with one intensifying screen.

2.14 PCR-MEDIATED MUTAGENESIS

Figure 1: Diagram indicating position of primers required for PCR-mediated mutagenesis



Four primers were required for PCR-mediated mutagenesis - see figure 1. Specific primers used are listed at the end of this section. In general primers R1 and R2 flank the promoter on either side of the region which is to be mutated and contain suitable enzyme restriction sites for cloning. The 5' and 3' ends of primer M are identical to the promoter while the middle section contains the new altered sequence. Primer rev is identical to the wild type 5' end of primer M. In step 1, 2 PCR reactions were performed in a volume of 25 µl with 20 ng wild type template plasmid, 10 X PCR buffer, 2.5 µl 2 mM dNTPs, 2 µl 20 µM primer R1 or M, 2 µl 20 µM primer rev or R2 and 0.25 µl Amplitaq (Perkin Elmer). The program used was 94°C for 2.5 minutes followed by 30 cycles of 94°C for 1 minute, 50°C for 1 minute and 72°C for 1 minute. The reactions were each carried out 4 times to generate enough product for step 2. The resulting fragments R1/rev and M/R2 were purified and quantified (see sections 2.2.4 and 2.2.5). In step 2 the fragments R1/rev and M/R2, which share a region of overlapping sequence, were annealed. 250 ng of each fragment were mixed in a 25 µl volume with 10 X PCR buffer, 2.5 µl 2 mM dNTPs and 0.25 µl Amplitaq. Annealing and then extension of the annealed product

were performed using the program 94°C for 2.5 minutes followed by 9 cycles of 94°C for 1 minute, 40°C for 1 minute and 72°C for 1 minute. In step 3 the extended heterodimers, comprising of the whole promoter sequence containing the mutation, were amplified by the addition of primers R1 and R2. To the same reaction from step 2, 2.5 µl 10 X PCR buffer, 2.5 µl 2 mM dNTPs, 2 µl 20 µM R1, 2 µl 20 µM R2, 15.75 µl of water and 0.25 µl Amplitaq were added. PCR was carried out using the same program as that which was used in step 1. The resulting fragment was purified and cloned into a suitable vector (see section 2). The primers used for PCR-mediated mutagenesis were as follows; the primers used in each construct are shown and their position in the promoter is indicated by the label R1, R2, rev or M (see figure 1).

pPUG5 mutations:

All mutations in pPUG5 utilised the primers pAI5 and pAI2 which served as primers R1 and R2.

R1 = 5' GCGGCAAGCTTAATATGTGATGATA 3' (pAI5)

R2 = 3' TATTCATCGTCGACGTCGTCCGT 5' (pAI2)

Construct name - 326MUT in pPUG5

M = 5' GTCTATTCAGGTTCTTGGAAGCCCATTATTTTAAAAATTTTGGTTCTC 3'

rev = 3' CAGATAAGTCCAAG 5'

Construct name - 290MUT in pPUG5

M = 5'CCTAAAATTATTTTAAAAATTTTGGTTCTCAGCGGGACTCCTCCG 3'

rev = 3' GGATTTTAATAAAATTTTTAAAACCAAGAGT 5'

Construct name - 284MUT in pPUG5

M = 5' CTCATATTTCAGAATAATCCCGGGAATAAATCC 3'

rev = 3' GAGTATAAAGACTTA 5'

pPUG6 mutations:

All mutations in pPUG6 utilised the primers pAI4 and pAI2 which served as primers R1 and R2.

R1 = 5' CTGAAAGCTTCGGTGCACCGAAT 3' (pAI4)

R2 = 3' TATTCATCGTCGACGTCGTCCGT 5' (pAI2)

For constructs 326MUT in pPUG6, 290MUT in pPUG6 and 284MUT in pPUG6 primers M and rev were identical to those used in the corresponding constructs in pPUG5, as above.

Construct name - 2XMUT in pPUG6

M = 5' CCTAAAATTATTTTAAAAATTTTGGTTCTCAGCGGGACTCCGAATCCCGGGA CGCCCTCCAAATAGACCG 3'

rev = 3' GGATTTTAATAAAATTTTTAAAACCAAGAGT 5'

2.15 TRANSIENT TRANSFORMATION OF BLACK MEXICAN SWEET (BMS) MAIZE SUSPENSION CULTURE CELLS WITH SILICON CARBIDE FIBRES

This method was based on that described by Kaeppler *et al.* (1990) with modifications according to Frame *et al.* (1994). All manipulations were performed aseptically. BMS cells were grown in the dark at 25°C, rotating at 120 rpm in BMS media (4.71 g MS media, 20 g sucrose, 2 mg 2,4 D dissolved in 1 l water, pH 5.6). Two days prior to the transformation 20 mls of cells were sub[cultured] into 50 mls fresh BMS media. To transform, 100 µl fresh BMS media were aliquoted into an eppendorf. 40 µl of a 5 % (w/v) silicon carbide fibre suspension (Silar SC-9, ARCO Metals), 10 µg plasmid DNA and 500 µl cell suspension (250 µl packed cell volume, 250 µl BMS media) were added. The tube was mixed at full speed on a vortex-2-genie (Scientific Industries International Inc.) for 60 seconds. The contents of the tube was transferred into a 50 mm petri dish. Any remaining solution was rinsed out with 2 X 750 µl of fresh medium and the washes were added to the petri dish. This was incubated at 25°C for 48 hours while rotating on a shaker at 60 rpm, before performing a histochemical GUS assay (see section 2.16.1). To analyse the effect of dichlormid safener on the transformed cells R-25788 was added to the media used in the transformation experiment, at a concentration of 40 ppm (100 µl R-25788 in 10 mls EtOH, 400 µl of that dilution into 100 mls BMS media).

2.16 GUS ASSAY

2.16.1 Histochemical GUS assay

Histochemical detection of GUS activity was performed essentially as described by Jefferson *et al.* (1987). Assay buffer consisted of 50 mls 0.1 M dibasic/monobasic sodium phosphate, pH 7.0, with 80 mg potassium ferricyanide, 105 mg potassium ferrocyanide, 0.06 % triton X-100 (1 drop), 0.1 % dimethyl sulphoxide (1 drop) and 25 mg X-Gluc (5,chloro-4,bromo-3,indoyl-β-D-glucoronic acid). To stain BMS cells, 2.5 mls assay buffer were added to the cells, which were then incubated for 24 hours at 37°C. The cells were poured onto Whatman No.1 filter paper, in a 90 mm petri dish, and observed under a binocular microscope (Nikon SMZ-2B) at 20X magnification. The number of visible blue spots were counted. To stain seedlings or leaf tissue the plant material was submerged in the assay buffer which was then vacuum infiltrated to allow good penetration of the solution into the tissue. The sample was incubated at 37°C for

24 hours. Chlorophyll was removed from stained tissue by incubating overnight in 70 % ethanol.

2.16.2 Fluorometric GUS assay

Fluorometric detection of GUS activity was performed essentially as described by Jefferson *et al.* (1987). Two leaves were sampled per plant. The leaves were frozen in liquid nitrogen and ground to a course powder using a pestle and mortar. The powder was transferred to a 50 ml Falcon tube and an equal volume of extraction buffer (50 mM $NaPO_4$, 10 mM DTT, 0.1 % Triton X-100, 0.1 % Sarkosyl, 10 mM EDTA) was added. From this time on the samples were kept on ice. The samples were centrifuged for 20 minutes at 4°C at 3,500 rpm (Sorvall RC3C centrifuge, H6000A rotor). 20 µl of the supernatant were transferred to a clear microtitre plate. 180 µl MUG assay buffer (22 mg MUG [4-methylumbelliferyl-β-D-glucoronide, Sigma] in 50 mls extraction buffer) were added to each sample. Each plate contained a positive control of assay buffer with 500 pmoles methylumbelliferone (MU) and a negative control of assay buffer only. The plate was incubated at 37°C. At 3 time points, typically 0, 60 and 120 minutes, 50 µl of the reaction solution were transferred to black microtitre plates containing 50 µl 0.4 M Na_2CO_3/well. After all the time points had been taken fluorescence of released MU was measured using a spectrofluorimeter (Titertek Fluoroscan II, Flow Laboratories) with excitation filters set at 365 nm and emission filters set at 455 nm. MU concentrations were calculated by comparison with the standards and the rate of MU release/min for each extract was calculated over the time points using software provided by the manufacturer of the spectrofluorimeter.

2.17 TECHNIQUES INVOLVED IN TOBACCO TRANSFORMATION

2.17.1 Cloning of constructs for transformation

Tobacco plants were transformed with seven constructs, all of which were based on the binary vector pBin400 (Spychalla and Bevan, 1993). Three 5' deletions of the *GST-27* promoter fused to the reporter gene GUS were prepared, containing 570, 378 and 217 bp of the promoter upstream from the TSP. These truncated promoter:GUS fusions were present in the constructs pPUG5 (570 bp), pPUG6 (378 bp) and pPUG7 (217 bp) which had been made previously at Zeneca Agrochemicals. The truncated promoters fused to GUS were cut out on a *Hind*III, *Eco*RI fragment from these three plasmids and cloned directly into pBin400, which had been linearised with the same enzymes. The resulting constructs were named pBin/5, pBin/6 and pBin/7. Two mutated forms of pBin/6 were prepared using the PCR-mediated mutagenesis technique with wild type pPUG6 as a template (see section 2.14). 3XMUTBin/6 contained 5 bp mutations to the footprinted G residues G-290, G-283/284 and G-275 and their flanking nucleotides and 4XMUTBin/6 contained 5 bp mutations to the footprinted G residues G-290, G-283/284, G-275 and G-325/326 and their flanking nucleotides. The primers used are given

below.

Primers used to create 3XMUT in pPUG6:

R1 = 5' CTGAAAGCTTCGGTGCACCGAAT 3' (pAI4)

R2 = 3' TATTCATCGTCGACGTCGTCCGT 5' (pAI2)

M =

5'CCTAAAATTATTTTAAAAATTTTGGTTCTCATATGGACTACGAATCAATGGACGAAA TCCAAATAGACCG 3'

rev = 3'GGATTTTAATAAAATTTTTAAAACCAAGAGT 5'

The fragment which resulted from the PCR-mediated mutagenesis was cloned into the pGEM-T vector (Promgea) and sequenced to ensure that the correct bases were mutated. The fragment was cut out of the pGEM-T vector with *Hind*III and *Pst*I and cloned into pPUG6 which had been linearised with the same enzymes. In this way the wild type promoter in pPUG6 was removed and replaced with the 3XMUT version, so creating the plasmid p3XMUT/PUG6. This was cloned into pBin400 using the same strategy as described above for the wild type pPUG6 plasmid, to form 3XMUT/Bin6. Further PCR mediated mutagenesis was performed using p3XMUT/PUG6 as template DNA in order to mutate the footprinted G residues G-325/236 and four flanking nucleotides. The primers used are given below.

Primers used to create 4XMUT in pPUG6:

R1 = 5' CTGAAAGCTTCGGTGCACCGAAT 3' (pAI4)

R2 = 3' TATTCATCGTCGACGTCGTCCGT 5' (pAI2)

M =5'GTCTATTCAGGTTCGGGGAAGCAAATTAT3'

rev = 3'CAGATAAGTCCAAG5'

The resulting fragment was cloned into pGEM-T and subsequently into pPUG6 as described above for the 3XMUT. An identical cloning strategy as was used for 3XMUT was also adopted for the 4XMUT in order to create 4XMUTBin/6. Finally, two constructs were engineered which contained 28 bp of the *GST-27* promoter (spanning the footprinted residues G-290, G-283/284 and G-275) fused to either a 90 bp truncated form of the 35S CaMV promoter or to a 60 bp truncated form of the 35S CaMV promoter. Two oligos were made (sequence is shown below), incorporating *Bam*HI and *Hind*III restriction sites, and were annealed and cloned (see section 2.2.9) into the multiple cloning sites of pBin421.9 and pBin421.8 so forming pWT9 and pWT8. These plasmids are identical to pBin400, but pBin421.9 contains 60 bp of the 35S CaMV promoter fused to the GUS reporter gene and pBin421.8 contains 90 bp of the 35S CaMV promoter fused to GUS. The multiple cloning sites for both of these plasmids is situated at the 5' end of the truncated 35S CaMV promoter.

The sequence of the oligos used in the construction of pWT9 and pWT8 is given below:

5'AGCTT ATATTTCAGAATCCGAAATTTCATAAAT G3'
3'A TATAAAGTCTTAGGCTTTAAAGTATTTA CCTAG5'

2.17.2 Transformation of *Agrobacterium*

An overnight culture of *A.tumefaciens* (strain T37SE, Monsanto) was set up in 10 mls YEP broth (1 % (w/v) Bactopeptone [Difco], 1 % (w/v) yeast extract [Difco], 0.5 % NaCl) with 50 µg/ml kanamycin sulphate and grown at 30°C. Four mls of this were used to inoculate 100 mls YEP broth, containing 50 µg/ml kanamycin sulphate, which was grown for 4-5 hours. The cells were pelleted at 2,500 rpm for 10 minutes (Sorvall RC3C centrifuge, H6000A rotor), resuspended in 2 mls YEP broth and chilled on ice for 5 minutes in 200 µl aliquots. Ten microlitres pBin DNA at 0.1-0.2 µg/µl were added to the cells. The cells were immediately frozen in liquid nitrogen for about 15 seconds followed by a heat shock of 37°C for 5 minutes. 1 ml YEP was then added and the cells grown at 30°C for 1-2 hours. A 100 µl aliquot from each transformation was spread onto YEP plates (YEP with 1.5 % (w/v) agar) containing 50 µg/ml kanamycin sulphate to select for the *Agrobacterium* and 50 µg/ml spectinomycin hydrochloride to select for the binary vector. The plates were inspected for growth after 48 hours incubation at 30°C. Single colonies were streaked out on Minimal T plates (350 mls water agar [1.5 % (w/v) agar], 20 mls 20X salts, 20 mls 20X T buffer, 20 % glucose), containing the selecting antibiotics, and grown for 3 days at 30°C. Bacterial colonies from these plates were used to inoculate liquid cultures for DNA preparation.

2.17.3 Plasmid DNA preps from *A.tumefaciens*

Ten mls YEP broth, containing antibiotic to select for the binary vector and the *Agrobacterium*, were inoculated with a single colony of *A. tumefaciens* and grown overnight at 30°C. The cells were collected by centrifugation at 2,500 rpm for 20 minutes (Sorvall RC3C centrifuge, H6000A rotor), then resuspended in 200 µl ice-cold solution I (solution I, II and III are identical to those described for DNA minipreps in section 2.2.1 except for the addition of 4 mg/ml lysozyme to solution I), allowed to stand for 30 minutes at room temperature and then vortexed. 200 µl solution II were added, mixed by gentle shaking and incubated at room temperature for 30 minutes. 150 µl ice-cold solution III were then added and the mixture was incubated on ice for 5 minutes. The sample was centrifuged for 5 minutes at 13,000 rpm (MSE Microcentaur benchtop microfuge). The supernatant was extracted with an equal volume of phenol/chloroform and then IPA precipitated. The pellet was resuspended in 50 µl $T_{10}E_1$. RNA was digested by the addition of 1 µl 1 mg/ml RNaseA, followed by incubation at 37°C for 30 minutes. Ten microlitres of this DNA were used in restriction enzyme digests and the identity of the binary plasmid was confirmed by agarose gel electrophoresis (see section 2.2.3).

2.17.4 Transformation of tobacco

Tobacco was transformed using the method developed by Horsch *et al.*(1985). The *Agrobacterium* containing the binary vector construct was grown in YEP broth, containing the appropriate antibiotics, at 30°C for 24 hours prior to the transformation. The cells were pelleted by centrifugation at 3,000 rpm for 20 minutes at room temperature (Sorvall RC3C centrifuge, H6000A rotor) and resuspended in 10 mls fresh YEP. Leaf discs were cut from leaves of *Nicotiana tabacum* var. Samsun using a cork-borer (7mm) and transferred to a 14 cm petri dish. The *Agrobacterium* was added to the petri dish which was left at room temperature for 30 minutes. Feeder plates were prepared before starting the experiment. One l NBM media (4.6 g MS salts [Flow Laboratories], 30 g sucrose, 0.1 mg EtOH NAA [Sigma], 0.1 mg BAP [Sigma], 8 g agar [Difco], 100 X B5 VITS, pH 5.9) was required for 40 plates. One ml of a tobacco cell suspension culture was spread evenly over the surface of the plates, and then covered with a sterile 9 cm Whatman No. 1 filter disc. The leaf discs were transformed to the feeder plates, lower epidermis uppermost. After 2 days co-cultivation with the *Agrobacterium* the discs were transferred to NMB plates containing 100 µg/ml kanamycin sulphate and 500 µg/ml carbenicillin sodium salt, in order to eliminate the *Agrobacterium* and select for transformants. The leaf discs were transferred every 2 weeks to fresh NMB plates containing the antibiotics. After approximately 4 weeks small shoots were visible on the leaf discs. These were transferred to MS medium (4.6 g MS salts, 30 g sucrose, 8 g agar, pH 5.9) containing 200 µg/ml carbenicillin sodium salt and 100 µg/ml kanamycin sulphate. Transformants rooted after approximately 14 days while untransformed plants bleached and died. When the transformed plants were established they were transferred to soil and grown in the greenhouse.

2.17.5 PCR screening of transformed tobacco

PCR screening of potentially transformed tobacco was used as a quick method to identify if a section of the *GST-27* promoter was present in the plants. Using a sterile yellow tip, a section of leaf was bored from the leaf of potentially transformed plants and placed in 10 µl of water in a microtitre PCR plate. This was subjected to vacuum infiltration for 1 minute. PCR reactions were then set up in the same microtitre plate in order to amplify the 217 bases of DNA from the *GST-27* promoter, upstream from the TSP. The primers sequence is given below:,

pAI2 3' TATTCATCGTCGACGTCGTCCGT 5'

pAI3 5' GTTAAAGCTTCGCAAGTCGCACCCCACTA 3'

An annealing temperature of 55 °C was used in 45 cycles of PCR. Products were analysed on an agarose electrophoresis gel.

2.17.6 Fluorometric analysis of transgenic tobacco leaves

Transgenic tobacco leaves, from plants which had been in the greenhouse for at least 7 days,

were analysed for GUS activity both before and after safener treatment. On day 1, 2 leaves were removed and analysed as described in section 2.16.2. In addition, both surfaces of 2 leaves on the plants were painted with a 5 g/l solution of R-29148 dichlormid safener. These were collected on day 2 and analysed as described in section 2.16.2. Subsequent statistical analysis of the fluorometric data was kindly performed by Keith Ward from Zeneca. The data was log transformed in order that the parametric test of Analysis of Variance could be applied.

2.17.7 Fluorometric analysis of transgenic tobacco roots

Roots were collected from tobacco plants which had not been treated with safener. This was achieved by digging under the surface of the soil and removing 1 or 2 roots to an eppendorf. Samples were subsequently kept on ice. Each root sample was placed in a sieve, washed with water to remove excess soil and placed in a liquisystem tube (Linbro) with 300 µl of extract buffer (see 2.16.2 for recipe) and a pinch of insoluble polyclar AT. They were then ground with an electronic grinder (Citenco, LC7) for 5 seconds. The samples were centrifuged in a Centuar 2 cenrifuge for 20 minutes at 3,500 rpm. Supernatant was removed and GUS assays were performed as described in section 2.16.2.

2.17.7 Histochemical staining of transgenic tobacco seedlings

Seeds were germinated on sterile filter paper in 9 cm petri dishes. The filter paper was moistened with 1 ml water containing 50 µg/ml kanamycin sulphate and the petri dish was sealed with parafilm. Seeds which had inherited the transgene appeared green and healthy; those without the transgene were bleached. After germination, and when the seedlings were approximately 5 mm in size, half of the healthy seedlings were transferred to fresh petri dishes, on to filter paper moistened with 1 ml water containing 50 µg/ml kanamycin sulphate and 30 ppm R-25788 safener (diluted in ethanol). The remaining half were transferred to moist filter paper in the absence of R-25788. After 96 hours the seedlings were histochemically stained as described in section 2.16.1.

Chapter Three

THE IDENTIFICATION OF SAFENER-RESPONSIVE ELEMENTS IN THE PROMOTER OF *GST-27*

3.1 INTRODUCTION

As discussed in detail in Chapter 1, the *GST-27* gene from *Zea mays* is induced significantly in the aerial parts of the plant following application of a herbicide safener (Holt *et al.*, 1995). A range of hormonal, environmental and physiological stimuli have been tested for their ability to induce this gene (Jepson *et al.*, 1994). An increase in *GST-27* mRNA was only observed following application of safener, in the late stages of leaf senescence and with treatments resulting in phytotoxic effects. As the promoter of this gene appears to be induced by only a few, specific stimuli it may have potential for use in an inducible gene expression system. The fact that the inducing agents, herbicide safeners, are currently registered for use in agriculture, and that methods are established for their use on field crops, adds to its potential. It may be assumed that the stimuli which can induce the gene, other than the safeners, should not prove a problem in an agricultural setting as treatments resulting in phytotoxic effects would naturally be avoided. The problem of senescence would not be encountered as the inducible gene expression system would be utilised before the plant reached that stage in its life cycle.

In order to produce an efficient inducible gene switch system utilising this promoter, it is desirable that the *cis* element conferring inducibility is identified. In this way, when the system is used in heterologous species, the minimum amount of foreign DNA will be introduced which may be important for the public acceptance of genetic engineering. By identifying the *cis* element, it could be concatamerised, or altered in some way, in order to increase the level of inducibility of the promoter. In addition, the element could be used together with other elements *i.e.* elements which confer tissue-specific expression, which may lead to the production of a more effective inducible gene switch system. The research discussed in the remainder of this introduction concerns the isolation and characterization of the *GST-27* promoter. The work was conducted at Zeneca Agrochemicals, UK, by Ian Jepson and co-workers. The research presented in this thesis has been carried out in close collaboration with Zeneca Agrochemicals.

3.1.1 Isolation of the *GST-27* promoter

Ian Jepson and co-workers from Zeneca Agrochemicals, UK, constructed a maize root cDNA library from plants which had been treated with the herbicide safener dichlormid. Clones corresponding to the *GST-27* gene were isolated by immunoscreening with a *GST-27*-specific polyclonal antiserum (Jepson *et al.*, 1994). Genomic clones, containing the 5' controlling region of the gene, were then isolated from a maize genomic library. Clone pGIE7 was sequenced and found to contain 3.8 kb of sequence upstream from the transcription start point (TSP) of the *GST-27* gene. This region of DNA was isolated by cutting the DNA with *Eco*RI at the 5' end of the fragment and with *Nde*I at the TSP, and was used in the production of a series of reporter gene constructs.

3.1.2 Initial characterization of the *GST-27* promoter in tobacco and maize plants

The 3.8 kb promoter fragment from pGIE7 was cloned upstream of the β-glucoronidase (GUS) reporter gene to form the plasmid pGSTTAK. This was used to stably transform transgenic tobacco via *Agrobacterium tumefaciens* mediated gene transfer. A thickened dichlormid safener formulation, R-29148 (2,2,5-Trimethyl-3-(dichloroacetyl)-1,3-oxazolidine), of a 10 g/l concentration, was painted onto the surface of the transgenic tobacco and inducible expression of the GUS reporter gene was observed. This result indicated that the promoter from a monocotyledonous species was active in a dicotyledonous species, and that it retained its safener-responsive inducibility. The 3.8 kb promoter fragment fused to the GUS reporter gene was also used to construct the plasmid pZM/RMS3. This was used in particle bombardment experiments to produce stably transformed maize plants. All maize transformants were generated by researchers at ICI Seeds, Iowa, USA. Second generation transformants were painted with the safener R-29148, at a concentration of 10 g/l and, again, the gene was shown to be induced by the application of safener.

In an attempt to identify the position of the inducible element within the promoter, a construct (RMS3) was engineered which carried 0.9 kb of the promoter region upstream from the TSP fused to the reporter gene GUS. Leaves from primary transformants of maize, containing the RMS3s construct, were painted with R-29148, at a concentration of 10 g/l, on days one and three and harvested on day four. Fluorometric analysis of GUS activity revealed that the truncated promoter retained its responsiveness to the safener. Basal levels of expression, without the addition of safener, were low but gene expression was increased by the addition of safener. Levels of induction varied between one to twelve fold with average non-induced levels of GUS measured at 10 nmoles/mg protein/hour, increasing to 60 nmoles/mg protein/hour after treatment with safener. This experiment indicated that the inducible element was contained within the 0.9 kb of sequence upstream of the TSP. Promoter analysis in whole plants is time consuming, both in the generation of transformed plants and in their subsequent analysis. In addition, the results obtained are highly variable due to the transgene integrating into the genome in a random manner with respect to position and copy number (Ow *et al.*, 1995). Variable results may also reflect the difficulties encountered when trying to standardise chemical application between plants. Therefore, to further dissect the promoter, a transient assay system was used.

3.1.3 Further characterization of the *GST-27* promoter in transient assay systems

3.1.3.i Transient assays in protoplasts

Initial investigations with a transient assay system were performed using protoplasts prepared

from cell suspensions of tobacco cells or Black Mexican Sweet (BMS) maize cells. The transformation procedure was based on that described by Negrutiu *et al.* (1987). Three constructs were engineered in the pUC plasmid, by fusing 5' deletions of the *GST-27* promoter to the reporter gene GUS. These were named pPUG1, pPUG2 and pPUG3 and contained 3.8 kb, 2.5 kb and 0.9 kb of the promoter respectively. When 30 ppm of R-25788 were added to the transiently transformed cells a two to three fold increase in the expression level of GUS was observed for all the constructs. However, there was an unacceptably high basal level of expression which was not observed in experiments conducted in whole plants. The process of preparing protoplasts is an invasive technique, and damaging to the plant tissue. This damage may lead to the accumulation of products which could induce the *GST-27* gene, as it is known that the gene is induced by treatments which result in phytotoxic effects. This may explain the observed high levels of basal expression.

3.1.3.ii Transient assays by particle bombardment

As transient assays in protoplasts proved to be an ineffective system for these experiments, transient transformation of BMS cells by particle bombardment was next investigated. However, using the construct pPUG1, no induction of gene expression was observed when safener was added as the basal levels of expression were high. Again, this may be due to the damage received by the BMS cells as a result of the transformation procedure. In addition, results between experiments were highly variable.

3.1.3.iii Transient assays using silicon carbide fibres

The third approach attempted in order to assay the deletion constructs was transient transformation of BMS cells by silicon carbide transfer (see Materials and methods, 2.15). The method had been used before at Zeneca and shown to be a simple but effective transformation procedure. Before detailed analysis of a number of deletion promoter constructs were carried out, the system was optimised by a masters student, Anne-Isobelle Michou. Conditions concerning the concentration, formulation and timing of application of the safener were optimised. The conditions finally chosen are given in section 2.15 of this thesis. Having gained promising results from the transformation of pPUG1, pPUG2 and pPUG3 into BMS cells using the optimised system, further 5' deletion promoter constructs were engineered. pPUG4, pPUG5, pPUG6 and pPUG7 were created which contained 760, 570, 378 and 217 bp of the promoter respectively. The silicon carbide transformation was performed under two conditions, with or without the addition of 40 ppm of the dichlormid safener R-25788 (*N,N*-diallyl-2,2-dichloroacetamide). Three replicates were performed for each condition and with each construct tested. Expression of GUS was determined by counting the number of blue stained BMS cells, referred to as colour forming units, after histochemical assay buffer had been added. For a summary of constructs see figure 1 and for a summary of results see table 1. All the constructs showed, on average, a four to five-fold induction of GUS expression with the addition of

safener, except for pPUG7 which contained the shortest promoter fragment of 217 bp. pPUG6, containing 378 bp of the promoter sequence, retained inducibility. This result implies that the element conferring inducibility to the promoter lies between 378 and 217 bp upstream of the TSP.

Table 1 Data of GUS expression from *GST-27* promoter deletion constructs transiently transformed into BMS cells using the silicon carbide fibre technique. 40 ppm R-25788 were added to induced cells. Cells expressing GUS were stained blue with histochemical assay buffer and are referred to as colour forming units (cfu). This data set was generated by Anne-Isobelle Michou, at Zeneca Agrochemicals, UK.

Construct	Average no. cfu (n = 3) non-induced	induced	Induction (n-fold)
pPUG1	38	184	4.84
pPUG3	10	59	5.90
pPUG4	14	59	4.84
pPUG5	4	59	4.21
pPUG6	18	61	3.38
pPUG7	8	8	0

3.1.4 Detailed analysis of the *GST-27* promoter by *in vivo* footprinting

To further define the position of the *cis* element which confers safener-responsiveness it was decided to use the *in vivo* footprinting technique as described by Hammond-Kosack and Bevan (1993). Deletion promoter analysis could have been continued, but *in vivo* footprinting has many advantages over transient transformation or *in vitro* techniques. The method allows the detection of protein-DNA interactions in the context of intact, transcriptionally active chromatin. Therefore, it is more accurate than *in vitro* studies, as factors such as DNA accessibility in chromatin, the methylation status of cytosine residues, protein factor modification, and the presence of competing factors or co-regulators, which all determine protein binding, remain in their natural positions and so if a promoter is footprinted it is likely to be a true representation of a naturally occurring process. In additon, as it is a direct approach it can indicate the position of a *cis* element more efficiently than transient assays.

Dimethyl sulphate (DMS) was used as the agent to chemically modify DNA which was unprotected by interacting proteins. This agent methylates guanosine (G) residues at the N-7 position and to a lesser extent adenine (A) residues at the N-3 position. Although this limits the

visualisation of protected DNA to G, and possibly A, residues, it is useful as it has the ability to penetrate intact plant tissues. In this respect it has an advantage over DNase I, which can cut any of the four DNA residues and so more accurately define DNA-protein interactions, but is too large an enzyme to penetrate plant tissue. After the tissue is treated with DMS, chromosomal DNA is prepared and modified residues are cleaved with piperidine. It is important to use a concentration of DMS that is sufficient to methylate statistically only one G residue per DNA molecule. If too high a concentration is used many G residues would be methylated which would result in the production of short DNA fragments when the modified residues are cleaved by piperidine, and this would prevent the analysis of the DNA which lay outside the range of the short fragments. A DMS concentration of 0.1 % was found to be sufficient for our purposes. Ligation mediated polymerase chain reaction (LMPCR) was then used in order to amplify the fragments so that they might be visualised on a sequencing gel. The maximum number of bp which can be visualised using this technique is approximately 400. Therefore, it was decided to footprint the promoter DNA which was present in the pPUG6 and pPUG7 constructs, a region spanning 348 bp.

3.2 RESULTS

Histochemical staining of safener induced RMS3s plants was performed and the results are shown in figure 2. The reporter gene, GUS, is not expressed in untreated plants, and so it can be assumed that the promoter is not normally active in this system. Twenty-four hours after painting both sides of the leaves with dichlormid safener (R-29148), at a concentration of 10 g/l, low levels of GUS expression are observed (visualised as blue staining). By 48 hours expression has increased and more blue staining is seen which indicates that the promoter is still active 24 hours after safener treatment. The GUS expression appears to be uneven in the leaf as the blue staining is not uniform. This is thought to be due to the problem of getting the histochemical assay buffer into the leaf cells, even though vacuum infiltration was used to help this process, and it probably does not reflect the expression pattern of the gene itself. Researchers using GUS as a reporter gene in transgenic tobacco found that a more even GUS staining was obtained if the cuticle of the leaves was removed by dipping the plant into chloroform (M. Slater, pers. comm.) which supports the hypothesis that uneven staining is due to the problems of getting the assay buffer into the cells. The histochemical data shown in figure 2 is in agreement with the fluorogenic data obtained previously from these plants, as described in section 3.1.2. The flourogenic data indicates that the levels of transcription achieved by the deleted 900 bp promoter are equivalent to those achieved by the full length (3.8 kb) promoter. Both data sets indicate that the 900 bp of promoter upstream of the TSP are sufficient to confer safener-responsiveness to a reporter gene. Therefore, it can be assumed that a *cis* element, or elements, which confer safener-responsiveness to the promoter, are situated within this region of DNA.

Northern blotting experiments were performed to assess the timing of the production of GST-27 mRNA after the application of dichlormid safener (R-29148), at a concentration of 10 g/l, to both sides of the plant leaves. An autoradiograph of the northern blot is shown in figure 3. The nine lanes contain RNA isolated from leaves harvested at 0, 4, 6, 8, 12, 20, 24, 32 and 48 hours after safener treatment respectively. At 0 hours no transcript is visible. Four hours after safener treatment the gene is being expressed. Expression appears to peak at approximately eight hours and transcript is still visible at 48 hours. The significance of this result is discussed in section 3.3.

Ligation mediated PCR (LMPCR), as described in section 2.4, was performed, using the primers indicated in figure 4, on maize chromosomal DNA, which had been treated with DMS *in vivo*. This region of DNA was amplified because the silicon carbide transient assays performed by Anne-Isobelle Michou at Zeneca Agrochemicals indicated that this region of DNA was able to confer safener inducibility to a reporter gene. Nested primers were used to increase specificity in the PCR reactions, and were designed such that the Tm of the third primer used was higher than the second primer, which was higher than the first primer. In order to determine the specificity of the primers for the *GST-27* gene, the primers were used to amplify maize genomic DNA in a standard PCR reaction; figure 5 shows the products of the PCR reactions after electrophoresis on an agarose gel. (a) shows the PCR products when an annealing temperature of 55 °C was used and (b) shows the products when an annealing temperature used was 60 °C. Lane 1 is the negative control in which water was used as a template. Lanes 2 to 5 contain chromosomal DNA amplified by the primer sets FOR1 with REV1, FOR1 with REV2, FOR2 with REV1 and FOR2 with REV2, respectively, of which the product sizes expected were 464, 433, 451 and 446 bp respectively. Lane 6 is the positive control in which pPUG1 plasmid DNA was used as the template, amplified by the primer set FOR2 with REV2. When an annealing temperature of 55°C was used in the PCR non-specific products are seen with each primer set, except for the primer set FOR1 with REV1 which gives one band of the predicted size. Therefore, an annealing temperature of 55 °C was chosen to anneal the primers FOR1 and REV1 to the template DNA in the LMPCR experiment. By using an annealing temperature of 60 °C in the PCR it can be seen in figure 5 (b) that all the primer sets produce one band which appears to be of the correct size, and so this was the temperature used in the amplification step of the LMPCR procedure, utilising primers FOR2 and REV2 individually with the linker primer Ib in order to amplify the sense and antisense strands of the gene.

When *in vivo* footprinting was performed on the *GST-27* promoter only the bottom, antisense strand of DNA revealed any protected G residues. In figure 6 (a) an autoradiograph of *in vivo* footprinted DNA from the sense strand of DNA is shown, in which the DNA was amplified

using the REV set of primers. The lanes represent DNA which was treated with DMS *in vivo* at 0, 24 and 48 hours after the leaves had been painted with dichlormid safener. Figure 6 (b) is an autoradiograph of *in vivo* footprinted DNA from the antisense strand of DNA, amplified using the FOR set of primers. The lanes represent DNA which was treated with DMS *in vivo* at 0, 6, 24 and 48 hours after the leaves had been painted with dichlormid safener. These time points were chosen to analyse the occupation of the promoter by transcription factors when the gene was inactive (0 hours), prior to maximum expression of the gene (6 hours), and at lower levels of gene expression (24 and 48 hours). It was expected that at 6 hours, all safener-inducible elements would be occupied by their cognate *trans* acting factors. In figure 6 (a) and (b) the G residues are numbered relative to the TSP. Protected G residues were only observed on the anti-sense strand of DNA. Single open circles indicate the G residues which are protected at 24 hours after the plant had been treated with safener. These residues are not protected at any other time point. Two open circles indicate the positions of two residues which are protected at 48 hours after the plant has been treated with safener. These residues are not protected at any other time point. The DNA sequence spanning the promoter region shown in (b) is indicated. Figure 7 is a densitometric scan of part of the autoradiograph shown in figure 6 (b), scanning G residues from -267 to -290. From this it can be seen that residues -267 and -268 maintain a relatively even density across all four time points. However, residues -275, -283, -284 and -290 show decreased densities at 24 hours, even when loading discrepancies between samples are taken into account. The results in figure 6 and 7 indicate that, at 24 hours after safener treatment, the G residues between -275 and -290 inclusive, on the anti-sense strand of DNA, are protected from DMS methylation. The protecting factor does not have an effect at 0, 6 or 48 hours and so the transient footprint may be associated with *GST-27* transcription, induced by safener. This footprint was reproducible. Figure 6 also indicates that the G residues at -326 and -325 on the anti-sense strand of DNA were protected, 48 hours after safener treatment. This result, although very clear in figure 6, could not be reproduced. The timing of the observed footprint and its correltation with the mRNA timecourse observed in the northern blot experiment is discussed in section 3.3.

3.3 DISCUSSION

Using a silicon carbide fibre transient assay system in BMS cells, Anne-Isobelle Michou at Zeneca defined the position of a *cis* element, or elements, which contribute to the safener-inducibility of a reporter gene, to between 217 and 378 nucleotides upstream of the TSP. This region of DNA was subjected to *in vivo* footprinting analysis. Using this method, it was found that some G residues present in the anti-sense strand of DNA prepared from plants which had been treated with safener appeared fainter when compared to the same bands derived from DNA prepared from untreated plants. Therefore, these G residues were protected, *in vivo*, from methylation by DMS. The *in vivo* footprint (figure 6) reveals two protected regions in the

promoter which may indicate that a protein, or several proteins, interact with at least two regions of the promoter, in a safener-dependent manner. These regions span G residues G-275 to G-290 inclusive and G-325 to G-326. The footprint encompassing residues G-275 to G-290 was a reproducible result, whilst the footprint encompassing G-325 and G-326 was only observed once. It is not clear why the G-325/6 footprint could not be reproduced. However, as a footprint can only be visualised if the majority of the cells are actively expressing the gene in question, it could be that external factors which may play a role in gene expression were active when this experiment was being conducted, which were not active when the experiment was repeated. These could include such factors as the ambient temperature in the greenhouse or the plants' condition *i.e.* they may have been the subject of pathogen attack at the time of the experiment. Such factors may interact synergistically with the effect of safener treatment as, for example, it has been noted that plants treated with safener appear to be deleteriously affected if they are treated during periods of hot weather, when necrotic patches may be observed on the leaf edges.

No footprinted G residues were identified on the sense strand of DNA which was subjected to *in vivo* analysis (see figure 6 a). Footprints may have been expected as some G residues present on the sense strand of DNA were close neighbours of footprinted G residues on the anti-sense strand. This is particularly true of G-282 on the sense strand which is directly adjacent to the footprinted G-283/4 residues on the antisense stand of DNA. However, the sequence obtained for the sense strand of DNA was normally only clearly visualised up to G-225 and so a footprint may have been present at G-282 which was not detected. The fact that no footprint was seen on the DNA which gave readable sequence does not necessarily mean that a protein was not interacting with the DNA. A footprint would not be seen if the protein did not closely interact with G residues, which is a limitation of this technique. In addition, the majority of cells in the tissue tested must be actively expressing the protein of interest or else a footprint will be masked by those G residues which are accessible to the DMS.

In figure 7 it can be seen that at 24 hours the intensity of bands which correspond to the G residues G-275, G-283/4 and G-290 were reduced compared to their intensity at 0, 6 or 24 hours. A flanking G residue, at position -267, maintained an even band intensity at all time points. As some bands maintain an even intensity, any alteration in the intensity of other bands can be assumed to their being protected, *in vivo*, from DMS treatment. The protection may be due to the presence of a *trans* acting factor which is interacting with the DNA, and so these regions of DNA can be thought of as putative safener-responsive *cis* elements. The DNA which was analysed by *in vivo* footprinting was present in the constructs pPUG6 and pPUG7. pPUG6 retains safener responsiveness when tested in transient assays, while the shorter promoter in pPUG7 loses the ability to respond to safener. All footprinted residues were observed in the DNA which was present in the responsive promoter of pPUG6, but which was

deleted in the non-responsive promoter of pPUG7. Therefore, the transient assay data is in agreement with the footprint data.

The footprinted residues can be regarded as four separate *cis* elements. By comparing the nine bp encompassing the footprinted G residues it was observed that these elements share some similarity. In figure 8 it can be seen that four out of the nine residues are identical in each element, as indicated by a capital letter in the consensus sequence. Another four residues are identical in three out of the four elements, as indicated by an asterisk in the consensus sequence. One element, around residue G-284, is shown in an opposite orientation to the other elements as in this orientation it exhibits greater homology. The similarity indicates that the footprinted regions may relate to four variant copies of the same element.

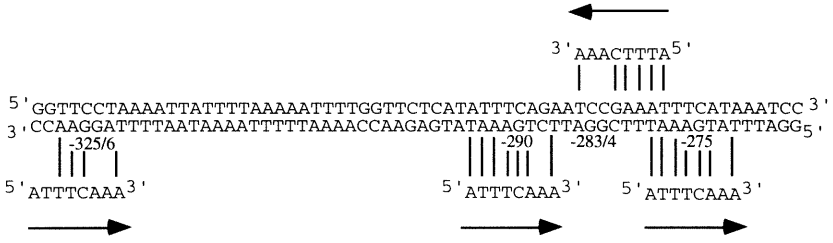
Figure 8 Similarities in the sequences surrounding the four footprinted G residues can be seen when sequences are compared 5' to 3', with G-284 in the 3' to 5' orientation. The footprinted residues are underlined. The putative ethylene responsive elements of the *GST1* gene in carnation (Itzhaki *et al.*, 1996) and E4 gene of tomato (Montgomery *et al.*, 1993) are also indicated (3' to 5').

G residue	8 bp encompassing the central footprinted G
consensus	* * A A G * T *
-326	5'C C A A G G A T T T3'
-290	5'T A A A G T A T T A3'
-284/285	3'T A A A G C C T A A5'
-275	5'T A A A G T A T T T3'
ERE	3'T A A A G T T T5'
E4	3'T T A A G T T T5'

In the introduction section 1.3.3. the controlling regions of the ethylene responsive *GST1* gene of carnation were described. Itzhaki *et al.* (1994) incubated promoter DNA with nuclear extracts isolated from carnation petals in their ethylene climacteric. Twenty-two nucleotides of DNA were protected from digestion by DNase I after the incubation period. This result indicates that this region is a *cis* element involved in ethylene responsiveness. Of these 22 nucleotides, eight (5'ATTTCAAA3') were found to share homology with part of a putative ethylene responsive element identified in the tomato E4 gene (5'AATTCAAA3') (Montgomery *et al.*, 1993). Comparing these sequences to those shown in figure 8, it can be seen that they show some

complementarity to the elements identified in this experiment. Figure 9, in which footprinted G residues are numbered, illustrates how eight bp of the carnation *GST1* element are complementary to the putative elements identified in the *GST-27* gene. The bases around footprinted residues G-325/6, G-290 and G-275 could be regarded as direct orientation copies of an element which is similar to the ERE while the bases around footprinted residues -284/5 could be regarded as an element which is similar to the ERE but which lies in an inverted orientation. It is relevant to note that the ERE has been shown to function in minimal promoters in an orientation independent manner (Itzhaki *et al.* 1994). Therefore, one may assume that these elements, which are similar to the ERE, could also direct transcription regardless of their orientation in the promoter.

Figure 9 The footprinted regions of the *GST-27* promoter are complementary to, or similar to, an eight base pair region of an ethylene responsive *cis* element (ERE) from the *GST1* gene of carnation (Itzhaki *et al.,* 1994). The footprinted region of the *GST-27* promoter DNA is shown and G residues which were footprinted are numbered. The sequence of the ERE is given above and below the *GST-27* DNA; lines indicate bases which are complementary to the ERE. The arrows indicate the orientation of the elements relative to the TSP.



Therefore, it could be argued that the elements identified in this experiment are equivalent to the ERE. However, the *GST-27* gene is not induced when maize plants are exposed to ethylene (Jepson *et al.*, 1994). Nevertheless, it may be that identical *cis* elements and their *trans*-acting factors are associated with different signal cascades in different species. Thus, the elements identified in the *GST-27* promoter, possibly involved in safener-responsiveness, could be functionally equivalent to the ERE of the *GST1* in carnation, which is involved in ethylene-

responsiveness. The fact that they may be functionally equivalent does not mean that they respond to the same signals.

The northern in figure 3 indicates that GST-27 is not expressed without the addition of safener. At the first time point tested after safener treatment (4 hours) the gene is expressed at relatively high levels. Expression peaks at 8 hours, after which expression decreases but is maintained until the last time point tested at 48 hours. The footprint data may lead one to expect the gene to show maximum expression at 24 hours. However, as was mentioned earlier, for a footprint to be visible, the majority of cells in the tissue tested need to be actively expressing protein and therefore have the *trans* acting factor bound to the *cis* element in the promoter. It may be that, at 6 hours, when gene expression is seen but a footprint is not visible, a small percentage of the cells are expressing the gene, but at high levels. The safener may not have penetrated the tissue to reach all cells and so a footprint would be masked. By 24 hours, the majority of cells may be expressing the gene at a lower level and so the footprint is observed. Gene expression is still observed at 48 hours when the main footprint has been lost. An explanation for the RNA levels observed after the promoter is assumed to be inactive, is that the mRNA is stable and has a long half life. Therefore, even if the promoter is inactive after 24 hours the mRNA would still be present. This seems plausible as the mRNA levels do not increase after the eight hour time point. In addition, in a similar time-course carried out by Ian Jepson low levels of mRNA were still visible eight days after safener treatment (pers. comm.). This indicates that the mRNA is relatively stable and would be expected to be present at 48 hours, even if the promoter was no longer actively driving transcription. Alternatively, the clear footprint observed at residues G-235/6 at 48 hours, may indicate the position of another *cis* element which is involved in prolonging the promoter's activity. If time allowed it would have been interesting to carry out a more detailed time-course between 6 and 24 hours for the *in vivo* footprint experiment, in order to define more accurately when the footprint appears and when it disappears.

In vivo DMS footprinting has been performed on the promoters of two maize alcohol dehydrogenase genes (*Adh-1* and *Adh-2*) (Garrity and Wold, 1992). These genes are induced by hypoxia stress (low oxygen levels). Two regions of constitutive binding activity and two regions of inducible binding activity were observed in the *Adh-1* promoter. It is interesting to note that the inducible footprints were found in regions of the promoter which are not essential for response to hypoxic stress, but which, when mutated, result in decreased transcription levels of the gene (Garrity and Wold, 1992). With these results in mind, one cannot assume that the elements identified in the promoter of *GST-27* are necessary and sufficient for safener-responsiveness.

Having identified putative *cis* elements it was decided to determine if these short regions of DNA could associate with nuclear protein factors *in vitro*. Electrophoretic mobility shift assays

(EMSA) were utilised to analyse the protein binding capabilities of ten probes. Eight corresponded to wild type and mutant versions of the four footprinted areas of the *GST-27* promoter. Two were designed to mimic wild type and mutant versions of the ethylene-responsive element from the *GST1* gene in carnation. These experiments are described in detail in Chapter 4.

Figure 1

A schematic diagram of the series of the *GST-27* 5' deleted promoter constructs engineered at Zeneca.

The deleted promoters were fused to the β-glucoronidase (GUS) reporter gene. These constructs were used in silicon carbide mediated transient transformation assays of Black Mexican Sweet (BMS) maize cells. GUS expression was analysed in the presence or absence of safener. The constructs were induced by safener as indicated. pPUG7, however, was not inducible.

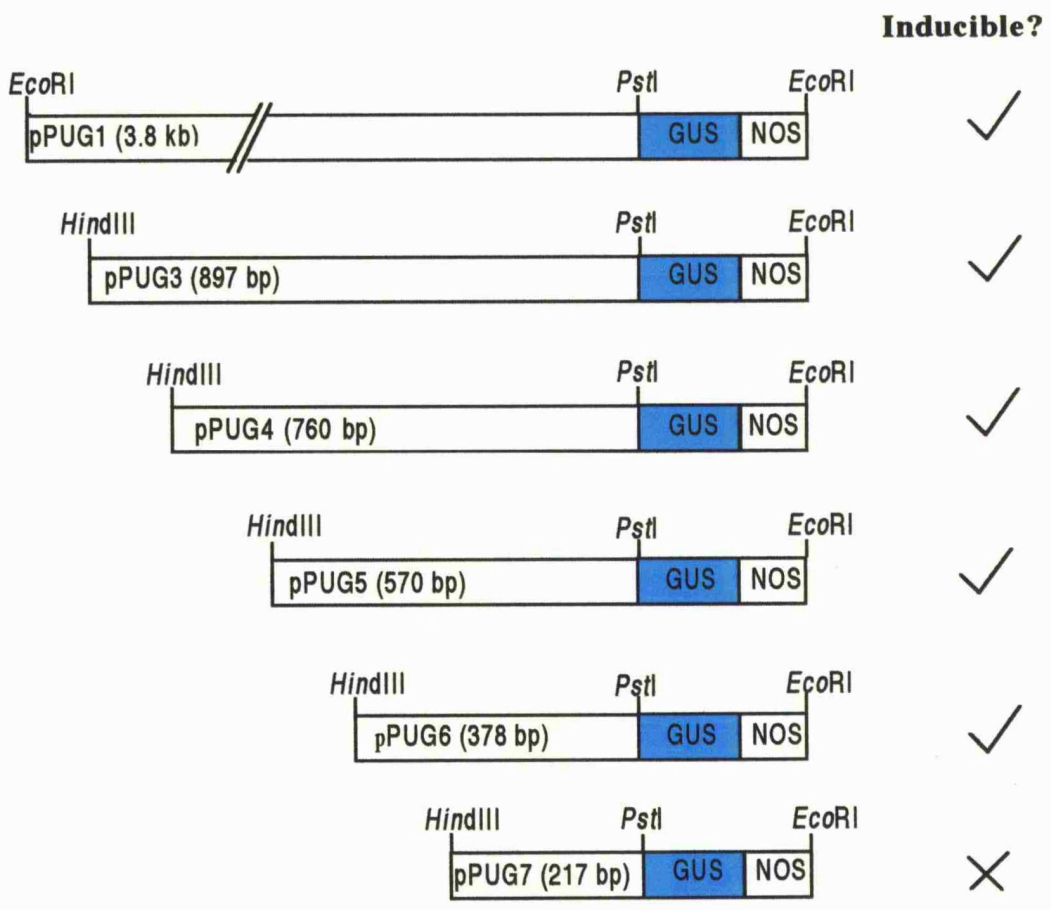

Inducible?
EcoRI
PstI
EcoRI
pPUG1 (3.8 kb)
GUS
NOS
HindIII
PstI
EcoRI
pPUG3 (897 bp)
GUS
NOS
HindIII
PstI
EcoRI
pPUG4 (760 bp)
GUS
NOS
HindIII
PstI
EcoRI
pPUG5 (570 bp)
GUS
NOS
HindIII
PstI
EcoRI
pPUG6 (378 bp)
GUS
NOS
HindIII
PstI
EcoRI
pPUG7 (217 bp)
GUS
NOS

Figure 2

A photograph of histochemically stained leaves from RMS3s maize at 0, 24 and 48 hours after the addition of safener.

The RMS3s maize plants, generated by Zeneca, contain 0.9 kb of the promoter upstream of the TSP, fused to the reporter gene GUS. No GUS activity is seen in untreated leaves. Twenty-four hours after a 10 g/l concentration of the safener formulation R-29148 was painted onto both sides of the leaves, GUS expression is observed. After 48 hours a higher level of GUS staining is obtained.

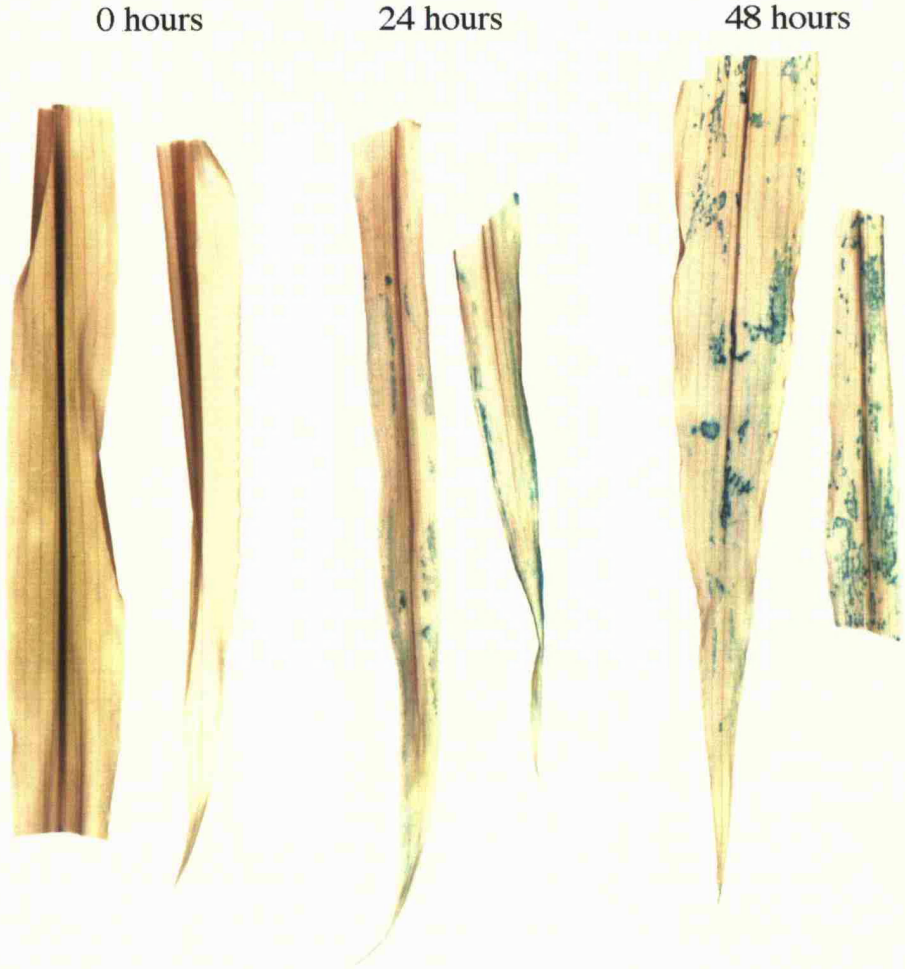

0 hours
24 hours
48 hours

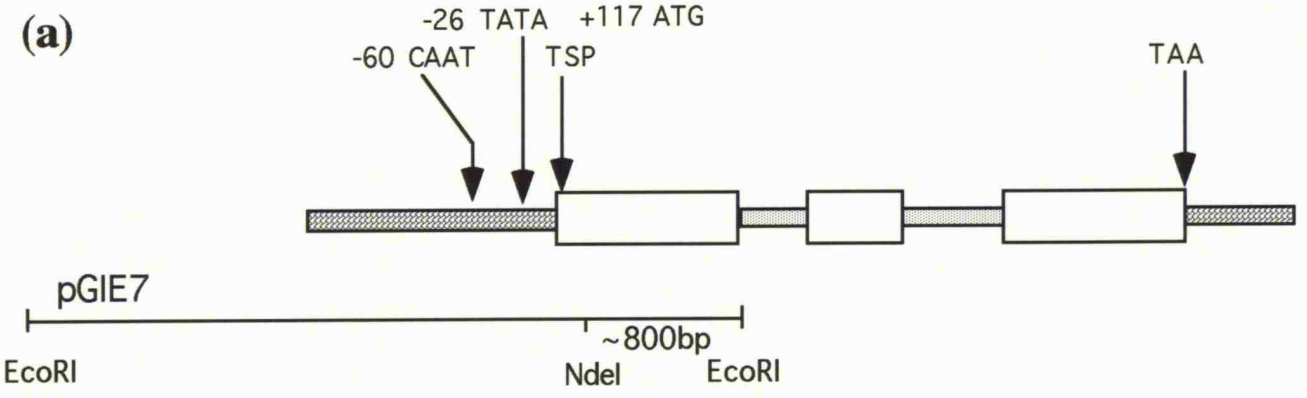
Figure 3

A schematic diagram of the *GST-27* gene.

(a) A schematic diagram representing the structure of the *GST-27* gene. It has three introns and two exons. The probe which was used for the northern blot analysis was cut from a cDNA clone of the gene, pIJ21, and contained 800 bp of DNA from the first exon.

Northern blot analysis of RNA isolated from leaves harvested at various time points after the application of safener.

(b) Both sides of maize leaves were painted with a 10 g/l concentration of the safener formulation R-29148. Total RNA was isolated at the time points indicated (in hours) and 10 μg of each sample were subjected to electrophoresis on an agarose gel. The RNA from the gel was blotted to a nylon membrane and hybridised with the probe (100 ng of probe labelled using 1 μl Klenow fragment and 5 μl α-^{32}P dCTP) to detect the presence of GST-27 mRNA.


(a)
-26 TATA
+117 ATG
-60 CAAT
TSP
TAA
pGIE7
~800bp
EcoRI
NdeI
EcoRI

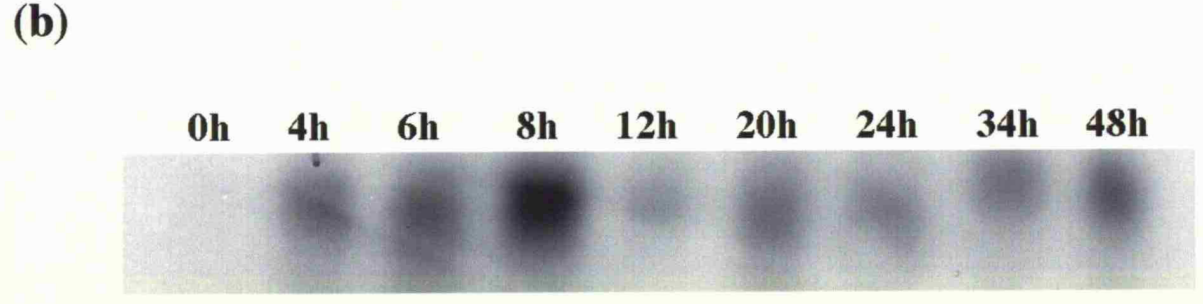

(b)
0h
4h
6h
8h
12h
20h
24h
34h
48h

Figure 4

A schematic diagram of the *GST-27* promoter and the primers used for the LMPCR technique.

A schematic diagram of 533 bp (5' to 3') of the *GST-27* promoter upstream from the TSP, indicating the position of the primers used for LMPCR. FOR primers were used to amplify the antisense strand of DNA; REV primers were used to amplify the sense strand of DNA. FOR1 and REV1 were annealed to the template DNA, using an anealing temperature of 55 °C, and first-strand extension was performed. After the linker had been ligated to the end of each DNA molecule, amplification of the sense strand was achieved using the primers FOR2 with linker specific primer Ib, and primers REV2 with Ib were used in order to amplify the antisense strand, using an annealing temperature of 60 °C. The amplified fragments were labelled by nine rounds of extension with the radiolabelled primers REV3 or FOR3, using an annealing temperature of 65°C. The promoter DNA present in constructs pPUG6 and pPUG7 is also indicated. The putative TATA box is shown in bold type as are the nucleotides ATG (translation start point).

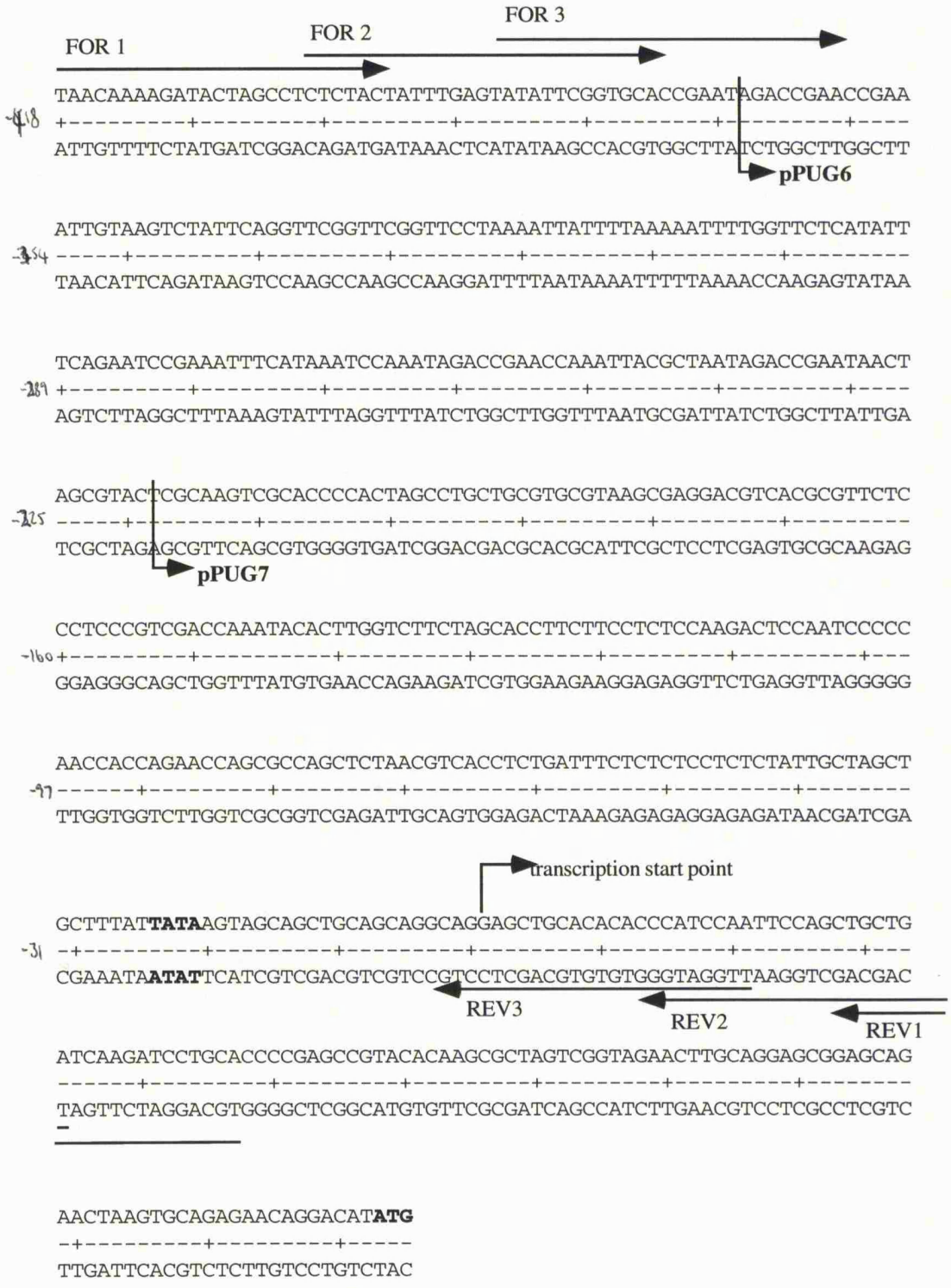

FOR 1
FOR 2
FOR 3
TAACAAAAGATACTAGCCTCTCTACTATTTGAGTATATTCGGTGCACCGAATAGACCGAACCGAA
ATTGTTTTCTATGATCGGACAGATGATAAACTCATATAAGCCACGTGGCTTATCTGGCTTGGCTT
pPUG6
ATTGTAAGTCTATTCAGGTTCGGTTCGGTTCCTAAAATTATTTTAAAAATTTTGGTTCTCATATT
TAACATTCAGATAAGTCCAAGCCAAGCCAAGGATTTTAATAAAATTTTTAAAACCAAGAGTATAA
TCAGAATCCGAAATTTCATAAATCCAAATAGACCGAACCAAATTACGCTAATAGACCGAATAACT
AGTCTTAGGCTTTAAAGTATTTAGGTTTATCTGGCTTGGTTTAATGCGATTATCTGGCTTATTGA
AGCGTACTCGCAAGTCGCACCCCACTAGCCTGCTGCGTGCGTAAGCGAGGACGTCACGCGTTCTC
TCGCTAGAGCGTTCAGCGTGGGGTGATCGGACGACGCACGCATTCGCTCCTCGAGTGCGCAAGAG
pPUG7
CCTCCCGTCGACCAAATACACTTGGTCTTCTAGCACCTTCTTCCTCTCCAAGACTCCAATCCCCC
GGAGGGCAGCTGGTTTATGTGAACCAGAAGATCGTGGAAGAAGGAGAGGTTCTGAGGTTAGGGGG
AACCACCAGAACCAGCGCCAGCTCTAACGTCACCTCTGATTTCTCTCTCCTCTCTATTGCTAGCT
TTGGTGGTCTTGGTCGCGGTCGAGATTGCAGTGGAGACTAAAGAGAGAGGAGAGATAACGATCGA
transcription start point
GCTTTATTATAAGTAGCAGCTGCAGCAGGCAGGAGCTGCACACACCCATCCAATTCCAGCTGCTG
CGAAATAATATTCATCGTCGACGTCGTCCGTCCTCGACGTGTGTGGGTAGGTTAAGGTCGACGAC
REV3
REV2
REV1
ATCAAGATCCTGCACCCCGAGCCGTACACAAGCGCTAGTCGGTAGAACTTGCAGGAGCGGAGCAG
TAGTTCTAGGACGTGGGGCTCGGCATGTGTTCGCGATCAGCCATCTTGAACGTCCTCGCCTCGTC
AACTAAGTGCAGAGAACAGGACATATG
TTGATTCACGTCTCTTGTCCTGTCTAC

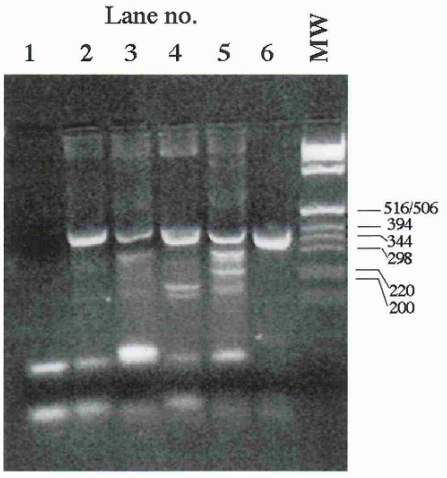
Figure 5

Photographs of agarose electrophoresis gels containing the products of standard PCRs performed with the LMPCR primers on the *GST-27* promoter.

(a) A photograph of an agarose electrophoresis gel of PCR products formed when 100 ng maize chromosomal DNA was amplified with LMPCR primers (1 µl of a 20 µM stock) using an annealing temperature of 55 °C. Lanes two to five, respectively, contain chromosomal DNA amplified by the primer sets FOR1 with REV1, FOR1 with REV2, FOR2 with REV1 and FOR2 with REV2. Lane one contains a negative control in which water was used as a template; lane six contains a positive control in which 20 ng pPUG1 plasmid DNA was used as a template. The controls were amplified with the primer set FOR2 with REV2. The size, in bp, of the molecular weight markers (MW) are indicated.

(b) As (a) but in the PCR reaction an annealing temperature of 60 °C was used.

(a)


Lane no.
1
2
3
4
5
6
MW
516/506
394
344
298
220
200

(b)

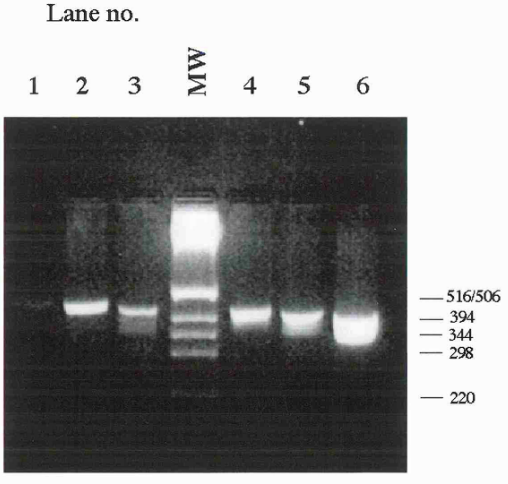

Lane no.
1
2
3
MW
4
5
6
516/506
394
344
298
220

Figure 6

Sections of autoradiographs of the *in vivo* footprinted *GST-27* promoter

(a) The three lanes contain chromosomal DNA which was treated, *in vivo,* with 0.1% DMS, 0, 24 and 48 hours after the plant had been treated with safener (as indicated). Two microgrammes of the DNA was amplified and radiolabelled with the REV set of primers in order to visualise the sense strand of DNA. The G residues are numbered in respect to their position upstream from the TSP.

(b) The four lanes contain chromosomal DNA which was treated, *in vivo,* with 0.1% DMS, 0, 6, 24 and 48 hours after the plant had been treated with safener (as indicated). Two microgrammes of the DNA was amplified and radiolabelled with the FOR set of primers in order to visualise the anti-sense strand of DNA. The G residues are numbered in respect to their position upstream from the TSP. Single open circles indicate the position of G residues which appear to be protected at 24 hours. Two open circles indicate the positions of G residues which appear to be protected at 48 hours. The sequence of DNA spanning this region of the promoter is indicated and the footprinted residues are labelled.

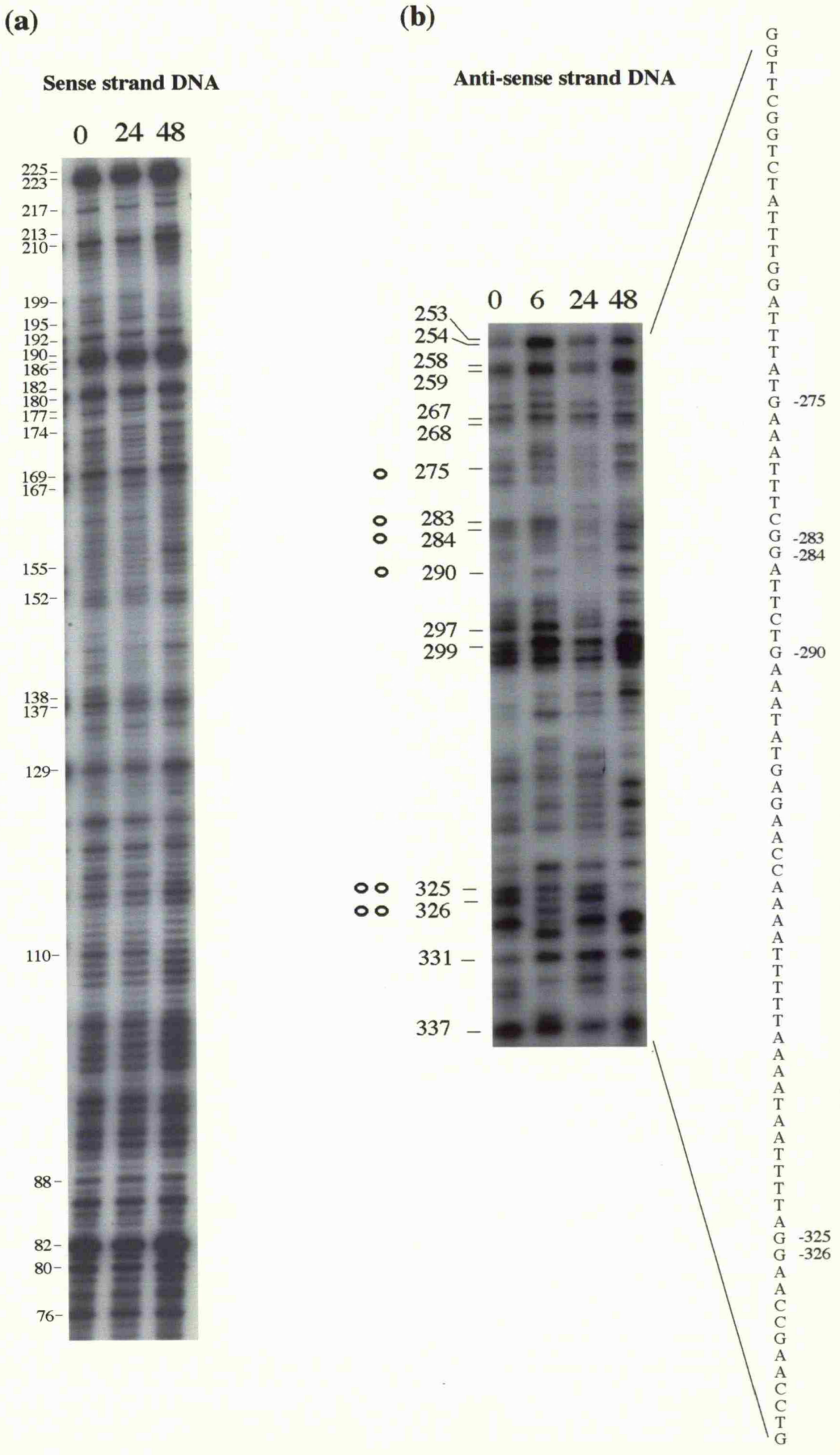
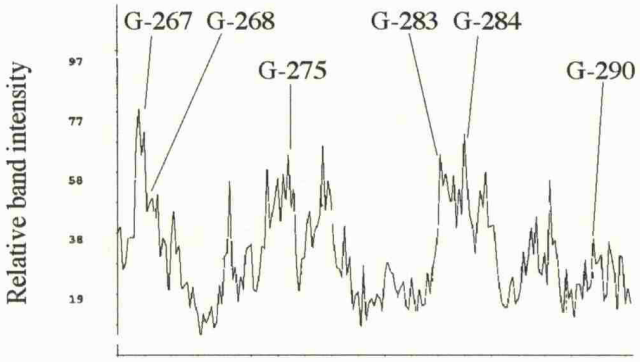

(a)
Sense strand DNA
0 24 48
225
223
217
213
210
199
195
192
190
186
182
180
177
174
169
167
155
152
138
137
129
110
88
82
80
76
(b)
Anti-sense strand DNA
0 6 24 48
253
254
258
259
267
268
275
283
284
290
297
299
325
326
331
337
GGTTCGGTCTATTTGGATTTATG -275
AAATTTTCGG -283 -284
ATTCTGAAATATGAGAACCAAAATTTTTAAAATAATTTTAGG -290 -325 -326
AACCGAACCTG

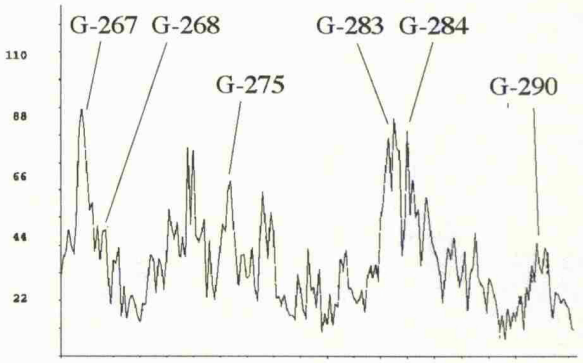
Figure 7

Densitometric scan of a section of the autoradiograph in figure 6 (b).

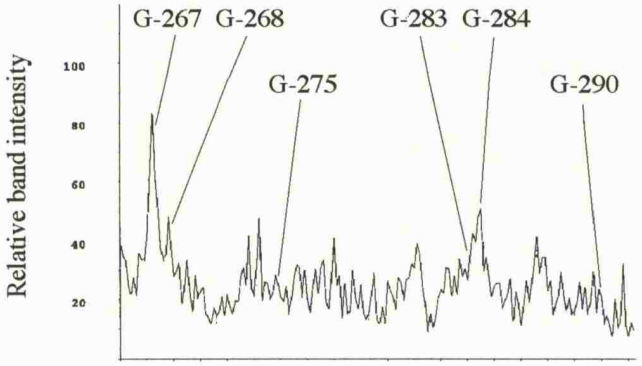
The relative intensities of the bands from the autoradiograph in figure 6 (b), between G residue -267 to -290, were quantified. The data for 0, 6, 24 and 48 hours corresponds to the four lanes indicated in figure 6 (b). Peaks which correspond to G residues are labelled. The peaks are visible, and relatively constant in their intensities, at time points 0, 6 and 24 hours. At 24 hours, however, the peaks are not well defined and the relative intensities of the bands have decreased by an approximate factor of two.



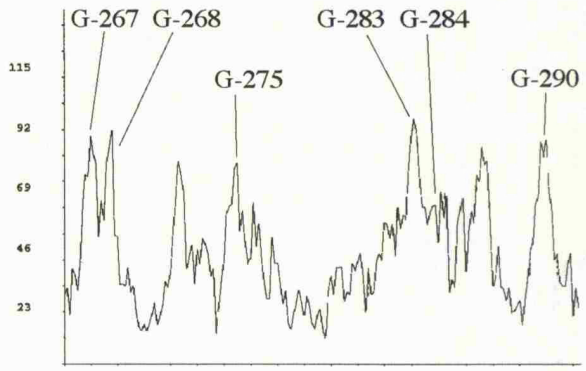
0 Hours



6 Hours



24 Hours



48 Hours

Chapter Four

IN VITRO ANALYSIS OF THE PUTATIVE SAFENER-RESPONSIVE *CIS* ELEMENTS

4.1 INTRODUCTION

As described in Chapter 3, four putative *cis* elements, implicated in safener-inducibility, were identified in the *GST-27* promoter using the *in vivo* footprinting technique. *Cis* elements can bind *trans*-acting protein factors *in vivo*, therefore, to test whether the elements identified in the *GST-27* promoter could interact with nuclear protein factors *in vitro*, electrophoretic mobility shift assays (EMSAs) were performed. This technique utilises the fact that a DNA probe which is bound by protein has a different mobility in a non-denaturing polyacrylamide gel compared to its free, unbound form. The protein can be in pure form or a component of a nuclear protein extract. In the latter situation, the extract may contain many non-specific binding proteins which can interfere with the required specific binding-activity, therefore an excess of non-specific competitor DNA has to be added in order to titrate out the non-specific binding proteins. In the experiments described in this chapter synthetic poly(dIdC) was used for this purpose. If a probe has been shown to associate with nuclear protein *in vitro*, mutant forms of the probe can be used to identify which residues are specifically required in the DNA-protein interaction. In addition, specificity of binding can be assessed by competition assays. If binding is highly specific the addition of an unrelated probe to the binding reaction mix would have no observable effect on the DNA-protein complex; only the addition of the wild type probe would be able to compete for the binding of a protein with the radiolabelled wild type probe. Although the EMSA technique can be used to show that a sequence of DNA has the ability to interact with nuclear protein, it does not necessarily mean that this interaction can or does occur *in vivo* (Rigaud *et al.*, 1991). However, when *in vivo* footprinting has indicated that DNA-protein interactions do occur in a cell, it is a useful technique to show that *trans*-acting factors exist which bind specifically to the footprinted regions of DNA.

4.2 RESULTS

Ten double-stranded DNA probes were designed for these experiments, eight based on the four putative elements identified by *in vivo* footprinting of the *GST-27* promoter (see Chapter 3) and two based on the ethylene responsive element (ERE) identified in the promoter of the *GST1* gene of carnation (Itzhaki *et al.*, 1994) which is similar to the putative elements (see Chapter 3). For each element a wild type and mutant probe was prepared. The wild type probe incorporated the footprinted G residue and the 11 nucleotides flanking that residue in the promoter. Eleven nucleotides were used in order to include all the bases which showed homology between the putative elements (see Chapter 3, figure 3.8). One G and a C residue were added to each end of the probe to increase its thermal stability and *Hin*dIII restriction sites were placed at both ends. One mutant probe was prepared to correspond to each wild type probe, which was identical to the wild type except that the five nucleotides including and flanking the footprinted G were mutated. If two adjacent G residues were footprinted, a total of six nucleotides including the

footprinted G residues were mutated. The number of nucleotides which were mutated was decided by the fact that in many other EMSA studies a mutation of five nucleotides in the wild type probe was shown to be sufficient to abolish the binding of a protein to the probe (Michael Hammond-Kosack, pers. comm.). In order to create effective mutant probes and a higher probability of abolishing protein binding, pyrimidines were changed to purines and purines were changed to pyrimidines *i.e.* T was converted to G and A converted to C . Figure 1 indicates the position of the probes in the *GST-27* promoter and depicts their sequence.

Initial studies were concerned with the optimisation of conditions in order that binding activities could be observed. Concentrations of $MgCl_2$, KCl and DTT in the binding reactions were varied in order that maximum binding could occur. The most abundant retarded bands were observed when 5 mM DTT, 50 mM KCl and 100 mM $MgCl_2$ were present. In addition, the use of a 6 % polyacrylamide gel with a 0.25 X TBE running buffer was found to aid the visualisation of binding activities. This may be because the low ionic strength of the running buffer helps to increase the binding affinity of the protein to the DNA, and the relatively high acrylamide concentration in the gel means that the gel matrix may have a caging effect. Therefore, if the DNA probe and protein disassociate they will remain in close proximity with each other and may re-associate. When conditions were optimised, 200 pg of each of the wild type and mutant probes were incubated for 20 minutes at room temperature with 1 µg nuclear protein extracts prepared from leaves from untreated maize plants (0 hours) or from safener-treated maize plants (24 hours). These time points were chosen in order to compare the binding activities of an extract prepared from a plant which had not been treated with safener to those from an extract prepared from a plant which had been treated with safener. The safener-treated extract was prepared 24 hours after safener treatment as the reproducible *in vivo* footprint was observed at this time point, and therefore the binding activity would be expected to be present and most abundant at that time. The results of these assays are shown in figure 2. A band, retarded with the same apparent mobility, was observed with all of the wild type probes. As the probes were of equal length they would be expected to have equal mobilities. The fact that the mobility appears to remain constant when protein is bound indicates that each probe may be bound by the same protein or proteins. The retarded band is abolished, or reduced in intensity, with all of the mutant probes, which indicates that the observed binding is sequence specific. The retarded band is not visible unless the probes are incubated with protein. However, even in the absence of protein, some higher mobility retarded bands are observed with probes WT326, WT-ERE and WT275. Repeated purification of these probes did not eliminate these bands so they are not due to uncleaved concatemers of the probe. It may be that they are due to a secondary structure which is adopted by these probes. However, as similar retarded bands are observed when using these probes in the presence of protein as are observed ~~with the probes which do not display these extra bands~~ in the absence of protein, it is assumed that the extra bands are artefacts and do

not interfere with the validity of the results. It is clear from figure 2 that all the wild type probes are capable of binding nuclear protein in a sequence specific manner as binding activity is reduced in the corresponding mutant probes. The activity binding to all the probes was present in nuclear protein extracts prepared from safener-treated (24 hours) or non-treated (0 hours) leaves.

To assess how specific the binding observed in figure 2 was, competition assays were performed. Two hundred picogrammes of radiolabelled WT290 probe were incubated with nuclear protein extracts prepared from untreated (0 hour) and safener-treated (24 hour) maize leaves; this probe concentration had previously been shown to be in excess of that required for maximum binding to occur. WT290, WT284, WT275, WT326 and WT-ERE were used individually in separate reactions as unlabelled competitor at concentrations of 10 X, 50 X and 100 X greater than the labelled WT290 probe. The results of the competition assay using nuclear protein extracts prepared from untreated leaves (0 hours) are shown in figure 3. Identical binding activities were observed in competition assays using nuclear protein extracts prepared from leaves which had been treated with safener 24 hours beforehand. It can be seen that all of the WT probes are effective competitors against the WT290 probe, as the probes' abilities to compete for the factor which is bound to WT290 can be assessed by judging the intensity of the retarded band which is visible in the presence of the competitor. In all cases the band is abolished or reduced to a faint band when a 10 X concentration of competitor probe is added. The bands are not very radioactive as in order to visualise them the gel had to be exposed to film for 14 days; an overnight exposure was sufficient to visualise the EMSAs shown in figure 2. Therefore, as all the wild type probes are effective competitors it can be assumed that the protein which is bound to the radiolabelled probe must have a high affinity for the competitor probes. The effectiveness of competition can be rated as WT290 and WT275 > WT-ERE > WT284 and WT326, where the WT290 and WT275 probes are the most effective competitor probes, as at a 10 X concentration these probes reduce the intensity of the retarded band to the greatest extent. The probes which differ most from the WT290 probes are the least effective competitor probes, which would be expected. Each probe is capable of interacting with the same nuclear protein factor as WT290 as all the probes can compete for binding of the protein which is interacting with this probe. It may be assumed that all the probes are competing for the same factor as each other.

4.3 DISCUSSION

The results in figure 2 indicate that all the wildtype probes have the ability to interact with protein *in vitro*. The binding appears to be sequence specific as it is abolished, or reduced, when the corresponding mutant probes are used. Therefore, the bases which were altered in the mutant probes must be important to the protein-DNA interaction. One band was observed with

each probe which indicates that only one protein, or group of proteins, was present which possessed the ability to bind these probes. The fact that a binding activity present in the extracts could interact with each probe resulting in a retarded band of identical mobility leads one to assume that each of the probes is bound by the same factor. This idea is supported by the results in figure 3, which indicate that each wild type probe can compete for the binding of the protein which is bound to the WT290 probe. Therefore, as similar binding was observed for the probes, all of which share homology, the data supports the idea suggested in Chapter 3 that these short regions of DNA are all variant copies of the same element, and that this element is also present in the ERE in the promoter of the *GST1* gene of carnation.

If it is assumed that the elements in the *GST-27* promoter are necessary for safener-responsiveness, it may be expected that their cognate *trans*-acting factor or factors would only be present in protein nuclear extracts prepared from safener-treated leaves. However, the EMSAs show retarded bands whether protein nuclear extracts are prepared from safener-treated or untreated leaves. The relative intensity of the retarded band observed when extracts were used which had been prepared from safener-treated or untreated leaves was constant, and any minor variations seen in the intensities of the bands is a reflection on the inaccuracies encountered when calculating the protein content of different extract preparations and when pipetting small volumes. Therefore, one could assume that, *in vivo*, the *trans*-acting factor is always present, but that after safener treatment it undergoes a post-translational modification, interacts with other proteins, or is translocated into the nucleus in order for it to gain the ability to bind to DNA. *In vitro* the mechanism used to modify the factor in order that it may bind to DNA may be affected, so that the factor appears to bind DNA in a safener-independent manner. Mitogen-activated protein (MAP) kinases are involved in many signal transduction pathways in plants, including ethylene signalling and auxin-induced cell proliferation (Jonak *et al.,* 1994). Therefore the *in vivo* modification could be one of phosphorylation of the factor; the control mechanism used to modify the factor may be lost *in vitro* because when the nuclear protein extract is prepared a factor which was not phosphorylated *in vivo* may become phosphorylated. To test if phosphorylation is required for the activity of the extract with these probes, the extract could be treated with a phosphatase in order to abolish all the occupied phosphorylation sites; if it is an important mechanism used to control the interaction of these factors with DNA, it would be expected that no retarded bands would be observed when the phosphatase-treated extract was used in EMSA studies. This experiment was not performed due to time constraints. It is interesting to note that a similar result was observed when EMSAs were performed using a longer fragment of the ERE, which is present in the promoter of the *GST1* gene in carnation (Itzhaki *et al.,* 1994). A retarded band was observed when the ERE probe was incubated with nuclear protein extracts prepared from carnation petals, whether the extract was prepared from petals at anthesis (low ethylene levels) or during the ethylene climacteric (high ethylene levels). In addition, other ethylene-inducible genes have been shown to interact with nuclear proteins in

an ethylene-independent manner (Eyal *et al.,* 1993; Montgomery *et al.,* 1993; Raghothama *et al.,* 1991). In all these cases an ethylene-regulated post-translational modification of the factor is proposed. In the light of these published results it is not unexpected that retarded bands were observed with nuclear extracts prepared from safener-treated or untreated leaves. Post-translational modification of a transcription factor which is already present in the nucleus is a rapid way to control the factor's activity compared to *de novo* synthesis. As GST-27 is a sub-unit of the GST II enzyme, which is involved in the rapid detoxification of xenobiotics such as herbicides and herbicide safeners, it is fitting that its transcription may be controlled by post-translational modification of an existing factor because the enzyme needs to react rapidly in response to a xenobiotic challenge in order for a plant to survive. The northern blot studies described in Chapter 3 indicate that as few as four hours after the application of safener the gene is transcribed at relatively high levels, and, although earlier time points have not been tested, the transcript may be present prior to this. Therefore, the northern data appears to agree with the EMSA data which suggests that the *GST-27* gene is activated rapidly after the application of safener because the *trans*-acting factor required for transcription of the gene is controlled by post-translational modification and does not require *de novo* synthesis.

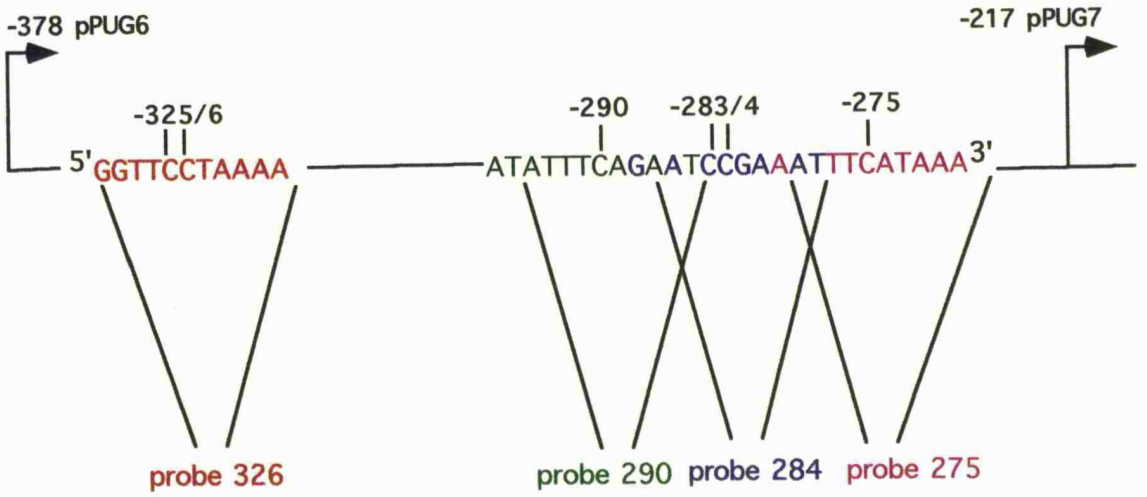
The *in vitro* binding observed in the EMSA studies supports the *in vivo* footprinting data, and it can be assumed that the proteins present in the retarded protein-DNA complexes represent *trans*-acting factors which are involved in safener-dependent transcription. In order to provide direct evidence linking the elements to safener-dependent transcription it was decided to perform transient assays, in the presence or absence of safener, with constructs in which these elements were mutated. These experiments are discussed in detail in chapter 5.

Figure 1

(a) A schematic diagram of the *GST-27* promoter indicating the position of the probes used in the EMSA studies. The region of promoter DNA between 378 base pairs and 217 base pairs upstream of the TSP is shown, which is the DNA present in the pPUG6 construct but not present in the pPUG7 construct (as indicated). The position of the probes relative to the numbered footprinted G residues are indicated.

(b) A schematic diagram of the wild type and mutant probes used in the EMSA studies. Wildtype (WT) and mutant (MUT) probes were prepared for each of the four footprinted regions in the *GST-27* promoter and, dependent on the footprinted G residue they contained, were named accordingly. The asterisks on the mutant probes indicate which nucleotides differ from the wildtype sequence. Wildtype and mutant probes were also prepared which corresponded to the region of the ERE in the promoter of the *GST1* gene from carnation which shows homology to the elements in the *GST-27* promoter (Itzhaki *et al.,* 1994).

(a)



(b)

326 WT

5'agcttGCGGTTCCTAAAAGCa3'
3'aCGCCAAGGATTTTCGttcga5'

284 WT

5'agcttGCGAATCCGAAATGCa3'
3'aCGCTTAGGCTTTACGttcga5'

326 MUT

5'agcttGCGGCGAAGCAAAGCa3'
3'aCGCCGCTTCGTTTCGttcga5'

284 MUT

5'agcttGCGACGAATCAATGCa3'
3'aCGCTGCTTAGTTACGttcga5'

290 WT

5'agcttGCTATTTCAGAATGCa3'
3'aCGATAAAGTCTTACGttcga5'

275 WT

5'agcttGCAATTTCATAAAGCa3'
3'aCGTTAAAGTATTTCGttcga5'

290 MUT

5'agcttGCTATGGACTAATGCa3'
3'aCGATACCTGATTACGttcga5'

275 MUT

5'agcttGCAATGGACGAAAGCa3'
3'aCGTTACCTGCTTTCGttcga5'

ERE WT

5'agcttGCTATTTCAAAATGCa3'
3'aCGATAAAGTTTTACGttcga5'

ERE MUT

5'agcttGCTATGGTCCAATGCa3'
3'aCGATACCAGGTTACGttcga5'

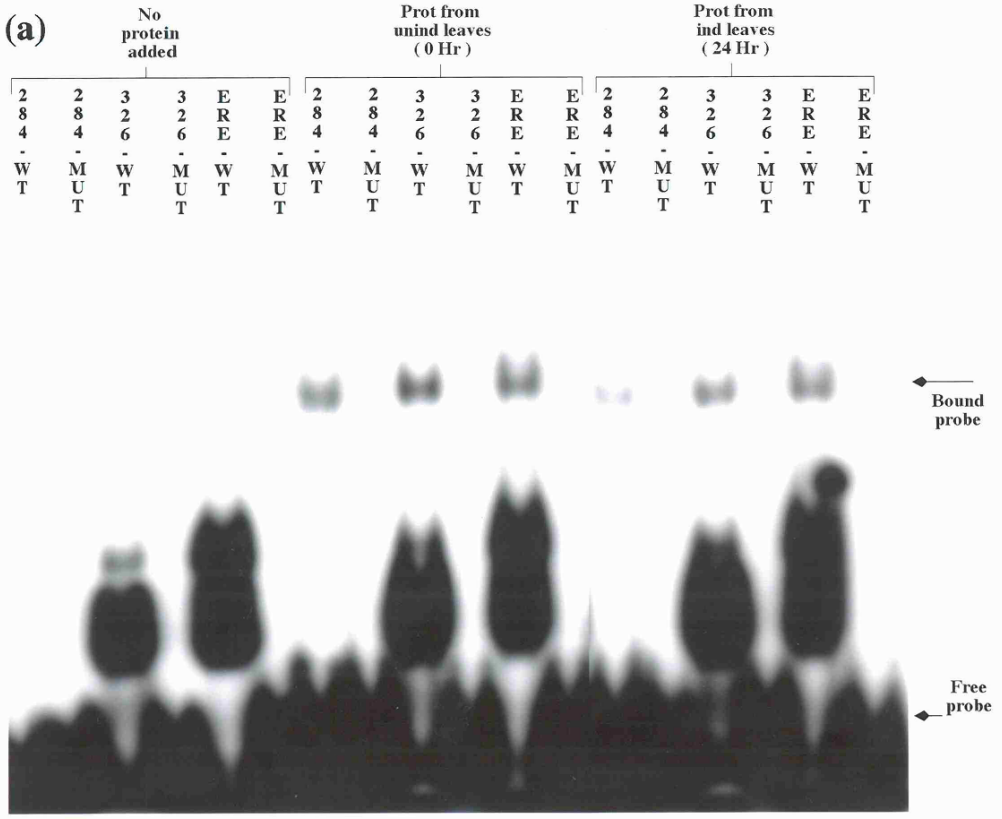
Figure 2

Autoradiographs of EMSA gels, using nuclear protein extracts from safener-treated and untreated leaves.

(a) An autoradiograph (overnight exposure) of an EMSA using 284WT (wild type), 284MUT (mutant), 326WT, 326MUT, ERE-WT and ERE-MUT probes, as indicated on the diagram. The first six lanes contain the free probes, with no protein added. In the middle six lanes the probes, prior to electrophoresis, were incubated for 20 minutes at room temperature with 1 μg nuclear protein extract prepared from maize leaves which had not been treated with safener (unind., 0 hours). In the last six lanes the same probes, prior to electrophoresis, were incubated for 20 minutes at room temperature with 1 μg nuclear protein extract prepared from maize leaves which had been treated with safener 24 hours earlier (ind., 24 hours). In each case 200 pg of probe were used mixed in a 10 μl volume with 5 mM DTT, 50 mM KCl, 1 μg poly-dIdC, 100 mM $MgCl_2$ and 1 μl gel retard buffer.

(b) An autoradiograph (overnight exposure) of an EMSA using 290WT (wild type) and 290MUT (mutant) probes, as indicated on the diagram. The first two lanes contain the free probes, with no protein added. In the middle two lanes the probes, prior to electrophoresis, were incubated for 20 minutes at room temperature with 1 μg nuclear protein extract prepared from maize leaves which had not been treated with safener (unind., 0 hours). In the last two lanes the same probes, prior to electrophoresis, were incubated for 20 minutes at room temperature with 1 μg nuclear protein extract prepared from maize leaves which had been treated with safener 24 hours earlier (ind., 24 hours). In each case 200 pg of probe were used mixed in a 10 μl volume with 5 mM DTT, 50 mM KCl, 1 μg poly-dIdC, 100 mM $MgCl_2$ and 1 μl gel retard buffer.

(c) An autoradiograph (overnight exposure) of an EMSA using 275WT (wild type) and 275MUT (mutant) probes, as indicated on the diagram. The first two lanes contain the free probes, with no protein added. In the middle two lanes the probes, prior to electrophoresis, were incubated for 20 minutes at room temperature with 1μg nuclear protein extract prepared from maize leaves which had not been treated with safener (unind., 0 hours). In the last two lanes the same probes, prior to electrophoresis, were incubated for 20 minutes at room temperature with 1 μg nuclear protein extract prepared from maize leaves which had been treated with safener 24 hours earlier (ind., 24 hours). In each case 200 pg of probe were used mixed in a 10 μl volume with 5 mM DTT, 50 mM KCl, 1 μg poly-dIdC, 100 mM $MgCl_2$ and 1 μl gel retard buffer.


(a)
No protein added
Prot from unind leaves (0 Hr)
Prot from ind leaves (24 Hr)
284-WT
284-MUT
326-WT
326-MUT
ERE-WT
ERE-MUT
Bound probe
Free probe

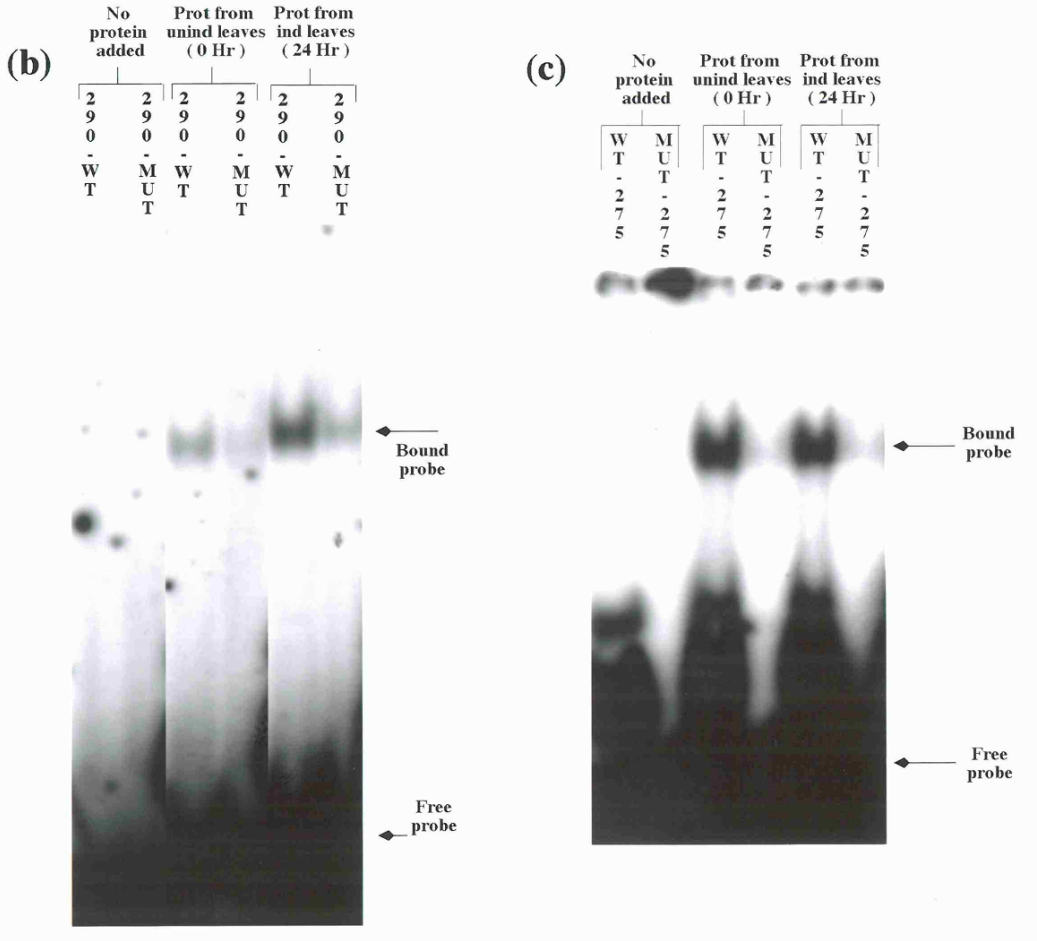
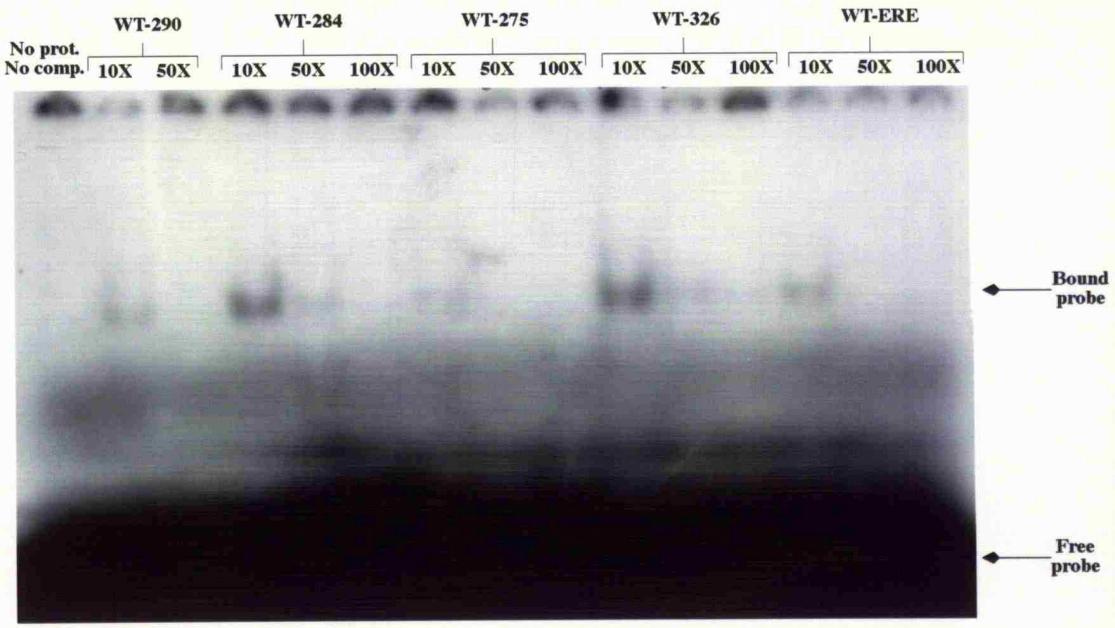

(b)
No protein added
Prot from unind leaves (0 Hr)
Prot from ind leaves (24 Hr)
290-WT
290-MUT
Bound probe
Free probe
(c)
No protein added
Prot from unind leaves (0 Hr)
Prot from ind leaves (24 Hr)
WT-275
MUT-275
Bound probe
Free probe

Figure 3

An autoradiograph of an EMSA competition assay gel (14 day exposure).

In this competition assay radiolabelled WT290 was used as a probe with WT290, WT284, WT275, WT326 and WT-ERE used individually as competitors. Prior to electrophoresis 200 pg of WT290 probe and the relevant amount of competing probe (as indicated on the diagram) were incubated for 20 minutes at room temperature with 1μg nuclear protein extract prepared from maize leaves which had not been treated with safener. The same result was obtained using nuclear protein extract prepared from maize leaves which had been treated with safener 24 hours earlier. The probes and protein were mixed in a 10 μl volume with 5 mM DTT, 50 mM KCl, 1 μg poly-dIdC, 100 mM $MgCl_2$ and 1 μl gel retard buffer.


WT-290
WT-284
WT-275
WT-326
WT-ERE
No prot.
No comp.
10X
50X
10X
50X
100X
10X
50X
100X
10X
50X
100X
10X
50X
100X
Bound probe
Free probe

Chapter Five

IN VIVO ANALYSIS OF THE PUTATIVE SAFENER-RESPONSIVE *CIS* ELEMENTS IN TRANSIENT ASSAYS

5.1 INTRODUCTION

Having identified four putative safener-responsive elements in the *GST-27* promoter by *in vivo* footprinting (see Chapter 3), and having shown that these elements could interact with nuclear proteins *in vitro* (see Chapter 4) it was decided to perform transient assays with constructs containing wild type or mutated forms of the elements. The *GST-27* 5' deletion promoter constructs engineered at Zeneca (as described in Chapter 3, section 3.1.3.iii) were used for this purpose, specifically pPUG5 and pPUG6, which contain 570 and 378 base pairs (bp) of the promoter upstream of the transcription start point (TSP), respectively. Using the silicon carbide fibre-mediated transformation procedure (see below) with Black Mexican Sweet (BMS) maize suspension culture cells, it was previously observed that the wild type truncated promoters contained in these constructs conferred safener-responsiveness to the reporter gene β-glucoronidase (GUS) (Anne-Isobelle Michou, Zeneca, unpubl., see Introduction to Chapter 3). By creating mutations in the putative safener-responsive elements present in these constructs it might be expected that the promoters could lose the ability to respond to safener, assuming that these elements were necessary and sufficient for this response. A series of constructs were engineered to test the effect of a ten base pair mutation to the individual elements around footprinted G residues G-326, G-290 and G-283/4 combined with G-275, and to test the effect of mutating all the bases including and between G-290 to G-275. By transiently transforming these constructs into BMS cells, in the presence or absence of safener, the elements role in safener-dependent transcription may be determined.

Transient transformation of BMS cells mediated by silicon carbide fibres is a relatively new technique (Kaeppler *et al.,* 1990) which was developed to provide a cheap, simple and routinely applicable method for DNA delivery into intact plant cells. Other techniques available for this purpose include removal of the plant cell wall followed by DNA uptake into protoplasts (Potrykus *et al.,* 1987), the use of *Agrobacterium tumifaciens* as a vector for DNA delivery (Horsch *et al.,* 1985) or physical penetration of the cell wall by microprojectile bombardment (Klein *et al.,* 1987). As described earlier (see Chapter 3), the techniques of protoplast transformation and microprojectile bombardment were considered unsuitable for testing the pPUG series of constructs, because background levels of the reporter gene were too high, which masked the induction effect of the safener. *Agrobacterium* mediated DNA transfer was considered unsuitable as it is a time-consuming technique which is not suitable for use in transient assays with BMS cells as its host range is limited mostly to dicotyledonous plants, although there have been some reports of its use in the transformation of monocotyledonous plants (Gould *et al.,* 1991; Hess *et al.,* 1990; Raineri *et al.,* 1988). Therefore, silicon carbide fibre-mediated DNA transfer appeared to be the best technique available for our purposes. This method (described in Chapter 2, 2.15) involves the vortexing of tissue culture cells and plasmid DNA with minute silicon carbide fibres (0.6 μm average diameter, 10 - 80 μm length). It is

thought that the DNA-coated fibres can penetrate the cell wall and so deliver the DNA into the cell. This method has wide-ranging applications and it has been used to transform monkey cells (Appel *et al.,* 1988), insect embryos (Kaeppler *et al.,* 1990), tissue cultures of monocotyledonous and dicotyledonous plants (Kaeppler *et al.,* 1992) and ~~in the production of~~ to produce transgenic maize plants (Frame *et al.,* 1994).

5.2 RESULTS

The mutated constructs which were used in the transient transformation procedure are illustrated in schematic form in figure 1. Four mutations in the context of pPUG6 are illustrated; the nucleotides shown in red indicate the 10 wild type base pairs which were mutated surrounding the footprinted G residues -325/6, the nucleotides shown in green indicate the 10 wild type base pairs which were mutated surrounding the footprinted G residue -290 and the nucleotides shown in purple indicate the 10 wild type base pairs which were mutated surrounding the footprinted G residues -275 and -283/4. In three separate constructs the nucleotides contained within one of these three regions were converted from G to T and from C to A, and the resulting constructs were named pPUG6/MUT326, pPUG6/MUT290 and pPUG6/MUT284. In a fourth construct all the nucleotides contained by the blue box shown in figure 1 were also converted from G to T and from C to A and the resulting construct was named pPUG6/2XMUT. Identical mutations for the single elements were also introduced into pPUG5 resulting in three constructs, namely pPUG5/MUT326, pPUG5/MUT290 and pPUG5/MUT284. The mutations introduced into the MUT326 and MUT290 plasmids are similar to those introduced into the mutant probes used in the EMSA studies, in which five or six nucleotides were mutated (see Chapter 3), but are extended to include ten nucleotides. As the mutations in the mutant probes used in the EMSA studies were sufficient to abolish interactions with the nuclear protein factor or factors, the 10 nucleotide mutations in the plasmids were expected to be sufficient to abolish the putative *cis* elements. The mutation introduced into the MUT284 plasmids, which included the footprinted residues G-283/284 and G-275 and the nucleotides between, was designed so that two of the putative elements could be tested in one construct, as only a limited number of plasmids can be analysed successfully at one time using this technique (see Discussion, 5.3). This effectively meant that only one half of these putative elements were mutated, but it was thought that this would be sufficient to abolish the activity of the elements. These mutations were generated by PCR-mediated mutagenesis (see Methods 2.14) and 10 μg of each plasmid were transformed into BMS cells using the silicon carbide fibre-mediated DNA transfer technique (see Methods 2.15). The pUC based plasmid p35-PAC was used as a positive control for the transformation procedure (kindly provided by Ian Evans, Zeneca). This plasmid contains the strong constitutive cauliflower mosaic virus 35S (CaMV 35S) promoter fused to the reporter gene GUS, and contains modifications to help achieve high levels of expression in BMS cells, namely the incorporation of the first intron from

the maize *Adh1* gene between the promoter and the GUS gene, and the inclusion of a dicot modified ATG consensus in the GUS gene.

As the technique of silicon carbide fibre-mediated DNA transfer is relatively new, figure 2 (a) shows a photograph of BMS cells which have been vortexed for 60 seconds in the presence of silicon fibres. An arrow indicates a fibre which may be piercing a cell and thereby delivering DNA into it. Figure 2 (b) shows a photograph of cells which were transformed using this technique with pPUG1, the construct containing 3.8 kb of the *GST-27* promoter fused to the reporter gene GUS. The transformation was performed in the presence of 40 ppm of the inducing agent, dichlormid safener R-25788, and two days later the cells were stained with histochemical assay buffer so that cells which were expressing GUS would be stained blue. In figure 2 (b) there are two cells expressing GUS which are easily distinguished from those cells which are not expressing the gene.

The results of the transformation of pPUG5 constructs containing mutations to individual elements are given in table 1. The results of the transformation of pPUG6 constructs containing mutations to individual elements are given in table 2. The results of the transformation of the pPUG6 construct containing mutations to three of the putative elements are given in table 3. If samples were microbially contaminated and could not be counted, this is indicated in the tables. In each set of experiments the appropriate wild type plasmid was transformed as a positive control to ensure that the inducing conditions were efficient and p35-PAC was transformed as a control to indicate the efficiency of the transformation procedure. The results of the three experiments are represented graphically in figure 3.

Table 1 The number of colour forming units (cfu) counted in BMS cells transformed with pPUG5, pPUG5/MUT326, pPUG5/MUT290, pPUG5/MUT284 and p35-PAC in the presence or absence of 40 ppm R-25788 safener are shown. The mean number of cfu (me.) and the standard deviation (st. dev.) is given for each construct in both of these conditions. N-fold induction is given, calculated per replicate and as a mean per construct.

Construct	cfu + safener	cfu - safener	n fold induction
p35-PAC	209	280	0.75
	306	255	1.20
	389	188	2.07
	(me.=301; st.dev.=90.09)	(me.=241; st.dev.=47.57)	(me. = 1.34)
pPUG5	28	14	2.00
	37	9	4.11
	45	24	1.88
	(me.=37; st.dev.=8.50)	(me.=16; st.dev.=7.64)	(me. = 2.66)
pPUG5/MUT326	49	13	3.76
	48	12	4.00
	45	12	3.75
	(me.=47; st.dev.=2.08)	(me.=12; st.dev.=0.58)	(me. = 3.84)
pPUG5/MUT290	31	18	1.72
	50	13	3.85
	67	21	3.19
	(me.=49; st.dev.=18.00)	(me.=12; st.dev.=4.04)	(me. = 2.92)
pPUG5/MUT284	21	3	7.00
	47	14	3.36
	31	11	2.82
	(me.=33; st.dev.=13.11)	(me.=9; st.dev.=5.69)	(me. = 4.39)

Table 2 The number of cfu counted in BMS cells transformed with pPUG6, pPUG6/MUT326, pPUG6/MUT290, pPUG6/MUT284 and p35-PAC in the presence or absence of 40 ppm R-25788 safener are shown.The mean (me.) number of cfu and the standard deviation (st. dev.) is given for each construct in both of these conditions. N-fold induction is given calculated per replicate and as a mean per construct.

Construct	cfu + safener	cfu - safener	n fold induction
p35-PAC	173	500	0.35
	181	506	0.36
	275	686	0.40
	(me.=210; st.dev.=56.72)	(me.=564; st.dev.=105.70)	(me. = 0.37)
pPUG6	72	11	6.55
	49	10	4.90
	47	8	5.88
	(me.=56; st.dev.=13.89)	(me.=10; st.dev.=1.53)	(me. = 5.78)
pPUG6/MUT326	57	24	2.38
	52	22	2.36
	contam.	11	-
	(me.=55; st.dev.=5.86)	(me.=19; st.dev.=2.31)	(me. = 2.37)
pPUG6/MUT290	26	8	3.25
	22	12	1.83
	27	15	1.80
	(me.=25; st.dev.=2.12)	(me.=12; st.dev.=1.41)	(me. = 2.29)
pPUG6/MUT284	41	13	3.15
	30	13	2.31
	32	9	3.56
	(me.=34; st.dev.=2.65)	(me.=9; st.dev.=3.51)	(me. = 3.00)

Table 3 The number of cfu counted in BMS cells transformed with pPUG6, pPUG6/2XMUT and p35-PAC in the presence or absence of 40 ppm R-25788 safener are shown. The mean (me.) number of cfu and the standard deviation (st. dev.) is given for each construct in both of these conditions. N-fold induction is given calculated per replicate and as a mean per construct.

Construct	cfu + safener	cfu - safener	n-fold induction
p35-PAC	contam.	224	-
	488	216	2.26
	127	288	0.44
	730	297	2.46
	(me.=448; st.dev.=303.45)	(me.=256; st.dev.=44.40)	(me. = 1.72)
pPUG6	31	23	1.35
	51	9	5.67
	21	15	1.40
	65	15	4.33
	(me.=37; st.dev.=15.61)	(me.=16; st.dev.=5.74)	(me. = 3.19)
pPUG6/2XMUT	52	25	2.08
	22	13	1.69
	35	24	1.46
	65	7	9.29
	(me.=44; st.dev.=18.88)	(me. = 17; st.dev.=8.73)	(me. = 3.63)

5.3 DISCUSSION:

From table 1 it can be seen that the single element mutations did not appear to abolish safener-responsiveness of the pPUG5 promoter (containing 570 base pairs of the *GST-27* promoter). Although the results are variable between replicates within one experiment and between repeat experiments (data not shown), all the constructs appear to retain a degree of inducibility similar to the wild type pPUG5 plasmid (between approximately two to seven fold; the variability is such that a construct showing two fold inducibility in one experiment may show four fold inducibility in the next experiment). The results of the effect of the single element mutations in the context of pPUG6 (containing 378 base pairs of the promoter) are given in table 2. Again,

the results are variable but this experiment, and repeats of it (data not shown), indicate that the mutated constructs retain a degree of inducibility similar to the wild type pPUG6 plasmid (between two and five fold). As the single mutations did not abolish the safener-responsiveness of either the pPUG5 or pPUG6 promoter it was reasoned that the other unmutated elements which were present in these constructs were sufficient to elicit a response to safener. The results of the transformation of the pPUG6/2XMUT construct into BMS cells are given in table 3. This experiment, and repeats of it, indicate that pPUG6/2XMUT containing mutations to three of the putative safener-inducible elements retains a degree of inducibility similar to the wild type pPUG6 plasmid (between two and nine fold). In this case it may be that the remaining unmutated putative element, around footprinted G residues -325/6, is sufficient on its own to confer safener-inducibility. The reason why a construct containing mutations to all four elements was not tested was because, at the time of these experiments, the importance of all four elements had not been recognised. EMSA studies had been carried out prior to these transient assay experiments, but the extensive EMSA studies, including a G-325/6 probe, were only carried out afterwards. Therefore, at this stage of experimentation, the G-325/6 footprint was not considered to be important in safener-responsiveness, as this footprint had only been observed once. However, the results from the pPUG6/2XMUT experiment are highly variable. In addition, it can be seen from table 3 that the positive control of p35-PAC, which is not considered to be responsive to safener, formed more cfu in the presence of safener than in its absence, such that it exhibited a 1.75 fold induction (the significance of this result is discussed in Chapter 6). As a promoter which is not responsive to safener was giving results which made it appear that it may be safener-responsive, it could not be clear if the mutated pPUG6 construct was not also exhibiting a false positive result. The positive control did not always give results which made it appear as though it was safener inducible as, for example, from the results in table 2 it can be seen that in this experiment more cfu were counted in the absence of safener than in its presence, so giving a fold induction of 0.37. Therefore, even when comparing results obtained from the positive control plasmid which was not considered to be responsive to safener, it can be seen that great variability is encountered with this technique, both between replicates in one experiment and between repeats of the same experiment. The high levels of variability make it difficult to obtain statistically significant results. A statistically significant result can be regarded as a result in which the mean number of cfu, counted in the presence of safener, plus or minus two standard deviations, gives a number which does not overlap with the mean number of cfu, counted in the absence of safener, plus or minus two standard deviations. Therefore, although the results in table 2 are statistically significant they could not be repeated, despite numerous attempts. The plasmid pPUG6 gave a statistically significant level of induction in one experiment (see table 2) but exactly the same construct transformed in identical conditions in a separate experiment did not give a significant level of induction (see table 3). Therefore, although one could draw conclusions from the results of one experiment, different conclusions would arise from subsequent repeats of the experiment. One way to

overcome the difficulty encountered when trying to repeat an experiment would be to increase the number of replicates within one experiment. In this way enough data could be collected in one experiment in order to eliminate the extra factor of variability between experiments. However, experience has shown that experiments which contain a large number of samples often give more variable results compared to experiments which contain fewer samples (Paul Drayton, pers. comm.). The maximum number of samples which can be successfully handled is approximately 30; it is thought that the length of time the cells are kept in anaerobic conditions when performing larger experiments may be deleterious and that this may adversely affect the results.

Another problem with this technique is that the results are qualitative, not quantitative. We were not successful in obtaining quantitative results as the levels of GUS were too low to detect fluorometrically. The problem with qualitative data is that differences between constructs which may be apparent in the intensity of blue colour expressed in the cfu could not be measured. It may be that even though the number of cfu is similar between a wild type and mutant plasmid, the mutant plasmid is unable to drive as high levels of GUS expression as the wild type plasmid. Subtle differences like these were not registered but may have been expected, especially with the constructs in which one of the four putative elements was mutated. Although techniques exist which can be used to amplify low levels of GUS to detectable levels it was decided, because of the variability of this transformation procedure, to test the mutant constructs in an alternative system. It was thought that the technique was useful in analysing deleted promoter fragment constructs but that it was not sensitive enough for the assays we were trying to perform.Therefore, it was decided to engineer constructs for use in stable transformation into tobacco. Although this method has disadvantages in that it is time-consuming and levels of expression can be variable between plants, it was felt that this system would provide more useful and dependable data than the transient assay could provide. It was thought to be advantageous to be able to analyse the effects of mutations to the potential *cis* elements in the context of a whole plant. In addition the wild type plasmids pPUG5, pPUG6 and pPUG7 had not been tested in whole plants and so new data on the effect of 5' deletions to the *GST-27* promoter *in vivo* would be generated. The subsequent transgenic plants could also be used to analyse the effect of the promoter deletions on root expression in seedlings as, although GST-27 is not normally expressed in maize or tobacco plants, it is expressed in the roots of tobacco and in the roots of young seedlings of maize. The transgenic tobacco work is described in detail in Chapter 6.

Figure 5.1

A schematic diagram to show the four mutations introduced into pPUG5 and pPUG6.

The 10 wild type nucleotides illustrated in red surround the footprinted residues G-325/326 and were mutated from G to T and C to A to give constructs pPUG5/MUT326 and pPUG6/MUT326. The 10 wild type nucleotides illustrated in green surround the footprinted residue G-290 and were mutated from G to T and C to A to give constructs pPUG5/MUT290 and pPUG6/MUT290. The 10 wild type nucleotides illustrated in purple include the footprinted residues G-283/284 and G-275 and were mutated from G to T and C to A to give constructs pPUG5/MUT284 and pPUG6/MUT284. The 26 nucleotides contained by the blue box were all mutated from G to T and C to A to give the construct pPUG6/2XMUT (the equivalent pPUG5 mutant was not engineered). Footprinted G residues are indicated by arrows. The mutations were generated with PCR mediated-mutagenesis (see Materials and Methods, 2.14).

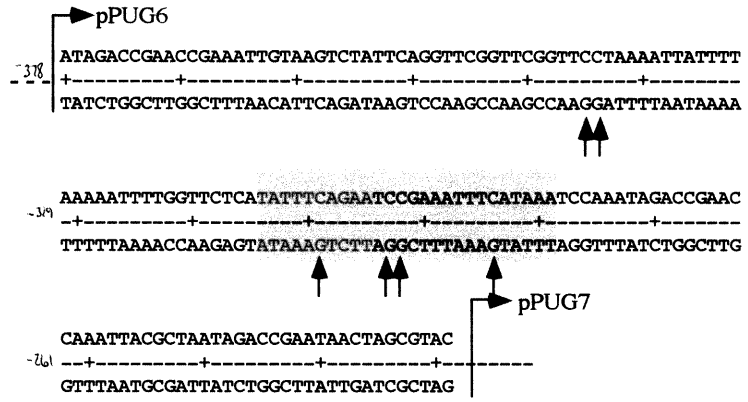


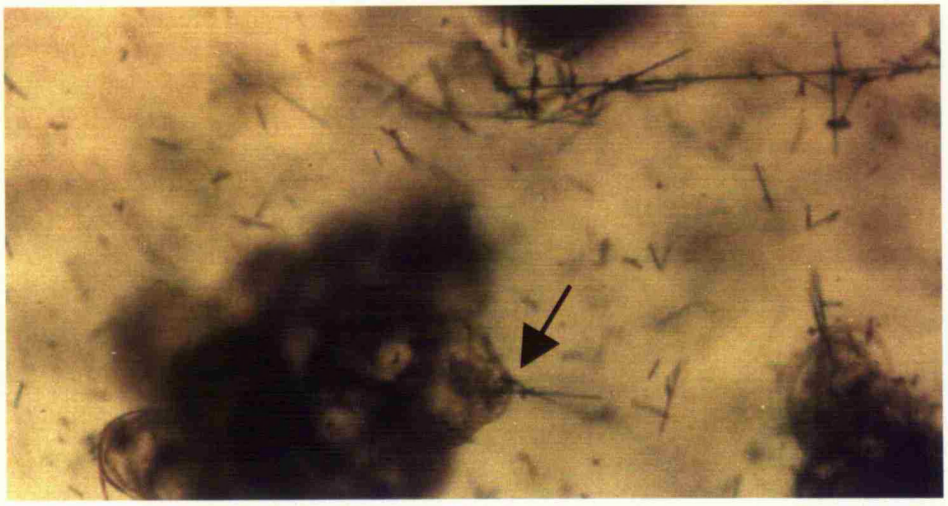
Figure 2

Photographs to illustrate the technique of silicon carbide fibre-mediated DNA transfer into BMS cells.

(a) A photograph of BMS cells (500 µls of a 50 % packed cell volume in BMS media) which have been vortexed for 60 seconds in the presence of silicon carbide fibres (40 µls of a 5 % [w/v] suspension) and 10 µg plasmid DNA. DNA transfer is thought to be achieved by DNA coated fibres piercing the cell; the arrow indicates a fibre which may be piercing a cell and thereby delivering DNA into it. The photograph was taken at 20 fold magnification with a camera attached to a Nikon SMZ-2B microscope.

(b) A photograph of histochemically stained BMS cells which were transformed by the silicon carbide fibre-mediated technique with the construct pPUG1, which contains 3.8 kb of the *GST-27* promoter upstream of the TSP fused to the reporter gene GUS. The transformation was performed in the presence of 40 ppm of the inducing agent, dichlormid safener (R-25788). The two cells expressing GUS in this photograph are clearly identifiable by their blue colour. The photograph was taken at 6 fold magnification with a camera attached to a Nikon SMZ-2B microscope.

(a)



(b)

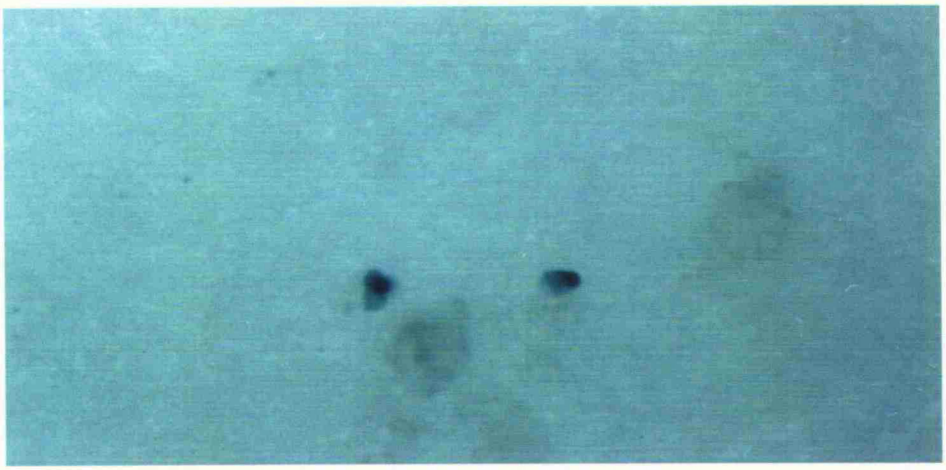
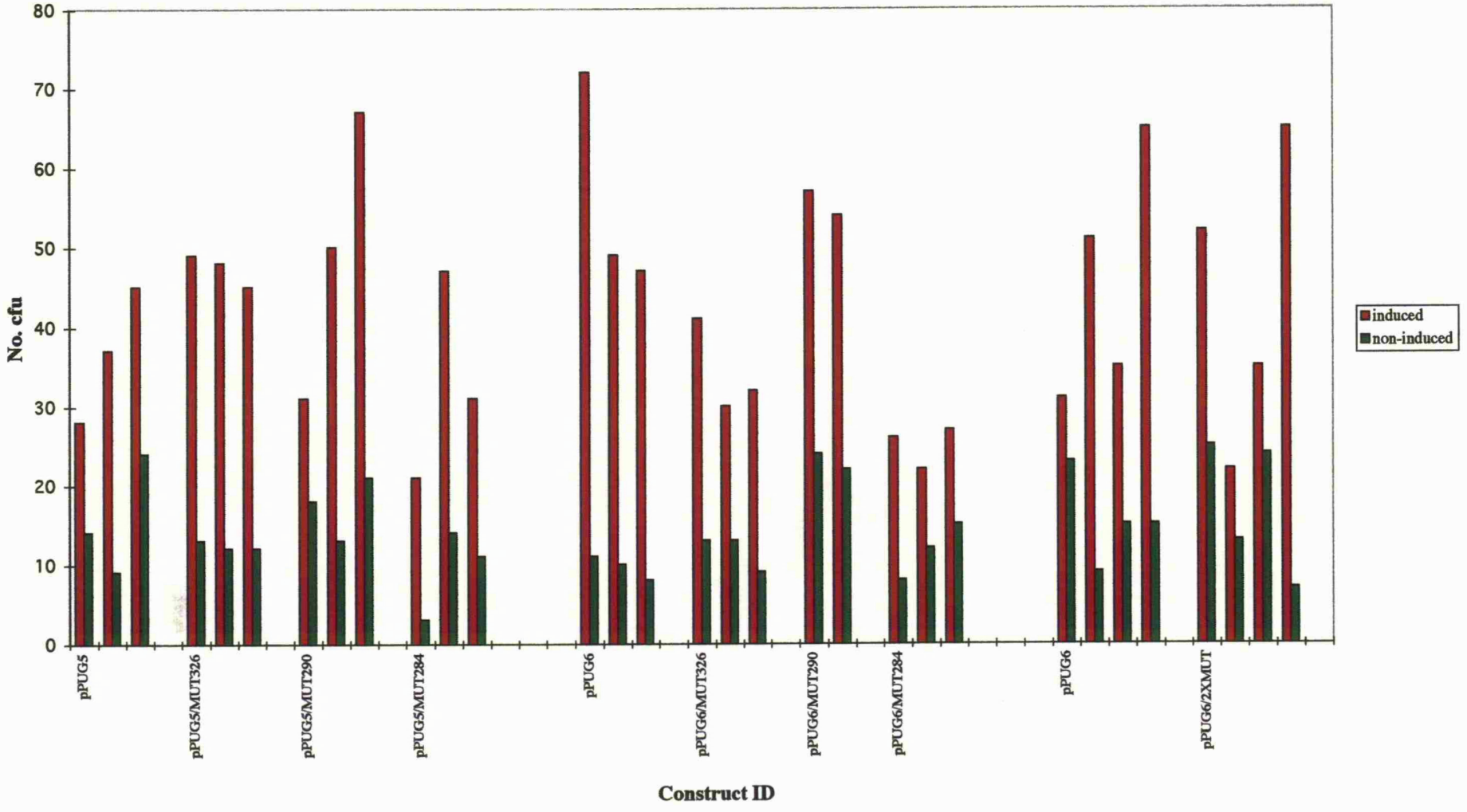


Figure 3

A graphical representation of the results from the transient transformation of BMS cells with the mutant pPUG5 and pPUG6 constructs.

BMS cells were transiently transformed in the presence or absence of 40 ppm dichlormid safener R-25788 with 10 µg plasmid DNA using the silicon carbide fibre-mediated transformation technique. Two days after the transformation the cells were histochemically stained in order to analyse GUS expression. Colour forming units were counted and the results are plotted on the graph; the construct name is given on the x-axis, and the number of colour forming units (cfu) it produced is given on the y-axis. For each construct three or four replicates in two conditions (with or without 40 ppm dichlormid safener [R-25788]) were performed in each transformation experiment. The three segments on the graph are to indicate that the results were obtained from three separate experiments. From the first four data sets, on the left hand side of the graph, the number of cfu obtained from wild type pPUG5 in the presence or absence of safener can be compared to the number of cfu obtained in the same conditions from pPUG5/MUT326, pPUG5/MUT290 and pPUG5/MUT284. From the middle four data sets the number of cfu obtained from wild type pPUG6 in the presence or absence of 40 ppm dichlormid safener (R-25788) can be compared to the number of cfu obtained in the same conditions from pPUG6/MUT326, pPUG6/MUT290 and pPUG6/MUT284. From the last two data sets, on the right hand side of the graph, the number of cfu obtained from wild type pPUG6 in the presence or absence of 40 ppm dichlormid safener (R-25788) can be compared to the number of cfu obtained in the same conditions from pPUG6/2XMUT.


80
70
60
50
40
30
20
10
0
No. cfu
induced
non-induced
pPUG5
pPUG5/MUT326
pPUG5/MUT290
pPUG5/MUT284
pPUG6
pPUG6/MUT326
pPUG6/MUT290
pPUG6/MUT284
pPUG6
pPUG6/2XMUT
Construct ID

Chapter Six

ANALYSIS OF THE PUTATIVE SAFENER-RESPONSIVE *CIS* ELEMENTS IN TRANSGENIC TOBACCO

6.1 INTRODUCTION

As described in the discussion section of Chapter 5, *in vivo* analysis of the putative safener-responsive elements in BMS cells using the silicon carbide fibre-mediated transformation technique produced inconsistent results. The data generated was highly variable between replicates in one experiment and repeats of an experiment, such that it was difficult to obtain reproducible, statistically significant data. In addition, it was felt that the technique may not be sufficiently sensitive to distinguish between promoters which were actively transcribing but at different rates; by counting colour forming units, distinctions were not made between varying intensities in the colour of the cells, which may have reflected different transcription rates achieved by the promoters in question. During the course of this study experiments were performed at Zeneca which indicated that the full length *GST-27* promoter was active and safener-responsive in transgenic tobacco plants, therefore it was decided to test the putative safener-responsive elements in stably transformed transgenic tobacco. Although transgenic tobacco give variable data, this is often due to positional effects caused by the random integration of the exogenous DNA into the plant genome (Ow and Medberry, 1995). Therefore, repeated testing of a plant should give reproducible results, and the observed variability of results between plants is explainable. It was also expected that transgenic tobacco would give results which could enable one to distinguish between different rates of transcription from the various promoters used in the experiment. Finally, the generation of transgenic tobacco containing short fragments of the *GST-27* promoter with extensive 5' deletions would enable their analysis in whole plants, which had not been performed previously. It was expected that these plants would help to identify the position of the element which is involved in driving the observed constitutive expression of the gene in the roots of tobacco (Ian Jepson, pers. comm.).

Seven constructs were engineered for transformation into tobacco (see Materials and Methods 2.17.1) as follows (see figure 1 for a schematic diagram of the constructs):

pBin/5 containing 570 bp of *GST-27* promoter upstream from the transcription start point (TSP) fused to the reporter gene β-glucoronidase (GUS)

pBin/6 as pBin/5 but containing 378 bp of the *GST-27* promoter

pBin/7 as pBin/5 but containing 217 bp of the *GST-27* promoter

3XMUT/Bin6 as pBIN6 but incorporating 5 bp mutations around the footprinted G residues G-290, G-283/284 and G-275

4XMUT/Bin6 as pBIN6 but incorporating 5 bp mutations around the footprinted G residues G-325/326, G-290, G-283/284 and G-275

pWT8 containing 26 bp of the *GST-27* promoter, including the footprinted G residues G-290, G-283/284 and G-275, fused to 90 bp from the 3' end of the 35S cauliflower mosaic virus promoter (CaMV) which was then

	fused to the reporter gene GUS
pWT9	as pWT8 but containing 60 bp of the 35S CaMV promoter

pBin5, pBin6 and pBin7 were engineered in order to test the effect of deletions to the *GST-27* promoter on the transcription rate of the reporter gene in whole plants which had been treated with safener, and to compare these results to those obtained in transient assays performed by Anne-Isobelle Michou at Zeneca (see Chapter 3, table 1). It was expected that the results would be similar to those obtained in transient assays *i.e.* that the promoters present in pBin5 and pBin6 would retain inducibility and that inducibility would be lost in pBin7. The mutations introduced to pBin6 to create 3XMUT/Bin6 and 4XMUT/Bin6 were identical to those present in the mutant probes which were used in EMSA studies (see Chapter 4), which were found to be sufficient to abolish the binding of nuclear protein. By mutating the putative safener-responsive elements it was expected that, if the elements were necessary and sufficient to confer safener-responsiveness, the promoter would not be induced to transcribe by the application of safener. Constructs containing three and four mutated areas were created to determine if the putative element situated around footprinted residues G-235/236 was important to the safener-responsiveness of the promoter; because this *in vivo* footprint was only observed once, there was some doubt about its role. The constructs pWT8 and pWT9 were engineered to determine if a short length of DNA, containing three of the putative safener-responsive elements, was sufficient to confer safener-responsiveness to a minimal promoter. It was considered to be important to use both a 60 bp and a 90 bp 35S CaMV minimal promoter, as, in some cases, expression is only seen from a heterologous promoter containing the 90 bp minimal promoter. However, it would be preferable if expression was observed when using the 60 bp minimal promoter, as the 90 bp minimal promoter contains an *as-1* element which can drive the expression of a reporter gene in the roots of transgenic tobacco (Lam *et al.*, 1989). By transforming tobacco with these constructs and analysing the expression of GUS in untreated and safener-treated transformants, it was hoped that the role of the putative safener-responsive elements in safener-dependent transcription could be determined.

6.2 RESULTS

6.2.i Fluorometric analysis of 5' deleted *GST-27* promoter constructs

When performing the tobacco transformation experiments it was our aim to produce at least 20 transformants per construct, as, to gain meaningful data, it is important to use as many individually transformed plants as possible. However, for some constructs this aim was not achieved; for the construct 3XMUT/Bin6 only nine transformants were created from two separate transformation experiments. For the construct 4XMUT/Bin6 no plants were transformed, despite repeating the transformation procedure twice. Mutations into these constructs were introduced by PCR-mediated mutagenesis and they were sequenced before use

so it is not clear why both of the constructs which contained introduced mutations were difficult to transform. The transformations were performed in parallel with those of the wild type constructs Bin5, Bin6 and Bin7, all of which transformed successfully after one attempt. Therefore the problem was probably not due to the use of an ineffective sample of *Agrobacterium*, operator error or the use of incorrect media. The problem may have been a chance effect, or it may be that the mutations present in these constructs were affecting the success of the transformation rate in some way. If these promoters were linked to a gene whose product may be harmful to the plant their low transformation rate may be explainable, but GUS should be a relatively harmless gene product. Ideally, these constructs should be remade and retransformed, but time constraints did not allow for this. Sixteen transformants were obtained with the Bin5 construct, 26 transformants were obtained with the Bin6 construct and 17 transformants were obtained with the Bin7 construct .

The transformants were grown on kanamycin sulphate containing medium in tissue culture until they were approximately 5 cm tall. At this stage healthy green plants (non-transformants bleached and died) were transferred to the greenhouse and left for at least one week; this allowed the plants to develop a thicker cuticle. When the plants were ready to be analysed a sample (two whole leaves) was removed from each plant before, and 24 hours after a 5 g/l solution of R-29148 dichlormid safener had been applied to the upper and lower surfaces of the plants' leaves. When samples had been collected, and during all subsequent procedures, they were kept on ice. A sample size of two leaves was chosen in an effort to minimise the effect of variations in GUS levels encountered across the leaf. In addition, as far as possible, leaves of similar size and from similar positions on the plant were sampled, as all these factors can affect GUS levels (pers. obs.). Standard fluorometric GUS assays were performed on the samples as described in Materials and Methods, 2.16.2. The results of the analysis of Bin5, Bin6, 3XMUT/Bin6 and Bin7 transformants are shown in figure 2; in figure 3 the same results are given as means (standard deviation bars are included).

From figures 2 and 3 it can be seen that there is great variability between transformants containing the same transgene; this can be expected when dealing with transgenic plants and can be explained by positional effects and by the fact that transformants may contain the transgene in different copy numbers. However, it can be seen that transformants containing the Bin5 construct show high levels of GUS expression, which generally appear to be increased after the application of safener. Although the level of induction is not statistically significant in this experiment, data from repeat experiments in transgenic tobacco indicates that this promoter, containing 570 base pairs of the *GST-27* promoter, retains inducibility in stably transformed tobacco plants. Figure 4, for example, shows GUS levels achieved by a small number of the Bin5 and 3XMUT/Bin6 transformants in a repeat experiment; the Bin5 transformants are clearly responsive to safener. This result is in agreement with data obtained in transient transformation

assays performed in BMS cells by me and by Anne-Isobelle Michou from Zeneca. The results for all the transformants are generally consistent between experiments. Where differences are seen in the levels of induction between one experiment and another, variables such as the age and condition of the plant should be taken into account. It appears that younger plants respond more readily to safener; this may be due to the softer leaf cuticle which allows the safener to penetrate into the leaf more easily.

From figure 2 and 3 it appears that the remaining transformants, containing the transgenes Bin6, 3XMUT/Bin6 and Bin7, all express GUS at low levels, whether the samples were harvested before or after the application of safener. Bin7 contains 217 base pairs of the *GST-27* promoter. In transient assays this promoter exhibited low levels of GUS expression and did not respond to safener treatment. The transient assay data and transgenic tobacco data together clearly indicate that this promoter does not respond to safener and it may be assumed that the element which confers safener-responsiveness has been removed from this truncated promoter. However, Bin6 transformants, containing 378 base pairs of the *GST-27* promoter, exhibit GUS levels which conflict with the results obtained when the same promoter is used in transient assays. In transient assays this promoter can drive safener inducible GUS expression, but it appears from figures 2 and 3 that in stably transformed tobacco the promoter is not responsive to safener. Identical results were obtained when 18 other transformants of Bin6, obtained from a separate transformation experiment and using a newly engineered version of the Bin6 construct, were analysed. As the same result has been obtained twice in separate experiments it is assumed that this is a significant, meaningful result. In the light of this, it is unexpected that a few of the 3XMUT/Bin6 transformants, containing the Bin6 promoter with mutations to three of the four putative safener-responsive elements, give marginally higher levels of GUS expression when compared to the wild type Bin6 transformants. In figure 2 it can be seen that of the nine 3XMUT/Bin6 transformants tested, two plants produce GUS levels which are higher than the average background GUS levels. It can also be seen that these GUS levels appear to be achieved in safener-independent manner. However, in figure four, the results of a repeat experiment on six of the 3XMUT/Bin6 transformants appears to indicate that these transformants do respond positively to the application of safener. However, of the six plants tested in figure 4, only two show significant levels of induction. Histochemical analysis of T2 seedlings obtained from the 3XMUT/Bin6 transformants indicate that these seedlings do not respond to safener (see figure 7). Therefore, the data obtained from this line of transformants is unclear, but suggests that this promoter may have the ability to respond to safener. The situation would be more clear if additional lines of this transformant were available for testing, but, as stated earlier, this construct yielded few transformants.

Generally, in figure 2, the uninduced and induced levels of GUS for the transformants containing the constructs Bin6, 3XMUT/Bin6 and Bin7 remain constant and low compared to

the transformants containing the Bin5 construct. However, some of the plants show higher levels of GUS expression, for example transformant Bin6, line 3 (Bin6.3), 3XMUT/Bin.7, 3XMUT/Bin.1 and Bin7.25. For the transformant 3XMUT/Bin.1 this higher level of expression could be explained by positional effects, as both non-induced and induced levels of GUS expression are slightly higher than the mean basal level of GUS expression. Therefore the transgene may have inserted to a site on the genome in such a way that it is always active at low levels. The other peaks of GUS expression observed in these plants can not be explained by positional effects as only one of the values, induced or non-induced, is unexpectedly high when compared to mean GUS levels obtained from the other transformants containing the same transgene. These peaks could be explained if the protein content of the samples was calculated to be lower than the actual protein levels. However, as discussed earlier, in the case of the 3XMUT/Bin6 transformants, these peaks in GUS expression may indicate that this promoter is responsive to safener.

The fact that all the transformants express the GUS gene at detectable levels indicates that the GUS gene is present in these plants; when the GUS gene is absent no detectable levels of GUS are observed (Michael Hammond-Kosack, pers. comm.). As the GUS gene is clearly present in all the transformants tested, it may be assumed that the promoter fused to the GUS gene is present in each of the transformants. However, to test this assumption Southern blot analysis could be performed on the plants; this could not only determine if the whole transgene was present but also indicate how many copies of the transgene were present. This is important as copy number can affect the expression of the transgene (Flavell, 1994). Time constraints did not allow this experiment to be performed, but it will be suggested for future work. PCR analysis was performed on some of the plants, the results of which are shown in figure 5. The PCR was performed using primers specific to the 217 base pairs of the *GST-27* promoter present in the Bin7 construct. Therefore, it can be assumed that at least this length of promoter is present in all these transformants, and suggests that each plant is transformed with a transgene to which the relevant promoter is still attached. A disadvantage in screening plants with the PCR technique is that *Agrobacterium* containing the transgene may be present in the leaves of untransformed plants, and so lead to false positive results. However, the GUS levels observed in all the plants suggest that this is not the case.

Levels of GUS expression in the roots of uninduced transformants were also analysed fluorometrically. It was discovered at Zeneca that tobacco transformants containing 3.8 kb of the *GST-27* promoter fused to GUS were responsive to safener, but that GUS expression was endogenous in the roots of these transformants in the absence of safener (pers. comm., Ian Jepson). This is not unexpected as GST-27 mRNA is observed in the roots of maize seedlings which have not been treated with safener. By testing for GUS expression in the uninduced roots of the truncated *GST-27* promoter transformants, data could be collected which may help

to determine the position of the element which confers root-specific expression. Figure 6 shows the mean GUS levels expressed in uninduced roots tested from Bin5, Bin6, 3XMUT/Bin6 and Bin7 transformants (10 plants per construct were analysed). It appears that GUS is expressed at high levels in the roots from all of the transformants, although there is a high degree of variability between transformants. The results in figure 6 were obtained in the same assay as those shown in figures 2 and 3. Therefore, these results are directly comparable and appear to indicate that GUS expression is approximately ten times higher in the roots of uninduced transformants when compared to GUS levels achieved from uninduced leaves. The expression levels are relatively uniform between constructs, although GUS levels are slightly reduced in Bin7 transformants. However, GUS expression in roots was not observed in the histochemical analysis of the T2 seedlings obtained from Bin7 transformants (see figure 7). It should be noted that soil contamination present in the root samples subjected to fluorometric analysis may have a positive effect on the GUS levels observed.

6.2.ii Histochemical analysis of 5' deleted *GST-27* promoter constructs

Histochemical analysis of seedlings of transgenic tobacco containing the full length *GST-27* promoter has clearly shown that GUS is expressed constitutively in the roots, but that expression in the cotyledons is only observed following safener treatment (Ian Jepson, pers. comm.). Therefore histochemical analysis of the T2 transgenic tobacco seedlings was performed in addition to the fluorometric analysis of T1 plants. In this way GUS expression in the roots and cotyledons of the seedlings could be analysed in the presence and absence of safener. Seeds were germinated on moist filter paper in the presence of kanamycin sulphate, so that seedlings which did not contain the transgene would bleach and die. After approximately 7 days, half of the healthy green seedlings were transferred to fresh moist filter paper, also containing kanamycin sulphate but with the addition of 30 ppm dichlormid safener, R-25788. The other half of the seedlings were transferred to filter paper in the absence of safener. After at least 4 days further growth in these conditions the seedlings were submerged in histochemical assay buffer in order to determine the levels of GUS expression. Seedlings from all lines were tested and the results are shown in figure 7, in which one safener-induced (+) and one untreated seedling (-) is shown; these seedlings were photographed as they were representative of all the transformants carrying the same transgene. As a positive control seedlings containing the full length 35S CaMV promoter fused to GUS were analysed and shown to produce a relatively intense, uniform blue colour both before and after the addition of safener. To test the inducing conditions used in this experiment seedlings containing the full length 3.8 kb *GST-27* promoter fused to GUS were analysed (seeds were kindly provided by Ian Jepson, Zeneca). It can be seen that, both in the presence and absence of safener, GUS levels are high in these seedlings (and comparable to levels achieved by the positive control 35S-GUS seedlings), although slightly increased levels of GUS expression were visible in those which were treated with safener. It was expected that the seedlings which were not treated with safener would show no

GUS expression in the cotyledons. However, it was thought that the stressful conditions the seedlings were subjected to *i.e.* grown for approximately 11 days with no carbohydrate source, and with the additional stress of kanamycin sulphate selection, may be sufficient to induce the *GST-27* gene. In addition, these seedlings were stained overnight; less prolonged staining may have shown a more marked difference in GUS levels observed in induced and non-induced seedlings. Therefore, in this experiment comments can be made on the relative expression of a promoter but not on its safener-inducibility. Ideally this experiment should be repeated in the absence of kanamycin sulphate selection so that safener effects can be more easily visualised; time constraints prevented a repeat experiment being performed during this study.

GUS expression in Bin5 transformants was observed both in safener-treated and untreated seedlings, although expression was more marked in the treated seedlings. GUS expression was clearly visible in the root tip of Bin5 transformants in the absence of safener. Therefore, it is clear that the 570 base pairs of the *GST-27* promoter, present in Bin5, is active, and may be responsive to safener, in transgenic tobacco seedlings and that it can confer root tip expression. The levels of GUS seen in Bin5 seedlings were slightly lower than the levels seen in the 3.8-GUS seedlings which contained the full length promoter. No seeds were available to test for GUS expression in Bin6 transformants which contained 378 base pairs of the *GST-27* promoter. However, as the T1 data for these transformants clearly indicates that this transgene is not responsive to safener in tobacco, it is highly probable that seedlings from the T2 generation will behave in a similar manner. The histochemical analysis of T2 seedlings containing the Bin6 transgene will be suggested for future work. Seedlings derived from 3XMUT/Bin6 transformants showed no GUS expression, either in the presence or absence of safener. The fluorometric analysis of the T1 leaves of 3XMUT/Bin6 transformants gave unclear results as to whether this promoter was responsive to safener in tobacco. However, in the histochemical analysis of T2 seedlings carrying this transgene none of the lines tested showed any GUS activity in the cotyledons of safener-treated or untreated seedlings. Very low levels of GUS expression could be detected in the safener-treated and untreated roots of these seedlings. T2 seedlings carrying the Bin7 transgene showed no GUS expression in the cotyledons or roots, either in the presence or absence of safener. In summary, the fluorometric and histochemical data indicate that 570 base pairs of the *GST-27* promoter (Bin5) are sufficient to confer safener-inducible expression to a reporter gene in stably transformed tobacco; however, if the promoter is further truncated to 378 base pairs (Bin6) safener-inducible expression is lost, although endogenous activity in the roots is retained. It appears that endogenous activity in the roots is also retained when the promoter is further truncated to 217 base pairs (Bin7), although this was not observed in histochemical analysis. The data concerning the non-inducibility of the Bin6 transformants conflicts with the data obtained from transient transformation of BMS cells, in which the same promoter was able to drive safener-inducible GUS expression.

6.2.iii Analysis of minimal promoter constructs

pWT8 transformants contained 26 base pairs of the *GST-27* promoter, including three of the four putative safener-responsive elements, fused to a 90 base pair minimal promoter from the 35S CaMV promoter. pWT9 transformants were identical to pWT8 transformants but only 60 base pairs of the 35S CaMV promoter were present in the transgene (see figure 1 for schematic diagram of the constructs). Fluorometric analysis of pWT8 and pWT9 plants was performed as described above for the deletion promoter transformants. In addition, controls carrying the minimal promoters (named as -60 and -90 transformants, containing the 35S CaMV 60 bp and 90 bp minimal promoters alone) were also analysed. The results of mean GUS levels expressed in safener-treated or non-treated leaves from the T1 plants is shown in figure 8. As a control to test that the inducing conditions used in this experiment were sufficient, five Bin5 transformants were also analysed. It can be seen that for the five Bin5 transformants tested, GUS levels were increased by the application of safener. Therefore, it can be assumed that the inducing conditions used in this experiment were sufficient to activate the *GST-27* gene. -60 transformants show low levels of expression both in the absence and presence of safener, which indicates that this promoter is not responsive to safener. pWT9 transformants appear to show marginally increased levels of GUS expression, both in the presence and absence of safener, if compared to the GUS levels obtained from the -60 transformants. Statistical analysis was performed on this data, in the form of an Analysis of Variance (ANOVA) test. A p-value of 18% was obtained when non-induced GUS levels of -60 transformants were compared to non-induced GUS levels of pWT9 transformants. To be statistically significant a p-value must be equal to or less than 5% *i.e.* the probability that an observed result is due to chance is equal to or less than 5%. Therefore, the higher levels of GUS seen in the uninduced WT9 leaves is, according to this statistical analysis, a chance effect, and not due the addition of the putative safener-responsive elements to the transgene. A p-value of 94.2% is obtained when uninduced and induced GUS levels from the -60 transformants are analysed by ANOVA, clearly indicating that, in this experiment, this promoter is not responsive to safener. Similar analysis, comparing uninduced and induced GUS levels from -90 transformants, gives a p-value of 81.8%, which again clearly indicates that, in this experiment, the -90 promoter is not responsive to safener. Levels of GUS appear to be higher in pWT8 plants, both in the presence and absence of safener, when compared to GUS levels expressed in -90 transformants. When GUS levels obtained from -90 uninduced leaves are compared to GUS levels obtained from pWT8 uninduced leaves, a p-value of 7.8% is obtained. This result, although not statistically significant, implies that there may be an important difference between the GUS levels observed for the -90 transformants and the pWT8 transformants. The difference between these plants is that pWT8 contains three of the four putative safener-responsive elements; therefore, the difference in observed GUS levels may be due to the presence of these elements. If this were the case, it would appear that the elements have an effect on GUS levels in the absence and presence of safener. As the elements have been removed from their natural context they may

affect transcription in a way which is different from that observed in their native promoter *i.e.* they may act as general enhancers of transcription. As their role in safener-dependent transcription has not been determined, this indeed may be their function in the *GST-27* promoter. Therefore, based on these results, it was decided to repeat the analysis of the -90 and pWT8 plants.

When working with T1 transformants it can be difficult to ensure that all plants are of a similar size at the time of analysis. This is because some transformants root more quickly than others. Therefore, as seed was available by this time for pWT8 and -90 transformants it was decided to repeat the analysis using T2 plants. The seeds were germinated in the presence of kanamycin sulphate to ensure that only plants which had inherited the transgene were analysed. The plants were analysed for GUS in the same way as the T1 transformants described above, and the results are shown in figure 9. The same results are shown in figure 10 as mean values (standard deviation bars are included). The results of this experiment clearly show that both pWT8 transformants and -90 transformants respond to the application of safener by producing higher levels of the reported gene, GUS. The levels of GUS expression achieved by the pWT8 plants after safener treatment does not exceed the levels of GUS expression achieved by the -90 plants after safener treatment. Therefore, the 35S CaMV 90 base pair minimal promoter appears to be responsive to safener and this response is not significantly altered by fusing the putative safener-responsive elements to the promoter. Roots from these plants were also analysed for GUS expression, as it is known that the -90 promoter can drive root expression (Lam *et al.*, 1989). The level of GUS expression achieved in roots which have not been treated with safener is similar for both the -90 and pWT8 plants (for -90 roots GUS activity = 2336.5 pmoles 4MU/mg/hr [standard deviation = 983.54, for pWT8 roots GUS activity = 2138.4 pmoles 4MU/mg/hr [standard deviation = 803.7]) . The levels are high, and similar to the GUS levels achieved in the induced leaf samples taken from the same plants.

6.3 DISCUSSION

Experiments performed at Zeneca indicate that a 5' deletion of the *GST-27* promoter containing 900 base pairs of DNA retained the ability to respond to dichlormid safener when tested in transgenic maize. From figure 2 it appears that Bin5 tobacco transformants, containing 570 base pairs of the *GST-27* promoter, also retain the ability to respond to dichlormid safener. Although the inducible activity of Bin5 in this experiment is not statistically significant, other experiments with this promoter have shown more convincingly that it is safener-inducible, for example, see figure 4. The results given for Bin5 are shown as they were obtained at the same time as the rest of the data given in figure 2, and it was felt to be important not to mix data from different data sets. In addition, the data from this data set, although consistent within itself, shows higher GUS levels compared to other data sets *e.g.* those represented in figures 4, 8 and 9. The same

mathematical formula was used throughout all of the experiments to calculate GUS levels. Therefore it may be that inconsistencies between experiments in observed GUS levels are due to differences in the reagents used. Researches at Zeneca have reported similar problems of inconsistent GUS levels between experiments (Ian Jepson, pers. comm.). However, as stated previously, although the absolute GUS levels observed in this data set are unusually high, the overall trend in GUS values is consistent with repeats of this experiment.

It is clear from figures 2 and 3, and previous repeat experiments, that the transformants containing the transgenes Bin6 and Bin7 cannot respond to safener. This is unexpected in the case of Bin6 as the promoter in this transgene was found to be responsive to safener in BMS cell transient transformation experiments. Bin5 transformants are safener-inducible both in BMS cells and stably transformed tobacco. This may imply that, in tobacco, the element which confers inducible expression lies between 570 and 378 base pairs upstream of the transcription start point. However, the putative safener-responsive elements, which were identified by *in vivo* footprinting, are present in the 378 base pairs of promoter, which is shown to have lost safener-responsiveness in transgenic tobacco plants. The footprinting experiments were performed in maize plants on the endogenous *GST-27* gene. It could be that the unexpected results obtained with transgenic tobacco reflect the fact that promoters can act differently in different species. This has been observed when transferring a promoter from a monocotyledonous species to a dicotyledonous species, for example, the 35S CaMV promoter is highly active in tobacco plants but not very active in monocotyledonous species (Wilmink *et al.,* 1995; Schledzewski and Mendel, 1994). At Zeneca, a 35S CaMV construct used in BMS cell transient transformation experiments is modified in order to promote high levels of expression in the monocotyledonous cells. One modification is the inclusion of the first intron from the maize *Adh1* gene. The addition of this intron has no effect on expression levels in dicotyledonous species, which indicates that the promoters function differently in monocotyledonous and dicotyledonous species.

Longer versions of the *GST-27* promoter retain safener-inducibility in transgenic tobacco, and therefore it can not be argued that the transcription factors responsible for this promoters activation do not exist in tobacco, assuming that the safener-inducible expression seen in tobacco is driven by the same element which drives safener-inducible expression in maize. It may be that a different element is driving the inducible response in the tobacco transformants. It would be interesting and informative to generate transgenic maize containing the same promoter constructs which were used to generate the tobacco plants. In this way the queries surrounding the fact that the constructs have been tested in a different species would be eliminated. It would also be useful to repeat the EMSA experiments, as described in Chapter 4, using a nuclear protein extract derived from tobacco leaves. If complexes of different mobility were observed with the tobacco nuclear protein extract compared to those observed with the maize nuclear

protein extract, evidence would be added to the argument that the promoter's response to safener was affected by which species it was being tested in.

An alternative explanation for the conflicting data obtained with the Bin6 promoter in transient and stable transformation experiments is that the nature of the assays themselves effects the results. In transient assays a very high copy number of plasmid DNA, which is autonomous from the nuclear DNA, is introduced into the BMS cells. In the tobacco plants the transgene is stably integrated into the host chromatin at lower copy numbers. The properties of plasmid DNA and DNA which is part of a chromatin structure are very different, and may affect the way a promoter functions. For example, the inducibility of the promoter may rely on the interaction of two factors which are brought into proximity by the bending of an intervening stretch of DNA. It may be that in transient assays the Bin6 promoter can achieve this, but not when it is stably integrated into the tobacco genome. The longer length of promoter present in Bin5 may enable such bending to occur, even in stable tobacco transformants. The difference in transgene copy number in the experiments may also effect promoter activity. As it is in high copy number in transient assays it may be better able to compete for *trans* acting factors, assuming that those factors are present in low abundance, compared to when it is present in low copy numbers in the transformed tobacco. As the Bin5 promoter retains inducibility in tobacco transformants while the Bin6 promoter loses inducibility it may be that additional *cis* elements and *trans* acting factors present in Bin5 act co-operatively to enable this promoter to compete more effectively for the factor/s which are necessary for the inducible response. To test the idea that the Bin6 promoter would be active in tobacco plants if it could effectively compete for its cognate *trans* acting factors, the promoter construct could be microinjected at high copy number into cotyledon cells of tobacco plants. Neuhaus *et al.* (1994) used this approach to prove that the *as-1* element in the -90 35S CaMV promoter is not active in cotyledon cells because its cognate *trans* acting factor is present at low levels; by increasing the copy number of the transgene to 50,000 copies per cell gene expression from this promoter was observed. As the transient assay data and stably transformed transgenic tobacco data obtained for the 378 base pair promoter conflict, one can not categorically say that this length of promoter has lost the ability to respond to safener. It is unfortunate that the data obtained in these experiments neither proves, nor disproves, the importance of the putative safener-responsive elements to *GST-27* expression in maize plants.

Even more unexpectedly, the data given in figure 4 indicates that the mutant form of the Bin6 transgene, in which the putative safener-responsive elements are abolished, is responsive to safener. However, combining all the fluorometric data derived from the 3XMUT/Bin6 transformants (see figures 2 and 4) it can be seen that only in two cases do transformants containing this gene show a significant response to safener (both in figure 4). One of these lines, X3MUT.11, does not show a significant level of induction in a repeat experiment (figure

2). Therefore, to clarify the situation, further experiments need to be performed on these transformants, and ideally, more transformants need to be produced. The histochemical analysis of the T2 3XMUT/Bin6 transformants indicates that these plants do not respond to safener and therefore, with the data accumulated so far, it appears more likely that this promoter is not responsive to safener. When a repeat GUS analysis was performed on the T2 generation of -90 and pWT8 transformants very clear data was obtained, and therefore, detailed testing of T2 transformants of Bin5, Bin6, 3XMUT/Bin6 and Bin7 may yield more meaningful data. The advantage of working with T2 transformants is that the plants are all of a similar size and because they can be tested whilst they are still young and the cuticle is not well formed, the safener treatment may be more effective. With T1 plants the difference in time between obtaining the first and last transformants can be a matter of weeks; therefore, in an effort to ensure that the plants are of a similar size, early rooting transformants need to be sub-cloned. By comparing plants which have been sub-cloned and grown in the greenhouse for considerable lengths of time with transformants which have only been out of tissue culture for a few weeks, variables are introduced into the experiment which may serve to confuse the final analysis.

If the 3XMUT/Bin6 promoter was confirmed to be an inducible promoter, it may be hypothesised that the elements which were *in vivo* footprinted are involved in the repression of transcription. This hypothesis would be compatible with the timing of the *in vivo* footprint, which was observed in one region of the promoter 24 hours after safener-treatment, and in another 48 hours after safener-treatment, when gene expression is known to peak at 8 hours after safener-treatment. In its native host it may be that when a threshold level of GST-27 is reached a factor is activated which prevents further synthesis of that gene product. This repressor of transcription may have been what was detected in the *in vivo* footprinting experiments. The EMSA data would be as valid with this hypothesis as they are with the hypothesis that the elements are involved in safener-dependent transcription. This idea would also explain why higher levels of GUS expression appear to be achieved by the mutant promoter compared to the wild type form in transgenic tobacco plants, because a repressor would be prevented from binding to its cognate *cis* element, if that element had been mutated. This would assume that in tobacco the repressing *trans* acting factor is always present and not activated by high levels of the gene product GST-27, and that the *cis* element involved in the activation of this promoter is situated upstream of the DNA which is present in the Bin6 promoter. However, this hypothesis is incompatible with the fact that this promoter, in numerous experiments, has been shown to be safener-inducible in transient assays. In addition, as demonstrated in Chapter 3, the putative elements show significant homology to an element which has been shown to be involved in inducible expression of the *GST1* gene from carnation (Itzhaki *et al*, 1994).

The data shown in figure 6 clearly indicates that GUS expression is maintained in the roots of all the transformants. Therefore, the element which drives root expression both in transgenic tobacco and maize seedlings may lie within 217 base pairs upstream from the transcription start point. However, in maize plants, constitutive root expression from this promoter is only observed in seedling plants while in tobacco root expression is observed in mature plants, so it is not clear if the root expression is similarly controlled at the molecular level. Histochemical analysis of the T2 generation seedlings did not reveal GUS expression in the roots of transformants containing the Bin7 transgene, which is in contrast to the result obtained by fluorometric analysis. A disadvantage with the fluorometric analysis of roots is that soil contamination is inevitable. As soil contains microorganisms, which may contain the native bacterial GUS gene, negative GUS levels in roots may not necessarily appear as negatives. Therefore, further experimentation is required to resolve this point, in which it would be useful to include a control of roots assayed from tobacco which did not contain the GUS gene. In this way background levels from the soil may be determined.

GUS expression was also high in the uninduced roots tested from the T2 generation of pWT8 and -90 transformants (see figure 11). Again, the same reservations about soil contaminants applies to this data, but it was expected to see expression in the roots of these plants as the -90 35S CaMV promoter is known to be active in the roots of tobacco (Lam *et al.,* 1989). However, it was unexpected that the -90 promoter would be responsive to safener in the leaves of treated plants (see figures 9 and 10). The addition of the putative safener-responsive elements did not significantly affect the inducibility of the promoter. However, this does not eliminate the possibility that these elements are involved in safener-responsiveness, but probably indicates that either their positioning in the minimal promoter was such that they failed to function or that other elements are also required for safener-dependent transcription. Considering the results in figure 9, the DNA sequence present in the 35S CaMV 90 base pair minimal promoter was searched for the presence of a sequence similar to the putative safener-responsive elements, which may be responsible for the observed inducibility. Figure 12 represents the sequence of DNA present in the -90 35S CaMV promoter. The DNA represented in bold type, $^{5'}$ATTTCA$^{3'}$, is identical to part of the putative safener-responsive element identified around the footprinted G residue -290, which has the sequence $^{5'}$ATTTCAGA$^{3'}$ (see figure 8, Chapter 3). Therefore, it may be that this region of DNA in the -90 promoter is sufficient to drive safener-inducible expression. However, if this region of DNA was acting as a *cis* element and conferring safener-inducibility to the reporter gene, it would be expected that the -60 minimal promoter would also be safener-inducible, as it also contains this DNA. From figure 8 it appears that this promoter is not safener-inducible, although it would be sensible to repeat this analysis on T2 transformants. Therefore, it may be assumed that the element conferring inducibility to the -90 minimal promoter lies between -60 and -90 base pairs upstream of the transcription start point.

Figure 12 Sequence of the DNA present in the -90 35S CaMV minimal promoter. Underlined sequence represents *as-1* elements (Qin *et al.,* 1994); the sequence shown in bold type represents a region of DNA which shares homology with the putative safener-responsive elements identified in the *GST-27* promoter.

-90ATCTCCACTGACGTAAGGGATGACGCACAT

-60TCCCACTATCCTTCGCAAGACCCTTCCTTC

-30TATATAAGGAAGTTC**ATTTCA**TTTGGAGAG

Loss-of-function and gain-of-function experiments have shown that the *cis* element in the -90 35S CaMV promoter which is responsible for driving root expression, with little or no expression in cotyledons, is the activation sequence-1 (*as-1*) (Lam *et al.,* 1989). This is present between -63 and -83 base pairs upstream of the TSP in the 35S CaMV promoter. It can bind the nuclear factor ASF-1 and the cloned transcription factor TGA1a (Katagiri *et al.,* 1989) at the sites which are underlined in figure 12. It has been shown that expression in the cotyledons is low because there are low levels of the cognate binding factor in this area of the plant; if 10^4 molecules of TGA1a are microinjected into a cotyledon cell, transgenes containing *as-1* linked promoters are activated (Neuhaus *et al.,* 1994). Similarly, if 50,000 copies of a -90 35S:GUS fusion gene are microinjected into a cotyledon cell, GUS expression is observed (Neuhaus *et al.,* 1994). These results may indicate that the application of safener to tobacco causes the plants to express either ASF-1 or a related transcription factor, which can activate this promoter via the *as-1* element. However, computer analysis of the *GST-27* promoter has indicated that an *as-1* like element is present at between -171 and -187 base pairs upstream of the TSP (sequence 5'CGTAAGCGAGGACGTGA3'). Bin7, contains 217 base pairs of DNA upstream from the TSP and includes the *as-1* element, but is not inducible in stable tobacco transformants or in transiently transformed BMS cells. This may indicate that the *as-1* element is not responsible for the safener-inducible expression observed in the -90 35S CaMV promoter as a similar element does not appear to be sufficient to drive safener-inducible expression in the *GST-27* promoter. However, it may be hypothesised that the *as-1* element is important for safener-inducibility in the *GST-27* promoter, but that other elements which are situated further upstream are also required for this function. This would explain why the shorter promoters present in transgenes Bin6 and Bin7 were not safener inducible in tobacco, but the longer promoter in transgene Bin5 retained inducibility. The fact that the *as-1* like element is situated in the DNA which was

subjected to *in vivo* footprinting but that no footprint was observed in this area is not definitive proof that protein/DNA interactions are not occurring at this site. In addition, it is relevant to note that the *as-1* element is implicated in the expression of some plant GSTs (Droog *et al.*, 1993) and that it is similar to the AP-1 element which is involved in the transcriptional regulation of many animal GSTs (Frilling *et al.*, 1992; Okuda *et al.*, 1990; Rushmore and Pickett, 1993). The -90 promoter has also been found to be induced by low levels (2 μM) of salicylic acid (Qin *et al.*, 1994). This activation was found to be via the *as-1* element. It is of interest to note that salicylic acid induces GST-27 mRNA in maize leaves when it is applied at high levels (100 mM) that lead to a phytotoxic effect (Jepson *et al.*, 1994). It is unclear if the inducibility is due to the salicylic acid treatment itself or due to the phytotoxic effects of the treatment. If it is found that salicylic acid can induce the *GST-27* promoter it would seem probable that inducibility was conferred via the *as-1* like element. Therefore, the safener-inducibility of the -90 35S CaMV plants leads one to conclude that the *as-1* like element in the *GST-27* promoter may be involved in safener-dependent transcription.

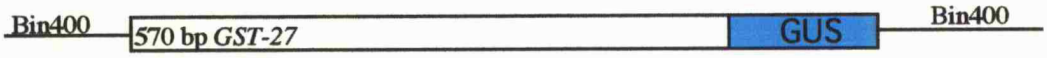
In summary, the stably transformed tobacco experiments have indicated that the region required for safener-inducible expression is situated between 378 and 570 base pairs upstream of the transcription start point. This is in contrast to transient assays performed in BMS cells in which the safener-inducible region was defined to be between 217 and 378 base pairs upstream of the transcription start point. The marked difference in the results obtained from transient assays and from stably transformed plants is thought to be due to the use of different test organisms in each case. Ideally, similar stable transformation experiments need to be performed in maize plants. These were not performed originally as maize transformation is a difficult, time consuming procedure. The mutations to the putative safener-responsive elements introduced into a truncated form of the *GST-27* promoter did not yield informative data, as the equivalent wild type promoter was not inducible in this system. Root expression from the *GST-27* promoter appears to be driven by a region of DNA contained within 217 base pairs upstream of the transcription start point. Finally, the -90 35S CaMV minimal promoter is induced by the application of safener. The addition of the putative safener-responsive elements to the minimal promoter did not increase its inducibility by safener, but this does not prove that these elements are not involved in safener-dependent transcription.

Figure 1

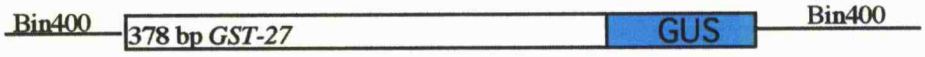
A schematic diagram of the Bin400 constructs used to stably transform tobacco.

pBin5, pBin6 and pBin7, respectively, contain increased 5' deletions of the wild type *GST-27* promoter, fused to the reporter gene GUS, in the context of the binary vector Bin400. Using PCR mediated mutagenesis, five base pair mutations to either three or four of the putative safener-responsive elements were introduced into pBin6 to form 3XMUT/Bin6 and 4XMUT/Bin6. Mutated regions are indicated by red boxes; the numbers of the footprinted G residues (determined by their position relative to the transcription start point) which are present in the putative safener-responsive elements are indicated. A 26 base pair fragment of the *GST-27* promoter, which includes three of the four putative safener-responsive elements, was fused to the -90 35S CaMV minimal promoter to give the construct pWT8. The same 26 base pairs were fused to the -60 35S CaMV minimal promoter to give the construct pWT9.

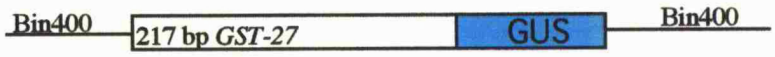
pBin/5



pBin/6

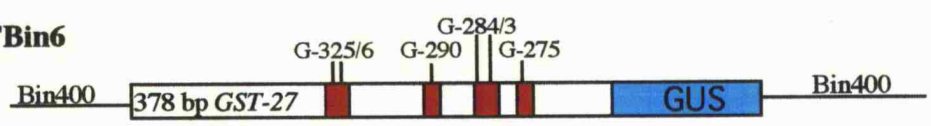


pBin/7

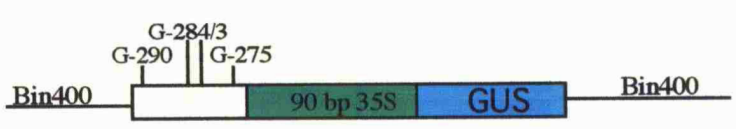


3XMUTBin6

4XMUTBin6



pWT8



pWT9

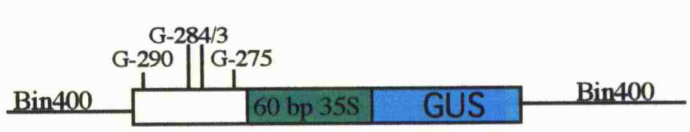


Figure 2

Levels of GUS present in the safener-treated and untreated leaves of T1 transgenic tobacco containing the deleted promoter constructs.

Two leaves from tobacco transformed with either pBin5, pBin6, 3XMUT/Bin6 or pBin7 were analysed for GUS activity both before, and 24 hours after, the application to both sides of the leaf of the dichlormid safener R-29148, at a concentration of 5 g/l. Levels of GUS are given in pmoles 4MU/mg protein/hour. The results for each plant tested are shown and the number identifying each plant and the transgene it contains are given on the x axis. Red bars represent GUS levels achieved in non-treated leaves and green bars represent GUS levels achieved in treated leaves. Bin5 transformants appear to be responsive to safener-treatment, while all other transformants appear to be unresponsive to safener-treatment.

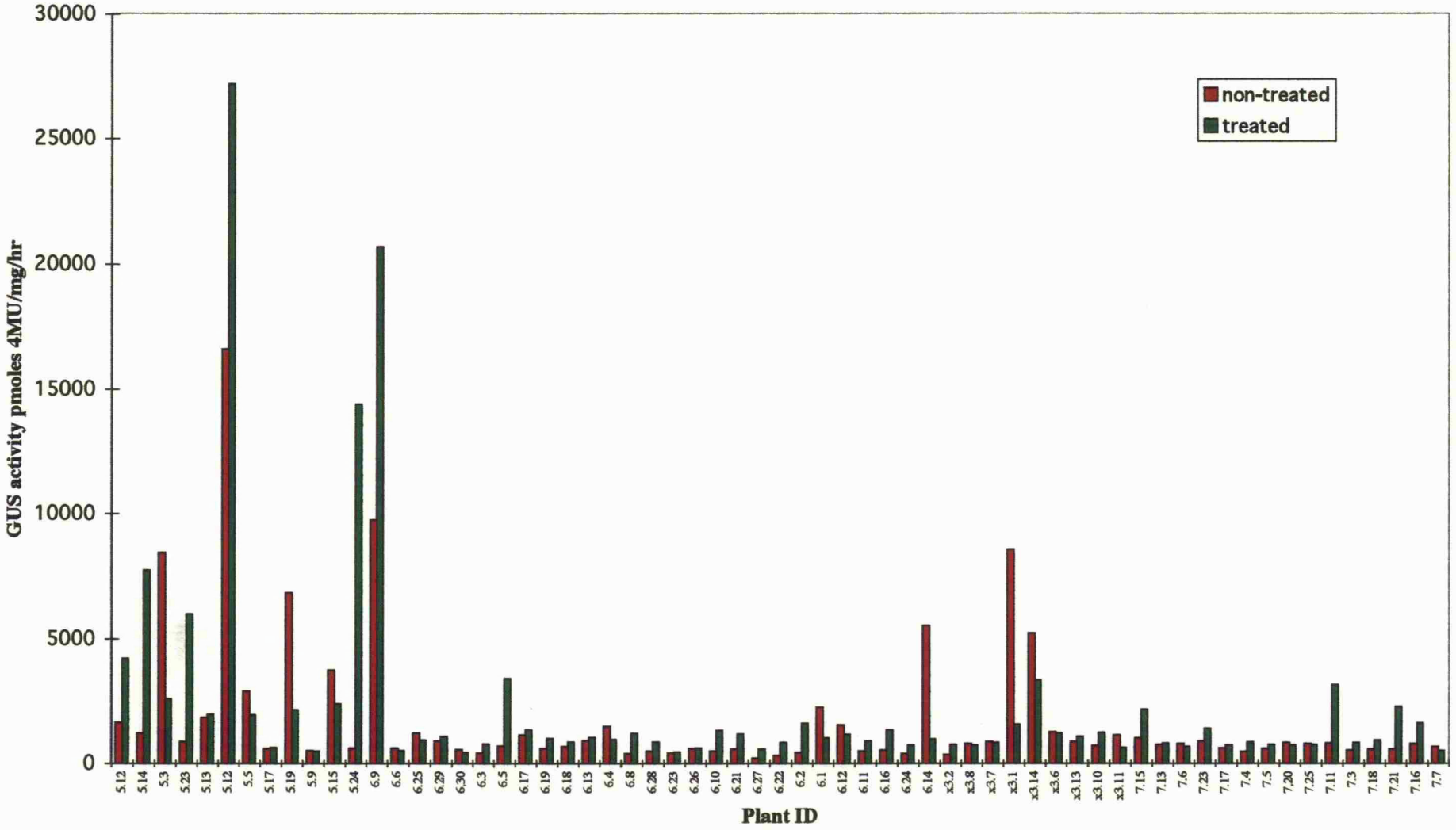

30000
25000
20000
15000
10000
5000
0
GUS activity pmoles 4MU/mg/hr
non-treated
treated
5.12 5.14 5.3 5.23 5.13 5.12 5.5 5.17 5.19 5.9 5.15 5.24 6.9 6.6 6.25 6.29 6.30 6.3 6.5 6.17 6.19 6.18 6.13 6.4 6.8 6.28 6.23 6.26 6.10 6.21 6.27 6.22 6.2 6.1 6.12 6.11 6.16 6.24 6.14 x3.2 x3.8 x3.7 x3.1 x3.14 x3.6 x3.13 x3.10 x3.11 7.15 7.13 7.6 7.23 7.17 7.4 7.5 7.20 7.25 7.11 7.3 7.18 7.21 7.16 7.7
Plant ID

Figure 3

Mean levels of GUS present in the safener-treated and untreated leaves of T1 transgenic tobacco (see figure 2 for individual transformant data). Standard deviation bars are indicated.

Levels of GUS are given in pmoles 4MU/mg protein/hour. Red bars represent GUS levels achieved in non-treated leaves and green bars represent GUS levels achieved in treated leaves. The transgene present in the transformants is indicated on the x axis. The standard deviation bars indicate that there is a high degree of variability in the results. However, when the data from figure 2 is shown as mean levels of GUS achieved per construct, it can be seen that GUS levels achieved in Bin5 transformants are considerably higher than those achieved by all other transformants, and that the promoter in these plants is induced by the safener-treatment. GUS levels in Bin6 and Bin7 transformants are low in comparison, both with and without safener-treatment, indicating that the promoters present in these plants do not respond to safener; GUS levels appear to be marginally higher in the 3XMUT/Bin6 transformants.

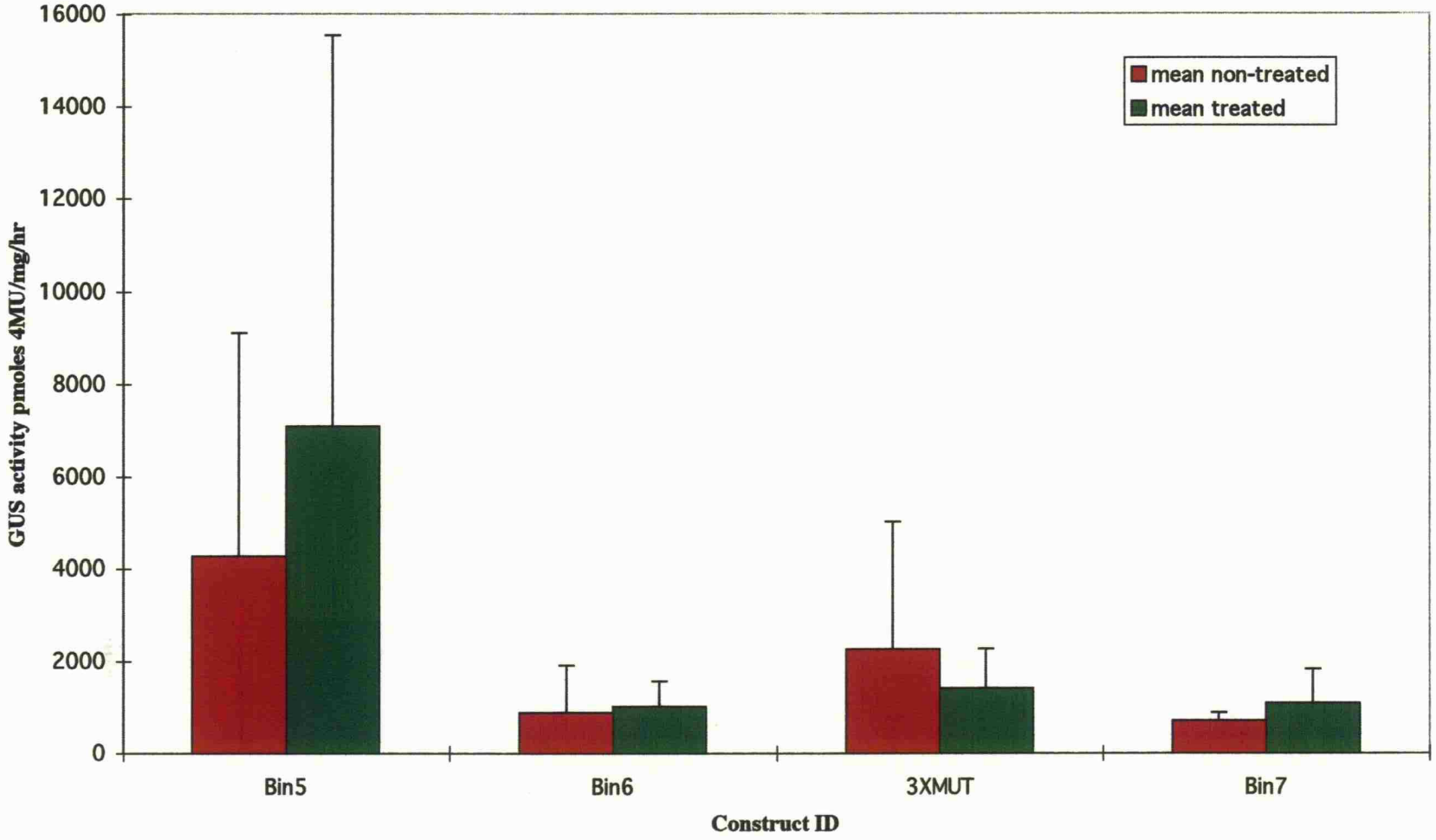

16000
14000
12000
10000
8000
6000
4000
2000
0
GUS activity pmoles 4MU/mg/hr
mean non-treated
mean treated
Bin5
Bin6
3XMUT
Bin7
Construct ID

Figure 4

A repeat experiment to analyse the levels of GUS present in the safener-treated and untreated leaves of T1 transgenic tobacco, containing the transgene Bin5 or 3XMUT/Bin6.

Two leaves from tobacco transformed with either pBin5 or 3XMUT/Bin6 were analysed for GUS activity both before, and 24 hours after, the application, to both sides of the leaves, of dichlormid safener R-29148, at a concentration of 5 g/l. Levels of GUS are given in pmoles 4MU/mg protein/hour. The results for each plant tested are shown and the number identifying each plant and the transgene it contains are given on the x axis. Red bars represent GUS levels achieved in non-treated leaves and green bars represent GUS levels achieved in treated leaves. On the right hand side of the graph the data is represented as the mean GUS level achieved by transformants containing the same transgene with standard deviation bars included. In this experiment, the limited number of Bin5 and X3MUT/Bin6 transformants tested appear to be responsive to safener-treatment.

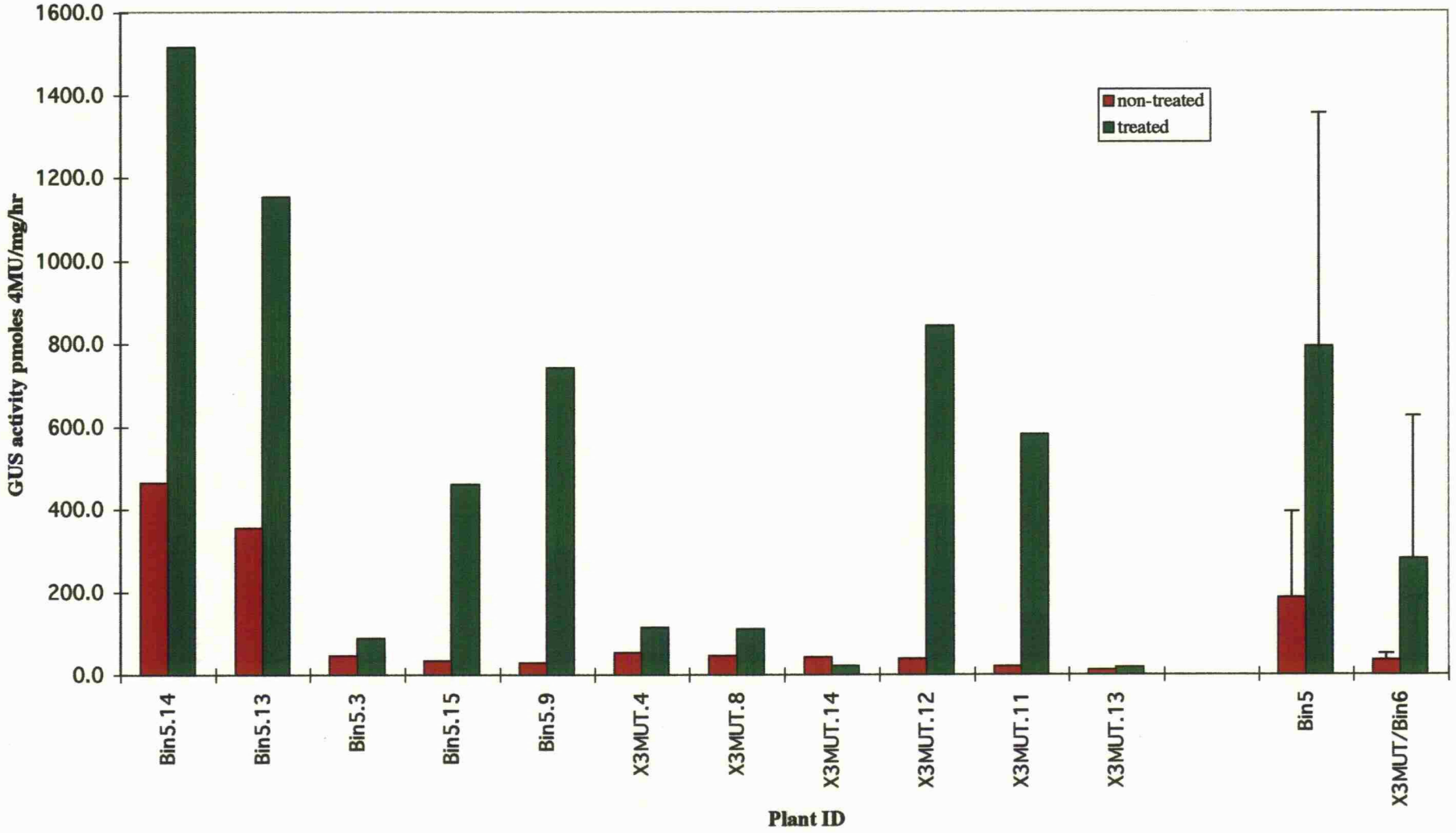
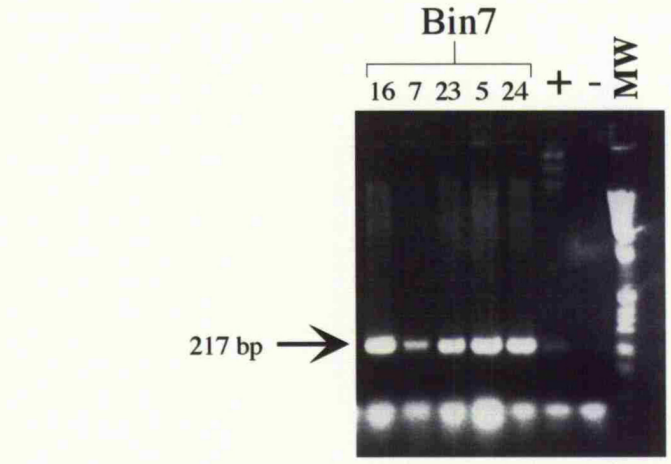

1600.0
1400.0
1200.0
1000.0
800.0
600.0
400.0
200.0
0.0
GUS activity pmoles 4MU/mg/hr
non-treated
treated
Bin5.14
Bin5.13
Bin5.3
Bin5.15
Bin5.9
X3MUT.4
X3MUT.8
X3MUT.14
X3MUT.12
X3MUT.11
X3MUT.13
Bin5
X3MUT/Bin6
Plant ID

Figure 5

Photographs of agarose electrophoresis gels containing the products of a PCR reaction performed on some of the transformed tobacco plants.

Discs of leaf tissue were removed from tobacco leaves using the end of a sterile yellow pipette tip as a borer, and placed into 10 µl of water in a PCR microtitre plate. The plate was twice subjected to a vacuum for 5 minutes. PCR reactions were performed in the same plate using primers pAI2 and pAI3 - these primers were supplied by Ian Jepson from Zeneca, and amplify 217 base pairs of DNA present at the 3' end of the *GST-27* gene. An annealing temperature of 55°C was used to perform 45 cycles of PCR. The positive and negative control lanes are indicated, in which 1 µl of 20 ng of pPUG7 plasmid DNA and 1 µl of water were added respectively. Above each lane the number identifying each transformant and the transgene it contains are given. A PCR product is observed in all of the plants tested, which indicates that they are transformed with a transgene containing part of the *GST-27* promoter.


Bin7
16 7 23 5 24 + - MW
217 bp

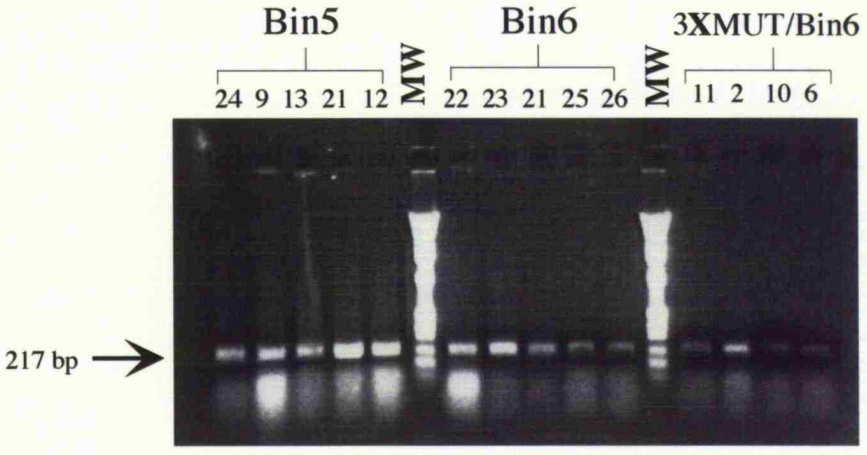

Bin5
Bin6
3XMUT/Bin6
24 9 13 21 12 MW 22 23 21 25 26 MW 11 2 10 6
217 bp

Figure 6

Mean levels of GUS present in the untreated roots of T1 transgenic tobacco. Standard deviation bars are indicated.

Root samples were taken from each of the transformants before they were treated with safener, and analysed for GUS activity. Levels of GUS are given in pmoles 4MU/mg protein/hour. The transgene present in the transformants is indicated on the x axis. The standard deviation bars indicate that there is a high degree of variability in the results, but it appears that GUS expression is present in the roots of all transformants. Therefore, the region of DNA conferring root-expression may be present in the shortest promoter containing 217 base pairs of the *GST-27* promoter.

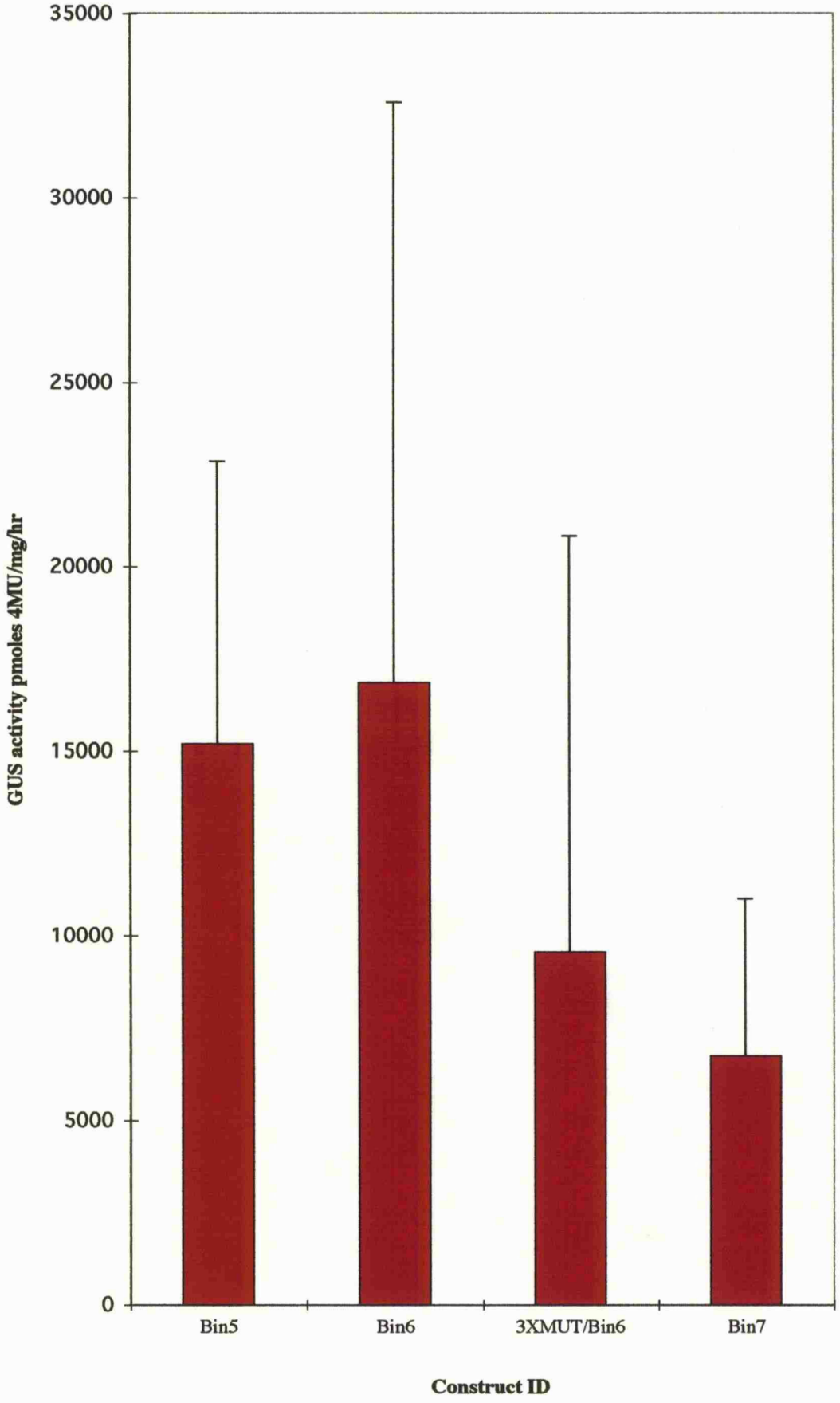

35000
30000
25000
20000
15000
10000
5000
0
GUS activity pmoles 4MU/mg/hr
Bin5
Bin6
3XMUT/Bin6
Bin7
Construct ID

Figure 7

A photograph of T2 tobacco transformants, either non-treated or treated with dichlormid safener, histochemically stained to detect GUS expression.

T2 seedlings from every line were analysed histochemically for GUS expression. Seedlings representative of all those carrying the same transgene were photographed. Those treated with safener are on the left hand side of the photograph (+), and untreated seedlings are on the right hand side of the photograph (-). The transgene present in the seedlings is indicated. Blue colour indicates GUS expression. The full length 35S CaMV promoter was used as a positive control and all parts of these seedlings are blue in the presence and absence of safener. The full length *GST-27* promoter was used as a control to test the inducing conditions in the experiment. It appears that even in the absence of safener the promoter is induced in the roots and cotyledons, possibly because of the stressful conditions the seedlings are subjected to. The Bin5 promoter is active in the cotyledons and the root, especially the root tip, in the presence and absence of safener, but at marginally higher levels in the safener-treated seedling. The only staining observed in the 3XMUT/Bin6 seedlings is very faint and located in the roots. No GUS expression was observed in Bin7 seedlings.

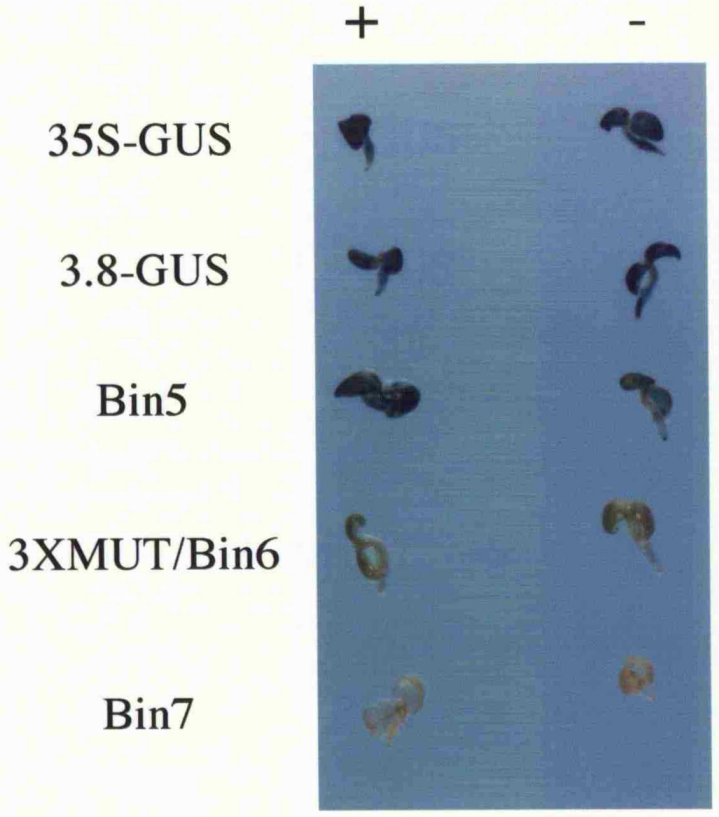

+
-
35S-GUS
3.8-GUS
Bin5
3XMUT/Bin6
Bin7

Figure 8

Mean levels of GUS present in the safener-treated and untreated leaves of T1 transgenic tobacco containing the minimal promoter constructs. Standard deviation bars are indicated.

Levels of GUS are given in pmoles 4MU/mg protein/hour. Red bars represent GUS levels achieved in non-treated leaves and green bars represent GUS levels achieved in treated leaves. The transgene present in the transformants is indicated on the x axis. The standard deviation bars indicate that there is a high degree of variability in the results. However, it is clear that, in this experiment, Bin5 transformants are responsive to safener. The -60 and -90 minimal promoters and the WT9 promoter (26 bp of the *GST-27* promoter including three of the four putative safener-responsive elements fused to the -60 minimal promoter) do not appear to be responsive to safener. The WT8 promoter (26 bp of the *GST-27* promoter including three of the four putative safener-responsive elements fused to the -90 minimal promoter) does show higher levels of GUS expression, both in the absence and presence of safener. These results prompted a retest of the -90 and WT8 transformants.

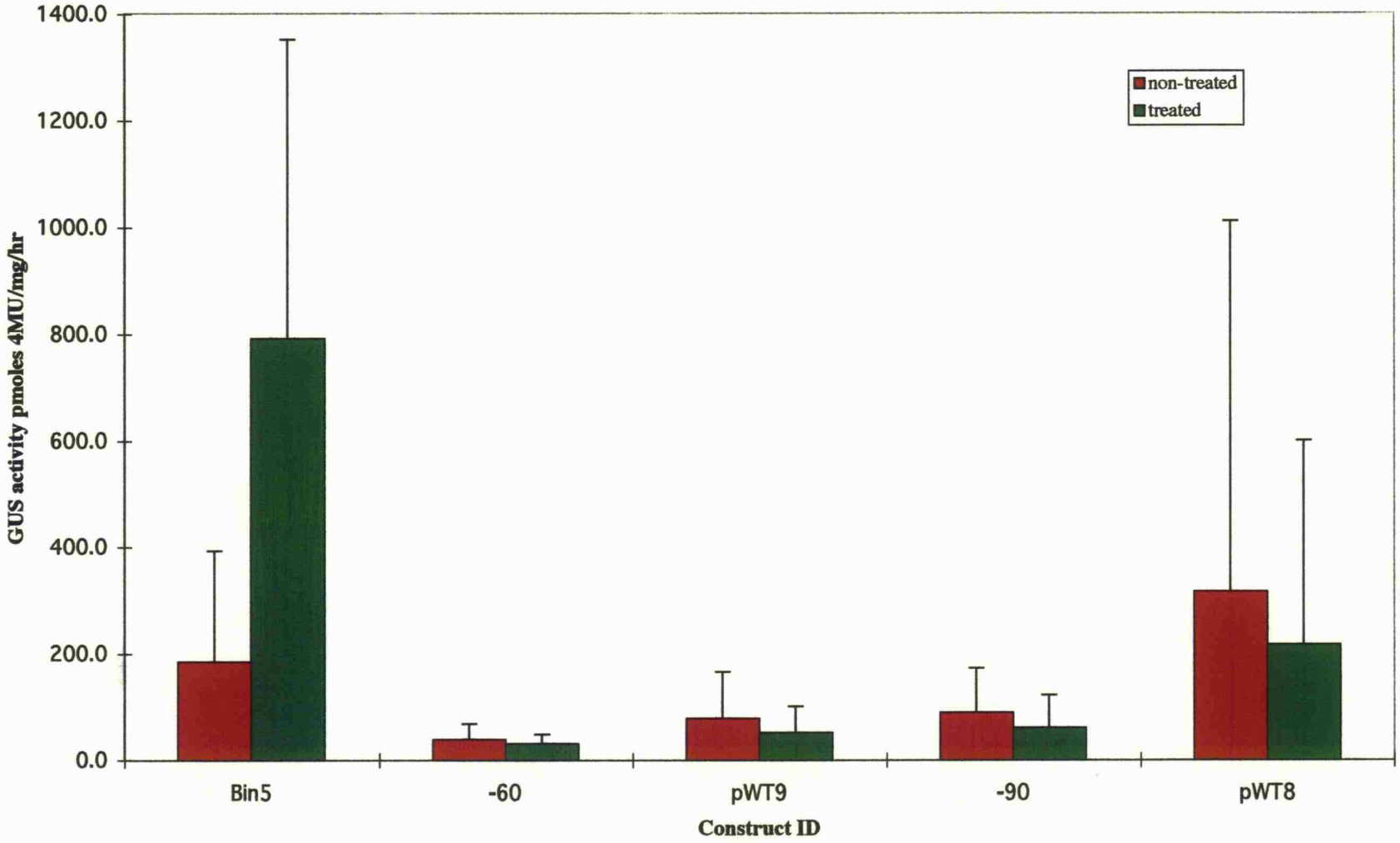

1400.0
1200.0
1000.0
800.0
600.0
400.0
200.0
0.0
GUS activity pmoles 4MU/mg/hr
non-treated
treated
Bin5
-60
pWT9
-90
pWT8
Construct ID

Figure 9

Levels of GUS present in the safener-treated and untreated leaves of T2 transgenic tobacco containing the minimal promoter constructs.

Levels of GUS are given in pmoles 4MU/mg protein/hour. Red bars represent GUS levels achieved in non-treated leaves and green bars represent GUS levels achieved in treated leaves. The number identifying the plant and the transgene it contains is given on the x axis. In this experiment both the WT8 promoter (26 bp of the *GST-27* promoter including three of the four putative safener-responsive elements fused to the -90 minimal promoter) and the -90 minimal promoter transformants appear to be induced to a similar extent by the application of safener.

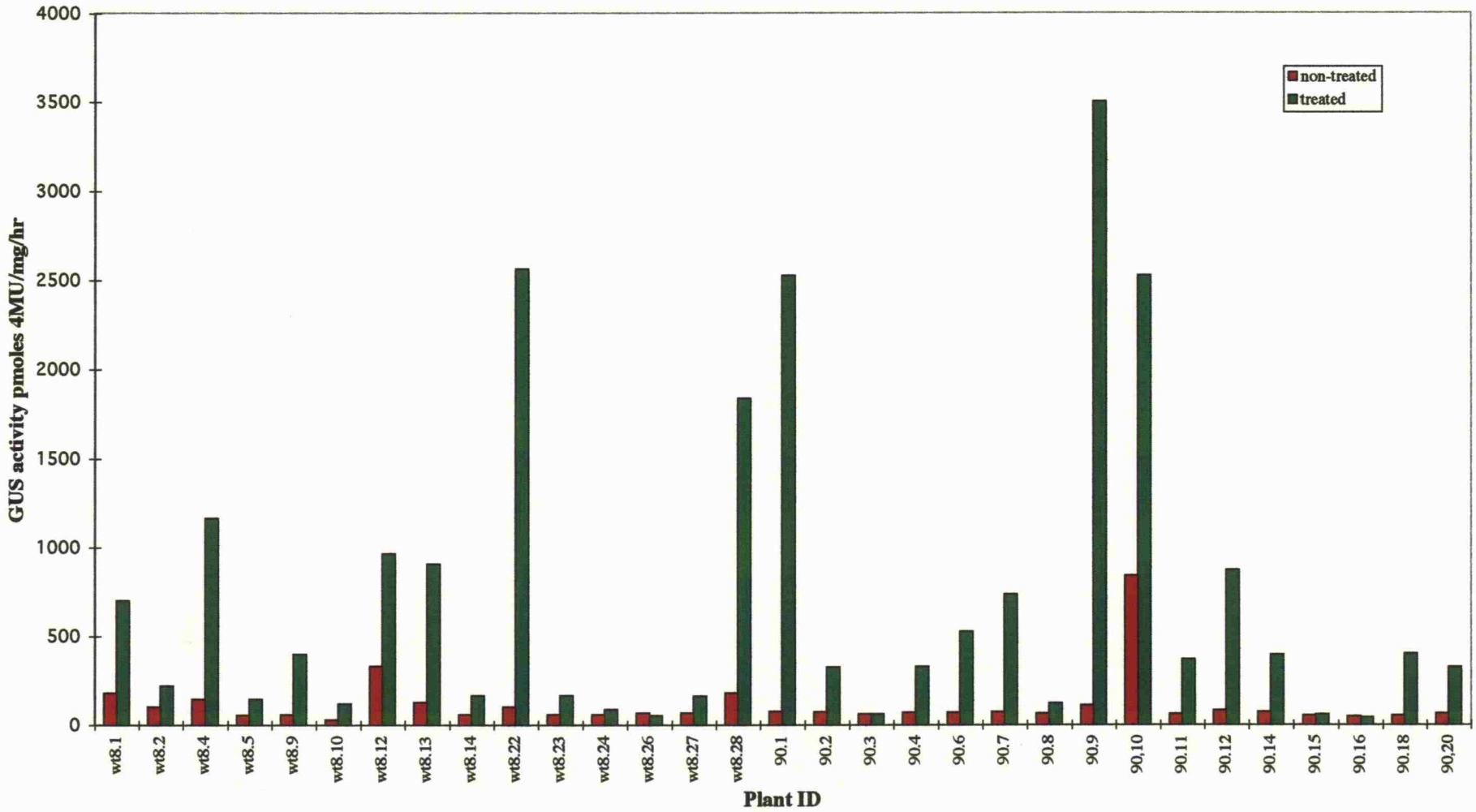
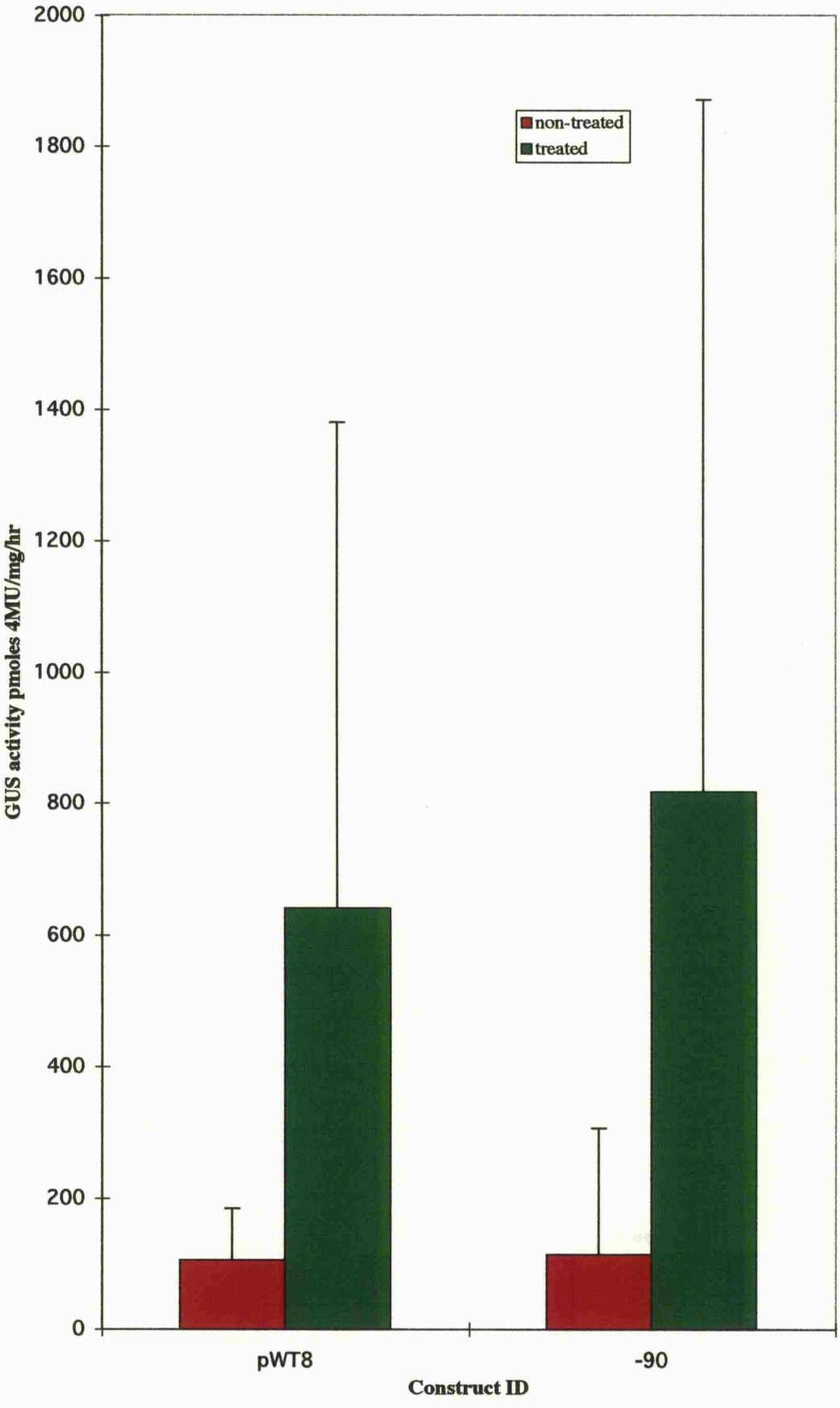

4000
3500
3000
2500
2000
1500
1000
500
0
GUS activity pmoles 4MU/mg/hr
non-treated
treated
wt8.1
wt8.2
wt8.4
wt8.5
wt8.9
wt8.10
wt8.12
wt8.13
wt8.14
wt8.22
wt8.23
wt8.24
wt8.26
wt8.27
wt8.28
90.1
90.2
90.3
90.4
90.6
90.7
90.8
90.9
90,10
90.11
90.12
90.14
90.15
90.16
90.18
90,20
Plant ID

Figure 10

Mean levels of GUS present in the safener-treated and untreated leaves of T2 transgenic tobacco containing the minimal promoter constructs (see figure 9 for individual transformant data). Standard deviation bars are indicated.

Levels of GUS are given in pmoles 4MU/mg protein/hour. Red bars represent GUS levels achieved in non-treated leaves and green bars represent GUS levels achieved in treated leaves. The transgene present in the transformants is indicated on the x axis. The standard deviation bars indicate that there is a high degree of variability in the results. It is clear that both of the promoters present in the transformants responds to safener. The addition of the safener-responsive elements to the WT8 promoter does not increase the response to safener treatment.


2000
1800
1600
1400
1200
1000
800
600
400
200
0
non-treated
treated
GUS activity pmoles 4MU/mg/hr
pWT8
-90
Construct ID

Chapter Seven

GENERAL DISCUSSION

7.1 GENERAL DISCUSSION

The endogenous *GST-27* gene is not normally expressed in the aerial organs of the maize plant but is induced to transcribe at high levels following treatment with herbicide safeners *e.g.* dichlormid (Holt *et al.*, 1995). Understanding the transcriptional regulation of this gene is important for a number of reasons. As this gene's expression is tightly correlated with safener treatment, its' promoter is a good candidate for use as a gene switch. This is particularly true as the inducing agent, dichlormid, is a herbicide safener which is registered for use in agriculture. The full length promoter can be used in gene switch systems, but it is more desirable that the element responsible for conferring inducibility to the gene is identified. In this way the element may be integrated into the natural promoter of the gene over which controlled expression is desired, thereby conferring safener-inducible expression to it. In addition, if the element is identified experiments designed to improve the efficiency of the switch system can be envisaged *e.g.* concatemerisation of the element may lead to higher levels of inducible expression. Although safeners are widely used in agriculture little is known about how they work and therefore another aim of this study was to increase the understanding of their mechanism of action. In animal systems commonality exists between the enzymes and substrates involved in oxidative stress and xenobiotic detoxification. This may be true in plant systems because safeners also induce enzymes and substrates involved in combating oxidative stress and pathogen attack. Therefore, the knowledge relating to safener induction of GST-27 may also be relevant to these other responses. Finally, because interest is growing in using GSTs as bioremediation enzymes an increased knowledge of their regulation would be useful.

In an effort to identify the element which confers safener-inducible expression to the GST-*27* gene a series of 5' deleted promoter constructs fused to the reporter gene β-glucoronidase (GUS) were engineered at Zeneca. These were transiently transformed into BMS cells, using the silicon carbide transformation technique. The results clearly indicated that elements within ~~a region~~ a region of 161 base pairs of the *GST-27* promoter were necessary for it to retain its characteristic safener-inducible expression. This DNA lies between -217 and -378 base pairs upstream of the transcription start point (TSP). Although further deletion promoter constructs could have been engineered in an effort to further localise the position of the safener responsive element, it was thought that an *in vivo* footprinting approach would be the best technique to use in order to locate the element directly in the context of transcriptionally active chromatin. The technique was applied to DNA in the leaves of untreated maize and maize which had been treated with dichlormid safener 6, 24 and 48 hours earlier. The results of the footprinting experiment are shown in Chapter 3, figure 6. It appears that four G residues are protected at 24 hours after safener treatment and that two G residues further upstream are protected at 48 hours after treatment. The footprint does not correlate with the timing of gene expression. The northern blot result shown in Chapter 3, figure 3 indicates that expression of the gene peaks at

eight hours after safener treatment of the leaves. However, as explained in Chapter 3, for a footprint to be visible the majority of cells tested need to be actively expressing the gene of interest *i.e.* the *trans*-acting factor which is involved in the gene's expression needs to be bound to the majority of that gene's promoters, otherwise the footprint will be masked. Therefore, it may be that at eight hours when gene expression is high but no footprint is observed, only a few cells are actively expressing GST-27, but at high levels. At 24 hours, when gene expression is slightly decreased, the footprint is visualised, which may indicate that the majority of the cells are expressing the gene but at lower levels. In Chapter 3, figure 8 it can be seen there is homology between the DNA immediately surrounding the footprinted G residues, which indicates that the *in vivo* footprinting experiment has detected the presence of four variant copies of the same element. The fact that these elements also share homology with ethylene-responsive elements identified in the *GST1* gene from carnation (Itzhaki *et al.*, 1994) and in the tomato *E4* gene (Montgomery *et al.*, 1993) supports the idea that the footprinted regions contain elements which are involved in the regulation of transcription. Although, *GST-27* does not respond to the application of ethylene (Jepson *et al.*, 1994) the elements in the promoter may be functionally equivalent to those in the *GST1* and *E4* promoters. The elements could be regarded as the effectors acting at the end-point of different signal cascades.

In order to determine if the elements identified *in vivo* could interact with nuclear proteins *in vitro* electrophoretic mobility shift assays were performed. The results are given in Chapter 4, figure 2, where it is clear that probes corresponding to the wild type elements interact with nuclear proteins. Mutations to these probes effectively abolish binding, therefore, the DNA/protein interaction is sequence specific. A probe which corresponds to the ethylene responsive element from the *GST1* promoter in carnation gives results which are similar to those obtained when using probes corresponding to the elements in the *GST-27* gene. As all the elements contain similar sequences, these results suggest that the protein/s which interact with the elements present in both promoters, are the same. The results of the competition assays, given in Chapter 4, figure 3, indicate that all the probes can effectively compete for the binding of the protein/s bound to the WT-290 probe. Again, this suggests that the probes are bound to the same protein/s. The fact that nuclear proteins can bind *in vitro* to the areas of DNA identified by *in vivo* footprinting strongly indicates that these regions are *cis* elements to which *trans* acting factors can bind. Although the interacting protein is present in a safener-independent manner, previous studies with other elements involved in inducible transcription have found that interaction with nuclear proteins occurs in the presence or absence of the inducing factor *i.e.* the ethylene responsive elements in the *GST1* promoter (Itzhaki *et al.*, 1994) and the ethylene responsive element in the *E4* gene of tomato (Montgomery *et al.*, 1993). It is thought that in these circumstances ethylene-dependent post-translational modification occurs; a similar process may be occurring with the *GST-27* elements.

The data derived from deletion analysis in transient assays, *in vivo* footprinting and EMSA studies with wild type and mutant probes all support the theory that the elements identified in the *GST-27* promoter may be involved in safener-dependent transcription. Taken together with the fact that the elements are similar to a known GST inducible *cis* element, this theory is even more compelling. However, to prove that this is the case *in vivo* studies in which mutation of the elements leads to a loss of inducibility, or addition of the elements to a minimal promoter leads to a gain of inducibility, needed to be performed. To this end, constructs were engineered for testing in transiently transformed BMS cells. Mutations were introduced into the promoters of pPUG5, which contains 570 base pairs of DNA upstream from the TSP and pPUG6, which contains 378 base pairs of DNA upstream from the TSP, both of which had previously been shown to be safener-responsive using this test system. In Chapter 5, figure 3 it can be seen that individual mutations to the elements surrounding footprinted G residues G-290, G-326 and G-284 combined with G-275, appear to have little or no effect on the safener-responsiveness of the promoter. However, because there are four elements, which from the EMSA studies are assumed to be able to interact with the same *trans* acting factors, when just one of the elements is mutated the other elements may continue to function. Therefore, it is not surprising that promoters in which one of the four elements is mutated retain safener inducibility. A decrease in the fold induction may have been expected, but the results obtained when using this assay system are so variable that subtle differences in promoter activity are not detectable. A construct in which three of the four elements are mutated, also appears to retain inducibility. Again, the remaining one element may be sufficient to drive inducible expression. However, in this last experiment, as a control plasmid also appeared to show safener-inducible expression, and because the results obtained with this assay were variable and rarely statistically significant, it was decided to continue testing in an alternative system.

Stable transformation of tobacco was chosen as the alternative system. As is discussed in the introduction section of Chapter 3, other transient assay systems, including tobacco protoplast transformation and particle bombardment of BMS cells, proved ineffective to assess the inducibility of this promoter because background levels of gene expression were too high. Although stable transformation of tobacco is time consuming and can give variable results, it was hoped that this system would yield results which would enable a more detailed analysis of the response of the various promoters to safener treatment. In addition, during the course of this study it had been discovered that the full length *GST-27* promoter was active and responsive to safener in this system. However, difficulties encountered when using this test system included high variability in gene expression between plants and obtaining results which were not always statistically significant. The fact that the results were often not statistically significant does not mean that they lack biological significance. Variable results were expected to some extent, as the transformation technique does not control the site of transgene integration in the genome, nor does it control how many copies of the transgene integrate. Both of these factors can affect the

subsequent transgene expression (Flavell, 1994; Ow and Medberry, 1995). However, the results presented in Chapter 6 indicate the general levels of gene expression which may be expected from the promoters tested, but repeat experiments would be sensible to consolidate the data.

It appears from figures 2, 3 and 4 in Chapter 6 that the 570 base pairs of *GST-27* promoter present in Bin5 are responsive to safener treatment in stably transformed tobacco; in figure 4 a four-fold induction in GUS expression is observed after safener-treatment of transformants containing this transgene. The 378 base pairs of promoter present in Bin6 do not appear to be responsive to safener when stable transformed into tobacco plants. This is in contrast to data shown in Chapter 5, figure 3 and in Chapter 3, table 1, in which this promoter is inducible when tested in transiently transformed BMS cells. The conflicting results obtained when testing the same promoter may be a reflection on the different assay systems used. In transient transformation the copy number of the transgene in each cell is high and it is present in a plasmid which is autonomous from the nuclear DNA. In stable transformation experiments the transgene is stably integrated into chromatin at low copy numbers. These differences may affect the way a promoter responds to safener treatment. For example, if the factor responsible for inducible expression of the *GST-27* promoter is present in low abundance, the high copy number of the promoter present in transient assays would allow it to effectively compete for its cognate factor. The fact that the longer promoter present in Bin5 is inducible in tobacco transformants, while the Bin6 promoter is not, may indicate that extra elements present in Bin5 act co-operatively leading to its inducible expression. These elements may include more copies of the safener responsive elements, although direct repeats of these elements were not identified upstream in the promoter. An alternative explanation for the conflicting results is the species difference in the two test systems used. In transient transformation assays the native monocotyledonous host, maize, was used, while the dicotyledonous tobacco species was used in stable transformation experiments. It is known that promoters from a monocotyledonous species can act differently in dicotyledonous species (Schledzewski and Mendel, 1994; Wilmink *et al.*, 1995), and this may reflect different *trans* acting factors which are present. It may even be that inducible expression in tobacco is via a different element to that which confers inducible expression in maize.

The mutations of the putative safener responsive elements were introduced into the Bin6 promoter, which, as explained above, was found to be non-inducible in tobacco transformants. Therefore, their effect on the safener-responsiveness of the promoter could not be analysed. However, if the mutations had an effect at all, it was to increase levels of expression of the reporter gene, as is indicated in chapter 6, figures 1, 3 and 4. These result may indicate that the elements are the binding site for a factor which represses the transcription of the *GST-27* gene. This factor may be active when a threshold level of GST-27 is reached, and may therefore

function as a negative feedback element on the gene's transcription levels. It may be important to control the activation and inactivation of this gene as high levels of GST-27 may lead to a depletion in the cell's glutathione supplies. As well as being involved in the detoxification of xenobiotic compounds, glutathione is important as an antioxidant (Field, 1996; Meister and Anderson, 1983) and its availability in the cell needs to be controlled. If these elements were repressors, the *cis* element which confers inducibility to the gene must lie between -378 and -570 base pairs upstream of the transcription start point. However, further analysis of the 3XMUT/Bin6 transformants needs to take place before it is confirmed whether this promoter is induced by the application of safener.

The minimal promoter experiments in stably transformed tobacco did not yield data which elucidated the role the elements play in the activation or repression of transcription. However, because the addition of the putative safener-responsive elements to a minimal promoter did not affect transcription levels compared to those achieved by the minimal promoter on its own, it does not mean that these elements do not have a role to play in transcription. The positioning of elements in a promoter is important to their function and it is likely that, in the context of the minimal promoters, the spatial organisation was such that the elements failed to function. It may also indicate that other elements are involved in safener-dependent transcription. However, the experiments did indicate that the -90 35S CaMV minimal promoter, on its own, was induced by the application of safener. It is not clear whether the element which confers inducibility is the activation sequence-1 (*as-1*) element present in this promoter (Qin *et al.*, 1994). An *as-1* like element is present in the shortest *GST-27* promoter fragments of Bin6 and Bin7, both of which are not inducible. This either means that the *as-1* element is not responsible for the safener-inducibility in the *GST-27* promoter or indicates that this element has to interact with upstream elements in the *GST-27* promoter in order to function. The fact the *as-1* like element was present in the DNA which was subjected to *in vivo* footprinting analysis but revealed no footprint is not definitive proof that protein/DNA interactions are not occurring at this site. Low levels (2 μM) of salicylic acid induce gene expression from the -90 minimal promoter and are known to achieve this effect via the *as-1* element (Qin *et al.*, 1994). High levels (100 mM) of salicylic acid have also been shown to induce expression of the *GST-27* gene (Jepson *et al.*, 1994), although it is not clear if this is due to the treatment or the treatment's phytotoxic effect. Therefore, two agents, safener and salicylic acid, can induce the *GST-27* and 35S CaMV -90 promoters. It is known that salicylic acid induction of the -90 promoter operates via the *as-1* element, and it could be reasoned that the same element is involved in this promoter's safener-responsiveness. Therefore, the *as-1* like element in the *GST-27* promoter may be involved in safener-dependent transcription. Further work will be required to assess the validity of this idea.

Transient transformation of BMS cells and stable transformation of tobacco plants have neither

proved nor disproved the role the putative elements play in safener-dependent transcription. Other researchers have been successful in fusing inducible *cis*-acting elements from plant GSTs to minimal promoters and thereby conferred inducibility to a heterologous system (Itzhaki *et al.*, 1994; van der Zaal *et al.*, 1996). However, promoters are complicated and their response may be the result of interplay between a number of *cis* elements (Benfey *et al.*, 1990; Puente *et al.*, 1996). The *GST-27* promoter is responsive to safeners, phytotoxic effects and senescence (Jepson *et al.*, 1994). In addition, it is spatially and developmentally controlled. As this promoter is responsive to a number of factors it may be expected to be complicated in terms of its *cis*-acting elements. Although only one *cis* element involved in the expression of the GST Ya subunit in mouse has been identified (Friling *et al.*, 1987) five elements have been identified in the promoter of the same gene in rats (Rushmore *et al.*, 1990; Rushmore and Pickett, 1990). Therefore, this suggests that in animal GSTs the mechanism of control of expression can be complicated. This is also true of some plant GSTs: for example, the promoter of the soybean *Gmhsp*26-A gene contains four putative *cis* elements. Nevertheless, although the control of GST expression can be complex, the data presented in this study (including deletion analysis, *in vivo* footprinting and EMSA studies on putative elements which share homology with an inducible element from the *GST1* promoter of carnation) provide strong evidence that these elements are involved in safener-dependent transcription.

7.2 FUTURE WORK

Time constraints on this research has meant that important experiments have been omitted. Initially, the transgenic tobacco should be screened using Southern blot analysis to ensure that the whole transgene is present in each plant as expected and to determine its copy number. This data may help to explain the results of GUS assays performed on the safener treated and untreated leaves. It would be useful to repeat the GUS assays on T2 tobacco transformants in order to confirm the results observed in T1 transformants, and especially in order to determine whether the 3XMUT/Bin6 promoter is inducible. If this promoter was found to be inducible it may indicate that the footprinted regions are involved in the repression of *GST-27* transcription. Therefore, to locate the position of the element/s which confer safener inducibility to the promoter, *in vivo* footprinting could be performed on the DNA present between the Bin5 and Bin6 promoters (*i.e.* -378 and -570 base pairs upstream of the TSP). If the 3XMUT/Bin6 promoter was found to be non-inducible it may be assumed that the *in vivo* footprinted elements are involved in safener-dependent transcription and the fact that the promoter in which they are present appears to be non-inducible in tobacco transformants reflects the fact that it is in a heterologous species. To assess if tobacco contains *trans* acting factors which can interact with the putative elements a series of EMSA experiments could be performed using probes identical to those used in the EMSA experiments described in Chapter 4. If the probes produced complexes of different mobility when incubated with nuclear extract prepared from tobacco

leaves compared to those visualised when incubated with nuclear extract prepared from maize leaves it may be assumed that the difference in the promoter behaviour observed in the two test systems was a result of the fact that the species contain different *trans* acting factors. This would justify an experiment in which maize plants were stably transformed with the series of deleted promoter constructs which were used in the transformation of tobacco, as described in Chapter 6. In order to test the theory that the safener-response of the transgenic tobacco carrying the 35S CaMV -90 minimal promoter operates via the *as-1* element, experiments could be performed on wild-type and mutant versions of this promoter in transgenic tobacco. If a -90 minimal promoter, containing a mutation of the *as-1* element, fails to respond to safener then it could be assumed that this element is important to safener-dependent transcription from this promoter. If this were found to be the case then future work on the safener-inducibility of the *GST-27* promoter should focus on the *as-1* like element which is present in it. The relative importance of the putative safener-responsive elements and the *as-1* like element may also be determined by fusing the 161 bp of DNA present between -217 and -378 upstream of the TSP (*i.e.* containing the putative *cis* elements but not the *as-1* like element) to a minimal promoter. If this construct is tested in transient assays in BMS cells and found to retain inducibility, this would indicate that the *as-1* like element is not important to the safener-responsiveness of the promoter. In this case further research should concentrate on these 161 bp of DNA.

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