REGULATION OF *ARABIDOPSIS* TGA TRANSCRIPTION FACTORS BY CYSTEINE RESIDUES: IMPLICATION FOR REDOX CONTROL

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ABSTRACT

The *Arabidopsis* TGA family of basic leucine zipper transcription factors regulate the expression of pathogenesis-related genes and are required for resistance to disease. Members of the family possess diverse properties in respect to their ability to transactivate and interact with NPR1, the central regulator of systemic acquired resistance in *Arabidopsis*. Two TGA factors, TGA1 and TGA2, have 83 % amino acid similarity but possess differing properties. TGA1 does not interact with NPR1 but is able to transactivate, while TGA2 interacts with NPR1 but is unable to transactivate. This study uses these two TGA factors to identify amino acids that are responsible for their function.

Four cysteines residues within TGA1 were targeted for study by site-directed mutagenesis and the resulting mutants were tested for interaction with NPR1 in yeast. The construct containing a mutation of cysteine 260 (Cys-260) interacted well with NPR1, while those with mutations at Cys-172 or Cys-266 interacted poorly. The Cys-260 mutant also displayed the greatest decrease in transactivation potential in yeast, while mutation of Cys-172 or Cys-266 resulted in smaller decreases. Mutation of Cys-287 had no effect on NPR1 interaction or transactivation. Combining various point mutations in a single protein did not increase NPR1 interaction or transactivation levels, indicating that Cys-260 is crucial for regulating TGA1 properties. Cysteines possess the unique ability of forming reversible disulfide bonds which have been shown to regulate several mammalian cellular processes. The observation that mutation of a single TGA1 cysteine (Cys-260) greatly alters the protein's properties provides a convincing

argument that oxidoreduction of this residue is important for its regulation, possibly through the formation of a disulfide bond with either Cys-172 or Cys-266.

To test whether other members of the TGA family could be regulated by oxidoreduction, several TGA2 constructs were created that introduced Cys at positions corresponding to those found in TGA1. When tested in yeast none were able to transactivate but continued to interact with NPR1.

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LIST OF ABBREVIATIONS

°C	degrees centigrade
%	percentage
µ	micro-
A	asparagine
ACD2	accelerated cell death2
ARD	ankyrin repeat domain
as-1	activating sequence 1
Asn	asparagine
BABA	β-aminobutyric acid
bla	β-lactamase gene
BTB/POZ	broad-complex, tramtrack, and bric-a-brac/pox virus and zinc finger
BTH	benzol (1,2,3) thiadiazole-7-cabothionic acid S-methyl ester
bZIP	basic leucine zipper
C	cysteine
c	centi-
CaMV	Cauliflower Mosaic Virus
cat	chloramphenicol acetyltransferase gene
Cruc	cruciferin
Cys	cysteine
DB	DNA-binding domain
DNA	deoxyribonucleic acid
EDTA	ethylenediamine-tetraacetic acid
EMSA	electromobility shift assay
f	femto-
F	phenylalanine
FD	Faraday
g	gram(s)
GST-6	glutathione S-transferase6
GSH	reduced glutathione

GSSG	oxidized glutathione
GUS	β-glucuronidase
HR	hypersensitive response
hr	hour(s)
INA	2,6-dichloroisonicotinic acid
ISR	induced systemic resistance
l	litre(s)
LacZ	β-galactosidase gene
Leu	leucine
Li	lithium
LS	linker scan
K	kilo-
m	milli-, meter(s)
M	molar
min	minute(s)
mol	moles
MU	methyl-umbelliferone
MUG	methyl-umbelliferyl-β-D-glucuronide
n	nano-
NH1	NPR1 homolog1
NIM1	non-inducible immunity1
NLS	nuclear localizing signal
npt	neomycin phosphotransferase gene
NPR1	non-expresser of pathogenesis-related genes1
nos	nopaline synthase
OBF	octopine binding factors
ocs	octopine synthase
OD_x	absorbance at wavelength (x)
PEG	polyethylene glycol
PCR	polymerase chain reaction
pH	percentage of hydrogen

PR	pathogenesis-related
pv	pathovar
ROS	reactive oxygen species
RNAi	RNA interference
rpm	revolutions per minute
S	serine
SA	salicylic acid
SAR	systemic acquired resistance
Ser	Serine
SD	synthetic dextrose
Sd	standard deviation
SDM	site-directed mutagenesis
SDS	sodium dodecyl sulphate
sec	second(s)
TA	transcriptional activation domain
TAE	Tris, sodium acetate, and EDTA
Ti	tumour-inducing
TE	Tris buffer and EDTA
Trp	tryptophan
UAS	upstream activator sequences
uidA	β-glucuronidase gene (GUS)
V	volt(s)
VIGS	virus-induced gene silencing
X-GAL	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

CHAPTER 1: INTRODUCTION

All plants possess a variety of mechanisms that prevent the establishment of disease by pathogens. The first line of defence includes constitutive barriers, such as waxes, lignin, suberin and phytoanticipins, which the pathogens must overcome in order to infect the plant tissue. In many cases these barriers are sufficient to repel the pathogen, but in the event that the constitutive barriers fail another line of resistance is required. Several forms of induced responses, triggered during or immediately after pathogen infection, exist and the results are wide and varied. These secondary defence reactions include the production of antimicrobial compounds, programmed cell death (in the form of the hypersensitive response, HR) and cell wall modifications (Mysore and Ryu, 2004; Veronese et al., 2003).

It is important to consider that the crux of induced disease responses lies in the ability of the plant to coordinate the transcription of perhaps thousands of genes with the sole purpose of achieving a disease resistant state (Katagiri, 2004). In *Arabidopsis* the identification of genes affected transcriptionally by disease or by chemical elicitors, which induce a disease response, has increased substantially by the advent of large-scale gene expression profiling studies (Katagiri, 2004; Wan et al., 2002). These studies indicate that as much as 25% of the genes identified in *Arabidopsis* are affected at the

transcriptional level by pathogen infection. Although many studies are underway to explore these genes, assigning a function, with regard to defence responses, will take many years.

The study of transcription factors that regulate these genes is just one of the many methods employed to study the phenomenon of transcriptional regulation in response to pathogen challenge. Plants devote many genes to the regulation of transcription, with over 1500 transcription factors encoded by the *Arabidopsis* genome alone (Riechmann et al., 2000). Numerous transcription factors have been identified as possessing a role in plant disease resistance (Euglem, 2005; Rushton and Somssich, 1998). Understanding the targets and the regulatory mechanisms of these transcription factors during pathogen infection should facilitate studies targeting key genes in defence response.

CHAPTER 2: LITERATURE REVIEW

2.1 Induced disease resistance

In addition to the immediate defence responses described in the introduction, exposure to a pathogen can also confer broad spectrum long-term resistance against subsequent infection. These responses have been termed induced disease resistance and have been observed and studied in plants for a century. Three forms of induced disease resistance have been identified in plants, each unique in the spectrum of pathogen protection and the gene expression cascade resulting in resistance.

2.1.1 Systemic acquired resistance (SAR)

The most commonly referred to and studied form of induced disease resistance is systemic acquired resistance (SAR). SAR, identified in a variety of monocots and dicots, occurs in response to microbes able to cause necrosis, be it the HR or as part of the disease process (Durrant and Dong, 2004; Sticher et al., 1997). The resulting enhanced resistant state of systemic plant tissues extends to a broad range of pathogens including bacteria, fungi and viruses (Sticher et al., 1997).

A key element to the establishment of SAR in a plant is the accumulation of the small stress molecule salicylic acid (SA; Ryals et al., 1996). Mutants in which SA production or accumulation is inhibited are compromised in their ability to mount SAR (Dewdney et al., 2000; Nawrath and Metraux., 1999). Conversely, exogenous

application of SA or SA analogs such as benzol (1,2,3) thiadiazole-7-cabothionic acid S-methyl ester (BTH) or 2,6-dichloroisonicotinic acid (INA) instigates a SAR-like reaction and will restore the SAR pathway in mutants deficient in SA (Parker et al., 1996; Uknes et al., 1992; Ward et al., 1991).

2.1.1.1 PR Genes

SAR is characterized at the molecular level by the accumulation of a group of proteins called pathogenesis-related (PR) proteins (Uknes et al., 1992; Ward et al., 1991). PR proteins are structurally diverse and possess various functions within, or outside of, the plant cell (Van Loon and Van Strien, 1999). While several PR proteins have been identified to possess antimicrobial properties (e.g. chitinases, β-1,3-glucanases), a function for the majority of PR proteins in response to disease has not yet been determined.

PR proteins begin to accumulate in local and systemic tissues soon after pathogen infection and levels may increase for several hours or days (Van Loon and Van Strien, 1999). Because they are reliably induced in pathogen infection, transcripts of PR proteins are widely used as molecular markers for SAR. It is important to note that the type of PR proteins and the level of their accumulation are specific to the plant-pathogen interaction; therefore marker genes must be determined on an individual plant-pathogen basis. In *Arabidopsis*, *PR-1* is widely used as a marker of SAR (Durrant and Dong, 2004). It is the coordinated expression of multiple *PR* genes that is thought to account for the broad-spectrum resistance observed as SAR. Mutants in which *PR* gene expression is abolished, like *npr1* (*non-expresser of PR genes1*; Cao et al., 1994), display increased susceptibility to disease whereas mutants that constitutively express

PR genes like *acd2* (*accelerated cell death2*; Dietrich et al., 1994), display enhanced resistance to disease.

2.2 NPR1

Screening for mutants has proven to be a useful technique for identifying genes involved in the SAR signalling pathway. The study of several SAR-deficient mutant phenotypes led back to the same gene designated *npr1* or *nim1* (*non-inducible immunity1*; Shah et al., 1997; Glazebrook et al., 1996; Delaney et al., 1995; Cao et al., 1994). In addition to being compromised in SAR, *npr1* mutants are also defective in basal resistance, Induced Systemic Resistance (ISR, see section 2.3), and gene-for-gene resistance against specific races of pathogens (Pieterse et al., 1998).

Two features of *npr1* mutants help place the gene in the complicated defence gene signalling cascade. The observation that *npr1* mutants accumulate normal levels of SA and that the mutant phenotype cannot be rescued by exogenous SA (Delaney et al., 1995; Cao et al., 1994) indicates that NPR1 is located downstream of this metabolite in the signalling pathway. *npr1* mutants also do not activate *PR* genes or accumulate PR proteins subsequent to treatment with SAR elicitors including pathogen infection (Delaney et al., 1995; Cao et al., 1994). This indicates that *NPR1* is located upstream of *PR* gene expression in the signalling pathway.

The *NPR1* gene encodes a protein containing two protein-protein interaction motifs: a BTB/POZ (Broad-Complex, Tramtrack, and Bric-a-brac/Pox virus and Zinc finger) as well as an Ankyrin Repeat Domain (ARD; Cao et al., 1997; Ryals et al., 1997). Several mutant alleles have been mapped to conserved amino acids found within the ARD of NPR1, suggesting an important role for this motif during disease resistance.

Only one mutant allele has been mapped to an amino acid within the BTB/POZ domain (*npr1-2*), but as the particular amino acid affected is located within a non-conserved region of the BTB/POZ (Després, unpublished observation) no conclusions can be drawn about the significance of the motif during disease resistance.

Overexpression studies of the *Arabidopsis NPR1* gene in *Arabidopsis*, tomato and rice all report plants with increased resistance to a range of pathogens (Lin et al., 2004; Chern et al., 2001; Friedrich et al., 2001; Cao et al., 1998). The levels of NPR1 in these transgenic lines do not necessarily correlate to the level of disease resistance observed, therefore it has been proposed that a threshold level of NPR1 may be required for enhanced disease resistance (Lin et al., 2004; Chern et al., 2001). Most of these transgenic lines do not constitutively express *PR* genes, therefore the observed enhanced resistance appears to be due to either stronger *PR* gene expression (Cao et al., 1998), or to faster *PR* gene expression (Friedrich et al., 2001) in response to pathogen infection. In contrast, rice plants overexpressing *NH1*, a homolog of *NPR1*, do constitutively express *PR* genes (Chern et al., 2005). Rice plants overexpressing *(At)NPR1* or *NH1* are unique in that under certain growth conditions, spontaneous disease-like lesions develop and hydrogen peroxide accumulates while SA levels may increase or decrease (Chern et al., 2005; Fitzgerald et al., 2004). The changes observed in the SA levels may indicate that NPR1 is involved in the perception and modulation of SA.

The lack of constitutive *PR* gene expression in *NPR1* overexpressors may be linked to the cellular localization of the protein in non-elicited cells. In resting cells NPR1 is found throughout the cell, but when the cell is treated with an elicitor NPR1 is translocated to the nucleus (Després et al., 2000; Kinkema et al., 2000). Nuclear

localization of NPR1, which is mediated by a Nuclear Localization Signal (NLS) found in the C-terminus, is required for *PR* gene expression (Kinkema et al., 2000). NPR1 does not contain a recognizable DNA binding motif suggesting that it does not function as a transcription factor. In fact interaction between NPR1 and a group of basic leucine zipper (bZIP) transcription factors called TGA factors is required for PR gene expression and SAR (Fan and Dong, 2002; Després et al., 2000; Zhou et al., 2000; Zhang et al., 1999; see section 2.4 for information on TGA factors). Translocation of NPR1 to the nucleus and interaction with TGA factors appear to be regulated posttranslationally through redox changes of conserved cysteine (Cys) residues (Mou et al., 2003; see section 2.5.2).

2.3 Other forms of induced disease resistance

Another well studied form of induced resistance is termed Induced Systemic Resistance (ISR). ISR occurs in response to soil borne rhizobacteria which triggers pathogen resistance in aerial plant parts (Van Loon et al., 1998). ISR has been identified in a variety of plant species and provides resistance against a variety of pathogens (Ton et al., 2002; Pieterse et al., 1998; Van Loon et al., 1998). ISR is also markedly different from SAR in that SA is not required for resistance; rather the response requires jasmonic acid and ethylene, and that *PR* gene expression is not observed. Interestingly, ISR does require functional NPR1 (Pieterse et al., 1998; Cao et al., 1994).

The third type of resistance was discovered relatively recently and therefore has not been as well studied as the former two. BABA resistance consists of a priming mechanism initiated by treating the plants with the non-protein amino acid β-aminobutyric acid (BABA; Jakab et al., 2005; Zimmerli et al., 2000). Plants treated with

BABA respond to pathogen infection by rapidly increasing the expression of *PR* genes. Another feature that sets BABA resistance apart from the other two types is the ability to not only protect the plant from biotic stress but also from abiotic stress (Jakob et al., 2005).

One of the underlying themes of induced disease resistance in the last few years is the cross-talk that exists between the three types of resistance. Although each appears to have its unique characteristics, genes and molecules that were once thought to be exclusive to one type of resistance appear to also possess a role in one or both of the other types (Pieterse and Van Loon, 2004).

2.4 TGA factors in SAR

2.4.1 The *as-1* element

TGA factors were first identified through their ability to bind to the *activating sequence 1* (*as-1*) element of the *Cauliflower Mosaic Virus* (*CaMV*) 35S promoter (Katagiri et al., 1989). It is from the *as-1* element, composed of two TGACG motifs spaced eight nucleotides apart, that TGA factors derive their name. Research done concurrently found that TGA factors were also able to bind a similar element found in the *octopine synthase* (*ocs*) promoter from the *Agrobacterium tumefaciens* tumour-inducing (Ti) plasmid (Fromm et al., 1989). Consequently some of the TGA factors were first referred to as Octopine Binding Factors (OBF) (Zhang et al., 1993); however they will be referred to as TGA factors in this thesis.

The *as-1* element has been found to be important in the regulation of *glutathione S-transferase 6* (*GST-6*) (Strompen et al., 1998) as well as *PR-1* (Lebel et al., 1998). It has also been found to be over-represented in the promoters of genes differentially

expressed under abiotic stress and pathogen infection (Mahalingam et al., 2003). The *as-1* element appears to respond to a number of phytohormones such as SA, methyl jasmonate and auxins (Xiang et al., 1996).

Further investigation into the *PR-1* promoter found that it contains two putative TGA factor binding targets termed *linker scan7* (*LS7*) and *LS5* (Lebel et al., 1998). These elements act as positive and negative regulators, respectively, of *PR-1* expression in response to INA or SA treatment. TGA2 is able to interact with both of these elements, and interestingly NPR1 enhances this binding *in vitro* (Després et al., 2000), which may provide the link between NPR1 and TGA factors relative to a role during disease. Thus far NPR1 does not appear to enhance the DNA-binding properties of those TGA factors in which protein interaction has not yet been established.

2.4.2 Structure and family of TGA factors

Sequence analysis of the first TGA factors isolated from tobacco identified a Basic Leucine Zipper (bZIP) (Jakoby et al., 2002; Katagiri et al., 1989). The bZIP is composed of ~16 basic amino acids, a spacer of nine amino acids followed by a heptad repeat of either leucines or any bulky hydrophobic amino acid (Jakoby et al., 2002). These structures, located on an α-helix, allow for the interaction between proteins via the hydrophobic sides of the helices. Therefore creating a coiled-coil structure otherwise called “the zipper”. As a dimer the proteins interact with the DNA through the basic region preferring sequences with an ACGT core, such as the A-box (TACGTA), C-box (GACGTC) and G-box (CACGTG).

Transcription factors containing a bZIP have been identified in plants and mammals (Riechmann et al., 2000) and participate in a variety of tasks in plants from

pathogen defence (Kim and Delaney, 2002) to floral development (Wigge et al., 2005). In *Arabidopsis* alone over 80 members have been identified. These genes have been compiled into ten groups based on sequence similarity of the basic region and the presence of additional conserved motifs; TGA factors make up one group (Jakoby et al., 2002).

The *Arabidopsis* TGA family consists of 10 members. Seven of these members have been grouped according to amino acid sequence similarity into three subclasses (Figure 2.1; Xiang et al., 1997). These subclasses possess little or no similarity to each other at the N-terminal end (before the bZIP) while a high degree of similarity is found at the C-terminal end. Furthermore the members within a subclass possess similar DNA-binding specificities, expression patterns, transactivational properties and protein-protein interactions (Schiermeyer et al., 2003; Després et al., 2000; Niggeweg et al., 2000a; Niggeweg et al., 2000b; Zhou et al., 2000; Zhang et al., 1999).

2.4.3 Interaction with NPR1

Interest in TGA factors with regard to their functions in the SAR pathway first emerged when they were identified to interact with NPR1 in yeast-two hybrid screens (Després et al., 2000; Zhou et al., 2000; Zhang et al., 1999). Using this system, four TGA factors (TGA2, 3, 5, 6 and 7) were found to interact with NPR1, while three (TGA1, TGA4 and PERIANTHIA) did not interact or interact only very weakly (Hepworth et al., 2005; Després et al., 2000; Zhou et al., 2000). The remaining two TGA factors have not yet been tested. For two *Arabidopsis* TGA factors (TGA2 and 5) interaction with NPR1 has been confirmed using *in vitro* binding assays (Després et al., 2000; Zhang et al., 1999). TGA2 has also been shown to interact with NPR1 *in planta*

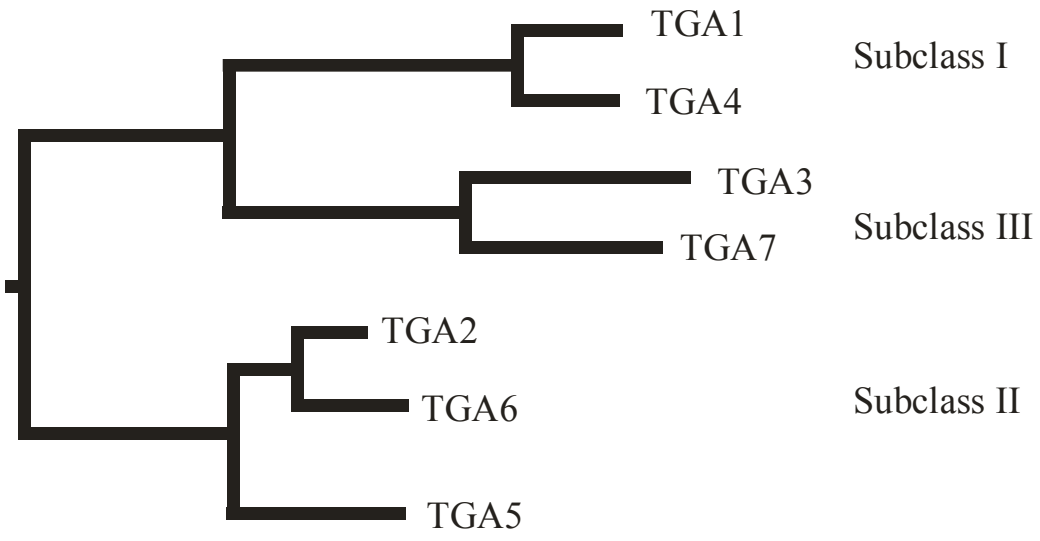

Figure 2.1 Dendrogram of *Arabidopsis* TGA factors.
Seven members of the TGA family have been divided into three subclasses based on amino acid sequence similarity.

(Fan and Dong, 2002; Subramaniam et al., 2001). Interestingly, the interaction between these two proteins was stimulated by treatment with SA, where it was localized primarily to the nucleus (Subramaniam et al., 2001). *Arabidopsis* TGA factors do not interact with the NPR1 mutants when tested either in yeast, *in vitro* (Després et al., 2000; Zhang et al., 1999) or *in planta* (Subramaniam et al., 2001). As these are the same NPR1 mutations that compromise SAR, these results provide an argument that TGA factors may play an integral role in the SAR pathway.

Although demonstrating stable interaction between NPR1 and TGA factors *in planta* has proven elusive there is compelling evidence that NPR1 moderates TGA binding to its cognate promoter. Chromatin immunoprecipitation (ChIP) studies confirmed that TGA2 and TGA3 bind to the *PR-1* promoter *in planta* (Johnson et al., 2003). This binding is observed only after SA treatment and is abolished in *npr1* plants. This not only provides evidence indicating that TGA factors possess a role in the SAR pathway but that a functional NPR1 is required for TGA function. Després et al. (2000) demonstrated this further by showing that binding of TGA2 to the *LS5* and *LS7* elements in the *PR-1* promoter *in vitro* is strengthened by the presence of NPR1. It is important to note that this increased binding affinity is not seen with either TGA1 or TGA4, the two TGA factors that do not interact with NPR1 in yeast, nor is it observed with NPR1 mutants that compromise SAR. Based on this evidence, it would appear that members of the TGA family are differentially regulated depending on their interaction with NPR1.

2.4.4. Functional analysis of TGA factors

Determining the function of TGA factors during SAR has proven to be a complex task. To date only one study has been published with meaningful data on the

overexpression of a single member of the family. Kim and Delaney (2002) found that by overexpressing *TGA5, Arabidopsis* plants displayed enhanced resistance to a virulent strain of the oomycete *Peronospora parasitica* while at the same time exhibited decreased levels of *PR* genes. This same study reported that overexpression of another member of the TGA family (*TGA2*), had no effect on disease resistance or *PR* gene expression.

Attempts to obtain results from plants containing mutations in an individual TGA factor have proven to be difficult. It appears that to effectively study TGA function using this approach, one cannot target a single member of the family but must instead include as many genes as are grouped in individual subclasses (see Section 2.4.2). Perhaps the most profound example of this was found in a study done on the triple loss-of-function mutant *tga2,tga5,tga6* (Zhang et al., 2003). This study also provides the most compelling evidence for the involvement of TGA factors in SAR. The triple mutant possesses many similarities to the *npr1* mutant in that exogenous application of SA or INA does not increase *PR* gene expression, and it is deficient in SAR against *P. parasitica* and the bacterial pathogen *Pseudomonas syringae.* These phenotypes were not observed in the loss-of-function mutants *tga6* or *tga2,tga5.* The compelling evidence that the function of members of Subclass II is not only redundant but also involved in SAR lies in the fact that either *TGA2* or *TGA5* is able to rescue the triple mutant phenotype.

The use of dominant-negative versions of TGA factors has been frequently utilized in the study of TGA function *in planta*. Dominant-negative studies rely on overexpressing a null variant of a chosen protein that by design supersedes the function

of the wild type protein. It would be expected that the resulting transgenic plant exhibits a phenotype similar to that of a plant containing a loss-of-function mutation in the gene encoding the chosen protein. The use of dominant-negatives is beneficial in studying protein families as the null variant may not only supersede the function of its intended protein but also that of proteins that are structurally similar or with which it interacts. The greatest drawback of using dominant-negatives has been the vastly conflicting results between studies which have yet to be convincingly rectified. Expression of dominant-negative forms of the *Arabidopsis TGA2* gene resulted in *Arabidopsis* plants that were compromised in basal resistance against *P. syringae* pathovar (pv) *maculicola* (Fan and Dong, 2002) while another study found that tobacco plants, also expressing dominant-negative *Arabidopsis TGA2*, possessed enhanced SAR against *P. syringae* pv. *tabaci* (Pontier et al., 2001). Each study evaluated the expression of SAR marker genes containing an *as-1* element in their promoters. These genes fall into one of two categories based on the timing of their expression subsequent to SA treatment; early genes or late genes. In the case where *Arabidopsis* plants were compromised in basal resistance levels of *PR-1*, a classical gene used for the late gene category, was reduced (members of the early gene category were not tested; Fan and Dong, 2002), whereas in tobacco, where enhanced SAR was observed, levels of *PR-1a* was increased while expression of early genes were decreased (Pontier et al., 2001). Expression of a dominant-negative version of the tobacco gene *TGA2.2* resulted in decreased expression of early and late genes (Niggeweg et al., 2000b), while transgenic plants overexpressing the wild type *TGA2.2* which displayed decreased expression of early genes and no change in the expression of *PR-1a*. Expression of a dominant-negative version of a

closely related tobacco gene *TGA2.1* resulted in decreased expression of early genes and transgenic plants overexpressing the wild type *TGA2.1*, resulted in decreased expression of early genes was observed. In both cases no change in the expression of *PR-1a* was observed (Kegler et al., 2004). A recurring result from most of these studies is the observation that TGA2 has a differential effect on early and late marker genes. Together results with dominant-negative TGA factors suggest that these proteins may possess both positive and negative roles in disease resistance (Pontier et al., 2001).

Gene silencing, a term that encompasses a variety of methods whose end result is to prevent the formation of target proteins, has been less commonly used to study TGA function. RNA-interference (RNAi), a method that targets degradation of the RNA of a chosen gene, revealed that reducing levels of TGA4 reduced, while reducing levels of TGA5 enhanced, the response of a transgenic reporter gene under the control of an *ocs* -containing promoter (Foley and Singh, 2004). These authors did not report any differences in the expression of endogenous genes containing *as-1* elements in their promoters, nor did they test the transgenic plants for changes in disease resistance. Using virus-induced gene silencing (VIGS), another method that targets the degradation of the RNA of a chosen gene, the tomato genes *TGA1a* and *TGA2.2* were shown to be required for *Pto*-mediated resistance to *P. syringae* pv. *tomato* harboring *avrPto* (Ekengren et al., 2003).

2.5 Redox regulation

Redox regulation of cellular processes is defined by the reduction and/or oxidation of molecules, including proteins. The most well known form of redox regulation is the devastating results that occur when Reactive Oxygen Species (ROS)

accumulate beyond the ability of the cell to buffer against these changes, hence producing a state of oxidative stress and resulting in the formation of non-specific disulfide bonds in cytoplasmic proteins that causes irreversible damage (Berlett and Stadtman, 1997; Sies, 1991). Traditionally disulfide bonds were thought to be stable and to help with protein folding and enhancing the stability of exported proteins (Wedemeyer et al., 2000; Darby and Creighton, 1995). Work in non-plant systems in the last ten years has revealed that the reversible formation of disulfide bonds is used extensively to regulate cellular reactions (Shelton et al., 2005; Toledano et al., 2004).

Disulfide bond formation within proteins is dependent on the redox status of the surrounding environment, resulting in their uneven distribution within cells. The cytoplasm is generally a reducing environment leading to minimal disulfide bond formation compared to the oxidative environments of the periplasm in prokaryotes or the endoplasmic reticulum in eukaryotes (Darby and Creighton, 1995). Two cytosolic pathways have been identified that regulate disulfide bond formation; the thioredoxin and glutaredoxin-glutathione pathways (Ortenberg and Beckwith, 2003; Ritz and Beckwith, 2001). The thioredoxin pathway reduces disulfide bonds by direct thiol-disulfide exchange reactions between the protein and small oxidoreductases known as thioredoxins (Ortenberg and Beckwith, 2003). The resulting oxidized thioredoxins are then reduced by thioredoxin reductase, which is reduced by NADPH. The glutaredoxin-glutathione pathway reduces disulfide bonds using a small reduced tripeptide glutathione (GSH) to form mixed glutathione-disulfide adducts (Ritz and Beckwith, 2001). These are then resolved by glutaredoxins to form oxidized glutathione (GSSG), which is then reduced by glutathione oxidoreductase. The ratio of GSH:GSSG is critical for

maintaining the reducing environment of the cytoplasm and it appears that it is this ratio that acts as the switch to regulate disulfide bond formation.

2.5.1 Redox regulation of transcription factors

Two proteins from *Escherichia coli*, OxyR and SoxR, have been instrumental in expanding the understanding of redox regulation of transcription factors. OxyR regulates antioxidant defence (Christman et al., 1989). Upon exposure to hydrogen peroxide (H_2O_2) OxyR induces transcription of regulatory proteins, including the small non-coding regulatory protein *oxyS*, as well as enzymes that degrade peroxides that balance the redox environment of the cell (Zheng et al., 2001; Aslund et al., 1999; Storz and Tartaglia, 1992). OxyR possesses an N-terminal DNA binding domain, a C-terminal regulatory domain and six conserved cysteines that confer redox sensitivity (Kullik et al., 1995a; Kullik et al., 1995b). Under normal reducing conditions, OxyR's cysteines are all reduced and the protein is bound to DNA in a tetrameric form that does not interact with RNA polymerase, thus preventing it from transactivating target genes. During oxidative stress OxyR cysteines are modified in such a way as to alter protein conformation and allowing interaction with RNA polymerase and initiating transcription. Of the six cysteines present only one (Cys-199) is crucial for transcription. Mutants at this residue are locked in a reduced state resulting in a H_2O_2 hypersensitive phenotype similar to *OxyR* knockouts. Of the remaining Cys residues only one, Cys-208, appears to possess properties that affect transcription (Zheng et al., 1998; Kullik et al., 1995b). Under strong oxidizing conditions caused by H_2O_2 a disulfide bond has been observed between Cys-199 and Cys-208 (Aslund et al., 1999; Zheng et al., 1998), but as

Cys-199 is the more important residue it has been hypothesized that it is the initial target of H_2O_2.

The R1R2R3 family of MYB transcription factors provides an interesting example of cross-kingdom redox regulation. The vertebrate R1R2R3 family and plant R2R3 family must be reduced for DNA-binding to occur (Williams and Grotewold, 1997; Grasser et al., 1992; Guehmann et al., 1992). In vertebrates this property has been attributed to a single Cys residue, Cys-130, that acts as a redox sensor, as it is required for DNA-binding and transactivation. This residue is conserved in the plant R2R3 family but does not appear to possess the same significance as in vertebrates. The corresponding residue, Cys-53, in maize P1, a regulator of flavenoid biosynthesis, is not required for the protein to bind DNA or to activate transcription (Heine et al., 2004). A second Cys residue, Cys-49, appears to be the residue that senses the redox state. Another difference between these families is the presence of a disulfide bond between Cys-49 and Cys-53 of P1 where the vertebrate family has only one Cys residue.

Other well-known mammalian transcription factors, including p53, NF-κB, AP-1 and nuclear receptors have also been shown to be regulated by redox conditions (Nishiyama et al., 2001). Typically, changing redox conditions affect the DNA-binding properties of these transcription factors, although transactivation and nuclear localization may also be affected.

2.5.2 Redox regulation of SAR

The best known form of redox involvement during disease is the oxidative burst that immediately follows pathogen infection (Lamb and Dixon, 1997). The oxidative burst is characterized by the accumulation of ROS which in turn trigger cross-linking in

the cell wall, HR, as well as act as signals for gene expression. During SAR, transient microbursts of H_2O_2 production have been detected in distal (systemic) tissues (Alvarez et al., 1998). These were shown to be required for SAR manifestation.

The use of redox through the formation of disulfide bonds to regulate SAR in plants has only recently been established (Mou et al., 2003). The use of non-reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) showed that in an unelicited sample, NPR1 is present throughout the cells in oligomeric form, whereas in an INA-treated sample, NPR1 is present mainly in the nucleus in a monomeric form. Site-directed mutagenesis confirmed two cysteines within NPR1, Cys-82 and Cys-216, were key for INA-induced monomerization. Mutation of these Cys residues mimics a reduced state where mutated NPR1 is constitutively found as monomer in the nucleus and *PR-1* gene expression is observed without any elicitors such as INA. Treatment with SA was also reported to lead to an increase in the amount of GSH, as well as an increase in the ratio of GSH:GSSG (Mou et al., 2003). Based on these results, it was proposed that pathogen-induced increases in SA lead to the reduction of NPR1 Cys-82 and Cys-216, triggering monomerization and the subsequent nuclear localization.

2.6 Study Goals

2.6.1 Previous research relevant to the project

Using NPR1 fused to the GAL4 DNA-binding domain (GAL4-DB) as bait seven *Arabidopsis* TGA factors fused to the GAL4 transactivation domain (GAL4-TA) have been tested for protein interaction (Després et al., 2000; Zhou et al., 2000). All factors, other than TGA1 and TGA4, were found to interact with NPR1 (Després et al., 2000;

Zhou et al., 2000). To determine the region of TGA factors required for interaction with NPR1, a series of chimeric proteins were constructed between TGA1 and TGA2, a protein that is similar to TGA1 in primary sequence but capable of interacting with NPR1 in yeast. A chimeric protein that consists of the N and C-terminal regions of TGA1 and only 30 amino acids of TGA2 was found to interact with NPR1, indicating that this 30 amino acid region contains sequences important for interaction with NPR1 (Després et al., 2003).

Testing of GAL4-DB fusions indicated that only TGA1 and TGA4 were capable of autonomous transactivation in yeast (Stonehouse, 2002). To determine the regions involved in mediating transactivation the same series of TGA1/TGA2 chimeric proteins referred to above were analyzed. The same chimeric protein that consists of the N and C-terminal regions of TGA1 and only 30 amino acids of TGA2 was unable to transactivate, indicating that the corresponding 30 amino acid region of TGA1 contains sequences important for transactivation. Interestingly, this is the same 30 amino acid region that was identified as being important for interaction with NPR1 (Després et al., unpublished).

In addition to the chimeric approach detailed above, deletion analysis of the *Arabidopsis* TGA1 was also performed to identify regions responsible for transactivation (Stonehouse, 2002; Fobert et al., unpublished data). The N-terminal region of TGA1a, a tobacco homolog, has been found to be sufficient for transactivation (Niggeweg et al., 2000), but Stonehouse (2002) found that the N-terminal of *Arabidopsis* TGA1 is necessary but not sufficient for transactivation. A chimeric protein that

consisted of the N-terminus of TGA2 and the C-terminus of TGA1 did not transactivate indicating that N-terminus of TGA1 contains a transactivation domain.

2.6.2 Research questions and objectives

The main objectives of this study were to identify regions, and if possible, individual amino acids required for TGA2 interaction with NPR1 as well as for transactivation of TGA1, by focusing on the 30 amino acid domain from the chimeric TGA1/TGA2 protein described above. Using site-directed mutagenesis I intend to mutate the two Cys residues found in the 30 amino acid region of TGA1 and test the mutant constructs in yeast for NPR1 interaction and transactivation. Because of cysteine's unique ability to form disulfide bonds I also intend to study the remaining two Cys residues found in TGA1 by using the same methods.

CHAPTER 3: MATERIAL AND METHODS

3.1 Chemicals

All chemicals for media and buffers were purchased from Sigma-Aldrich (St. Oakville, ON) or DB-Canada (Oakville, ON) unless otherwise stated. All amino acids and antibiotics were purchased from Sigma-Aldrich. X-GAL (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and X-Gluc (5-bromo-4-chloro-3-indolyl-ß-D-glucuronic acid) were purchased from Rose Scientific (Edmonton, AB).

3.2 Bacteria and yeast cell methods

3.2.1 Bacterial and yeast strains

For transformation and propagation of bacterial plasmids the *E. coli* strain DH12S (Ø80d*lac*ZΔM15 *mcr*A Δ[*mrr-hsd*RMS-*mrc*BC] *ara*D139 Δ[ara, leu] 7697 Δ*lac*X74 *gal*U *gal*K *rps*L *deo*R *nup*G *rec*A 1/F' *pro*AB^{+} *lacI*^{q} ZΔM15) was purchased from Invitrogen (Burlington, ON).

All yeast methods used the *Saccharomyces cerevisiae* strain YPB2 (*MAT*a, *ura*3-52, *his*3-200, *ade*2-101, *lys*2-801, *trp*1-901, *leu*2-3, 112can^{R}, *Gal4*-542, *gal*80-538, *lys*2::*Gal*$_{UAS}$-*leu*2$_{TATA}$-his3, *ura*::*GAL*4$_{17\ mer(x3)}$, *CYC*$_{TATA}$-*LACZ*; Bartel et al., 1993) obtained from Dr. W. Crosby (Plant Biotechnology Institute, Saskatoon, SK; currently in the Department of Biological Sciences, University of Windsor, Windsor, ON).

3.2.2 Bacterial media

Liquid and solid media (Table 3.1) were prepared and autoclaved before use. Appropriate antibiotics were added to media after autoclaving and cooling (for solid media), or immediately prior to use (liquid media). Stock solutions of all antibiotics (Table 3.2.) were stored at -20°C.

3.2.3 Yeast media

Synthetic dextrose (SD) medium was used for all experiments (Table 3.1). Drop-out powder stocks contained all amino acids except those used for plasmid selection.

3.2.4 Cell growth and storage conditions

All *E. coli* and yeast cell cultures were grown at 37°C and 30°C, respectively. Liquid cultures were shaken constantly at ~250 revolutions per minute (rpm). When required, cultures were temporarily stored at 4°C, or permanently stored at -80°C in 30% glycerol.

3.2.5 Plasmid transformation into bacterial cells

3.2.5.1 Preparation of electro-competent bacterial cells

Prewarmed 2YT medium, supplemented with 0.2% glucose was inoculated from an overnight preculture so that an OD_{600} (absorbance reading at 600 nm; measured with a Beckman model DU-65 spectrophotometer) of between 0.5 and 1.5 was reached after 2 hours (hrs). Cells were harvested by centrifugation for 15 minutes (min), 2500 rpm at 4°C with a GSA rotor (Sorvall, DuPont, Mississauga, ON). The resulting pellet was washed twice with an equal volume of ice-cold sterile water and once in $1/50^{th}$ the volume of ice-cold sterile 10% glycerol before being resuspended in $1/100^{th}$ the initial

Table 3.1 Media used in this study.

Culture	Type[a]	Components[b]
Bacteria	2YT	1.7% tryptone, 1% yeast extract, 0.5% NaCl
Bacteria	SOC	2% tryptone, 0.5% yeast extract, 8.5 mM NaCl, and 2.5 mM KCl, (10 mM $MgCl_2$ and 200 mM glucose after autoclaving)
Yeast	SD	2% glucose, 0.67% yeast nitrogen base without amino acids, 0.15% of the appropriate dropout powder[c]

[a] Media was autoclaved at 20 psi (pounds per square inch) at 121°C for 30 min on the liquid cycle.
[b] For bacteria and yeast solid media, 1.5% and 2 % agar was added respectively.
[c] Prepared as per instructions (Kohalmi et al., 1997).

Table 3.2 Antibiotics used in this study.

Antibiotic	Stock solutions*	Final concentration in media
Carbenicillin	50 mg/ml in water (filter sterilized)	12.5 mg/L
Chloramphenicol	25 mg/ml in 100% ethanol	25 mg/L

*Stock solutions stored at -20°C.

culture volume of cold sterile 10% glycerol. Aliquots of competent cells were stored at –70°C until needed.

3.2.5.2 Electroporation of electro-competent bacterial cells

A 50 µl volume of competent cells was added to an equal volume of water containing ~80 fmols plasmid DNA. The mixture was transferred to a chilled 0.2 cm gap electroporation cuvette (BTX®-Genetronics, San Diego, CA) ensuring no bubbles were created. The cells were electroporated using a Gene Pulsar (BioRad, Hercules, CA) set at:

Capacitance – 25 µFD

Resistance – 200 ohms

Volts – 2.5 Kvolts

The cells were then transferred to 1 ml of SOC medium and incubated at 37°C for 1 hr with shaking. Aliquots were plated on selective media to isolate transformants.

3.2.6 Plasmid transformation into yeast cells

3.2.6.1 Preparation of chemically competent yeast cells

SD +all medium was inoculated from an overnight preculture using the following formula:

$$\text{Inoculation volume} = \frac{\text{desired cell density}}{\text{current cell density}} \times \frac{\text{culture volume}}{2^{(\#\text{ of generations})}} \qquad (3.1)$$

where: desired cell density is 0.6 OD_{600}
2 hrs is required to obtain each successive generation
the current cell density was calculated by measuring the absorbance at OD_{600}

The cells were harvested by centrifugation for 5 min, 5000 rpm at room temperature with a GSA rotor (Sorvall). The cells were washed with $1/10^{th}$ volume

sterile water, and resuspended with 1/100th the initial culture volume in lithium (Li) acetate solution (0.1 M Li Acetate, pH 7.5, in 1 x TE buffer, pH 7.5 [10 mM Tris, pH 7.5, 1 mM ethylenediamine-tetracetic acid (EDTA), pH 7.5]), before being incubated for 1 hr at 28°C with constant shaking. The now-competent cells were used for plasmid transformation or aliquoted and stored at –70°C until needed.

3.2.6.2 Transformation of chemically competent yeast cells

To 200 µl of competent yeast cells, 5 µg of plasmid DNA and 20 µl of sheared salmon sperm carrier DNA (10 mg·ml^{-1} stock solution) were added and incubated for 30 mins at 28°C with shaking (Schiestl and Gietz, 1989). One point two millilitres of polyethylene glycol (PEG) solution (40% PEG 4000, 0.1 M Li acetate, pH 7.5, 1 x TE buffer, pH 7.5) was added, gently mixed, and the cells were returned to 28°C for 30 min. The cells were then heat-shocked at 42°C for 15 min, washed twice with 500 µl of 1 x TE buffer (pH 7.6) and plated on SD media lacking the appropriate amino acid(s).

3.3 DNA methods

3.3.1 Bacterial Plasmids

Plasmids pBCSK^{+} (Stratagene, La Jolla, CA), and pUCBM21 (Roche, Laval, QC) were used for general cloning. pBCSK^{+} contains the *chloramphenicol acetyltransferase* gene (*cat*) gene which provides resistance to the antibiotic chloramphenicol. pUCBM21 contains the *β-lactamase* (*bla*) gene which provides resistance to the antibiotic carbenicillin.

PCR fragments were cloned directly into the PCR®2.1-TOPO® (Invitrogen) plasmid which contains the *neomycin phosphotransferase* (*npt*) and *bla* genes which provide resistance to the antibiotics kanamycin and carbenicillin, respectively.

3.3.2 Yeast Plasmids

The yeast plasmids pBI771, pBI880 and pBI881 (Kohalmi et al., 1997) were obtained from Dr. W. Crosby. These vectors contain both the CEN6/ARS and ColE1 origins of replication for propagation in yeast and *E. coli* respectively. All carry the *bla* gene, allowing for selection of bacteria cells in carbenicillin containing medium, and a yeast amino acid prototrophic marker, *Leu2* in pBI880 and *Trp1* in pBI771 and pBI881, allowing for selection of yeast cells in media lacking these amino acids. pBI880 contains the coding sequence for the GAL4 DB (DNA-binding) domain, while pBI771 and pBI881 contains the coding sequence for the GAL4 TA (transcription activation) domain. pBI771 and pBI881 differ only in the reading frame of the *SalI* restriction site in the multiple cloning region relative to the GAL4 DB coding region.

A pBI771-derived plasmid (pFL759-1, referred to here as CaMV 35S:GUS), used for transactivation studies, contains the CaMV 35S promoter (starting at position –343 from the transcriptional start site) fused to the β-glucuronidase (*GUS*) gene and nopaline synthase (*nos*) terminator (Jefferson et al., 1987; Stonehouse, 2002). This plasmid was used as a template to create another plasmid used for transactivation studies, rsL-20(5), referred to here as CaMV $35S_{mut}$:GUS, in which point mutations were introduced at each TGACG motif, changing them to TGCTG and TCTCG respectively (Stonehouse, 2002).

3.3.3 Extraction of plasmid DNA from bacteria

The Qiagen® Plasmid Mini Kit (Qiagen, Mississauga, ON) or the QIAPrep® 8 Turbo Miniprep Kit (Qiagen) was used according to manufacturer's instructions to isolate plasmid DNA from bacteria.

3.3.4 Restriction enzyme digest

A typical reaction mixture consisted of 10 – 15 Units of each of the appropriate restriction enzymes, not exceeding 10% of the final reaction volume (New England BioLabs, Beverly, MA), 2.0 µl of the manufacturer recommended reaction buffer, 0.5 – 5.0 µg of DNA, and sterile water to a final volume of 20 µl. The reaction mixture was incubated at 37°C for 2 hr before addition of 1.0 – 2.0 µl of stop/loading buffer (water, 50% glycerol, 100 mM EDTA, 1% sodium dodedyl sulphate (SDS), 0.1% bromophenol blue) to stop the reaction.

3.3.5 Agarose gel electrophoresis and recovery of DNA

Approximately 20 µl of restriction enzyme-digested or polymerase chain reaction (PCR) amplified DNA fragments (see sections 3.3.4 and 3.3.7) were loaded on a 0.8% agarose gel in 1x TAE buffer (12 mM Tris, 6 mM sodium acetate, 0.3 mM EDTA, pH 8.0), including 0.5 µg ml^{-1} ethidium bromide. The DNA fragments were separated at 90 V for about 20 – 30 min with a minisub^{TM} DNA cell (BioRad) in 1x TAE buffer. The DNA was visualized using a GENE GENIUS Bio Imaging System (Syngene, Frederick, MD), the desired DNA bands were excised from the agarose and purified using the QiaexII Agarose Gel Extraction Protocol (Qiagen) as per manufacturer's instructions. The resulting DNA was stored at -20°C if not used immediately.

3.3.6 Ligation of DNA fragments into vector DNA

A typical ligation consisted of approximately 20 fmols of vector DNA, three times the molar amount (~60 fmols) of insert DNA, 1 Unit of the T4 DNA ligase enzyme (Invitrogen), 4 µl of 5x ligase buffer (Invitrogen), and water to a final volume of

20 µl. The ligation mixture was incubated overnight in a 12°C water bath. To minimize the salts that may interfere with the transformation, a desalting step of the ligation mixture was performed prior to electroporation. The ligation was diluted with 400 µl of sterile water and loaded onto a desalting ultrafree-MC column (Millipore, Bedford, MA), which was centrifuged at 8000 rpm for 10 min. The desalted DNA was eluted from the column with 20 µl of sterile water.

PCR products were cloned into the pCR®2.1-TOPO® vector (Invitrogen) as per manufacturer's instructions.

3.3.7 PCR (Polymerase Chain Reaction) amplification

To confirm the presence of a plasmid in transformed bacterial cells a PCR was performed using aliquots of isolated colonies suspended in 50 µl of sterile water. A typical PCR amplification mixture consisted of 5 µl of cell suspension, 10 mmols of 2'-deoxy-nucleotide 5'-triphosphates (Pfizer, New York, NY), 25 pmols of each oligonucleotide primer (synthesized by the DNA technology service at PBI/NRC), 1.25 Units of Taq polymerase (Pfizer), 5 µl of 10x Taq reaction buffer (Pfizer), and water to a final reaction mixture volume of 50 µl.

The PCR amplifications were performed using a PTC-200 Peltier Thermalcycler (MJ Research, Waltham, MA). An initial denaturing step was performed at 95°C for 5 min. Each PCR consisted of 25 cycles containing a 1 min. denaturation step at 94°C, a 1 min. annealing step at 55°C, and a 1 min. extension step at 72°C. At the end of the PCR amplification, the samples were incubated for 7 min at 72°C to ensure complete strand extension.

3.3.8 Introduction of point mutations

3.3.8.1 Site-directed mutagenesis (SDM)

Point mutations were introduced using QuikChange™ Site-Directed Mutagenesis Kit (Stratagene) using mutagenic oligonucleotides listed in Table 3.3A and B.

3.3.8.2 Stitching

Stitching was utilized to simultaneously mutate two amino acids located within close proximity to each other in a gene (Sambrook and Russell, 2001). Three separate PCR reactions were performed to create an altered gene. Each PCR consisted of a single denaturing step at 94°C for 4 min. followed by 15 cycles each containing a 30 sec denaturation step at 94°C, a 30 sec. annealing step at 55°C, and a 30 sec. extension step at 72°C. The PCR amplification mixture consisted of 5-10 ng of DNA, 10 nmols of 2'-deoxy-nucleotide 5'-triphosphates (Pfizer), 50 pmols of each oligonucleotide primer, 1.25 Units of XTaq polymerase (Takara Mirus Bio. Inc., Madison, WI), 5 µl of 10x Taq reaction buffer, and water to a final reaction mixture volume of 50 µl.

Four oligos were needed to complete the procedure: 5' and 3' oligonucleotides located in the vector upstream and downstream of the start and stop codons respectively (5'-vector, 3'-vector); as well as 5' and 3' gene specific oligonucleotides that span the site of the desired mutations (5'-mut, 3'-mut; Table 3.3C). The gene specific oligonucleotides include the desired mutations and contain ~70% overlap to each other. The initial two PCR reactions, performed concurrently, used a plasmid containing the wild type coding sequence of the gene as template DNA. The first reaction used the 5'-vector and 3'-mut oligonucleotides to amplify the 5' end of the gene to the desired mutations. The second reaction used the 5'-mut and 3'-vector

Table 3.3 Oligonucleotides used for PCR amplification and sequencing.

Primer Name	5' to 3' sequence	Primer Target	Purpose
A. Oligos previously designed			
TGA1 P1	GCGTCGACCATGAACTCGAC ATCGACACATTTTGTG	TGA1	Cloning, adds Sal1 site prior to start codon.
TGA1 P2	ATGCGGCCGCTACGTTGGTTC ACGATGTCG	TGA1	Cloning, adds Not1 site subsequent to stop codon.
TGA2 P1	GCGTCGACTATGGCTGATAC CAGTCCGAGAAC	TGA2	Cloning, adds Sal1 site prior to start codon.
TGA2 P4	ATGCGGCCGCTCACTCTCTGG GTCGAGCAAGCC	TGA2	Cloning, adds Not1 site subsequent to stop codon.
BC293	GAATAAGTGCGACATCATC	pBI880	Vector specific sequencing primer.
BC304	CTATTCGATGATGAAGATAC C	pBI881	Vector specific sequencing primer.
JN069	TTGATTGGAGACTTGACC	pBI880/881	Vector specific reverse sequencing primer.
UP	CCCAGTCACGACGTTGTAAA ACG		Universal sequencing primer.
RP	AGCGGATAACAATTTCACAC AGG		Reverse sequencing primer.
B. Oligos designed for SDM			
TGA1-M1	GGGTTGAAGAACAGAACAGA CAGATATCCTGAACTAAGAA CAG	TGA1	Changes Cys172 to Ser172
TGA1-M2	CTGTTCTTAGTTCAGATATCT GTCTGTTCTGTTCTTCAACCC	TGA1	Changes Cys172 to Ser172
TGA1 S-P1	GCAATCTAAAACAATCGTCG CCAGCAAGCAGAAGACGCGT TG	TGA1	Changes Cys266 to Ser266
TGA1 S-P2	CAACGCGTCTTCTGCTTGCTG CGACGATTGTTTTAGATTGC	TGA1	Changes Cys266 to Ser266
TGA1 N-P1	CTTCTAGATGTAAACAATCTA AAACAATCGTGTCAGCAAGC AGAAGACGCG	TGA1	Changes Cys260 to Asn260
TGA1 N-P2	CGCGTCTTCTGCTTGCTGACA CGATTGTTTTAGATTGTTTAC ATC	TGA1	Changes Cys260 to Asn260
TGA1 S (1C)-P1	CTTCTAGATGTATCCAATCTA AAACAATCGTGTCAGCAAGC AGAAGACGCG	TGA1	Changes Cys260 to Ser260
TGA1 S (1C)-P2	CGCGTCTTCTGCTTGCTGACA CGATTGTTTTAGATTCGATAC ATCTAGAAG	TGA1	Changes Cys260 to Ser260
TGA1 ADS-P1	CACACCCTTGCGGACTCCGTT GCAGCGGGACAA	TGA1	Changes Cys287 to Ser287
TGA1 ADS-P2	TTGTCCCGCTGCAACGGAGT CCGCAAGGGTGTG	TGA1	Changes Cys287 to Ser287
TGA2 FC-1	GGAAAACACCAGCTGAGAGA TTCTTCTTGTGGCTCGGTGG	TGA2	Changes Cys186 to Phe186
TGA2 FC-2	CCACCGAGCCACAAGAAGAA TCTCTCAGCTGGTGTTTTCC	TGA2	Changes Cys186 to Phe186

Table 3.3 Oligonucleotides used for PCR amplification and sequencing continued.

Primer Name	5' to 3' sequence	Primer Target	Purpose
B. Oligos designed for SDM (continued)			
TGA2 NC-1	CAGTTGATGGGCATATGTAA CCTGCACAGACATCGCAGC	TGA2	Changes Asn218 to Cys218
TGA2 NC-2	GCTGCGATGTCTGTTGCAGGT TACATATGCCCATCAACTG	TGA2	Changes Asn218 to Cys218
TGA2 SC-1	CCTGCAACAGACATGCCAGC AGGCTGAAGATGCTTTGTCTC AAGG	TGA2	Changes Ser224 to Cys224
TGA2 SC-2	CCTTGAGACAAAGCATCTTC AGCCTGCTGGCATGTCTGTTG CAGG	TGA2	Changes Ser224 to Cys224
TGA2 C130-M1	GAAAAGAACAAGCAAATGTG CGAGCTGAGGTCTGCTCTG	TGA2	Changes Asn130 to Cys130
TGA2 C130-M2	CAGAGCAGACCTCAGCTCGC ACATTTGCTTGTTCTTTTC	TGA2	Changes Asn130 to Cys130
TGA4 S3	CGGATCAACAACTTTTGGAT GTATCCAATCTGAGGCAATC ATGTCAACAAGC	TGA4	Changes Cys256 to Ser256
TGA4 S4	GCTTGTTGACATGATTGCCTC AGATTGGATACATCCAAAAG TTGT	TGA4	Changes Cys256 to Ser256
TGA4 S5	CCAATCTGAGGCAATCATCC CAACAAGCAGAAGATGCG	TGA4	Changes Cys256 and Cys262 to Ser256 and Ser262
TGA4 S6	CGCATCTTCTGCTTGTTGGGA TGATTGCCTCAGATTGG	TGA4	Changes Cys256 and Cys262 to Ser256 and Ser262
C. Oligos designed for stitching			
TGA1 NS-P1	GATGTAAACAATCTAAAACA ATCGTCTCAGCAAGCAGAAG ACGCG	TGA1	Changes Cys260 and Cys266 to Asn260 and Ser266
TGA1 NS-P2	CTGAGACGATTGTTTTAGATT GTTTACATCTAGAAGTTGTTG	TGA1	Changes Cys260 and Cys266 to Asn260 and Ser266

oligonucleotides to amplify from the desired mutations to the 3' end of the gene. Each PCR reaction was subjected to agarose gel electrophoresis, the desired DNA bands were excised and purified using the QiaexII Agarose Gel Extraction Protocol (Qiagen) as per manufacturer's instructions. The final PCR used the two gel-purified fragments of the gene as template DNA and the 5'-vector and 3'-vector oligonucleotides to 'stitch' the complete gene together, including the newly introduced mutations.

3.4 Plasmid construction

The constructs desired for this study included point mutations within *TGA1* or *TGA2*, chimeric genes including the coding region from these genes, and the chimeric genes with point mutations. Dr. Charles Després previously constructed all of the chimeric genes through stitching. Plasmids containing the full length coding region of *TGA1*, *TGA2*, or the chimeric genes, site mutations were introduced through site-directed mutagenesis or stitching as described above. The resulting plasmid DNA was purified and assessed for the presence of desired mutations by sequence analysis. Sequencing was performed by the DNA Technologies Unit at PBI/NRC and analyzed using LaserGene (DNAstar^{TM}, Madison, WI) Mapdraw program.

In every case, a *Sal1* restriction site directly before the start codon, and a *Not1* restriction site directly after the stop codon flanked the full-length coding region of the genes. Using these restriction enzyme sites the coding regions were transferred from the cloning vectors into the appropriate yeast plasmids and the integrity of the resulting GAL4 fusion was confirmed by sequence analysis.

3.5 Enzymatic assays

3.5.1 β-galactosidase assay

To display β-galactosidase activity, three isolated colonies of each transformation experiment were transferred to a fresh plate and grown overnight at 28°C. The colonies were lifted from the plate using a 0.45 micron nylon transfer membrane (GE Osmonics, Trevose, PA), which was then submerged in liquid nitrogen for 5-10 seconds to lyse the cells. The thawed membrane was laid on a piece of Whatman #1 filter paper saturated with Z-buffer (100 mM Na_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 0.27% β-mercaptoethanol and 0.024 mM X-GAL), and incubated at 28°C overnight (Kohalmi et al., 1997).

3.5.2 Quantitative β-glucuronidase (GUS) assay of yeast cells

An overnight preculture was used to inoculate a fresh culture so that the OD_{600} was 0.5-0.8 after 2-3 hrs incubation at 28°C. A 1.9 ml aliquot of cell suspension was pelleted in a microcentrifuge and 200 µl of GUS extraction buffer (50 mM $NaPO_4$, pH 7.0, 10 mM Na_2EDTA, pH 8.0, 10 mM β-mercaptoethanol, 0.1% Sarcosyl, 0.1% Triton X-100) and 100 µl volume of 425-600 micron, acid-washed glass beads (Sigma, G-8772) was added to the pellet. The cells were lysed by seven repetitions of vortexing for 30 sec. and cooling on ice for 30 sec., and cell debris was removed by centrifugation. At this point the cell extract was used for GUS analysis or frozen at -80°C for future use.

To 450 µl of 1 mM methyl-umbelliferyl-β-D-glucuronide (MUG) solution, 50 µl of cell extract was added, followed by incubation at 37°C. At various time points (0, 1, 2, and 3 hrs) 100 µl aliquots were taken and mixed with 900 µl of 0.2 M Na_2CO_3 stop buffer. GUS activity was determined by measuring methyl-umbelliferone (MU)

fluorescence by a Perkin Elmer LS50 Luminescence Spectrometer (Wellesley, MA) using an excitation wavelength of 365 nm and an emission wavelength of 455 nm. The luminescence spectrometer was calibrated with 100 nM and 1 µM MU standards made in 0.2 M Na_2CO_3 stop buffer.

3.5.3 Quantitative protein assay

To standardize the results observed in the GUS quantitative assay, 20 µl of the cell extract was used to quantify the total protein level using the Microassay Procedure of the BioRad Protein assay as instructed by the manufacturer. Protein levels were correlated to a standard curve composed from measuring six bovine albumin (BSA) standards ranging from 2 µg/ml to 20 µg/ml.

The results of the GUS and protein quantitative assays were combined to determine a value that standardized the amount of GUS activity in µM of MU per µg of protein per minute (µM MU·µg protein^{-1}·min^{-1}).

3.5.4 Statistical analysis of quantified GUS activity

To test whether the differences in GUS activity observed between yeast cells with different effector and reporter plasmids were significantly different, a Student's *t*-test was performed. The formulas used to derive the observed t_{value} were:

$$\text{Standard Deviation}^2\ (Sd^2) = \frac{(n_1-1)S_1^2 + (n_2-1)S_2^2}{n_1 + n_2 - 2} \qquad (3.2)$$

$$t_{value} = \frac{\pm\ X_1 - X_2}{Sd\ [\text{sqrt}\ (1/n_1 + 1/n_2)]} \qquad (3.3)$$

Where: n_1 and n_2 are the sample sizes of the two groups respectively
S_1 and S_2 are the standard deviations of the two sample groups
X_1 and X_2 are the mean GUS activities of the two sample groups

Sd is the modified standard deviation that incorporates size and standard deviations of both of the sample groups.

In this study, the 10 degrees of freedom and a 95% confidence level ($p = 0.05$) was used to determine if the activity observed in the yeast cells was significantly different.

CHAPTER 4: RESULTS

4.1 Role of cysteine residues in controlling transactivation by TGA1/TGA4 and the interaction of TGA factors with NPR1

4.1.1 Role of TGA1 cysteines within the 30 amino acid domain

Previous research identified a 30 amino acid region of TGA2 that, when used to replace the corresponding sequences from TGA1, enabled the resulting chimeric protein to interact with NPR1 while abolishing transactivation in yeast (Després et al., 2003; see also Figure 4.1 and section 2.6.1). To identify the residues within the 30 amino acid region that are important for these functions, a rational site-directed mutagenesis approach was taken based on a multiple alignment of seven *Arabidopsis* TGA factors between the region corresponding to amino acids 236 to 266 of TGA1 (Figure 4.2). The selection criteria for targeting an amino acid were that the residue needed to be conserved between TGA1 and TGA4 but had to differ from that of the conserved residues in the remaining TGA factors analyzed. Cysteine 266 (Cys-266) is the only residue that conforms to these criteria (Figure 4.2). It is conserved between TGA1 and TGA4, while in all other TGA factors a conserved serine (Ser) residue is present at the corresponding position. Since Cys residues possess the unique ability to form a bond between their sulfhydryl side chains, called a disulfide bond, the other Cys residue found within the 30 amino acid region, at residue 260 (Cys-260), was also targeted for mutagenesis. Cys-260 conforms to the first criterion as it is conserved between TGA1

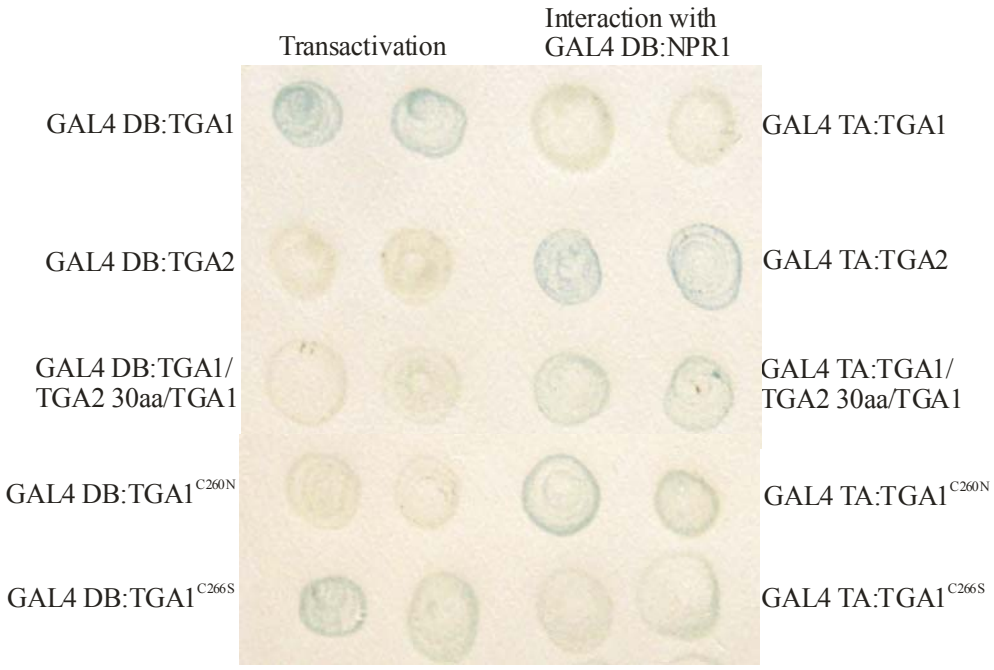

Figure 4.1 Transactivation and NPR1-interaction properties of TGA1 containing mutations within the 30 amino acid region.
β-galactosidase assays were performed on yeast cells transformed with TGA constructs fused to the domains indicated. Labels at top indicate the property tested. Each spot represents an independent transformation event. A blue colour observed with GAL4:DB fusions to TGA factors indicates autonomous transactivation of the construct. Blue colour observed with GAL4:TA fusions of TGA factors in cells also containing DB:NPR1 indicates interaction between the test protein pair.

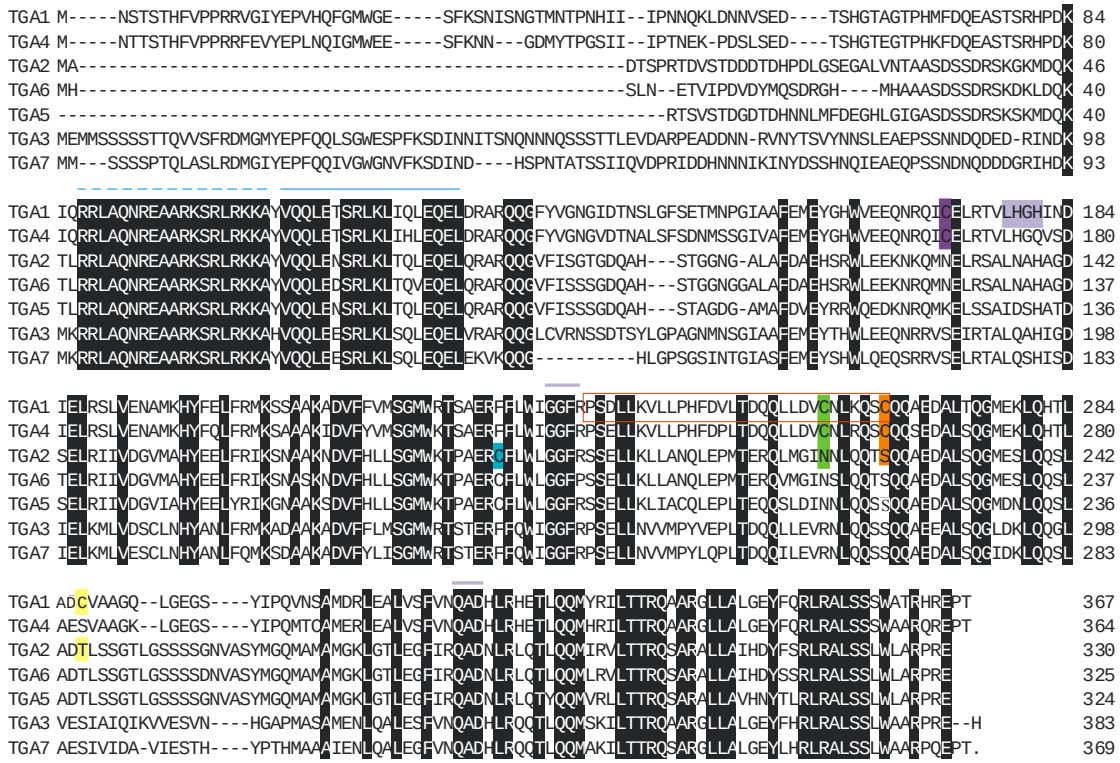

Figure 4.2 Alignment of seven of the *Arabidopsis* TGA factors.

Dashed and solid blue lines indicate the basic and leucine zipper domains respectively. The dark purple box indicates cysteines of TGA1 and TGA4 at positions 172 and 168 respectively. The blue/green closed box indicates cysteine of TGA2 at position 186. The green closed box indicates residues or TGA1, TGA4, and TGA2 at positions 260, 256, and 218 respectively. The orange closed box indicates residues of TGA1, TGA4, and TGA2 at positions 266, 262, and 224 respectively. The yellow closed boxes indicate residues of TGA1 and TGA2 at positions 287 and 245 respectively. The light purple closed box and solid lines indicate regions used to mark changes for chimeric proteins. The red open box indicates the 30 amino acid span between position 236 and 266 of TGA1 that was replaced with the corresponding region from TGA2. Dashes indicate gaps introduced to facilitate proper alignment.

and TGA4, but the residue is not strictly conserved in the remaining TGA factors (Figure 4.2).

The complete coding region of *TGA1* in pBC-SK+ was used as a substrate for site-directed mutagenesis (SDM). The first mutant gene created encodes a full-length protein in which Cys-260 and Cys-266 were replaced with asparagine (Asn) and Ser, the corresponding residues from TGA2, respectively ($TGA1^{C260N,C266S}$; Figure 4.3). The mutant gene was subsequently ligated into pBI880 and pBI881 (Kohalmi et al., 1997) to generate N-terminal fusions to the GAL4 DNA binding and GAL4 transactivation domains, respectively. The resulting GAL4 TA fusion protein was co-expressed in yeast with DB:NPR1 and the interaction between proteins was assessed using β-galactosidase assays. If the test proteins interact, the GAL4 TA domain is brought in close proximity to the $GAL4^{UAS}$ in the promoter of the *β-galactosidase* gene (*LacZ)* and initiates transcription, resulting in a blue colour when cells are treated with the colorimetric substrate X-GAL (Kohalmi et al., 1997). This assay revealed that TA:TGA1 and DB:NPR1 do not interact (Figure 4.1), while TA:$TGA1^{C260N,C266S}$ and DB:NPR1 do interact (Figure 4.3).

The mutant GAL4 DB fusion protein (DB:$TGA1^{C260N,C266S}$) was assessed for transactivation abilities using the β-galactosidase assay by coexpression with TA:cruciferin (TA:Cruc). Cruciferin is a seed storage protein that does not interact with TGA factors (Stonehouse, 2002). Therefore the GAL4 TA domain is not recruited to the $GAL4^{UAS}$ and is unable to transactivate the *LacZ* gene. Consequently, any blue colour formed during the β-galactosidase assay is due solely to the autonomous transactivation capabilities of the TGA factor. Results of this test indicate that the DB:$TGA1^{C260N,C266S}$

Figure 4.3 β-galactosidase assay results for TGA1 and TGA4 site-directed mutants used in this study.
Schematic representation of TGA1 (orange), TGA2 (blue), TGA4 (green) constructs showing the bZip domain of each (lighter shade) and location of point mutations (coloured bars). β-galactosidase assay results for transactivation and NPR1 interaction indicated by – (white), +/- (faint blue), and + (blue) after 24 hours. The +/- symbol is used to indicate when the blue colour observed was considerably less than the positive control (interaction between NPR1 and TGA2). Transactivation results were obtained from mutant constructs fused to the GAL4 DB domain in yeast cells coexpressing cruciferin fused to the GAL4 TA domain. NPR1 interaction results were obtained from mutant constructs fused to the GAL4 TA domain in yeast cells coexpressing NPR1 fused to the GAL4 DB domain. Drawings are not to scale.

construct was able to transactivate weakly (Figure 4.3). The above results indicate that the Cys residues within the 30 amino acid region of TGA1 are important for its ability to interact with NPR1.

To resolve the role of the individual Cys residues, Cys-260 and Cys-266 were mutated independently and yeast cells were transformed with the resulting genes in pBI880 or pBI881. Results shown in Figure 4.1 and summarized in Figure 4.3 indicate that TA:TGA1^{C260N} interacts with DB:NPR1 while the DB:TGA1^{C260N} construct transactivated weakly. In contrast, TA:TGA1^{C266S} interacts poorly with DB:NPR1 while DB:TGA1^{C266S} transactivates (Figure 4.1; 4.3). Cys-260 was also changed to a serine (TGA1^{C260S}), a structurally similar amino acid, and tested for NPR1 interaction and transactivation (Figure 4.3). TA:TGA1^{C260S} interacts with NPR1 while the same mutant fused to GAL4:DB (DB:TGA1^{C260S}) transactivates weakly (Figure 4.3). Although the above results are not quantitative, they clearly indicate that modifying Cys-260 has more significant consequences on TGA1 properties than modifying Cys-266, suggesting a more prominent role for the former amino acid in controlling the TGA1 functions tested.

4.1.1.1 Quantitative analysis of transactivation for TGA1 mutants with altered cysteines within the 30 amino acid region

β-galactosidase assays possess the limiting factor that they are restrictive to a qualitative, or at best semi-quantitative, observation based on the presence and intensity of the blue product. Also, testing of GAL4 DB fusions with the GAL4^{UAS} promoter element may not accurately reflect the ability of mutated proteins to interact with their cognate promoter binding sites. To help resolve these problems, quantitative experiments were performed using a *GUS* (*uidA*) reporter gene under the control of one

of the best characterized targets of the TGA factors, the CaMV 35S *as-1* element (Lam et al., 1989), which has been previously shown to be active in yeast (Rüth et al., 1992).

DB:TGA1, DB:TGA2, and GAL4DB fusions to different *TGA1* SDM constructs were transformed into yeast cells harbouring a pBI881 derivative containing the CaMV 35S:*GUS* gene (pFL759-1; Stonehouse et al., 2002). In these assays, the TGA factors should interact with the CaMV 35S promoter through the *as-1* element (see results below from control experiments with the CaMV 35S promoter containing a mutant *as-1* element). If the factor possesses transactivational properties the *GUS* gene will be expressed and the activity of the resulting enzyme can be quantified by measuring fluorescence of the 4-methyl umbelliferone (MU) product after the addition of the 4-methyl umbelliferyl glucuronide (MUG) substrate (Jefferson, 1987).

Background levels of GUS activity in the yeast cells, assessed by measuring MU levels in cells containing a DB:Cruc fusion along with the CaMV 35S:GUS reporter construct, averaged 2.82 pM MU/µg protein/min (Figure 4.4). Cells containing DB:TGA1 and the CaMV 35S:GUS plasmid displayed the highest levels of GUS activity measured in this study, averaging 32.21 pM MU/µg protein/min. A Student's *t*-test at a 95% confidence level ($p = 0.05$), indicates that GUS activity in these cells is significantly different from background levels. This result is consistent with β-galactosidase assays where DB:TGA1 transactivated (Figure 4.1). Levels of GUS activity measured in cells containing the DB:TGA2 were lower than those observed in cells containing DB:Cruc /CaMV 35S:GUS (averaging 0.23 pM MU/µg protein/min). A Student's *t*-test ($p = 0.05$) revealed that this difference is statistically significant, which suggests that TGA2 may be repressing transactivation.

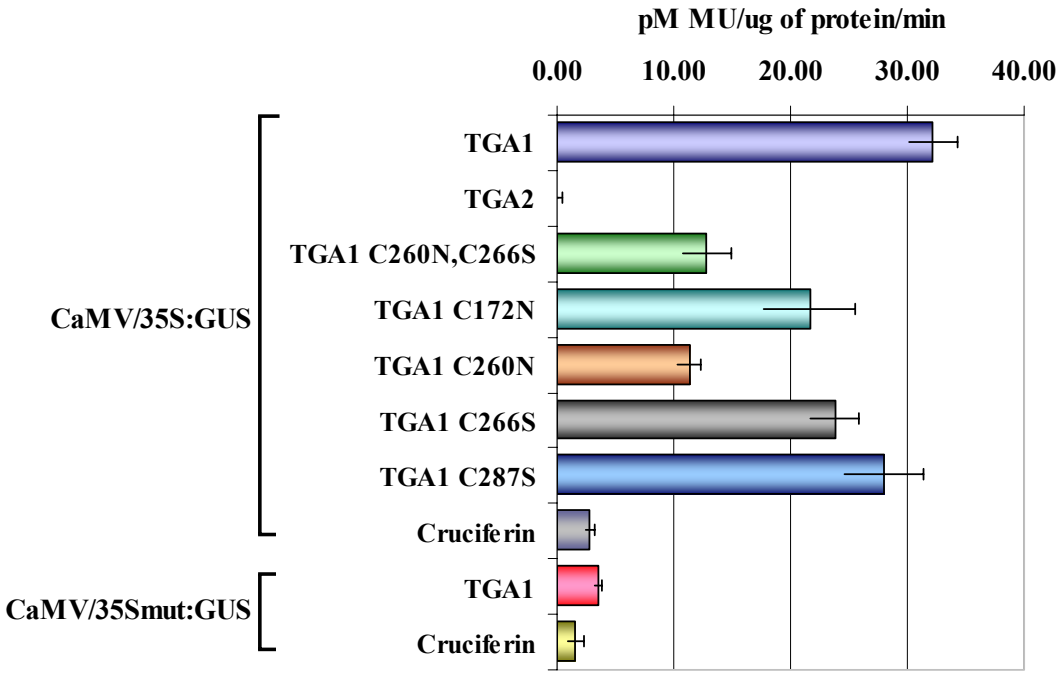

Figure 4.4 Quantitative GUS assay of transactivation of TGA1 with mutation of the cysteine residues.

Transactivation levels of TGA constructs and cruciferin when measured using the wild type CaMV 35S promoter and a mutated version of the promoter. Each bar represents the average of six independent GUS assays from a total of two independent transformations ± SE.

To ensure that GUS activity observed in cells containing DB:TGA1 is attributed to binding of the *as-1* element, this GAL4 DB fusion was tested against a CaMV 35S:GUS reporter gene with a mutant *as-1* element (pRSL20(5), hereafter referred to as CaMV $35S_{mut}$:GUS) (Stonehouse, 2002). This mutant element possesses point mutations at each TGACG motif, changing them to TGCTG and TCTCG, respectively (Stonehouse, 2002). TGA factors are unable to bind to this mutant *as-1* element (Després et al., 2000; Rüth et al., 1992). The levels of GUS activity measured in cells containing CaMV $35S_{mut}$:GUS with either DB:TGA1 or DB:Cruc, or containing DB:Cruc and CaMV 35S:GUS were not statistically different from the background fluorescence (Figure 4.4), indicating that TGA1 is unable to bind to the mutant *as-1* element.

When tested in combination with CaMV 35S:GUS, DB:$TGA1^{C260N}$ and DB:$TGA1^{C260N,C266S}$ displayed the greatest reduction in transactivation properties relative to DB:TGA1, dropping to an average of 11.32 pM MU/µg protein/min and 12.78 pM MU/µg protein/min, respectively (Figure 4.4). Using the Student's *t*-test ($p = 0.05$) these numbers were not found to be significantly different from each other but are significantly different than values obtained with DB:TGA1. Transactivation levels of the DB:$TGA1^{C266S}$ construct (averaging 23.8 pM MU/µg protein/min) were also significantly reduced ($p = 0.05$) relative to wild type DB:TGA1 supporting a role for Cys-266 in transactivation. However, GUS activity levels in cells expressing DB:$TGA1^{C266S}$ are still significantly greater than those observed with $TGA1^{C260N}$. These results are consistent with the β-galactosidase assays showing that the mutation of Cys-260 affects the properties of TGA1 to a greater extent than the mutation of Cys-266 and

further indicates that Cys-260 plays a critical role in mediating TGA1 transactivation. It may be important to note that although transactivation is reduced in cells containing the mutant TGA factors it is not entirely abolished to background levels. This suggests that additional amino acids or regions of TGA1 are involved in transactivation.

4.1.2 Role of TGA4 cysteines within the 30 amino acid region

TGA4 possesses 83.8% similarity to TGA1 at the amino acid level, the highest degree of similarity to TGA1 among the seven *Arabidopsis* TGA factors (Figure 4.2). In particular, TGA4 is the only other TGA factor with cysteines at positions corresponding to Cys-260 and Cys-266 of TGA1 (Figure 4.2). Furthermore both transactivate but do not interact with NPR1 in yeast. Given these similarities, both proteins may be regulated in a similar fashion; accordingly I decided to extend my survey of the effects of Cys residues within the 30 amino acid region to include TGA4.

Using an approach similar to the one utilized with TGA1, a mutant gene was created which encodes a full-length protein with Ser residues instead of cysteines at positions 256 and 262 ($TGA4^{C256S,C262S}$; Figure 4.3). The altered Cys residues correspond to Cys-260 and 266 of TGA1 (Figure 4.2). The mutant gene was ligated into pBI880 and pBI881 and introduced into yeast cells along with plasmids encoding TA:Cruc and DB:NPR1, respectively. Results from cells co-expressing TA:$TGA4^{C256S,C262S}$ and DB:NPR1 confirmed that they do interact and are therefore similar to those obtained with $TGA1^{C260N,C266S}$ in this respect (Figure 4.2). Furthermore DB:$TGA4^{C256S,C262S}$ is also similar to DB:$TGA1^{C260N,C266S}$ in that it transactivated weakly (Figure 4.2).

The codon for Cys at position 256 of TGA4 was also changed to encode a Ser and the resulting gene was introduced into pBI880 and pBI881. The β-galactosidase assays revealed that TA:TGA4^{C256S} interacts weakly with DB:NPR1 while DB:TGA4^{C256S} transactivated weakly. Together these results suggest that the Cys-256 contributes substantially to the properties seen in TGA4. A TGA4^{C262S} mutant has not been created or tested. Although the Cys residues within the 30 amino acid region of TGA4 do affect the protein's properties, the change in properties observed in these mutants is not identical to the change observed in the TGA1 mutants. These differences may be resolved when a complete analysis of the remaining two Cys residues is accomplished; however the results thus far do indicate that a slight difference in the mode of regulation may exist between TGA1 and TGA4.

4.1.3 Role of TGA1 cysteines outside of the 30 amino acid domain

Disulfide bonds may occur between cysteines located in the same protein (intramolecular) or between different proteins (intermolecular), causing conformational changes of the protein (see for example, Delaunay et al., 2002; Mahoney et al., 1996). Accordingly, it is tempting to speculate that Cys-260 of TGA1 may participate in forming such a bond. The mutation to Ser or Asn would preclude the formation of this bond, causing a conformation change of TGA1, hence affecting its ability to transactivate and interact with NPR1. In support of this hypothesis, the electrophoretic mobility of *in vitro*-produced TGA1, but not TGA1^{C260S}, has recently been shown to change under different redox conditions, suggesting TGA1 may contain an intramolecular disulfide bond (Després et al., 2003). As only one of the Cys residues within the 30 amino acid region (Cys-260) profoundly affects TGA1 properties, analysis

of the Cys residues outside of the 30 amino acid domain was undertaken to identify its potential partner in the intramolecular disulfide bond.

Two cysteines are present outside of the 30 amino acid region of TGA1 at residues 172 and 287 (Figure 4.2). The codons for these residues were respectively mutated to encode for Asn and Ser, the corresponding residues from TGA2. The resulting genes were ligated into pBI880 and pBI881 and introduced into yeast cells. β-galactosidase assays revealed that TGA1^{C172N} interacts weakly with NPR1 while it is still able to transactivate (Figure 4.3). In addition to mutating Cys-172 to Asn, it was also changed to a Ser, the corresponding residue from TGA3 and TGA7 (Figure 4.2). This mutant produced results identical to those with TGA1^{C172N} in that it was capable of both transactivation and interaction with NPR1 (Figure 4.3). These results show that Cys-172 influences the ability of TGA1 to interact with NPR1, but not its transactivation properties.

Mutation of Cys287 to Ser resulted in a protein that still transactivated and was unable to interact with NPR1 (Figure 4.3). Therefore, TGA1^{C287S} behaves the same as wild type TGA1, indicating that Cys-287 does not play a critical role in regulating transactivation or interaction with NPR1 in yeast. Together, results with Cys residues outside of the 30 amino acid domain suggest that Cys-172 is more likely than Cys-287 at partnering with Cys-260 to form an intramolecular disulfide bond.

4.1.3.1 Quantitative analysis of transactivation of TGA1 mutants in cysteines outside the 30 amino acid region

The results for transactivation obtained above by the qualitative β-galactosidase assay were quantified using the CaMV 35S:GUS reporter gene. The DB:TGA1^{C287S}

construct showed a slight decrease in transactivation levels, averaging 27.95 pM MU/μg of protein/min, compared to DB:TGA1 (Figure 4.4). However, a Student's t-test (p = 0.05) indicates that this difference in GUS activity is not significantly different.

The DB:TGA1^{C172N} construct showed a decrease in transactivation levels to an average of 21.68 pM MU/μg of protein/min compared to the DB:TGA1 (Figure 4.4). The transactivation levels of DB:TGA1^{C172N} is significantly lower than DB:TGA1 according to the Student's *t*-test (p=0.05). This value is not significantly different to that obtained with DB:TGA1^{C266S} (p=0.05).

4.1.4 Simultaneous mutation of multiple cysteines in TGA1

Although Cys-260 appears to make the more substantial contributions to TGA1's properties, a possible role for the other Cys residues cannot be entirely ignored based solely on the analysis of proteins with mutations at single Cys residues. To further probe the potential roles each residue and identify possible interactions between the four Cys residues, mutant genes were created that encode proteins containing two or more mutated Cys residues (Figure 4.3). As previously described for the single mutants, the complete coding region of *TGA1* in pBC-SK+ served as a template for creating genes with multiple mutations (see section 3.3.8), which were subsequently ligated into pBI880 and pBI881 and introduced into yeast cells.

To further probe the role of Cys-266, two double mutant constructs encoding proteins containing Asn instead of Cys at residue 172 and either a Ser instead of Cys at residue 266 (TGA1^{C172N,C266S}) or a Asn instead of Cys at residue 260 (TGA1^{C172N,C260N}) were created and tested (Figure 4.3). Results indicate that TA:TGA1^{C172N,C266S} interacts

weakly with DB:NPR1, while TA:TGA1^{C172N,C260N} interacts with DB:NPR1 (Figure 4.3). TGA1^{C172N,C266S} was able to transactivate, while TGA1^{C172N,C260N} does not transactivate (Figure 4.3). These constructs confirm that Cys-260 is an important residue in TGA1's properties.

The TGA1^{C172N,C260N,C266S} and TGA1^{C172N,C260N,C266S,C287S} mutants were created to determine if Cys-287 affects the properties of TGA1 when none of the other Cys residues are present. Both TA:TGA1^{C172N,C260N,C266S} and TA:TGA1^{C172N,C260N,C266S,C287S} were able to interact with DB:NPR1 while the corresponding GAL4:DB fusions were unable to transactivate (Figure 4.3). These results indicate that Cys-287 does not affect the properties of TGA1 either in the presence of the other cysteines (Figure 3.3, see section 4.1.3) or in their absence (Figure 4.3).

A last construct was created to further investigate the significance of Cys-260. A construct where all of the cysteines but Cys-260 were mutated (TGA1^{C172N,C266S,C287S}) was created and tested. This construct interacts weakly with NPR1 and is capable of autonomous transactivation (Figure 4.3). This again supports a major role for Cys-260 in TGA1's properties.

4.1.4.1 Quantitative analysis of transactivation for TGA1 with mutations at multiple cysteine residues

Quantitative transactivation analysis was also completed on several constructs containing the mutation of multiple cysteines. DB:TGA1^{C172N,C266S} showed a decrease in transactivation to an average of 21.64 pM MU/μg of protein/min compared to DB:TGA1 (Figure 4.5). This value is significantly different to that obtained with DB:TGA1 ($p=0.05$) thus corroborating the β-galactosidase assay results. This value is

not significantly different to that obtained with DB:TGA1^{C172N} (p=0.05; Figure 4.4). These quantitative data indicate that mutating Cys-172 and Cys-266 simultaneously does not have a cumulative effect on TGA1 transactivation.

DB:TGA1^{C172N,C266S,C287S} showed a decrease in transactivation to an average of 25.20 pM MU/μg of protein/min compared to DB:TGA1 (Figure 4.5). This value is not significantly different from DB:TGA1 (p=0.05). It is significantly higher than DB:TGA1^{C260N} and DB:TGA1^{C260S,C266S}. (p=0.05). However, it is noteworthy that there was a large amount of variability in results obtained with DB:TGA1^{C172N,C266S,C287S}.

In the GUS assay, DB:TGA1^{C172N,C260S,C266S} and DB:TGA1^{C172N,C260S,C266S,C287S}, transactivation levels averaged 10.94 pM MU/μg of protein/min and 13.62 pM MU/μg of protein/min respectively (Figure 4.5). These values are not significantly different to one another, but are significantly different to that of DB:TGA1, according to Student's *t*-test (p=0.05). The transactivation levels of these constructs are also not significantly different than those of DB:TGA1^{C260S,C266S} or DB:TGA1^{C260S} (Figure 4.4). This appears to point to Cys-260 as possessing a key role in TGA1 transactivation, as mutating more cysteines does not affect transactivation any further. Also of note is the fact that the transactivation levels of DB:TGA1^{C172N,C260S,C266S} and DB:TGA1^{C172N,C260S,C266S,C287S} are significantly different than those of the DB:Cruc control (p=0.05; Figure 4.5). Since a certain level of transactivation exists even without the presence of the cysteines, another domain must also be involved in TGA1 transactivation.

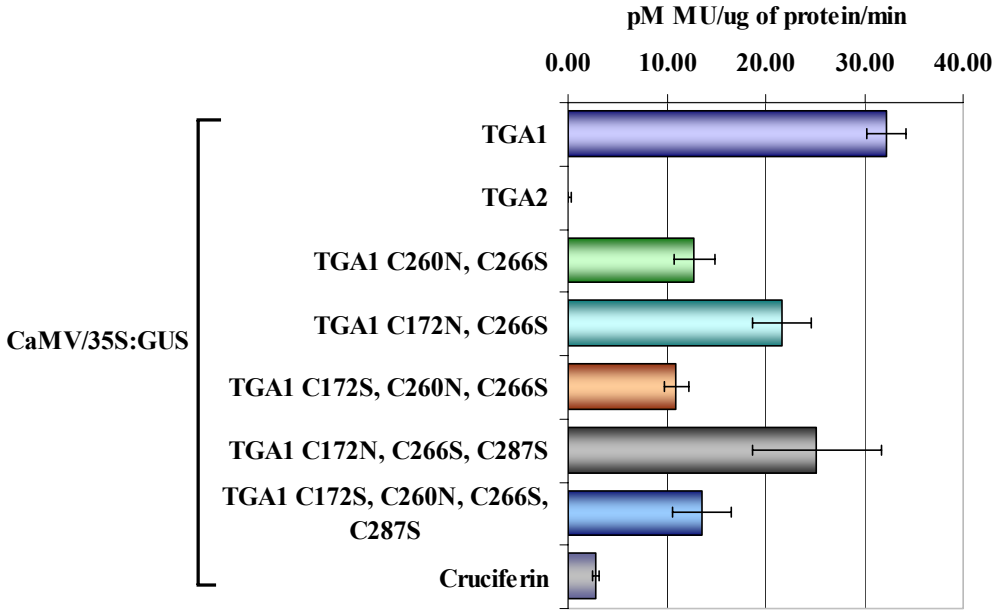

Figure 4.5 Quantitative GUS assay of transactivation of TGA1 with mutation at multiple cysteine residues.
Transactivation levels of TGA constructs and cruciferin when measured using the wild type CaMV 35S promoter. Each bar represents the average of six independent GUS assays from a total of two independent transformations ± SE.

4.2 Attempts to alter transactivation and NPR1 interacting properties of TGA2

Unlike TGA1, TGA2 does not transactivate in yeast but interacts strongly with NPR1 (Figure 4.2; 4.3; 4.4; Després et al., 2000). Therefore, in order to further study the influence of TGA1 Cys-172 and Cys-260 in TGA factor properties, a series of chimeric genes and site-directed mutants were created using *TGA2* as template. These constructs were then tested for their potential to alter the properties of this transcription factor.

As a first step, a *TGA2* mutant encoding a protein containing cysteines instead of Asn-218 and Ser-224 ($TGA2^{N218C,S224C}$; Figure 4.2, Figure 4.6), the corresponding positions to TGA1 Cys-260 and Cys-266, was created and introduced into yeast cells. β-galactosidase assay results indicate that this construct still interacts with NPR1 and is unable to transactivate (Figure 4.6). Therefore, this mutant behaves the same as TGA2, indicating that the addition of Cys residues at positions corresponding to Cys-260 and Cys-266 of TGA1 is not sufficient to alter TGA2 properties.

Given that the N-terminal domain of TGA1 has also been implicated in mediating TGA1 transactivation properties (Stonehouse, 2002), I tested the influence of this domain on TGA2 properties using a previously synthesized plasmid encoding the N-terminal of TGA1 including the bZIP domain and the C-terminal of TGA2 (TGA1 bZip/ TGA2). This construct interacts with NPR1 and is unable to transactivate (Figure 4.6). Therefore, this mutant behaves the same as TGA2, indicating that the N-terminal of TGA1 is not sufficient to confer transactivational properties onto TGA2 or prevent its interaction with NPR1 (see section 2.6.1; Figure 4.6).

I next created genes that introduced Cys residues found within the 30 amino acid region of TGA1 into the TGA1/TGA2 chimeric protein, ligated them into pBI880 and

Figure 4.6 β-galactosidase assay results for TGA1/TGA2 chimeric and site-directed mutants used in this study.
Schematic representation of TGA1 (orange), TGA2 (blue) constructs showing the bZip domain of each (lighter shade) and location of point mutations (coloured bars). β-galactosidase assay results for transactivation and NPR1 interaction indicated by – (white), +/- (faint blue), and + (blue) after 24 hours. The +/- symbol is used to indicate when the blue colour observed was considerably less than the positive control (interaction between NPR1 and TGA2). Transactivation results were obtained from mutant constructs fused to the GAL4 DB domain in yeast cells coexpressing cruciferin fused to the GAL4 TA domain. NPR1 interaction results were obtained from mutant constructs fused to the GAL4 TA domain in yeast cells coexpressing NPR1 fused to the GAL4 DB domain. Drawings are not to scale.

pBI881 and transformed them into yeast cells. None of these constructs, TGA1bZip/TGA2^{N218C,S224C}, TGA1bZip/TGA2^{N218C}, or TGA1bZip/TGA2^{S224C} transactivated while all still interacted with NPR1 (Figure 4.6).

TGA2 possesses a single Cys residue, located at amino acid 186 (Figure 4.2). To test for the possibility that this residue participates in a disulfide that is affecting the properties of TGA2, it was targeted for mutation to phenylalanine (F), the corresponding residue from TGA1 (Figure 4.6). This construct, TGA1bZip/ TGA2^{C186F}, was transferred into pBI880 and pBI881 which were transformed into yeast cells. Similar to TGA2, This construct did not transactivate but did interact with NPR1.

If a disulfide bond between Cys-172 and Cys-260 of TGA1 is crucial for the changes observed in TGA1 (see section 4.1.3.1), then perhaps the presence of a Cys in the corresponding residues may be necessary to see the equivalent change in TGA2. The final plasmid created encodes a protein that includes both of the corresponding TGA1 Cys-172 and Cys-260 residues, TGA1bZip/TGA2^{N130C,N281C}, which was transferred into pBI880 and pBI881 (Figure 4.6). This construct interacted with NPR1 and did not transactivate (Figure 4.6). These results indicate that the presence of the N-terminal of TGA1 in conjunction with the Cys residues implicated in the having the greatest affect on TGA1 function is not sufficient to alter TGA2 properties.

4.3 Identification of a *trans*-repression domain in TGA2

Attempts to change the properties of TGA2, using the domains identified in TGA1 as being important for functionality, have thus far failed. This suggests that amino acids other than the cysteines and those in the N-terminal play a key role in determining TGA2's properties.

One possibility to explain lack of transactivation is the presence of a repressor domain within TGA2. In an attempt to identify such a domain, I tested additional chimeric *TGA1/TGA2* genes that had been previously created (Figure 4.7). I ligated these genes into pBI880 and pBI881, and introduced the resulting plasmids into yeast and tested colonies for *LacZ* expression by β-galactosidase assays.

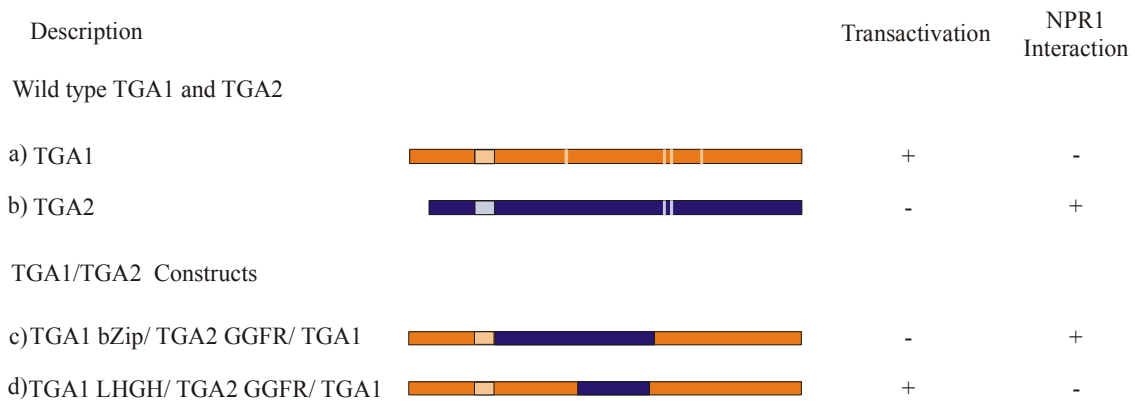

Figure 4.7 β-galactosidase assay results for TGA1/TGA2 chimeric and site-directed mutants used in this study.
Schematic representation of TGA1 (orange), TGA2 (blue) constructs showing the bZip domain of each (lighter shade) and location of point mutations (coloured bars). β-galactosidase assay results for transactivation and NPR1 interaction indicated by – (white), +/- (faint blue), and + (blue) after 24 hours. The +/- symbol is used to indicate when the blue colour observed was considerably less than the positive control. (interaction between NPR1 and TGA2). Transactivation results were obtained from mutant constructs fused to the GAL4 DB domain in yeast cells coexpressing cruciferin fused to the GAL4 TA domain. NPR1 interaction results were obtained from mutant constructs fused to the GAL4 TA domain in yeast cells coexpressing NPR1 fused to the GAL4 DB domain. Drawings are not to scale.

One gene encoded a chimeric protein that included the N-terminal of TGA1 up to the bZip domain, TGA2 up to the GGFR sequence and the C-terminal of TGA1 (TGA1 bZip/ TGA2 GGFR/ TGA1; see Figure 4.1 for location of GGFR sequence). TA:TGA1 bZip/ TGA2 GGFR/ TGA1 interacted with DB:NPR1 while DB:TGA1 bZip/ TGA2 GGFR/ TGA1 is unable to transactivate (Figure 4.7). The section of TGA1 replaced by TGA2 in the above chimeric (TGA1 bZip/ TGA2 GGFR/ TGA1) includes only one Cys (Cys-172). Earlier experiments had indicated that modification of this Cys enables the mutant protein to interact with NPR1, and reduced the transactivation properties (see $TGA1^{C172N}$, Figure 4.2, 4.4). The observation that DB:TGA1 bZip/ TGA2 GGFR/ TGA1 cannot transactivate suggested that the area of TGA2 between the bZIP and the GGFR may contain a repressor domain. To further localize this putative domain, I tested another chimeric protein in which the TGA1 sequences extend past the bZIP and Cys-172 until the residues LHGH (TGA1 LHGH/ TGA2 GGFR/ TGA1). TA:TGA1 LHGH/ TGA2 GGFR/ TGA1 is unable to interact with DB:NPR1 while DB:TGA1 LHGH/ TGA2 GGFR/ TGA1 is able to transactivate (Figure 4.7). These results suggest that the region between the bZIP and LHGH of TGA2 (Figure 4.1) may contain a putative repressor domain. This domain is also important for mediating the interaction with NPR1, since substitution of the corresponding region from TGA1 abolishes the interaction between TGA2 and NPR1 (compare results of TGA1 LHGH/ TGA2 GGRF/ TGA1 and TGA1 bZip/ TGA2 GGFR/ TGA1, Figure 4.7).

CHAPTER 5: DISCUSSION

Following the identification of TGA factors as proteins capable of binding the *as-1* DNA element in 1989 (Katagiri et al., 1989; Lam et al., 1989), much research has focused on determining their function *in planta*. A breakthrough came in the late 1990s, when TGA factors were found to interact with the disease resistance protein NPR1 (Zhang et al., 1999; Després et al., 2000; Zhou et al., 2000). Subsequent research established that NPR1 is an important regulator of TGA factor function *in vitro* and *in planta* (Després et al., 2000; Fan and Dong, 2002; Johnson et al., 2003). Very recently, analysis of a mutant lacking all three group II *Arabidopsis* TGA factors (TGA2, TGA5, TGA6) confirmed the need for these transcription factors for SA-induced *PR-1* expression and SAR (Zhang et al., 2003).

Although the above studies clearly indicate a role for TGA factors in disease resistance, little attention has been paid to identifying protein domains required for function or modes of regulation. My project not only identifies elements necessary for protein function more precisely, but also proposes a method by which they may be regulated.

Despite the fact that they share over 50% amino acid identity, the *Arabidopsis* TGA1 and TGA2 differ in their ability to interact with NPR1 and transactivate in yeast. This prompted the laboratory to create chimeric proteins between these two TGA factors, in an effort to identify functionally relevant protein domains. I subsequently

created additional chimeric genes and used site-directed mutagenesis to identify individual amino acids regulating TGA function.

5.1 Cysteines 260 and 266 of TGA1 affect interaction with NPR1

Arabidopsis TGA1 and TGA4 are unique within the TGA family as they are the only members unable to interact with the key disease regulatory protein, NPR1, in the yeast two-hybrid system (Zhang et al., 1999; Després et al., 2000; Zhou et al., 2000). Using a domain swapping approach a thirty amino acid region in TGA2 was identified as being crucial for interaction with NPR1 (Després et al., 2003). Within this region many residues are conserved between two or more TGA factors, with several being conserved in all seven of the *Arabidopsis* TGA factors examined. The conserved nature of TGA factors leads to the assumption that their divergent properties are due to slight variations in amino acid sequence. To examine this possibility, the region was scanned for residues conserved within TGA1 and TGA4 but different than the conserved residue found in the remaining TGA factors. Only one residue fulfilled the parameters of the search; a Cys residue corresponding to amino acid 266 of TGA1 and 262 of TGA4 (Figure 4.1). Since cysteines possess the unique ability to form disulfide bonds between their sulfhydryl side chains, the other Cys present in the 30 amino acid region, corresponding to residue 260 of TGA1 and 256 of TGA4, was also chosen for further study.

Using site-directed mutagenesis, mutant *TGA1* genes were created to encode proteins containing the corresponding amino acids found in TGA2 (asparagine and serine, respectively) in place of cysteines 260 and 266 ($TGA1^{C260N,C266S}$ and $TGA4^{C256S,C262S}$). Each of these SDM constructs, unlike the Cys-containing wild type

protein, interacted with NPR1 in yeast. Using a novel transient expression assay conceptually similar to the yeast two-hybrid system, the TGA1 SDM was also shown to interact with NPR1 when plasmids encoding both genes were transfected into *Arabidopsis* leaves by biolistics (Després et al., 2003). When each Cys was mutated separately and tested in yeast, the TGA1^{C266S} SDM construct appeared to interact with NPR1 weakly whereas the TGA1^{C260N} SDM construct interacted with NPR1 strongly. These results indicate that while both residues affect the ability of TGA1 to interact with NPR1, Cys-260 may play a more prominent role. These results demonstrate that it is possible to dramatically change the properties of TGA1/TGA4 by modifying key Cys residues and imply that these proteins possess all of the structural elements necessary for interaction with NPR1.

If TGA1 and TGA4 possess all of the elements required for NPR1 interaction, the question then arises: how do the Cys residues regulate these proteins' properties such that they are not capable of interacting with NPR1 in yeast? The answer is most likely found in the property unique to Cys residues; disulfide bond formation. Reversible disulfide bond formation has been shown to regulate the properties of a number of cytosolic and nuclear proteins including transcription factors. One well-studied example is the prokaryotic transcription factor OxyR (for review, see Kim et al., 2002). The cysteines of OxyR exist in a reduced state under normal conditions, but in the presence of oxidative conditions an intramolecular disulfide bond is formed. The disulfide bond changes the conformation of the protein to allow for interaction with RNA polymerase and thus initiates transcription of its target genes (Storz and Tartaglia, 1992; Zheng et al., 2001).

Since no other amino acids can be oxidized to form a disulfide bond, it is generally accepted that mutation of cysteines to other amino acids will mimic the reduced state of this residue. Thus, the observation that TGA1/TGA4 SDM are capable of interacting with NPR1 in yeast, while the wild types cannot, suggests that (1) only the reduced TGA1/TGA1 interact with NPR1 and (2) TGA1/TGA4 exist in an oxidized form in yeast cells. Using an *in vitro* assay that distinguishes between sulfhydryls and disulfides, Després et al. (2003) confirmed that TGA1 is predominantly oxidized in yeast cells. Furthermore, this group demonstrated that the reduced and oxidized forms of TGA1 produced *in vitro* displayed slightly different mobilities during gel electrophoresis under non-reducing conditions. This change in mobility was not observed when Cys-260 was mutated. Together, these results suggest that TGA1 forms an intramolecular disulfide bond involving Cys-260 under oxidizing conditions. Formation of this disulfide bond in yeast or plant cells could mask or alter a binding surface in TGA1 required for interaction with NPR1. Because the TGA1/TGA4 SDMs lack the critical cysteines, they cannot form the disulfide bond, regardless of redox conditions, and thus constitutively interact with NPR1.

Assuming that the redox state of TGA1 and/or TGA4 is crucial for interaction with NPR1, the next question of relevance is: how is the redox state of these proteins regulated in plants? By combining the assay that distinguished between protein sulfhydryls and disulfides with western blot detection using antibodies specific to TGA1/TGA4, Després et al. (2003) found that unelicited (resting) *Arabidopsis* leaves contain roughly equal proportions of oxidized and reduced forms of these TGA factors. In contrast, leaves treated with SA for 24 hours contained almost exclusively reduced

TGA1/TGA4. These results indicate that SA treatment leads to reduction of TGA1/TGA4 cysteines. This is consistent with the findings of Mou et al. (2003) who measured a net increase in the ratio of reduced to oxidized glutathione, the major redox buffer present in plant cells, in *Arabidopsis* leaves following treatment with INA, a biologically active analog of SA. Therefore, it appears that treatment with SA or an SA analog leads to a net reduction in the cytoplasmic redox status, which in turn brings about the reduction of TGA1/TGA4 cysteines.

Using the transient plant two-hybrid assay, Després et al. (2003) tested interaction of NPR1 and TGA1 in *Arabidopsis* leaves. In resting leaves very little interaction between NPR1 and TGA1 was observed while in leaves treated with SA a significant amount of interaction was detected. When the experiment was repeated using the TGA1 SDM, interaction between the two proteins was high regardless of SA treatment. This is consistent with the model proposed above, in which only the reduced state of TGA1 is competent to interact with NPR1 and that mutagenesis of the cysteines mimics the reduced state of TGA1.

What then is the functional significance of the redox-mediated interaction between TGA1 and NPR1? NPR1 was previously reported to enhance the DNA-binding activity of interacting TGA factors *in vitro* (Després et al., 2000) and *in vivo* (Fan and Dong, 2002). *In vivo*, NPR1, together with SA, is both required for the binding of TGA2 and TGA3 to the *PR-1* promoter (Johnson et al., 2003) and for TGA2-mediated gene activation (Fan and Dong, 2002). Després et al. (2003) first tested whether the DNA binding activity of TGA1, in the absence of NPR1, was altered by redox conditions. Using electrophoretic mobility shift assays (EMSAs) they found no differences in

binding affinity, regardless of excess presence of reducing or oxidizing agents. Addition of NPR1 to the oxidized form of TGA1 also did not alter DNA-binding. Only when NPR1 was added to the reduced form of TGA1 was a stimulation of DNA binding observed.

Together, my results along with those of Després et al. (2003) are consistent with a model wherein the pool of TGA1/TGA4 in resting *Arabidopsis* cells is substantially oxidized. TGA1 forms an intramolecular disulfide bridge which alters its conformation, preventing interaction with NPR1. In the absence of NPR1 interaction, binding of TGA1 to cognate *cis*-elements is low. When SA accumulates inside *Arabidopsis* cells, as occurs following pathogen infection, TGA1 becomes reduced and adopts a conformation allowing interaction with NPR1. Binding of NPR1 to reduced TGA1 stimulates its DNA binding activity to cognate *cis*-elements leading to the activation of defence genes and ultimately to enhanced disease resistance (Figure 5.1).

5.2 Cysteine 260 and cysteine 266 affect TGA1's ability to transactivate

In addition to differing in ability to interact with NPR1, *Arabidopsis* TGA factors also possess differing abilities in regard to autonomous transactivation (Stonehouse, 2002). Therefore, the domain swapping constructs between TGA1 and TGA2 were exploited once again to identify regions important for transactivation. Instead of fusing the chimeric proteins with the GAL4-TA (as used to determine interaction with NPR1) they were fused with the GAL4-DB and tested in yeast for their ability to initiate transcription of the *β-Galactosidase* reporter gene. The same thirty amino acid region identified as being important for interaction with NPR1 was also identified to be crucial for transactivation. Again the two cysteines located within this region were found to be

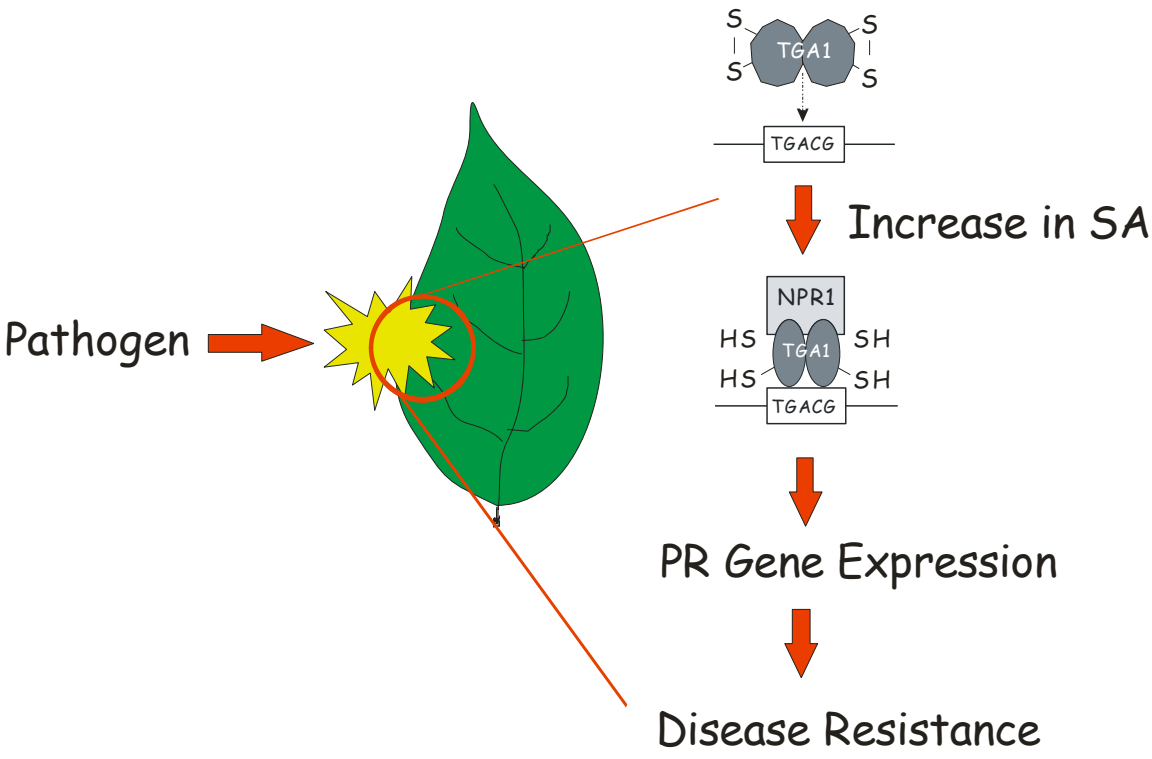

Figure 5.1 A model depicting redox control of TGA1 on binding activity (modified from Després et al., 2003).
In resting Arabidopsis cells TGA1 forms intramolecular disulfide bridges, which prevents interaction with NPR1. In response to a redox signal, such as pathogen-induces SA (Mou et al., 2003), TGA1 is reduced which leads to a conformation change that allows interaction with NPR1.

critical for TGA1 function. When the SDM TGA1^{C260N,C266S} and TGA4^{C256S,C262S} constructs were fused to the GAL4-DB an observable decrease in transactivation was detected compared to the wild type. This confirmed that the cysteines do play a role in controlling the proteins' transactivational ability. When each Cys was mutated separately and analyzed using the β-galactosidase assay, the TGA1^{C266S} SDM construct displayed the same transactivational level as the wild type, whereas the TGA1^{C260N} SDM construct appeared to only transactivate weakly compared to the wild type.

Since the β-galactosidase assay is not quantitative, the same GAL4-TA constructs were transferred into yeast cells containing the GUS reporter gene under the control of the cognate TGA factor promoter element (*as-1*) and quantitative assays were performed to assess transactivation. These tests revealed that simultaneous mutation of both Cys-260 and Cys-266 statistically reduced transactivation (2.5-fold) of TGA1, thus confirming the findings of the β-galactosidase assay that the cysteines are important for TGA1 transactivation. Furthermore, quantitative tests confirmed that mutation of Cys-266 had only minor effects (albeit statistically significant) on TGA1 transactivation, while mutation of Cys-260 affected transactivation to the same extent as when both cysteines are mutated. Therefore, it appears that the reduction in transactivation in the TGA1^{C260N,C266S} SDM can be attributed predominantly to Cys-260.

The results described above do not entirely dismiss a potential role of Cys-266 in regulating transactivation. Indeed there is a significant decrease in transactivation in the TGA1^{C266N} SDM compared to that of the wild type. At this time it can only be said that the contribution of Cys-266 to TGA1's transactivation potential in yeast is not as great as that of Cys-260. It is also important to point out that although the TGA1^{C260N,C266S} and

TGA1^{C260S} SDMs display a great decrease in transactivation, this property is not entirely abolished in either assay exploited. The continued presence of transactivation therefore leads to the conclusion that while the cysteines contribute significantly to transactivation, other elements must also be involved.

Deletion mutations of TGA1 determined that the N-terminal of TGA1 up to the basic domain is crucial for transactivation (Stonehouse, 2002). This is supported by studies of tobacco TGA factors TGA1a and TGA2.1 where the N-terminal was found to be required for transactivation (Pascuzzi et al., 1998; Niggeweg et al., 2000). Together with the above information, my results indicate that TGA1 contains a bipartite transactivation domain. Although we understand that the N-terminal and Cys-260 and Cys-266 participate in TGA1 transactivation in yeast, their relevance in plants has yet to be studied. As a first step, the SDM constructs are being tested for transactivation using a transient assay similar to the one described in Després et al. (2003) (C. Després, personal communication). The constructs are also being expressed in the context of transgenic plants to determine the consequences on *PR* gene expression and disease resistance (P. Fobert, personal communication). In the case of transgenic plants, determining the function of these proteins in the wild type genetic background has been greatly hindered by the presence of the redox-sensitive, wild type TGA1 and TGA4 proteins. Loss-of-function mutations in the genes encoding these proteins may facilitate analysis. However, functional redundancy within the TGA family, as demonstrated for group II factors (TGA2, TGA5, TGA6; Zhang et al., 2003), is also likely to be problematic for TGA1 and TGA4.

Based on the hypothesis that mutation of Cys residues mimic the reduced state of this amino acid, my data in yeast suggest that TGA1 would transactivate preferentially under oxidizing conditions. The demonstration that almost all TGA1 cysteines are oxidized in yeast cells (Després et al. 2003) and that TGA1 transactivates in these cells is consistent with the hypothesis. Preliminary results indicate that TGA1 does in fact transactivate in resting *Arabidopsis* cells (C. Després, unpublished). Based on the observation that SA leads to reduction of TGA1 cysteines (Despres et al., 2003), a reduction in transactivation when SA levels increase, such as following pathogen challenge, is expected to be observed. Therefore the $TGA1^{C260N,C266S}$ and $TGA1^{C260S}$ constructs would be predicted to mimic the reduced state in plants and would be unable to transactivate in the plant system. Therefore under these circumstances TGA1 may act as a transcriptional repressor following SA treatment.

5.3 The effect of the other cysteines in TGA1

In addition to the two Cys residues present in the 30 amino acid NPR1-interaction domain, TGA1 contains two other Cys residues. In the light of the results discussed in sections 4.2 and 4.3, it seemed relevant to test whether these additional Cys residues could also be involved in regulating TGA1 properties. To this end, site directed mutagenesis was used to change each Cys individually to encode the corresponding amino acids found in TGA2 (asparagine and serine) ($TGA1^{C172N}$ and $TGA1^{C287S}$). These mutant constructs were placed in a GAL4 TA containing vector, co-transformed into yeast cells with GAL4 DB:NPR1 and tested for protein interaction using the *β-galactosidase* reporter gene. While the mutation of Cys-287 does not appear to affect protein interaction, as no interaction is observed, mutation of Cys-172 enabled the

protein to interact with NPR1. The significance of Cys-172 in protein function versus that of Cys-287 is not entirely unexpected as Cys-172 is conserved between TGA1 and TGA4 whereas Cys-287 is not. This again raises the question of the possibility of a disulfide bond forming between Cys-172 and Cys-260 which may mediate TGA1:NPR1 interaction. However, additional research will be required to further assess the potential role of these Cys residues in regulating TGA1 function. For example, although Cys-260 has been shown to participate in a disulfide bond *in vitro* (Després et al., 2003), this has not yet been demonstrated for Cys-172. Furthermore, neither Cys residue has been shown to form a disulfide bond *in planta*.

To test whether the mutation of multiple cysteines would have an additive effect on protein interaction with NPR1, several constructs containing simultaneous mutation of multiple cysteines were created. An emphasis was placed on studying the effects of the two cysteines identified to influence TGA1:NPR1 interaction the greatest, Cys-172 and Cys-260. All of the mutant constructs created, $TGA1^{C172N,C260S}$, $TGA1^{C172S,C260N,C266S}$, and $TGA1^{C172S,C260N,C266S,C287S}$, interacted with NPR1 to the same extent as the $TGA1^{C172N}$ and $TGA1^{C260N}$ constructs. Therefore mutating multiple cysteines within TGA1 does not affect NPR1 interaction to a greater extent. This may be because the contribution to protein interaction of the remaining cysteines residues is marginal compared to the two key cysteines. However, it is important to remember that the β-galactosidase assay is only qualitative and may not be sensitive enough to detect slight variances in protein interaction. Quantitative tests in yeast or in plants will be necessary to resolve such changes. Although I did attempt such tests in yeast, they proved difficult to establish and were discontinued.

The mutant constructs were also placed in a vector containing the GAL4 DB and co-transformed with GAL4 TA:cruciferin into yeast cells and tested for transactivation. Using the *β-galactosidase* reporter gene it was found that the $TGA1^{C172N}$, $TGA1^{C287S}$, $TGA1^{C172N,C266S}$ SDM and $TGA1^{C172S,C266S,C287S}$ constructs transactivate, indicating that Cys-172 and Cys-287 are not crucial for TGA1 transactivation. In contrast, the $TGA1^{C172S,C260N,C266S}$, $TGA1^{C172S,C260N,C266S,C287S}$ SDM constructs do not transactivate. To confirm the observations from the β-galactosidase assays the same constructs were transferred into yeast cells containing the GUS reporter gene under the control of the *as-1* element and quantitative assays were performed to assess transactivation. These tests revealed that Cys-172, Cys-266, and Cys-287 affect transactivation slightly and only Cys-260 affects transactivation to a large extent. Any of the constructs containing mutation of multiple cysteines, that included the mutation of Cys-260, only had a decrease in transactivation equal to what is seen in the Cys-260 single SDM. Any multiple mutant constructs that did not include the mutation of Cys-260 had transactivation levels similar to the levels seen in the single mutation constructs. This confirms that while three of the four Cys residues of TGA1 (172, 260 and 266) affect the protein's ability to transactivate, Cys-260 possesses the greatest ability to affect transactivation. This also confirms that mutation of more than one Cys does not appear to interfere any further with transactivation capabilities than with only one Cys mutated. This is important to note because TGA1 appears to only create intramolecular disulfide bonds (Després et al., 2003). These results indicate that only Cys-260 is key to the formation of this disulfide bond as transactivation is only slightly reduced when either Cys-172 or Cys-266 are mutated. It is not known if either of Cys-172 or Cys-266 is the

preferred partner of Cys-260 in disulfide bond formation. It is possible that Cys-260 is capable of forming disulfide bonds with both of these residues, or preferentially interacts with one or the other under certain conditions. Furthermore, it is not known whether Cys-260 is capable of forming a disulfide bond with an alternative Cys if its preferred partner is mutated, nor is it known what the functional consequences of the novel bonding may have on TGA1 properties.

In addition to disulfide bond formation, it has been recently proposed that conjugation of Cys residues with glutathione moieties (glutathionylation) represents an alternative post-translational modification for protein cysteines (Shelton et al., 2005). Of note, glutathionylated Cys residues would behave as oxidized cysteines in the assays performed by Després et al. (2003). Thus, glutathionylation of Cys-260 would not require the participation of any other TGA1 cysteines which may explain why mutation of these other cysteines has only minor consequences on TGA1 function (compared to mutation of Cys-260).

Throughout this study, discrepancies were occasionally observed between results of β-galactosidase assay and MUG tests. In such instances, I typically sides with results from the latter assay, since it is quantitative and more sensitive, being based on the production of a fluorescence product. It is possible that some of the discrepancies may also be attributed to changes in DNA binding properties of TGA factors, which would only be detected in the MUG assays, since it relied on binding to the cognate *cis*-element of TGA factors.

5.4 TGA2 properties

Redox regulation through the reversible formation of disulfide bonds between Cys residues may not be a phenomenon limited to TGA1 and TGA4. Each subgroup of the TGA family possesses unique conserved Cys residues; members of subgroup I contain three conserved Cys residues, whereas members of subgroups II and III both contain one conserved Cys residue. The conserved nature of these residues is consistent with the theory that they are essential for protein function, perhaps through redox regulation. The possibility that all TGA factors may be controlled by redox conditions does not exclude the possibility that the regulation may differ between the subgroups or even between the factors in each group. TGA1 forms intramolecular disulfide bonds, most likely between Cys-260 and either Cys-172 or Cys-266, where the other TGA factors probably form intermolecular disulfide bonds due to the limited number of conserved Cys residues present in the protein. TGA2 possesses a single Cys residue and it is impossible for intramolecular disulfide bonds to form.

In yeast, TGA1 transactivates but is unable to interact with NPR1. By virtue of mutating only one Cys residue TGA1 was able to interact with NPR1 proving that it possesses all the requirements necessary for interaction. TGA2 however does not transactivate in yeast, but it does interact with NPR1. If regulation by redox status is conserved among TGA factors then by extension TGA2 may also possess all the elements necessary for transactivation it only needs its redox state switched for the property to be observed. To test this we applied the knowledge gained from TGA1's properties against that of TGA2. Initially only the Cys residues identified in TGA1 to participate in the disulfide bond were targeted to be studied in TGA2. A construct that

incorporated the corresponding Cys residues from TGA2 ($TGA2^{N218C,S224C}$) interacted with NPR1 and did not transactivate, indicating that the presence of these cysteines is not sufficient for transactivation. The largest variation in amino acid sequence between TGA1 and TGA2 is the presence of a forty five amino acid region at the N-terminal end of the protein. As this region has been shown to be crucial for transactivation in TGA1 (Stonehouse, 2002) its absence in TGA2 may preclude transactivation in yeast. Therefore a series of constructs were created in which the N-terminal of TGA1 was swapped for that of TGA2 and different combinations of residues were mutated to encode that of a Cys residue (TGA1bZip/$TGA2^{N218C}$, TGA1bZip/$TGA2^{S224C}$, TGA1bZip/$TGA2^{N218C,S224C}$, and TGA1bZip/$TGA2^{N130C,N218C}$). None of these constructs where able to transactivate in yeast indicating that other features are required.

At least two possibilities remain that may affect the ability for TGA2 to transactivate in yeast. The first is that the conserved Cys residue located at residue 186 may be forming a disulfide bond which is inhibiting the protein to transactivate. To account for this a construct containing the N-terminal of TGA1 and the C-terminal of TGA2 including the mutation of this Cys to encode the amino acid phenylalanine, the corresponding residue in TGA1 (TGA1bZip/$TGA2^{C186F}$) was created, but like the other constructs did not transactivate in yeast and still interacted with NPR1. Thus, I was unable to obtain experimental evidence to support the participation of cysteine residues in the regulation of TGA2.

A second possibility is that a transactivational repression domain is present within TGA2. This is apparent in the quantitative assay as TGA2 displays a decrease in transactivation to a level significantly lower than background levels. Two constructs

may be important in identifying a repression domain, TGA1 LHGH/ TGA2 GGFR/ TGA1 and TGA1 bZip/ TGA2 GGFR/ TGA1. The chimeric construct TGA1 LHGH/ TGA2 GGFR/ TGA1 does not interact with NPR1 but does transactivate whereas the chimeric construct TGA1 bZip/ TGA2 GGFR/ TGA1 interacts with NPR1 and does not transactivate in yeast. The only difference between these two constructs is the presence of a 34 amino acid region of TGA1 found in the TGA1 LHGH/ TGA2 GGFR/ TGA1. To confirm that the region does contain a repression domain constructs would need to be made that swapped this region between TGA1 and TGA2. If a repression domain does exist it would be expected that the transactivational levels of a TGA2 construct lacking the domain would rise to at least background levels whereas the transactivational levels of a TGA1 construct containing the domain would dramatically decrease.

CHAPTER 6: REFERENCES

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