Regulation of Systemic Acquired Resistance through the Interaction of *Arabidopsis*thaliana Transcription factors TGA1 and TGA2 with NPR1

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ABSTRACT

Arabidopsis thaliana is an established model plant system for studying plant-pathogen interactions. The knowledge garnered from examining the mechanism of induced disease resistance in this model system can be applied to eliminate the cost and danger associated with current means of crop protection.

A specific defense pathway, known as systemic acquired resistance (SAR), involves whole plant protection from a wide variety of bacterial, viral and fungal pathogens and remains induced weeks to months after being triggered. The ability of Arabidopsis to mount SAR depends on the accumulation of salicylic acid (SA), the NPR1 (non-expressor of pathogenesis related gene 1) protein and the expression of a subset of pathogenesis related (*PR*) genes. NPR1 exerts its effect in this pathway through interaction with a closely related class of bZIP transcription factors known as TGA factors, which are named for their recognition of the cognate DNA motif <u>TGA</u>CG.

We have discovered that one of these transcription factors, TGA2, behaves as a repressor in unchallenged Arabidopsis and acts to repress NPR1-dependent activation of *PR1*. TGA1, which bears moderate sequence similarity to TGA2, acts as a transcriptional activator in unchallenged Arabidopsis, however the significance of this activity is unclear. Once SAR has been induced, TGA1 and TGA2 interact with NPR1 to form complexes that are capable of activating transcription. Curiously, although TGA1 is capable of transactivating, the ability of the TGA1-NPR1 complex to activate transcription results from a novel transactivation domain in NPR1. This transactivation domain, which depends on the oxidation of cysteines 521 and 529, is also responsible for the transactivation ability of the TGA2-NPR1 complex.

Although the exact mechanism preventing TGA2-NPR1 interaction in unchallenged Arabidopsis is unclear, the regulation of TGA1-NPR1 interaction is based on the redox status of cysteines 260 and 266 in TGA1. We determined that a glutaredoxin, which is an enzyme capable of regulating a protein's redox status, interacts with the reduced form of TGA1 and this interaction results in the glutathionylation of TGA1 and a loss of interaction with NPR1.

Taken together, these results expand our understanding of how TGA transcription factors and NPR1 behave to regulate events and gene expression during SAR. Furthermore, the regulation of the behavior of both TGA1 and NPR1 by their redox status and the involvement of a glutaredoxin in modulating TGA1-NPR1 interaction suggests the redox regulation of proteins is a general mechanism implemented in SAR.

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LIST OF ABBREVIATIONS

2,4-D - 2,4-dichlorophenoxyacetic acid

acd - accelerated cell death

AIP - 2-aminoindan-2-phosphoric acid

as-1 – activation sequence 1

Avr - avirulence

BA2H – benzoic acid 2-hydroxylase

BTB/POZ - Broad-Complex, Tramtrack, and Bric-a-brac/Pox virus and Zinc finger

BTH – benzo-1,2,3-thiadiazole-7-carbothioic acid S-methyl ester or benzothiadiazole

bZIP – basic domain/leucine zipper

CaMV – cauliflower mosaic virus

ChIP - chromatin immunoprecipitation

CHX – cycloheximide

cim – constitutive immunity

cv - cultivar

Cys – cysteine

DB – DNA binding domain

DIR1 - defective in induced resistance 1

DTT – dithiothrietol

eds – enhanced disease susceptibility

EH1 – epoxide hydroxylase 1

FAD – flavin adenine dinucleotide

GAL – galactosidase

GR – glutathione reductase

Grx - glutaredoxin

GRX480 – Arabidopsis glutaredoxin At1g28480

GRX030 – Arabidopsis glutaredoxin At5g63030

GS• – thiyl radical

GSH - reduced glutathione

GS-NO – S-nitrosoglutathione

GSSG - oxidized glutathione

GST – glutathione S-transferase

GTase – glycosyl transferase

HED – β -hydroxyethylene disulfide

H₂O₂ – hydrogen peroxide

HO' – hydroxyl radical

HR – hypersensitive response

ICS – isochorismate synthase

IEGT1 – immediate-early-induced glucosyl transferase 1

INA – 2,6-dichloroisonicotini acid

IP – immunoprecipitation

IPL – isochorismate pyruvate lyase

ISR – induced disease resistance

JA – jasmonic acid

k_{cat} – catalytic constant

k_d – dissociation constant

k_M - Michealis-Menton constant

LS – linker scanning

lsd – lesion simulating disease

MALDI TOF MS – matrix assisted laser desorption ionization time-of-flight mass spectroscopy

MAP - mitogen-activated protein

MeJA – methyl jasmonate

MeSA – methyl salicylate

MPB – 3-(*N*-maleimido-propionyl) biocytin

MYB - myeloblastosis

NADPH - nicotinamide adenine dinucleotide phosphate

NEM – *N*-ethylmaleimide

NES – nuclear export sequence

NIM1 – non-inducible immunity 1

NLS - nuclear localization sequence

NMR – nuclear magnetic resonance

NO – nitric oxide

nos – nopaline synthase

NPR1 – non-expressor of pathogenesis related genes 1

O₂ - superoxide radical

ocs – octopine synthase

ONPG – ortho-nitrophenol- β -D-galactopyranoside

PAGE – polyacrylamide gel electrophoresis

PAL – phenylalanine ammonia lyase

PDF1.2 – plant defensin 1.2

PIOX – pathogen-induced oxygenase

PR – Pathogenesis related

PTM – post-translation modification

pv – pathovar

R – resistance

ROS - reactive oxygen species

SA – salicylic acid

SAI1 – salicylic acid insensitive 1

SAR – systemic acquired resistance

SDS – sodium dodecyl sulfate

SH - sulfhydryl

sid – SA-induction deficient

SNI1 – suppressor or *npr1-1*, inducible 1

S-OH - sulfenic acid

 $S-O_2H$ – sulfinic acid

S-O₃H - sulfonic acid

S-S - disulfide

TA - transactivation domain

t-CA - trans cinnamic acid

TF – transcription factor

Thi2.1 – thionin

TMV - tobacco mosaic virus

TNV - tobacco necrosis virus

Trx – thioredoxin

TrxR - thioredoxin reductase

UAS - upstream activation sequence

VIGS - virus induced gene silencing

 V_{max} – maximum velocity

Vir – virulence

VP16 - viral particle 16

X-Gal – 5-bromo-4-chloro-3-indoyl- β -D-galactopyransoide

CHAPTER 1 – INTRODUCTION

Plants are sessile beings that have evolved intricate constitutive and inducible defense mechanisms to protect themselves from invading pathogens. Constitutive defenses, which include physical barriers such as cuticle and waxes as well as pre-formed proteins and secondary metabolites, provide the plant's first level of defense (Osbourn, 1996). However if this initial level of defense is breached, inducible defense pathways can be rapidly triggered.

When an induced defense pathway is initiated, a series of events take place at the site of pathogen attack including localized cell death, known as the hypersensitive response (HR), and the accumulation of metabolites such as salicylic acid, jasmonic acid and ethylene (Greenberg, 1997, Dong, 1998). However, the key to restricting further pathogen growth and maintaining a resistant state during induced defense is the specific induction and coordination of numerous genes and proteins. A collection of genes that are either up- or down-regulated as a result of induced disease resistance have been identified through a multitude of microarray studies (Schenk et al., 2000; Reymond et al., 2000; Maleck et al., 2000). Regulation of disease resistance proceeds via de novo synthesized proteins or pre-existing proteins whose activity is altered as, a result of the addition or removal of post translational modifications (PTMs) (Kovtun et al., 2000; Després et al., 2003; Kang and Klessig, 2005). By studying the proteins responsible for regulating gene expression and the post-translational control that regulates them, it will be possible to elucidate key steps in the signaling cascade that leads to disease resistance. Ultimately, this elucidation will result in the identification of key genes and pathways that can be engineered to enhance disease resistance in crop plants.

Systemic acquired resistance (SAR) is the most widely studied inducible defense pathway in plants. It is defined as being a broad-range, long-lasting defense pathway induced by avirulent pathogen attack and is typified by the accumulation of salicylic acid (SA) and the induction of a suite of pathogenesis related (*PR*) genes (Glazebrook, 2001). One of the most salient features of this defense pathway, aside from its systemic nature, is that once induced, the plant is primed such that subsequent pathogen attack, whether virulent or avirulent, is unsuccessful (Ward et al., 1991). While key genes involved in regulating SAR have been identified over the past fifteen years, details concerning their regulation and mode of action remain elusive.

1.1 Outline

The goal of the research presented in this thesis is to better understand the role of TGA transcription factors during SAR, specifically focusing on their interaction with NPR1 and the behavior of this complex.

Chapter 2 is comprehensive literature review that provides background on the SAR pathway including details concerning key proteins and metabolites as well as signaling pathways and timing of events.

Chapter 3 is a published manuscript that establishes the role of the TGA2 transcription factor as a repressor and explores the nature of the TGA2-NPR1 complex as a regulator of *PR1* gene expression. Protein domains critical for NPR1's co-activator function are identified including a novel transactivation domain, found in the C-terminus, regulated by the oxidation of two cysteine residues.

Chapter 4 is a manuscript in preparation that explores the role of the TGA1 transcription factor with respect to its interaction with NPR1. TGA1 is redox regulated so

that in the oxidized state it will transactivate but not interact with NPR1, while in the reduced state, it interacts with NPR1 but does not transactivate. TGA1 also interacts with a novel glutaredoxin leading to the glutathionylation of TGA1 and a loss of interaction with NPR1. This is one of the first instances where an atypical redox switch in plants is described and where a specific activity and target has been defined for a novel glutaredoxin.

Chapter 5 examines the similarities and differences between TGA1 and TGA2 as closely related transcription factors involved in SAR. Also examined in this chapter is the redox regulation of TGA1 and NPR1 and how they represent a general mechanism for gene activation in the SAR pathway.

CHAPTER 2 – LITERATURE REVIEW – SYSTEMIC ACQUIRED RESISTANCE

2.1 Induced Disease Resistance

As plants do not have the ability to physically evade pathogens, they have evolved an extensive network of defense mechanisms, both constitutive and induced, to aid in their survival.

Induced disease resistance is a specific response and is not always initiated upon pathogen challenge. However all plants are able to evoke constitutive defense mechanisms which are present to slow down or prevent the spread of a pathogen. These include; (i) an oxidative burst, which creates reactive oxygen species (ROS) that have a broad range of effects (reviewed by Lamb and Dixon, 1997), (ii) deposition of callose (reviewed by Kauss, 1987) and physical thickening of the cell wall by lignification (reviewed by Vance et al., 1980), which provides a physical barrier to the pathogen, and (iii) various pre-existing antibiotic small molecules, such as phytoalexins (reviewed by Dixon, 1986) and proteins, such as cell wall hydrolases (reviewed by Bowles, 1990) that are often toxic or destructive to the invading pathogen. While these initial constitutive and mostly non-pathogen-specific defenses offer some level of protection, many pathogens are able to overcome these defenses to successfully invade the plant. However in certain instances, when genetic factors in both the host and the pathogen are present, an additional, pathogen-specific disease resistance pathway is induced.

2.1.1 Plant-Pathogen Recognition

The specific recognition of a pathogen by a plant requires the presence of unique factors in both organisms and results in the induction of a distinct signal transduction pathway. This leads to the expression of a subset of genes providing a resistance tailored to the invading pathogen. These factors were originally identified by Flor in the 1940s through his studies of the host/pathogen interaction between flax (*Linum ultissimum*) and flax rust fungi (*Melampsora lini*). He discovered that disease resistance would only occur during an incompatible interaction when the plant possessed a dominant resistance gene (R) and the pathogen expressed the complementary dominant avirulence gene (Avr), a phenomenon he termed gene-for-gene resistance (Flor, 1971; Hammond-Kosk and Jones, 2000). However in a compatible interaction, either the R gene, Avr gene or both are recessive resulting in a diseased state (Flor, 1971; Hammond-Kosk and Jones, 2000). Interestingly, in the instance of a compatible interaction, the Avr gene from the pathogen is required for pathogen virulence making it a Vir (virulence) gene (Leach and White, 1996; White et al., 2000).

2.1.2 Systemic Acquired Resistance

Systemic acquired resistance (SAR) is defined as a broad-spectrum disease resistance pathway induced in plants after attack by avirulent pathogens. The response results in the induction of a distinct signal transduction pathway at the site of infection and in distal cells. SAR is distinguished from other induced defense pathways by the spectrum of pathogen protection that is observed and the subset of defense genes whose expression is induced (Ward et al., 1991). Chester (1933a and b) was the first to identify

the phenomenon of acquired resistance in plants. But it was not until the 1960s when this induced immunity was clearly defined and termed SAR. Ross (1961 and 1966) observed that tobacco, which would react hypersensitively to tobacco mosaic virus (TMV), would also induce resistance in uninfected parts of the plant. This systemic induction of defense genes resulted in decreased symptom severity from secondary infection from several different pathogens.

The induction of SAR relies on the accumulation of the metabolite salicylic acid (SA) and the expression of a suite of pathogenesis-related (PR) genes through the action of the protein NPR1 (non-expressor of pathogenesis related gene 1) and TGA transcription factors.

2.1.3 Alternative Defense Pathways and Induced Systemic Resistance

An alternative systemic inducible defense pathway was identified in Arabidopsis and characterized by its SA-independent induction of a different subset of *PR* genes, namely the antimicrobial peptide defensin (PDF1.2) and thionin (Thi2.1). In this induced disease resistance pathway, *PDF1.2* and *Thi2.1* expression is induced by non-host fungal pathogens via a signaling pathway that involves jasmonate (JA) and ethylene (Penninckx et al., 1996; Eppel et al., 1997; Penninckx et al., 1998).

Another inducible defense pathway, known as induced systemic resistance (ISR), has also been found that leads to increased resistance in secondary tissue after pathogen attack. This pathway, which is also SA-independent, is induced by plant growth-promoting rhizobacteria and relies on a pathway regulated by JA and ethylene. Interestingly, this pathway does not result in the induction of *PDF1.2* (Pieterse et al., 1996; van Loon et al., 1998; Pieterse et al., 1998).

The interplay between SA and JA/ethylene in defense response pathways is not well understood (Maleck and Dietrich, 1999). Lawton et al. (1994) and Xu et al. (1996) found evidence to demonstrate that these signaling molecules work synergistically to induce defense responses. However evidence, including the inhibition of JA biosynthesis by SA, suggests that these molecules may also work antagonistically (Doares et al., 1995; Harms et al., 1998). The existence of positive and negative interconnections between SA and JA/ethylene provides an excellent control mechanism for the plant such that the accumulation of specific signaling molecules would not only induce the correct defense pathway(s), but would also be able to down-regulate or turn off pathways. While the detailed relationships between the induction of disease resistance pathways and their signaling molecules is outside the scope of this thesis, reviews by Dong (1998) and Glazebrook (2001), and more recently Loake and Grant (2007) and Flors et al. (2008), provide excellent insight into the subject.

2.2 The Role of Salicylic Acid in SAR

The accumulation of the metabolite salicylic acid (SA) is required for the establishment and deployment of SAR. The role of SA in SAR was first described by White and colleagues who discovered that tobacco leaves treated with SA or acetyl salicylic acid (aspirin) displayed heightened resistance to TMV and increased *PR* gene expression (White, 1979; Antoniw and White 1980).

Evidence for an endogenous role of SA in SAR came ten years later from analyses of SA levels in pathogen infected tobacco and cucumber plants. Malamy et al. (1990) observed that SA levels increased 20- to 50-fold following TMV infection of tobacco and these increases coincided with or preceded *PR* gene expression.

Additionally, a 10-fold increase in SA levels was observed in uninoculated leaves that coincided with the accumulation of *PR* transcripts. In the phloem sap of cucumber infected with *Colletotrichum lagenarium*, *Pseudomonas syringae* or tobacco necrosis virus (TNV), a 10- to 100-fold increase in SA was observed prior to the development of SAR (Métraux et al. 1990; Rasmussen et al., 1991; Smith et al., 1991).

Evidence for the requirement of SA in SAR came from plants expressing a bacterial salicylate hydroxylase gene (nahG) that converted SA into inactive catechol, which prevented the activation of SAR. Transgenic tobacco and Arabidopsis expressing nahG fail to accumulate high levels of SA after pathogen infection and also fail to develop SAR or to express PR genes in uninoculated leaves (Delaney et al., 1994; Vernooij et al., 1994; Gaffney et al., 1994).

The SAR pathway has been identified and studied in many dicotyledonous plant species where it is activated in response to a wide variety of plant pathogens (Malamy and Klessig, 1992; Ryals et al., 1996; Shah and Klessig, 1999). However the role of SA in the SAR defense pathway of monocots such as rice and maize is not as well established (Matsuta et al., 1991; Morris et al., 1998; Vallélian-Bindschedlir et al., 1998).

2.2.1 SA Analogs

To date, SA is the only plant-derived substance that has been demonstrated to be an inducer of SAR (White, 1979; Antoniw and White, 1980; Ward et al., 1991). Activation of SAR by SA (Figure 1), provides a very attractive means for an alternative form of disease control. In order for a compound to be considered as an activator of SAR, it needs to exhibit the following three qualities: (i) the compound or its significant metabolites should not exhibit direct antimicrobial activity, (ii) it should induce resistance

Figure 1. Three Inducers of Plant Defense

SA is the only plant derived substance known to be an inducer of SAR while INA and BTH are synthetic SAR inducers.

to the same spectrum of pathogens as in biologically activated SAR, and (iii) it should induce the expression of the same marker genes observed in pathogen-activated SAR (Kessmann et al., 1995). The chemical 2,6-dichloroisonicotinic acid and its methyl ester (both referred to as INA; Figure 1) were the first synthetic compounds shown to activate SAR (Métraux et al, 1991; Vernooij et al., 1995). However both SA and INA do not warrant practical use as plant protection compounds due to low crop tolerance resulting from phytotoxicity (Friedrich et al., 1996). Recently, the synthetic SA analog benzo-1,2,3- thiadiazole-7-carbothioic acid S-methyl ester (BTH; Figure 1) was demonstrated to be a potent activator of SAR (Friedrich et al., 1996; Gorlach et al., 1996; Lawton et al., 1996) and is marketed for use on a limited number of crops by Syngenta.

2.2.2 Biosynthesis of SA

Considering the significance of SA in the SAR pathway, a great deal of research has focused on the biosynthetic pathway leading to SA. Currently, there are two competing pathways proposed for SA biosynthesis. In 1993, Yalpani et al. observed that TMV-infected tobacco synthesize SA through a side branch of the phenylpropanoid pathway. Production of SA through this pathway (Figure 2) is unclear although it is believed to occur via an oxidative (Alibert and Ranjeva, 1971) or non-oxidative, chain shortening mechanism (Yazaki et al., 1991; Schnittzler et al., 1992). The first step toward SA biosynthesis is the conversion of phenylalanine to *trans*-cinnamic acid (*t*-CA), catalyzed by the enzyme phenylalanine ammonia lyase (PAL). When Mauch-Mani and Slusarenko (1996) used the PAL inhibitor AIP (2-aminoindan-2-phosphoric acid) as a pre-treatment on Arabidopsis to block general phenylpropanoid metabolism, they

Figure 2. Proposed Phenylpropanoid Pathway for SA Biosynthesis in Plants

The oxidative and non-oxidative pathways for the conversion of *trans*-cinnamic acid to benzoic acid leading to the formation of SA. Solid arrows indicate established biochemical reactions whereas broken arrows indicate possible steps not yet described. PAL – phenylalanine ammonia lyase; BA2H – benzoic acid 2-hydroxylase (Adapted from Ryals et al., 1996).

observed that Arabidopsis, which is normally resistant to *Peronospora parasitica* was now susceptible. Furthermore, Pallas et al. (1996) discovered that sense suppression of the *PAL* gene prevented both SAR development and *PR* gene expression in the uninoculated leaves of TMV-infected tobacco. Interestingly, Shadle et al. (2003) did not observe a change in resistance to TMV or an increase in SA levels in tobacco overexpressing PAL.

The final step in the biosynthesis of SA, as illustrated in Figure 2, involves the conversion of benzoic acid to SA via a cytochrome P450 monooxygenase called benzoic acid 2-hydroxylase (BA2H). Léon et al. (1993) observed that BA2H activity in cell free tobacco leaf extracts was 10-fold higher after inoculation with TMV and 6-fold higher after infiltration of healthy plants with benzoic acid (Léon et al., 1993; Yalpani et al., 1993).

Interestingly, a second pathway, typically found in mycobacteria, has been identified as a potential source of SA in plants (Figure 3). Expression of the bacterial enzymes isochorismate synthase (ICS1) and isochorismate pyruvate lyase (IPL1) in tobacco and Arabidopsis resulted in an accumulation of SA and an increase in pathogen resistance (Mauch et al., 2001; Verberne et al. 2000). In 1999, Nawrath and Métraux identified the SA-induction deficient mutants *sid1* and *sid2* in Arabidopsis, which are unable to accumulate SA after SAR induction. Alleles of *sid1* and *sid2*, called *eds5* and *eds16* (enhanced disease susceptibility) respectively, were identified independently with an enhanced disease susceptibility phenotype (Glazebrook et al., 1996; Dewdney et al., 2000). In 2001, Wildermuth and colleagues cloned SID2/EDS16 and found that it encoded a putative chloroplast-localized ICS whose expression is induced by infection in

Figure 3. Proposed Pathway for SA Biosynthesis in Plants Modeled from Mycobacteria

Bacterial pathway for biosynthesis of SA from chorismic acid also believed to operate in plants. ICS – isochorismate synthase; IPL – isochorismate pyruvate lyase (Adapted from Wildermuth et al., 2001).

both local and systemic tissue. Although no function has been assigned to SID1/EDS5, it has sequence similarity to a transporter protein which might suggest a role in transport of SA or a phenolic precursor outside of the plastid (Nawrath et al.,2002).

Recently, Catinot et al. (2008) examined virus induced gene silencing (VIGS) of ICS in tobacco and found that SA accumulation was compromised but not completely abolished. Combined with the observation that SA still accumulates in *sid1leds16*, it would appear that both the phenylpropanoid and isochorismate pathways may both be involved in SA biosynthesis. It has been postulated that SA biosynthesis via the phenylpropanoid pathway is responsible for the rapid accumulation of SA at the site of infection associated with localized cell death while SA biosynthesis from isochorismate is more important for sustained SA biosynthesis during SAR (Wildermuth et al., 2001; Ferrari et al., 2003).

2.2.3 The Systemic Signal in SAR

While pathogen attack at the site of infection can trigger induced disease resistance in primary cells, a signal is required to relay this information to distal, uninfected parts of the plant. It was initially postulated that the elicitor from the invading pathogen diffused from the site of infection to other cells throughout the plant to transduce the signal via the R-avr interaction. Studies by Dorey et al. (1997) found this not to be the case. The levels of SA increase at the site of infection in distal tissues and in phloem sap of cucumber and tobacco prior to *PR* gene expression (Métraux et al., 1990; Yalpani et al., 1991; Malamy et al., 1991). The more popular theory is that SA acts as the long distance signal in SAR. *In vivo* SA-labeling studies using [¹⁴C]-benzoic acid (Mölders et al., 1996) and [¹⁸O]-SA (Shulaev et al., 1995), showed that leaves of TMV-

infected tobacco and TNV-infected cucumber transport the label from the site of infection throughout the plant where it accumulates in uninfected tissues. Despite this evidence, two key experiments contradict the role of SA as the long distance signal molecule in SAR. When primary cucumber leaves infected with P. syringae were removed six hours after inoculation, before SA accumulates in the phloem sap, neither the systemic increase in SA nor the expression of PR genes was affected (Rasmussen et al., 1991). Similarly, experiments with grafted tobacco plants showed that TMV-inoculated nahG rootstocks, which accumulate little SA, were still able to transmit a systemic signal to wild-type scions to induce PR gene expression and resistance comparable to ungrafted wild-type plants (Vernooij et al., 1994). Criticism of these studies was that SA accumulation was not totally abolished with a low undetectable level of SA likely occurring in the cucumber phloem sap while nahG rootstocks still accumulated a small amount of SA. It is therefore possible that SA may be the long distance signaling molecule if only small amounts are required to induce a response. However in experiments by Willet and Ryals (1998), where they were able to control the gradient of free SA produced in TMVinoculated leaves of tobacco, they observed that the level of free SA in the inoculated leaf is directly proportional to the level of SAR in the upper, uninoculated leaf. This would suggest that a high accumulation of SA is required to induce SAR to provide pathogen protection.

2.2.3.1 A Lipid Transfer Protein as a Systemic Signal

In 2002, Maldonado and colleagues identified the Arabidopsis mutant *dir1-1* (defective in induced resistance) which exhibits wild-type local resistance to avirulent *P. syringae*, but fails to develop SAR or induce *PR* gene expression in distal tissue. *DIR1*

encodes a putative apoplastic lipid transfer protein and is proposed to interact with a lipid derived molecule to promote long-distance signaling. Similarly, Nandi et al. (2004), identified the Arabidopsis mutant *sfd1* (suppressor of fatty acid desaturase) which is compromised in the accumulation of SA and expression of *PR* genes in distal tissue but local disease resistance is not affected. *SFD1* encodes a putative dihydroxyacetone phosphate reductase, which is postulated to be involved in creating the lipid derived molecule that interacts with DIR1 (Nandi et al., 2004; Truman et al., 2007).

2.2.3.2 Methyl Salicylate as a Systemic Signal

Methyl salicylate (MeSA), which results from the methylation of the carboxyl group in SA (refer to Figure 1), is a common conjugated form of SA observed in several plants species (Lee et al., 1995). In 1997, Du and Klessig identified an SA binding protein (SABP2) that has esterase activity and is responsible for converting MeSA to SA (Sesker et al., 1998). Silencing of SABP2 results in the suppression of local resistance to TMV and SAR development (Kumar and Klessig, 2003) and its activity is induced 4- to 5-fold in wild-type plants after treatment with SA (Park et al., 2007). Forouhar et al. (2005) established that SABP2 possesses specific esterase activity toward MeSA and that SA binds the active site of SABP2 with a dissociation constant (k_d) of 90nM. These findings, in combination with competition assays between SA and MeSA, suggest that SA is a potent inhibitor of SABP2's MeSA esterase activity providing a mechanism for feedback inhibition (Forouhar et al., 2005). Park et al. (2007) observed that when the SA feedback inhibition of SABP2 was compromised, SAR was not established after inoculation with TMV. This suggests that if the conversion of MeSA to SA is not inhibited by the corresponding increase in SA, MeSA fails to accumulate and act as the

mobile signal. Additionally, the esterase activity of SABP2 was only required for the development of SAR in systemic tissue. As a complementary experiment, Park et al. (2007) performed a grafting experiment where they found that the SA methyl transferase responsible for MeSA production from SA (NtSAMT1; Dudareva et al., 1998) is only required in the rootstock of a TMV-infected tobacco graft and not in the systemic tissue (scion).

While the results of Park et al. (2007) are very convincing, they do not disprove any of the observations of Maldonado et al. (2002) or Nandi et al. (2004) suggesting a lipid derived signal molecule acts as the long distance signal. Conversely, SA accumulation to wild-type levels in *dir1-1* mutants and the lack of induction of SAR through DIR1 overexpression has led to the hypothesis that DIR1 and SFD1 function in cooperation with MeSA as the long distance signal in SAR (Maldonado et al., 2002; Park et al., 2007).

2.2.4 SA Modes of Action

In order to understand how an increase in SA, whether local or systemic, is able to control pathogen resistance, it is necessary to understand how SA exerts its control.

2.2.4.1 SA, Catalase and Hydrogen Peroxide

In 1991, Chen and Klessig identified a soluble SA binding protein (SABP) in extracts of tobacco leaves. They found that this protein strongly and reversibly bound SA and its biologically active analogs but not to structurally similar SA analogs lacking biological activity (Chen and Klessig, 1991; Chen et al., 1993). Sequence analysis of the SABP cDNA clone revealed high sequence similarity to the antioxidant catalase and *in*

vitro purified SABP showed activity in converting hydrogen peroxide (H₂O₂) to water and oxygen. The addition of SA or its biologically active analogs to this enzyme assay resulted in inhibition of activity both *in vitro* and *in vivo* (Conrath et al., 1995; Durner and Klessig, 1996; Wendehenne et al., 1998). Aside from tobacco, SA inhibition of catalase has also been observed in the leaves of tomato, cucumber and Arabidopsis (Sánchez-Casas and Klessig, 1994) and the roots of rice (Chen et al., 1997).

The mode of inhibition of catalase by SA is quite interesting; it centers on the iron in catalase which is Fe(III) in the native form. Fe(III) initially undergoes a two electron oxidation in the presence of H_2O_2 generating the Fe(V) intermediate which then accepts two electrons from another molecule of H_2O_2 resulting in the release of O_2 and a return to the native Fe(III) state. This catalytic activity is very rapid. However after the intermediate Fe(V) state is formed, catalase can follow another route, the peroxidase route, to regenerate the native Fe(III) state. This involves two sequential one-electron reduction steps where SA can act as the electron donor. This results in shunting the normally rapid catalase cycle to the peroxidase cycle which is approximately 1000-fold slower and effectively inhibits catalase activity (Durner and Klessig 1995 and 1996).

However, despite the almost twenty years that have passed since the identification of catalase as an SA-binding protein, there is yet to be a consensus on the role of H_2O_2 as a second messenger for SA. This is mainly due to several studies that show that SA acts as a second messenger of H_2O_2 (Bi et al., 1995; Neuenschwander et al., 1995; Chamnongpol et al.; 1998). In an attempt to reconcile the conflicting observations, interdependence between SA and H_2O_2 has been proposed. (Léon et al., 1995). For example, Rao et al. (1997) established that SA requires H_2O_2 to potentiate lipid

peroxidation, induce PR gene expression and establish SAR. Their results also supported observations by Neuenschwander et al. (1995) who determined that H_2O_2 requires SA to induce PR genes. An alternative hypothesis to explain the SA- H_2O_2 relationship is that the role of SA as a catalase inhibitor is only important at the site of infection as a means to initiate and help maintain the oxidative burst. The dissociation constant (k_d) for SA binding to catalase is approximately 14 μ M (Chen and Klessig, 1991) and this concentration of SA has only been observed at the site of infection. In distal tissue, the concentration of SA is 10- to 100-fold lower suggesting SA's inhibition of catalase could not function in this tissue (Enyedi et al., 1992; Neuenschwander et al., 1995).

The SA inhibition of catalase is an attractive model for the inhibition of SA because this would result in an accumulation of H₂O₂, a molecule known to directly affect proteins through post-translational modifications (PTMs). Changes in gene expression could be brought about by changing the redox status, and therefore activity, of transcription factors (discussed in detail in Section 2.5.1). Links between SA and nitric oxide (NO) (Durner et al., 1998; Dolledonne et al., 1998; Klessig et al., 2000) and SA and a mitogen activated protein (MAP) kinase (Conrath et al., 1997; Zhang and Klessig, 1997; Zhang et al., 1998) have also been observed and offer additional means by which proteins could be post-translationally modified to alter their activity.

2.3 Proteins Involved in SAR

Screens of Arabidopsis mutants provided some of the first glimpses into the proteins that are involved in the SAR pathway. Several mutants were identified with constitutive SAR and are characterized by spontaneous cell death in the absence of pathogen challenge (Ryals et al., 1996). These mutants, such as *lsd* (lesion simulating

disease) and *acd* (accelerated cell death), display elevated SAR gene expression, high concentrations of SA and resistance to normally virulent pathogens (Dietrich et al., 1994; Greenberg et al., 1994; Weymann et al., 1995). An Arabidopsis mutant known as *cim* (constitutive immunity) has also been characterized and found to behave in a similar manner to *lsd* and *acd* mutants however it does not display any obvious cell death phenotype (Lawton et al., 1993; Ryals et al., 1996). Although these mutants have enhanced diseases resistance, a major drawback is that the gene products of these mutants have poorly defined functions and do not provide much information to further our understanding of the signaling cascade that leads to SAR.

2.3.1 Pathogenesis Related Proteins

Pathogenesis related (PR) genes, whose coordinate expression correlates with the onset of SAR after pathogen challenge or treatment with either SA or its analogs, were the first genes identified to have a role in SAR. Although the identity of several of the proteins encoded by these PR genes has been known since 1987 (reviewed in Ward et al., 1991) the function of many PR proteins has yet to be identified. Some PR genes have been found to encode proteins such as β -1,3-glucanase and chitinase, however others, such as PRI, the archetypical PR gene for SAR in Arabidopsis, has no known function. In 1991, Ward and colleagues, studied the expression of nine classes of PR genes that correlated with the onset of SAR in tobacco treated with TMV, SA and INA. They observed a maximal expression of these PR genes both locally and systemically at six and twelve days after TMV infection, respectively.

A loss of SAR is often correlated with a loss of PR gene expression however overexpression of a PR gene almost never enhances disease resistance. Many groups

observed enhanced tolerance against fungal infection when they overexpressed two PR genes encoding, β -1,3-glucanase and chitinase, in model (Broglie et al., 1991; Zhu et al., 1994) and crop plants (Grison et al., 1996). However these groups were often only looking at pathogens that were known to be sensitive to chitinase and/or glucanase, while in reality, most plant pathogens were not significantly inhibited by the overexpression of these PR genes. The primary reason for this may be due to the large suite of PR genes that must be expressed to observe SAR (Ward et al., 1991).

2.3.2 Non-expressor of Pathogenesis Related Gene 1 (NPR1)

An Arabidopsis mutant compromised in SAR was identified by Cao et al. (1994) through screening mutants that were non-responsive to inducers of SAR. This mutant, named nprI (non-expressor of pathogenesis related genes 1), was also identified independently through different genetic strategies by two other groups and is also known as nimI (non-inducible immunity 1; Delaney et al., 1995) and saiI (salicylic acid insensitive 1; Shah et al., 1997). Treatment of nprI mutants with either SA or INA fails to activate PR gene expression or SAR suggesting that SA and INA signaling require the function of the NPR1 protein (Delaney et al., 1995; Shah et al., 1997).

Cloning of the Arabidopsis *NPR1* gene revealed a novel protein containing two protein-protein interaction domains. The BTB/POZ (Broad-Complex, Tramtrack, and Bric-a-brac/Pox virus and Zinc finger) domain is found in the N-terminus while the ankyrin repeat domain is located toward the central portion of NPR1 (Cao et al., 1997; Aravind and Koonin, 1999). The characterization of several *npr1* mutants revealed a single point mutation in either protein-protein interaction domain eliminated NPR1 function and demonstrated their critical role (Cao et al., 1994; Delaney et al., 1995; Shah

et al., 1997). Also critical for its function during SAR is the ability of NPR1 to localize to the nucleus. An NPR1-GFP fusion protein was detected exclusively in the nucleus after SA or INA treatment whereas it was mostly located in the cytoplasm in untreated cells (Kinkema et al., 2000). More recent studies have shown that some NPR1 is localized in the nucleus prior to SA treatment (Després et al., 2000 and 2003). The significance of nuclear localization was demonstrated by Kinkema et al. (2000) who showed that *PR* gene expression was only observed when NPR1 localized to the nucleus. Additionally, simply allowing nuclear localization of NPR1 was not sufficient for *PR* gene expression. Instead, nuclear localization of NPR1 in combination with INA treatment was required (Kinkema et al., 2000).

NPR1 overexpression in Arabidopsis is able to confer enhanced disease resistance without the detrimental side effects of the *lsd*, *acd* or *cim* mutants, providing an ideal target for genetic engineering in crop plants (Ryals et al., 1996; Cao et al., 1998). To this end, overexpression of the NPR1 homolog in tomato (Lin et al., 2004) and apple (Malnoy et al., 2007) has been shown to enhance disease resistance. Although NPR1 overexpression is beneficial for crop protection, the exact mechanism by which NPR1 exerts its effects is unclear. One key issue with the regulation of *PR* gene expression by NPR1 is the fact that NPR1 does not contain an obvious DNA binding domain.

2.3.3 TGA Transcription Factors

TGA transcription factors (TF), named for their recognition of the cognate DNA motif TGACG, contain a basic region/leucine zipper motif (bZIP) that is responsible for DNA binding and protein dimerization, respectively (Hurst, 1995). In plants, TGA TFs have been characterized most extensively in tobacco and Arabidopsis. Jakoby et al.

(2002) have identified over 80 bZIP TFs in Arabidopsis, which is approximately four times more than the number identified in yeast, worms or humans (Riechmann et al., 2002).

Characterization of a bZIP TF in tobacco, named TGA1a, revealed that the bZIP domain is necessary and sufficient for sequence specific DNA binding (Katagiri et al., 1992). Additionally, the basic portion of the bZIP region is also required for nuclear localization and constitutes a nuclear localization signal (NLS) (Vanderkrol and Chua, 1991). TGA1a will bind to a cis-element known as activation sequence 1 (as-1) in the cauliflower mosaic virus (CaMV) 35S promoter (Lam et al., 1989). It also binds the ciselements found in the ocs (octopine synthase) and nos-1 (nopaline synthase) promoter regions of Agrobacterium genes (Fromm et al., 1989; Lam et al., 1990). Interestingly, an as-1-like element (discussed further in Section 2.4.2.2) has been identified in the promoter of several PR genes suggesting TGA1a, as well as other TGA TFs, might act to regulate PR gene expression (Strompen et al., 1998). In fact, Strompen et al. (1998) determined that TGA1a bound the tobacco PR1a as-1-like element with the same affinity as the CaMV as-1 element and the same mutations in the PR1a as-1 element that affected PR1a inducibility also impaired binding of TGA1a in vitro. These results suggest that the level of expression of PR1a in tobacco is controlled through the as-1-like element and TGA1a. Around the same time, Lebel et al. (1998) characterized cis-acting regulatory elements in the Arabidopsis PRI promoter that were involved in SA and INA inducibility. They identified two regions upstream of the transcription start site, termed LS5 and LS7 (linker scanning), whose mutation was found to elevate or abolish the induction of PRI, respectively. A closer analysis of the two sequences revealed the

presence of the same *as-1-like* element observed in tobacco *PR1a* (Lebel et al., 1998; Strompen et al., 1998). Després et al. (2000) have shown that the Arabidopsis TGA2 TF is able to bind both LS5 and LS7 further supporting a role for TGA TFs in the regulation of *PR* gene expression.

2.3.3.1 TGA TFs and NPR1

The significance of TGA TFs binding the promoter of PR genes was not realized until several groups independently identified that NPR1 interacts with several TGA TFs providing NPR1 with means to regulate PR gene expression (Zhang et al., 1999; Després et al., 2000; Zhou et al., 2000).

Ten TGA TFs have been identified in Arabidopsis and seven (TGA1 to TGA7) have been assayed for their ability to interact with NPR1. There is very little sequence similarity between the seven TGA TFs in the N-terminus and varying levels of similarity in the rest of the protein, which has resulted in the clustering of the seven TGA TFs into three clades: (i) TGA1 and 4, (ii) TGA2, 5 and 6, and (iii) TGA3 and 7. Members within a clade possess similar DNA-binding specificities, expression patterns and transactivation properties (Zhang et al., 1999; Després et al., 2000; Zhou et al., 2000; Niggeweg et al., 2000a and b; Schiermeyer et al., 2003). A multiple alignment of the seven TGA TFs is illustrated in Figure 4. Yeast two-hybrid experiments identified that two clades, containing TGA2, 3, 5, 6 and 7, are able to interact with NPR1 while the third clade, containing TGA1 and 4, does not (Zhang et al., 1999; Després et al., 2000; Zhou et al., 2000).

Després et al. (2000) observed that binding of Arabidopsis TGA2 to either the CaMV 35S as-1 element or the LS5 or LS7 elements of the PR1 promoter is enhanced in

the presence of NPR1. Furthermore, mutations in NPR1 which result in a compromised ability to induce SAR also abolished interaction with TGA2, 3, 5, 6 and 7 and these NPR1 mutants also failed to enhance TGA2 binding to the *as-1* element (Zhang et al., 1999; Després et al., 2000; Zhou et al., 2000). The fact that mutations in NPR1 that abolish the development of SAR also prevent NPR1-TGA interaction suggests that NPR1-mediated DNA binding of TGA TFs is required for the activation of SAR associated defense genes.

In 2002, Fan and Dong provided further evidence to support the above observation when they showed that TGA2 and NPR1 interact in planta and this interaction is enhanced by SA or INA treatment. They also observed, using a modified TGA2Gal4 chimeric construct, that a reporter gene under the control of the Gal4 upstream activating sequence (UAS) was activated in response to SA and this activation was abolished in an npr1 mutant (Fan and Dong, 2002). Johnson et al (2003) made similar observations when they performed a gel shift assay using the LS7 element and leaf nuclear protein extracts from wild-type or nprl mutant plants with or without SA. While TGA2 binding to LS7 occurred in the absence of SA, the binding was markedly increased after SA treatment while there was no enhancement of binding observed in extracts from the npr1 mutant after SA treatment. Johnson et al. (2003) also observed an interaction NPR1 planta between TGA TFs through chromatin inand immunoprecipitation (ChIP) experiments; TGA2 and TGA3 were found to bind the PR1 promoter only in the presence of SA and NPR1. Both of these results underscore the importance of NPR1 in the SA-inducible binding of TGA TFs to the as-1-like element.

```
TGA1
      ---MNSTS--THFVPPRRVGIYEPVHQFGMWGESFKSNIS----NGTMNTPNHIIIPNNQ 51
      ---MNTTS--THFVPPRRFEVYEPLNQIGMWEESFKN-----NGDMYTPGSIIIPTNE 48
TGA4
      -----MADTSPRTDVSTDD 14
TGA2
TGA5
      -----MGDTSPRTSVSTDG 14
      ------MADTSSRTDVSTDG 14
TGA6
      MEMMSSSSSTTQVVSFRDMGMYEPFQQLSGWESPFKSDINNITSNQNNNQSSSTTLEVDA 60
TGA3
TGA7
      ---MMSSSSPTOLASLRDMGIYEPFOOIVGWGNVFKSDIN----DHSPNTATSSIIOVDP 53
      KLDNN-----VSEDTSHG--TAGTPHMFDQEASTSRHPDKIQRRLAQNREAARKSRLRKK 104
TGA1
TGA4
      KPDS-----LSEDTSHG--TEGTPHKFDQEASTSRHPDKIQRRLAQNREAARKSRLRKK 100
TGA2
      DTDHP-----DLGSEGALVNTAASDSSDRSKGKMDQKTLRRLAQNREAARKSRLRKK 66
      DTDHN-----NLMFDEGHLGIGASDSSDRSKSKMDQKTLRRLAQNREAARKSRLRKK 66
TGA5
      DTDHR-----DLGSDRGHMHAAASDSSDRSKDKLDQKTLRRLAQNREAARKSRLRKK 66
TGA6
TGA3
      RPEADDNN-RVNYTSVYNNSLEAEPSSNNDQDED-RINDKMKRRLAQNREAARKSRLRKK 118
TGA7
      RIDDHNNNIKINYDSSHNQIEAEQPSSNDNQDDDGRIHDKMKRRLAQNREAARKSRLRKK 113
                                 ..: . : :*
      AYVQQLETSRLKLIQLEQELDRARQQGFYVGNGIDTNSLGFSETMNPGIAAFEMEYGHWV 164
TGA1
      AYVQQLETSRLKLIHLEQELDRARQQGFYVGNGVDTNALSFSDNMSSGIVAFEMEYGHWV 160
TGA2
      AYVQOLENSRLKLTQLEQELQRARQQGVFISGTGD----QAHSTGGNGALAFDAEHSRWL 122
      AYVOOLENSRLKLTQLEQELQRARQQGVFISSSGD----QAHSTAGDGAMAFDVEYRRWQ 122
TGA5
      AYVQQLENSRLKLTQLEQELQRARQQGVFISSSGD----QAHSTGGNGALAFDAEHSRWL 122
TGA6
TGA3
      AHVQQLEESRLKLSQLEQELVRARQQGLCVRNSSDTSYLGPAGNMNSGIAAFEMEYTHWL 178
TGA7
      AYVQQLEESRLKLSQLEQELEKVKQQG------HLGPSGSINTGIASFEMEYSHWL 163
      *:**** ***** :****
      EEONROICELRTVLHGHINDIELRSLVENAMKHYFELFRMKSSAAKADVFFVMSGMWRTS 224
TGA1
TGA4
      EEQNRQICELRTVLHGQVSDIELRSLVENAMKHYFQLFRMKSAAAKIDVFYVMSGMWKTS 220
      EEKNKQMNELRSALNAHAGDSELRIIVDGVMAHYEELFRIKSNAAKNDVFHLLSGMWKTP 182
TGA2
TGA5
      EDKNROMKELSSAIDSHATDSELRIIVDGVIAHYEELYRIKGNAAKSDVFHLLSGMWKTP 182
TGA6
      EEKNRQMNELRSALNAHAGDTELRIIVDGVMAHYEELFRIKSNAAKNDVFHLLSGMWKTP 182
TGA3
      EEONRRVSEIRTALOAHIGDIELKMLVDSCLNHYANLFRMKADAAKADVFFLMSGMWRTS 238
      QEOSRRVSELRTALQSHISDIELKMLVESCLNHYANLFQMKSDAAKADVFYLISGMWRTS 223
      AERFFLWIGGFRPSDLLKVLLPHFDVLTDQQLLDVCNLKQSCQQAEDALTQGMEKLQHTL 284
TGA1
TGA4
      AERFFLWIGGFRPSELLKVLLPHFDPLTDQQLLDVCNLRQSCQQAEDALSQGMEKLQHTL 280
TGA2
      AERCFLWLGGFRSSELLKLLANQLEPMTERQLMGINNLQQTSQQAEDALSQGMESLQQSL 242
TGA5
      AERCFLWLGGFRSSELLKLIASOLEPLTEOOSLDINNLOOSSOOAEDALSOGMDNLOOSL 242
TGA6
      AERCFLWLGGFRSSELLKLLANQLEPMTERQVMGINSLQQTSQQAEDALSQGMESLQQSL 242
TGA3
      TERFFQWIGGFRPSELLNVVMPYVEPLTDQQLLEVRNLQQSSQQAEEALSQGLDKLQQGL 298
      TERFFQWIGGFRPSELLNVVMPYLQPLTDQQILEVRNLQQSSQQAEDALSQGIDKLQQSL 283
      :** * *:****.*:**:::
                           .: :*::* : : .*:*:.***:**:**: *
      ADCVAAGQ--LGEGS-----YIPQVNSAMDRLEALVSFVNQADHLRHETLQQMYRILTT 336
TGA1
TGA4
      AESVAAGK--LGEGS-----YIPOMTCAMERLEALVSFVNOADHLRHETLOOMHRILTT 332
TGA2
      ADTLSSGT--LGSSSSGNVASYMGQMAMAMGKLGTLEGFIRQADNLRLQTLQQMIRVLTT 300
TGA5
      ADTLSSGT--LGSSSSGNVASYMGQMAMAMGKLGTLEGFIRQADNLRLQTYQQMVRLLTT 300
      ADTLSSGT--LGSSSSDNVASYMGQMAMAMGQLGTLEGFIRQADNLRLQTLQQMLRVLTT 300
      VESIAIQIKVVESVN-----HGAPMASAMENLQALESFVNQADHLRQQTLQQMSKILTT 352
TGA3
      AESIVIDA-VIESTH-----YPTHMAAAIENLQALEGFVNQADHLRQQTLQQMAKILTT 336
TGA7
                         : : *: .* :* .*: *** :* *** :: ***
TGA1
      RQAARGLLALGEYFQRLRALSSSWATRHREPT 368
      RQAARGLLALGEYFQRLRALSSSWAARQREPT 364
TGA4
      RQSARALLAIHDYFSRLRALSSLWLARPRE-- 330
TGA2
TGA5
      RQSARALLAVHNYTLRLRALSSLWLARPRE-- 330
TGA6
      RQSARALLAIHDYSSRLRALSSLWLARPRE-- 330
TGA3
      RQAARGLLALGEYFHRLRALSSLWAARPREHT 384
      RQSARGLLALGEYLHRLRALSSLWAARPQEPT 368
TGA7
      **:**.*** :* ***** * :* :*
```

Figure 4. Alignment of Seven Arabidopsis TGA TFs

Clustal W amino acid alignment of TGA1 (NP_851273), TGA2 (NP_001078539), TGA3 (ABG48390), TGA4 (ABD85167), TGA5 (NP_974745), TGA6 (ABL66771) and TGA7 (Q937E2). The conserved basic portion of the bZIP domain is boxed in light grey while

the conserved leucines of the leucine zipper are boxed in dark grey. Conserved amino acids in all seven TGA TFs are indicated by an asterisks (*), conserved substitutions are indicated by two dots (*) and semi-conserved substitutions are indicated by one dot (•). The order of TGA TFs is based on their division into different clades.

SA is believed to enhance the interaction between TGA2 and NPR1 leading to enhanced binding of the *PR1* promoter, but the exact mechanism of SA-mediated enhancement is not clear. It is possible that SA either enhances the affinity of NPR1 for TGA2 or increases the availability of NPR1 for TGA2 (Fan and Dong, 2002). The second hypothesis is supported by the observation that SA or INA treatment increases the distribution of NPR1 to the nuclear fraction where TGA2 is found (Kinkema et al., 2000; Mou et al., 2003).

2.3.3.2 Functional analysis of TGA TFs

Determining the function of TGA TFs during SAR has proven to be a complicated task. Although an activity related to interaction with NPR1 for several TGA TFs is clear, it has been difficult to establish if any of the TGA factors have any NPR1-independent roles. These difficulties may be due to the redundancy of TGA TFs that diminish the values of mutants to describe their putative function. As a result, overexpression has been used to examine the effect of TGA5*overexpression on SAR in Arabidopsis. These plants were significantly more resistant to the normally virulent pathogen *P. parasitica* whereas an overexpressing TGA2 line had no obvious effect. Since the TGA5-accumulating line did not express higher levels of *PR1* and it was not compromised in an *npr1* mutant or in Arabidopsis lines expressing *nahG*, the observed resistance might involve an SA- and SAR-independent mechanism (Kim and Delaney, 2002). Using a different approach, Foley and Singh (2004) examined the effect of

decreased expression of either TGA4 or TGA5 in Arabidopsis. They observed that while neither had any effect on phenotype, both seemed to affect the responsiveness of an *ocs* (an element bearing sequence similarity to the *as-1-like* element) element to SA and H₂O₂ in roots. TGA4-silenced lines showed enhanced responsiveness of the *ocs* element to SA and H₂O₂ while TGA5 silenced lines reduced this response. These results would suggest TGA5 is possibly involved in at least two defense related signaling pathways, acting as a positive activator in both instances, whereas TGA4 may act as a negative regulator (Kim and Delaney, 2002; Foley and Singh, 2004).

Dominant-negative experiments have provided an alternative means to establish the function of TGA TFs, however with vastly conflicting results. Dominant-negative studies rely on overexpressing a mutant whose gene product adversely affects the normal, wild-type gene product. For example, Fan and Dong (2002) created a dominant-negative mutant of TGA2 where the DNA binding domain has been removed. This mutant is still able to interact with NPR1. Plants expressing this dominant-negative mutant of TGA2 had a phenotype similar to *npr1* mutants (Fan and Dong, 2002). These plants also displayed a reduction in PR1 and PR2 expression after INA treatment and infection resulted with a normally avirulent pathogen. Furthermore, Zhang et al. (2003) observed that INA treatment of a triple TGA2, 5, 6 knock-out Arabidopsis lines did not induce PR1 and did not protect this line from a normally avirulent pathogen. Taken together, these data suggest that the TGA2, 5, 6 clade positively regulates PR1 gene induction. However, the triple knock-out line also accumulated higher basal levels of PR1 compared to wildtype plants in the absence of INA treatment suggesting a negative regulation by these TFs of basal PR1 expression. The role of TGA2 as a negative regulator is also supported by

Pontier et al. (2001) who observed that a TGA2 dominant-negative mutant displayed enhanced expression of *PR1a* by SA in tobacco.

The *as-1-like* element, aside from being responsive to SA and INA, also responds to environmental cues and stresses. The hormone auxin, for instance, mediates at least some of its effect by enhancing the expression of specific genes through the *as-1-like* element through TGA TFs. Pascuzzi et al. (1998) and Johnson et al. (2001a) showed that TGA1a can activate the expression of a reporter gene fused to an *as-1-like* element after treatment with the synthetic auxin 2,4-D (2,4-dichlorophenoxyacetic acid). *In vitro* studies found that TGA1a is able to bind the *as-1-like* element in the promoter of *GNT1* and *GNT35*, two tobacco glutathione S-transferase (GST) genes. These results were confirmed by ChIP experiments *in vivo* where binding was only observed after treatment with 2,4-D (Johnson et al., 2001b). Interestingly, although TGA1a was observed to bind the tobacco *PR1a as-1-like* element *in vitro*, ChIP experiments did not detect TGA1a bound to the *PR1a* promoter before or after 2,4-D treatment (Johnson et al., 2001b).

One should be cognizant of the fact that the *cis*-acting elements being studied in most instances are not in the context of the full length promoter they were taken from. This means that while a specific type of responsiveness to one TGA factor over another might be relevant, it might not reflect the *in vivo* situation that would occur in the context of the full length promoter.

Using a reverse genetics approach, Kesarwani et al. (2007) recently provided extensive detail concerning the role of TGA TFs during disease resistance. They found that TGA1 and 4 play partially redundant roles in regulating basal resistance and have only a moderate effect on *PR* gene expression after INA treatment. They further

examined the TGA2, 5 and 6 clade and determined that TGA2 has repressor activity on *PR* gene expression even though it can act as a positive regulator in the *tga5-1 tga6-1* null mutant background. Also, an activation-tagged mutant of TGA6 was able to increase basal as well as induced expression of *PR1* suggesting a positive role in *PR* gene expression. These data have led to the hypothesis that single TGA TFs have dual functions and can act as both positive and negative regulators of SAR (Pontier et al., 2001; Kesarwani et al., 2007).

2.4 Timing of SAR

The events that take place during SAR are timed and coordinated to ensure that disease resistance pathways are induced early enough and sustained to prevent pathogen infection. PR gene expression is usually induced six to eight hours after pathogen infection which, on the time scale of induced disease resistance, is considerably rapid (Horvath et al., 1998). However on the time scale of a molecular response to a stimulus, it is quite slow. Several studies have examined the inducibility of genes by SA and other molecules and this has resulted in the identification and classification of two different groups of genes in disease resistance: immediate-early and late genes (Horvath and Chua, 1996; Horvath et al., 1998). These two groups are distinguished by their kinetics of induction and by the fact that expression of immediate-early genes is not affected by the protein synthesis inhibitor cycloheximide (CHX) while the expression of late genes is (Horvath and Chua, 1996). This suggests that expression of immediate-early genes involves latent or pre-existing pools of TFs while de novo synthesis is required for the induction of late genes (Horvath and Chua, 1996; Horvath et al., 1998; Uquillas et al., 2004).

One very interesting feature of both immediate-early and late genes is the presence of the *as-1-like* element in their promoter (Droog et a., 1995; Lebel et al., 1998; Strompen et al., 1998). As already discussed, TGA TFs bind the *as-1-like* element of several different genes (Després et al., 2000; Fan and Dong, 2002; Johnson et al., 2003). While the number of identified immediate-early genes is large, *PR* genes are the only genes identified as late genes and a role for TGA TFs in their expression has already been discussed.

2.4.1 Immediate-Early Genes

Initially, several glutathione S-transferases (GSTs) were identified as immediate-early genes in tobacco (Nt103-1 and Nt103-35; Droog et al., 1995 and GNT35; Xiang et al., 1996) and Arabidopsis (GST1; Chen et al., 1996) by their rapid induction after pathogen challenge and/or treatment with one or more of SA, INA, H₂O₂, 2,4-D and methyl jasmonate (MeJA). GSTs catalyze the nucleophilic addition of the thiol of reduced glutathione (GSH) to a large variety of electrophilic compounds with the main goal of detoxifying xenobiotic compounds (Armstrong, 1997). The postulated role of GSTs during pathogen infection is to limit tissue damage to neighboring cells (Levine et al., 1994).

Horvath et al., (1996) identified a glucosyl transferase, *IEGT1* (immediate-early-induced glucosyl transferase) in Arabidopsis whose expression was induced 30 minutes after SA treatment, peaked at three hours and then decayed. Treatment with CHX had no effect on *IEGT1* induction or timing, however, the decay of *IEGT1* was abolished suggesting that *de novo* protein synthesis is required to turn off expression of *IEGT1* (Horvath et al., 1996). *GST6* is another immediate-early gene in Arabidopsis whose

expression is rapidly induced by SA treatment (Chen et al., 1996) and is insensitive to CHX (Uquillas et al., 2004). Interestingly, TGA4 and TGA5 bind the promoter of *GST6* (Chen et al., 1996), however, the expression of *GST6* is not compromised in an *npr1* mutant (Uquillas et al., 2004). This would suggest that TGA-dependent induction of *GST6* occurs in an NPR1-independent manner.

EH-1 (epoxide hydroxylase 1; Guo et al., 1998) and PIOX (pathogen-induced oxygenase; Sanz et al., 1998) were also identified as immediate-early genes based on their responsiveness to SA. Similar to GSTs, EH-1 is also believed to play a role in protecting cells from oxidative damage during pathogen infection while PIOX has homology to cyclooxygenase. In mammalian systems, cyclooxygenases catalyzes the first committed step in the formation of prostaglandins and thromboxanes, both of which are lipid derived signal molecules involved in the immune response (Sanz et al., 1998). By analogy, PIOX may be involved in the generation of lipid-derived signal molecules that activate defense responses in plants (Dempsey et al., 1999).

Recently, Blanco et al. (2005) identified twelve genes in Arabidopsis that were induced by SA as early as 30 minutes to around 2 hours and classified these as immediate-early genes. They split these genes into two groups based on the proteins they encoded. The first group of genes are involved in cell protection (glycosyl transferases (GTases) and GSTs) while the second group of genes are involved in signal transduction (protein kinases and TFs). The first group of genes was characterized by their rapid response to SA (30 minutes), sensitivity to a lower concentration of SA, dose-dependent response to increasing concentrations of SA and independence of NPR1. The second group responded less rapidly to SA (2 hours), was insensitive to lower concentrations of

SA, showed less of a dose-dependent response and required NPR1. Curiously, the dependence of any of these genes on *de novo* protein synthesis was not tested. When the promoters of the two groups of genes were analyzed, it was noted that the *as-1-like* element was over-represented in the first group only, with little to no representation in the second group. This would suggest that the expression of the second group of genes is mediated in an NPR1-dependent but TGA-independent manner whereas induction of the first group is the complete opposite.

2.4.2 Gene Expression Controlled by the as-1-like Element

The fact that the *as-1-like* element occurs in the promoter of genes that are regulated so differently in terms of kinetics as well as dependence on *de novo* protein synthesis indicates the regulation by NPR1 and TGA TFs complex.

2.4.2.1 Chemical Signals

Aside from SA, the induction of many immediate-early genes containing an as-1-like element also occurs through treatment with INA, 2,4-D, H₂O₂, and MeJA although the timing and degree of induction sometimes varies (Qin et al., 1994; Xiang et al., 1996; Horvath and Chua, 1996). One way a common cis-acting element can be differentially regulated is by the dissimilar effects that varying chemicals have on TFs. The spectrum of TGA hetero- and homodimers that results from SA treatment is likely drastically different from those produced by treatment with 2,4-D or MeJA. For example, the expression of a reporter gene containing the upstream activating sequence for Gal4 by a TGA2::Gal4 fusion construct was activated by SA but not by 2,4-D or MeJA (Fan and Dong, 2002).

2.4.2.2 The Sequence and Arrangement of the as-1-like Element

The as-1 element found in the Cauliflower mosaic virus (CaMV) 35S promoter is twenty base pairs in length. It contains two perfect palindromes with a conserved sequence separated by four nucleotides where the center of each palindrome is separated by twelve base pairs (Krawczyk et al., 2002). The as-1-like element identified in plant promoters is the same except for the presence of an imperfect palindrome. In the promoters of immediate-early genes, the twelve base pair spacing between the palindromes is strictly conserved; an increase or decrease in spacing affects the DNA binding and transactivation of several tobacco TGA TFs (Krawczyk et al., 2002). In contrast, the twelve base pair spacing in the as-1-like element in the PR1 and PR1a promoters is not strictly conserved. However in both immediate-early and late genes, the sequence of the four nucleotides between the two palindromes was found to influence the binding affinity of TGA TFs (Krawczyk et al., 2002). Additionally, the nucleotides flanking the as-1-like element also dictate preference for binding of certain TGA TFs over others. There is also the possibility that binding will only occur on one palindrome of the as-1-like element as opposed to both. This was observed by Niggeweg et al. (2000a) who found that tobacco TGA1a and TGA2.2 bound to only a single TGACG motif on the as-1 element at low protein concentrations. At higher protein concentrations however, simultaneous occupation of both motifs was observed for TGA2.2 but not for TGA1a.

The accessibility of the *as-1-like* element to TGA TFs also affects regulation. Butterbrodt et al. (2006) observed that histone acetylation, which correlates with an increase in gene expression through increased DNA accessibility, occurs on the *PR1a*

promoter after SA treatment. On the other hand, histone acetylation of a minimal *as-1* element from CaMV 35S was not influenced by SA.

2.5 Redox Regulation

Reactive oxygen species (ROS) result from the incomplete reduction of oxygen. All organisms have evolved protective mechanisms, both enzymatic and non-enzymatic, to minimize ROS-mediated damage of DNA, proteins and lipids. However there are instances, such as the immune response in vertebrates and the oxidative burst in plants, where ROS accumulate past the point where normal buffering mechanisms are not effective (Lamb and Dixon, 1997; Alvarez et al., 1999). While these unmanageable increases in ROS are detrimental to cells, organisms have also managed to utilize these ROS as signals to regulate gene expression.

Cysteines are the most sensitive of all amino acids to oxidation and normally exist in a reduced state due to the reducing environment of the cytoplasm (Schafer and Buettner, 2001). However, under oxidative stress, several different cysteine modifications are possible. Research in the past ten years has focused on the redox regulation of cysteines in non-plant systems (Toledano et al., 2004; Shelton et al., 2005) while more recent work has identified similar mechanisms in plants (Let et al., 2004a; Fobert and Després, 2005; Michelet et al., 2005; Wang et al., 2006). The most common forms of oxidative stress include the superoxide radical (O2*), hydrogen peroxide (H2O2) and the hydroxyl radical (HO4) (D'Autréaux, and Toledano, 2007). The difference in reactivity, half-life and lipid solubility of ROS translates into varying effects on biological targets (Halliwell and Gutteridge, 1999; D'Autréaux and Toledano, 2007). For instance, HO4 has

indiscriminant reactivity whereas $O_2^{\bullet,-}$ has high reactivity with iron-sulfur clusters and H_2O_2 with cysteines (Toledano, 2007).

The formation of a disulfide bond constitutes one of the most common forms of cysteine redox regulation although nitrosylation (S-NO), glutathionylation (S-SG) and hydroxylation [S-OH (sulfenic); S-O₂H (sulfinic); S-O₃H (sulfonic)] have also been observed (Shelton et al., 2005). It was initially believed that most cysteine modifications, other than disulfide bonds, were simply transient intermediates, however extensive research has provided evidence that most of the above mentioned modifications are stable and have biological significance (Kim et al., 2002; Poole et al., 2004; Giustarini et al., 2004; Filomeni et al., 2005).

Most cysteine modifications are reversible and even interconvertible (i.e. once a cysteine has been oxidized to S-OH, it can still form a disulfide bond or become glutathionylated; Shelton et al., 2005) however S-O₃H is believed to be an irreversible modification (Dawson et al., 1991). Although this might not necessarily be the case given that S-O₂H was initially believed to by an irreversible oxidation until Biteau et al. (2003) discovered an ATP-dependent reduction activity for S-O₂H in *Saccharomyces cerevisiae*.

Two cytosolic pathways exist that maintain the reducing environment of the cell: thioredoxins (Trx) and glutaredoxins (Grx) (Ritz and Beckwith, 2001; Ortenberg and Beckwith, 2003). These two systems operate in a similar manner, via a dithiol mechanism, to reduce disulfide bonds (illustrated in Figure 5A and B). Trx uses the reducing power of NADPH while Grx employs glutathione (GSH), although ultimately the reducing power is derived from NADPH (Qin et al., 2000). Both Trx and Grx share a classical active site defined as the CXXC-type (for example CPYC). The N-terminal

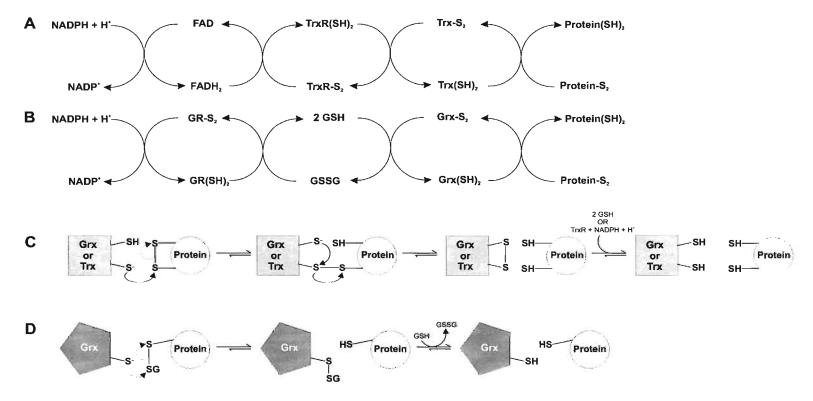


Figure 5. Enzymatic Reaction Schemes for the Thioredoxin and Glutaredoxin Systems

(A) Mechanism of NADPH dependent protein disulfide reduction by the thioredoxin system. TrxR – thioredoxin reductase; Trx – thioredoxin; S₂ – oxidized disulfide; (SH)₂ – reduced disulfide (Adapted from Meyer et al., 1999). (B) Mechanism of GSH dependent glutaredoxin reduction of a protein disulfide. GR – glutathione reductase; Grx – glutaredoxin; S₂ – oxidized disulfide; (SH)₂ – reduced disulfide (Adapted from Fernandes and Holmgren, 2004). (C) The thioredoxin (Trx) and glutaredoxin (Grx) dithiol mechanism for reducing a protein disulfide. The N-terminal cysteine (denoted as S') is deprotonated by stabilizing neighboring amino acids and attacks the protein disulfide bond. A transient glutaredoxin-protein disulfide is formed while the other cysteine from the protein disulfide deprotonates the C-terminal cysteine in the Grx/Trx active site. The C-terminal Grx/Trx cysteine then attacks the mixed disulfide, liberating a reduced target protein. The reduced forms of Grx and Trx are regenerated via 2 molecules of GSH and thioredoxin reductase (TrxR) plus NADPH and H⁺, respectively (Adapted from Fernandes and Holmgren, 2004). (D) The glutaredoxin (Grx) monothiol mechanism for reducing a mixed disulfide with GSH. In this instance, only the deprotonated N-terminal cysteine (S') is involved. It attacks the cysteine in glutathione leading to a reduced protein target and the glutaredoxin oxidized by glutathione. A second GSH molecule is involved in regenerating the reduced form of Grx (Adapted from Fernandes and Holmgren, 2004).

cysteine in the active site has a lower pK_a and is the first to attack the protein disulfide bond resulting in a mixed disulfide between Trx or Grx and the protein being reduced (Figure 5C). The C-terminal cysteine then attacks the mixed disulfide releasing the reduced protein and generating an oxidized Trx or Grx, which is regenerated through NADPH-dependent thioredoxin or GSH plus glutathione reductase, respectively (Meyer et al., 1999; Fernandes and Holmgren 2004). Grx can additionally reduce mixed disulfides between GSH and a protein (Figure 5D) (Fernandes and Holmgren 2004). In this monothiol reduction mechanism, only the N-terminal cysteine is present in the active site, the CXXS-type (for example CGFS), although a dithiol active site is also able to perform a monothiol reduction. The N-terminal cysteine attacks the cysteine of glutathione in the mixed disulfide and the reduced Grx is regenerated by one molecule of GSH.

Trx have been extensively studied and characterized in *E. coli*, yeast and mammals whereas Grx are less well characterized. Mammalian systems have one mitochondrial Trx and Grx and one cytoplasmic Trx and Grx while Arabidopsis has 26 Trxs (Meyer et al., 2002) and 31 Grxs (Rouhier et al., 2004), all with varied localization within the cell. Currently, very few plant Grxs have been characterized in terms of their expression, localization, targets or biochemical activity (see Table 1 in the Appendix) whereas the function of many Trxs has been characterized in various plant subcellular compartments (Rouhier et al. 2005).

2.5.1 Redox Regulation of Proteins

The reducing environment of the cytoplasm, which regulates the redox status of cysteines, is influenced by the ratio of reduced to oxidized glutathione (GSH/GSSG) as

well as the presence of ROS. Changes in the redox status of cysteine residues results in modifying the electronic and steric conformation of the amino acid thereby affecting protein structure. This in turn will influence their activity, localization, DNA-binding affinity, transactivation or repression properties and/or protein-protein interaction.

OxyR was one of the first TFs to be studied in detail in terms of the redox regulation of its activity. OxyR is an E. coli TF that activates the expression of antioxidant genes in response to H₂O₂ (Storz et al., 1990). In the reduced form, OxyR is inactive and does not transactivate. The oxidized form, which has been postulated to result from an intramolecular disulfide bond, is capable of activating transcription (Zheng et al., 1998; Choi et al., 2001). This mode of regulation depicts the classical redox switch where the reduced form constitutes the OFF or inactive form and the oxidized form is the ON or active form. However a simple redox switch involving a disulfide bond does not appear to explain the behavior of OxyR (Storz et al., 1990; Kullik et al., 1995). Instead, Kim et al. (2002) proposed that OxyR is involved in a more intricate sensing mechanism for oxidative stress where differential and graded responsiveness is observed. To this end, they observed several stable PTMs of a single OxyR cysteine in vivo including S-NO, S-OH and S-SG. Each of these modified forms of OxyR were still transcriptionally active, so the redox switch model is still applicable, however the modifications affect structure, DNA-binding affinity and promoter activity. This elaborate response to varied oxidative stress suggests that OxyR is able to process different signals into distinct transcriptional responses (Kim et al., 2002).

Yap1 (Yeast AP-1) is a bZIP TF in *S. cerevisiae* responsible for regulating genes encoding cellular enzymatic and non-enzymatic processes for the oxidative stress defense

systems (Jamieson, 1998; Toone and Jones, 1998; Carmel-Harel and Storz, 2000). Yap1 shuttles in and out of the nucleus under non-stress conditions, however, the nuclear export of Yap1 is inhibited by H₂O₂ resulting in its nuclear accumulation and transcription of its target genes (Kuge et al., 2001). Yap1 contains a C-terminal nuclear export sequence (NES) that contains three cysteines. The presence of an intramolecular disulfide bridge in the NES was observed *in vivo* after H₂O₂ treatment and corresponded with nuclear localization of Yap1. There is also evidence that sustained nuclear localization and maximal transcriptional activation requires redox regulation of cysteines at the N-terminus of Yap1 (Kuge et al., 2001; Paget and Buttner, 2003).

Aside from *E. coli* and yeast, there are many mammalian proteins whose DNA-binding affinity, localization and activity are regulated by redox conditions including p53 (Jayaraman et al., 1997), NF-κB (Xanthoudakis and Curran, 1992), Pax5 and Pax8 (Tell et al., 1998a and 1998b) and Jun and Fos (Abate et al., 1990). More recently, plant proteins have shown to be regulated by the redox status of cysteines. The R2R3 MYB domain proteins in plants comprise one of the largest known families of TFs. Vertebrate *Myb* genes encode proteins with MYB domains formed by three MYB repeats (R1R2R3) whereas the majority of plant *Myb* genes encode proteins with only two MYB repeats (R2R3) (Lipsick, 1996; Rosinski and Atchley, 1998; Braun and Grotewold, 1999; Stracke et al., 2001). The R1R2R3 MYB domain in vertebrates contains a single cysteine residue that is reduced for DNA-binding and transcriptional activity while the R2R3 MYB domain in plants contains two cysteines. By studying the maize P1 regulatory protein, a typical R2R3 MYB domain protein, Heine et al. (2004) discovered that under non-reducing conditions, these two cysteines form an intramolecular disulfide bond that

prevents the R2R3 MYB domain to bind DNA (Heine et al., 2004). While the exact mechanism of P1 redox regulation still needs to be elucidated, the redox regulation of the MYB domain (vertebrate or plant) provides an interesting example of a redox switch that appears to work in reverse. The oxidized protein, which is normally not supported in the reducing environment of the cell, is the OFF or inactive form while the reduced protein is the ON or active form required for DNA binding and transactivation.

2.5.2 Redox Regulation of SAR

The oxidative burst that occurs at the onset of SAR is responsible for an accumulation in ROS (Halliwell and Gutteridge, 1999). A change in the ratio of GSH/GSSG has also been observed after INA treatment and pathogen challenge in Arabidopsis (Mou et al., 2003) which, along with the oxidative burst, constitutes an ideal signal for redox responsive proteins.

In the absence of SA, NPR1 is retained in the cytoplasm (Kinkema et al., 2000) and this retention is the result of oligomerization of NPR1 via intermolecular disulfide bonds involving Cys82 and Cys216 (Mou et al., 2003). Upon SAR induction, NPR1 is reduced to its monomeric form which subsequently accumulates in the nucleus to induce *PR* gene expression. Inhibiting the reduction of NPR1 prevent's *PR* gene expression while mutation of either Cys82 or Cys216 leads to constitutive monomerization, nuclear localization and *PR* gene expression (Mou et al., 2003). Interestingly, by incubating purified NPR1 with increasing ratios of GSH/GSSG, Mou et al. (2003) observed a shift in the oligomeric to monomeric form of NPR1.

Yeast two-hybrid screens initially identified TGA1 and TGA4 as the only TGA TFs that did not interact with NPR1 (Zhang et al., 1999; Després et al., 2000; Zhou et al.,

2000) however a plant two-hybrid assay revealed that TGA1 was able to interact with NPR1 upon SA treatment (Després et al., 2003). Chimerics of TGA1 and TGA2 revealed a 30 amino acid region that appeared to be responsible for preventing TGA1's interaction with NPR1. Closer examination of this region identified two cysteines in TGA1, Cys260 and Cys266. These are conserved in TGA4 but are not present in TGA2 or the other four TGA TFs (Després et al., 2003). Mutants of TGA1 and TGA4, where Cys260 and Cys266 were changed to the corresponding amino acids in TGA2, were able to interact with NPR1 in a yeast two-hybrid assay and the mutant of TGA1 was able to interact with NPR1 in a plant two-hybrid assay whether or not SA was present (Després et al., 2003). An in vivo labeling assay determined that SA treatment reduced Cys260 and Cys266 suggesting that SA regulates TGA1's redox status such that interaction with NPR1 only occurs when these cysteines are reduced (Després et al., 2003). Cys260 and Cys266 are oxidized in yeast which explains why no interaction with NPR1 was observed in the original yeast two-hybrid assays (Després et al., 2003; Zhang et al., 1999; Després et al., 2000; Zhou et al., 2000). The DNA binding affinity of TGA1 is not influenced by its redox status, however NPR1 only enhances the binding of reduced TGA1 to the as-1 element (Després et al., 2003).

Recently, Ndamukong et al., (2007) identified an Arabidopsis glutaredoxin, GRX480, that interacts with several TGA TFs and is involved in the suppression of *PDF1.2* expression. The expression of *GRX480* was found to be SA-inducible and dependent on the presence of the TGA2, 5, 6 clade of TFs as well as NPR1 (Ndamukong et al., 2007).

CHAPTER 3 – The Co-Activator Function of the Arabidopsis NPR1 Requires the Core of its BTB/POZ and the Oxidation of C-terminal Cysteines

Contributions

This manuscript stems from a secondary research project I became involved in as a result of my interest and familiarity with the topic and expertise in laboratory techniques. I performed the experiments, including DNA and protein preparation, for Figures 1F through J, Figure 3A and B, Figure 4A through C, Figure 5A through G and Supplemental Figure 2. I was involved in data analysis as well as editing and for the purpose of this thesis, I have expanded sections of the manuscript for clarification; specifically, I have expanded areas in the methods and materials and results sections.

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¹These authors contributed equally to this work.

3.1 ABSTRACT

NPR1 is the regulator of systemic acquired resistance (SAR) in Arabidopsis. Current models propose that following treatment with the SAR-inducing metabolite salicylic acid (SA), Cys82 and Cys216 of NPR1 are reduced, leading to nuclear import. Through an unknown mechanism, interaction of nuclear-localized NPR1 with TGA transcription factors results in the activation of defense genes, including the SAR marker PATHOGENESIS-RELATED 1 (PR1), and deployment of SAR. Aside from the fact that they interact with each other, there is no biochemical evidence indicating how TGA TFs or NPR1 regulate transcription or whether a TGA-NPR1 complex forms on DNA. Here, we show by chromatin immunoprecipitation that TGA2 and NPR1 are recruited to the PRI gene independently of each other and of SA-treatment. Consistent with published data indicating that a triple knock-out in TGA2/5/6 derepresses PR1, in vivo plant transcription assays revealed that TGA2 is not an autonomous transcriptional activator, but is a transcriptional repressor in both untreated and SA-treated cells. However, after stimulation with SA, TGA2 is incorporated into a transactivating complex with NPR1 forming an enhanceosome. Genetic and biochemical data demonstrate that transactivation of the TGA2-NPR1 enhanceosome requires the core of the NPR1 BTB/POZ domain (residues 80-91) and the oxidation of NPR1 Cys521 and Cys529. These cysteines are found in a new type of transactivation domain that we term cysteine-oxidized transactivation domain. The data presented further our understanding of the mechanism by which TGA2 and NPR1 activate the Arabidopsis *PR1* gene.

3.2 INTRODUCTION

Plants, unlike animals, do not possess specialized cells for protection against invading pathogens. Instead, every plant cell must be capable of perceiving pathogens and mounting effective defense responses if the organism is to successfully protect itself from infection. Upon detection of an invading microbe, plant defense responses arise from the activation of signal transduction pathways that lead to global transcriptional reprogramming (Dangl and Jones 2001; Durrant and Dong 2004). Among the induced genes figure pathogenesis-related (PR) genes which are activated both at the site of infection and in uninfected parts of the plant in response to the pathogen-induced accumulation of salicylic acid (SA) (Ryals et al., 1996). Local and distal SA accumulations are mandatory for the deployment of a systemic long-lasting and broadspectrum plant disease resistance response called systemic acquired resistance (SAR) (Durrant and Dong 2004; Pieterse and Van Loon 2004; Ryals et al., 1996). Exogenous application of SA, or SA analogs, including 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole (BTH), termed chemical SAR, also triggers PR gene induction and SAR deployment (Ward et al., 1991).

The NPR1 (non-expressor of pathogenesis related gene 1) protein is the key regulator of SAR (Cao et al., 1994; Delaney et al., 1995). In resting cells of wild-type Arabidopsis, NPR1 is found in both the cytoplasm and the nucleus (Després et al., 2000). However, in an *npr1-1* mutant line of Arabidopsis overexpressing an NPR1:GFP fusion protein, the NPR1 fusion is sequestered in the cytoplasm and only localizes to the nucleus after INA treatment (Kinkema et al., 2000). The cytoplasmic NPR1:GFP fusion protein is contained within an oligomer complex held together by intermolecular disulfide bonds

(Mou et al., 2003). Upon INA treatment, Cys82 and Cys216 in NPR1 are presumably reduced and NPR1:GFP is released from this complex, resulting in accumulation of protein monomers inside the nucleus (Mou et al., 2003).

Activation of PR genes during SAR, which requires the nuclear localization of NPR1 (Kinkema et al., 2000), is also dependent on a functionally redundant clade of three basic leucine zipper TGA transcription factors (TFs), TGA2, TGA5, and TGA6, that interact with NPR1 (Després et al., 2000; Zhang et al., 1999). A triple knock-out of these TGA genes abolished PRI induction by INA, indicating that the gene products could act as transcriptional activators (Zhang et al., 2003). This conclusion is supported by a report in which a chimeric TGA2-GAL4:DB protein was used to study gene regulation and proposed to act as a transcriptional activator (Fan and Dong 2002). However, in a finding that appears to be contradictory to the previous one, Zhang et al., (2003) showed that whether unstimulated or INA-treated, the triply knocked-out plants displayed higher levels of PRI (when compared to levels found in wild-type without INA), which could indicate that the proteins of the TGA2-containing clade act as repressors of PRI, presumably by binding to its promoter (Zhang et al., 2003). Furthermore, chromatin immunoprecipitation (ChIP) experiments have demonstrated that TGA2 physically interacts with the PR1 promoter in an SA- and NPR1- dependent manner (Johnson et al., 2003), which would also contradict the hypothesis that TGA2 binds to the PRI promoter in the absence of SA (Zhang et al., 2003). It is thus not clear whether TGA2 is a transcriptional activator or a repressor. PR1 is also positively regulated in an SA-dependent, but NPR1-independent fashion by the transcription factor AtWhy1 (Desveaux et al., 2004). Furthermore, PR1 is negatively regulated by

SUPPRESSOR OF NPR1 INDUCIBLE1 (SNI1; Li et al., 1999) and ChIP experiments have shown an increase in histone H3 acetylation and methylation at the *PR1* promoter in *sni1* mutant plants (Mosher et al., 2006). These data implicate chromatin structure in the regulation of *PR1* expression.

NPR1 and TGA TFs (TGA1 and TGA2) show a direct physical interaction within the nucleus and *in vitro* (Després et al., 2003; Fan and Dong 2002; Subramaniam et al., 2001). This interaction stimulates the DNA-binding activity of TGA TFs to their cognate *cis*-acting element *in vitro* (Després et al., 2000; Després et al., 2003) and *in vivo* (Fan and Dong 2002). However, despite the fact that NPR1 and TGA2 can form a ternary complex on DNA (DNA-TGA2-NPR1 complex) in yeast (Weigel et al., 2005), it is unclear whether, when inside a plant nucleus, they will only interact in the nucleoplasm or whether they will form such a ternary complex. To date, there is no experimental evidence indicating that NPR1 is actually recruited to the *PR1* gene *in vivo*. Therefore, aside from its DNA-binding enhancement activity on TGA TFs, the biochemical role of NPR1 in NPR1-TGA complexes, if any, remains speculative.

NPR1 contains two protein-protein interaction motifs: ankyrin repeats (Cao et al., 1997; Mosavi et al., 2004; Ryals et al., 1997) and a BTB/POZ (Broad-Complex, Tramtrack, and Bric-a-brac/Pox virus and Zinc finger) domain (Aravind and Koonin 1999; Bardwell and Treisman 1994). The ankyrin repeats mediate interactions with TGA TFs and their mutation abolishes NPR1-TGA complex formation, *PR* gene expression, and SAR (Cao et al., 1997; Després et al., 2000; Després et al., 2003; Ryals et al., 1997; Zhang et al., 1999). The functional requirements of the NPR1 BTB/POZ in disease resistance are not yet understood.

Here, we demonstrate that TGA2 is not a transcriptional activator in resting or SA-treated cells, as it is unable to activate transcription when expressed on its own. We show that TGA2 and NPR1 can, independently of one another, physically interact with the PR1 promoter in both resting and SA-treated cells. We also show that NPR1 contains an autonomous transactivation domain in its C-terminus and acts as a co-activator in SAtreated cells where it associates with TGA2 to create a transcriptional activating complex. NPR1 and TGA2 are sufficient to activate gene expression after stimulation of the cells with SA and thus the DNA-TGA2-NPR1 ternary complex constitutes an SA-dependent enhanceosome. We demonstrate that the co-activator function of NPR1 requires the presence of the BTB/POZ core and the oxidation of Cys521 and Cys529, located in the transactivation domain of NPR1. Finally, using an in vivo labeling technique capable of distinguishing between reduced and oxidized cysteines, we determined that Cys521 and Cys529 are oxidized in both resting and SA-treated cells. The data presented here provide a mechanistic understanding of transcriptional regulation mediated by the TGA2-NPR1 complex. Additionally, they help to elucidate the biochemical function of TGA2, a repressor of NPR1-mediated de-repression, NPR1, a co-activator, and to unravel the existence of a new type of eukaryotic transactivation domain that we term: cysteineoxidized transactivation domain.

3.3 METHODS AND MATERIALS

3.3.1 Chemicals

All consumables were purchased from Sigma (St. Louis, MO) unless otherwise stated.

3.3.2 Bacterial Strains and General Cloning Vectors

The *E. coli* strain DH12S (GIBCO-BRL, Grand Island, NY) was used for plasmid maintenance and production. Cloning plasmids used were pBC SK(+) (Stratagene, La Jolla, CA) and pCR2.1 (Invitrogen, Carlsbad, CA). Large-scale plasmid purification was performed using Endo-Free GigaPrep Kits (Qiagen, Mississauga, ON). All constructs used were verified by sequencing.

3.3.3 Plasmid Constructs for Transactivation Assays

The GALA DB, from the yeast two-hybrid vector pBI880, the VP16 TA (provided by Brian Miki, Agriculture and Agri-Food Canada, Ottawa, Ontario, Canada), and the Renilla luciferase from plasmid pRL-null (Promega, Madison, WI) were amplified by PCR using appropriate primers and ligated into the expression vector pBI524 (Datla et al., 1993) containing a modified double Cauliflower mosaic virus 35S promoter with the Alfalfa mosaic virus translational enhancer. The coding sequence for all genes tested were amplified by PCR using gene specific primers and ligated into pBI524 containing GALA DB or VP16 TA in the appropriate reading frame to permit the expression of Nterminal fusion proteins. The unfused versions (i.e. no DB or TA) were cloned into pBI524 lacking the GAL4DB or VP16 TA. The 5X UASGAL4:luciferase reporter construct was provided by Masaru Ohme-Takagi National Institute of Advanced Industrial Science and Technology (Tsukuba, Japan; Fujimoto et al., 2000). To create the PRI promoter-luciferase reporter gene fusion, the -1293 promoter fragment (Lebel et al., 1998) was amplified by PCR and used to substitute the 5X UASGAL4 fragment in the luciferase-nopaline synthase (nos) polyadenylation signal reporter plasmid. To create the

LexA activated synthetic promoter, the double stranded binding domain for LexA, created as two single stranded oligomers annealed together with appropriate restriction enzyme overhangs, was ligated into the *5X UASGALA:luciferase* reporter construct such that 2 of the UAS of Gal4 were removed. Spacing between the Gal4 UAS, the LexA binding domain and the TATA box are depicted in Supplemental Figure 1A and were decided based on results from Tiwari et al. (2004). The DNA binding domain of LexA was ligated into pBI524 containing *VP16 TA* in the appropriate reading frame to permit the expression of an N-terminal fusion protein.

3.3.4 ChIP of NPR1 and TGA2

ChIP was performed as was described previously (Chakravarthy et al., 2003). Briefly, 2g of *Arabidopsis thaliana* was fixed in a 1% formaldehyde solution for 30 minutes at room temperature (RT) with stirring. The fixed tissue was washed 3X and dried before being ground in an equal volume of IP buffer (0.1% SDS, 1% Triton X-100, 50mM HEPES pH 7.9, 150mM NaCl, 1mM EDTA) plus 10X protease inhibitor cocktail (P2714) and centrifuged to remove leaf debris. The supernatant (SN) was sonicated (Sonic Dismembrator Model 100; Fisher Scientific, Ottawa, ON) at level 10 for 6 × 15 seconds of continuous blasts with 15 second rests on ice in between to shear DNA. The samples was then filtered through a PVDF filter (09-720-3, Fisher Scientific, Ottawa, ON) adapted to a 3mL syringe pre-packed with glass wool. The specificity of the anti-NPR1 antibody has been demonstrated (Després et al., 2000) and the anti-His antibody for TGA2 was from Santa Cruz Biotechnology (sc-803 AC). Antibody-coupled Protein A agarose beads (153-6153; Affi-gel Protein A Gel, Bio-Rad, Mississauga, ON) were incubated with the fixed tissue overnight at 4°C on a rotating wheel (Roto-torque, Cole-

palmer instrument company, Montreal, QC). The crosslinking was then reversed and the DNA eluted, precipitated and subjected to PCR. The PCR primer pair specific to the PR1 promoter was as follows: 59-ATGGGTGATCTATTGACTGTTT-39 and 59-GTAGCTTTGCCATTGTTGAT-39. To confirm that the PCR product generated was indeed a fragment of the PR1 promoter, it was gel excised, cloned, and sequenced.

3.3.5 Plant Growth Conditions and Transformation

Conditions for growth of Arabidopsis thaliana (Columbia) and *npr1-3* (Cao et al., 1997) and methods for plant transformation, the selection of transgenic individuals and northern blot hybridization were previously described (Liu et al., 1995).

3.3.6 Plant Two-Hybrid Assays

Arabidopsis thaliana (Columbia) leaves were harvested from four week old plants grown at 21°C (day) and 18°C (night) with a ten hour photoperiod and placed on a medium containing MS salts and micronutrients supplemented with B5 vitamins, 1% sucrose and 0.8% agar at a pH of 5.8. Two leaves were transformed per bombardment. Bombardment and sample processing were as described by Fujimoto et al. (2000). Briefly, sample preparation for bombardments consisted of one microcentrifuge tube containing enough DNA-gold mixture for 5 bombardments. With continuous vortexing, 44.4μL of 60mg/mL 1.0 micron gold (165 2263, Bio-Rad, Mississauga, ON) was mixed with 13μL of DNA consisting of the reporter gene, internal standard and the vector(s) for the protein or protein pair being tested. DNA was then precipitated onto the gold particles with 42μL of 3M CaCl₂ and 20μL of 0.1M spermidine. The DNA-gold mixture was then washed once with 70% and 100% EtOH before being resuspended in 48μL of 100% EtOH. 8μL of

DNA-gold mixture was pipeted for each bombardment on macrocarriers (165 2335; Bio-Rad, Mississauga, ON) and allowed to dry. The Model PDS-1000/He Biolistic® Particle Delivery System (165 2257; Bio-Rad, Mississauga, ON) was used to deliver the DNA-gold mixture to the Arabidopsis leaves. Rupture disks (156 2329; Bio-Rad, Mississauga, ON) of 1100psi were used in combination with ultra high purity (5.0) helium at 1500psi. When required, filter-sterilized salicylic acid was sprayed on the leaves at a concentration of 1mM for the indicated length of time. Leaves were left for a period of 24 hours before being assayed. The assay was performed using the Dual-Luciferase® Reporter Assay system (PRE 1960; Fisher Scientific, Ottawa, ON) following the manufacturers instructions. Fluorescence was measured as photon emission (EG and G Berthold Lumat LB9507 Luminometer, Bad Wildbad, Germany) and the value obtained represents the value of the reporter gene divided by the value of the internal standard (as relative luciferase units). Every bar in each graph represents five bombardments repeated five times (n = 25).

3.3.7 Yeast Two-Hybrid Assays

Plasmids pBI880 (DB) and pBI881 (TA) (Kohalmi et al., 1997) in conjunction with the yeast strain YPB2 of *Saccharomyces cerevisiae* were used throughout this study. Yeast cells were transformed using S.c. EasyComp™ Transformation Kit (K505001; Invitrogen, Carlsbad, CA) and grown on synthetic dextrose using the appropriate amino acid drop-out(s). Yeast cells were co-transformed with the modified DB and TA plasmids and selected for on synthetic dextrose without leucine or tryptophan for protein-protein interaction assays. Either the activity of the *lacZ* reporter gene was monitored visually by

using the X-gal filter assay or β -galactosidase activity was determined spectrophotometrically.

3.3.8 Molecular modeling and Wirplot diagram

The model of the NPR1 BTB/POZ domain was generated through comparative modeling by submitting the entire NPR1 amino acid sequence to the automated approach of the 3DJIGSAW v.2.0 modeling server (http://www.bmm.icnet.uk/servers/3djigsaw). The server's domain fishing module (Contreras-Moreira and Bates, 2002) initially identified the presence of a BTB/POZ domain spanning residues 36-190 of NPR1. The crystal structure of the PLZF BTB/POZ domain (PDB accession code: 1CS3) was then used as a template to construct a preliminary model for the corresponding NPR1 domain. To alleviate an unrealistic steric interference and generate the final model, a portion of L2 (residues 96 to 112) was refined using Quanta v.4.1 followed by energy minimization with the CHARMM force field v.27.1 (Accelrys, San Diego, CA) (Brooks et al, 1983). Images of the models were generated with the POV-Ray module (http://www.povray.org) of the Swiss-Pdb Viewer v3.7SP5 (Geux and Peitsch, 1997). The two-dimensional secondary structure diagram was generated using the WIRPLOT module of the PROCHECK program v.3.4.5 (Laskowski et al, 1993) and the colors of the resulting postscript file were edited using Ghostscript v.5.50 and visualized using GSview v.2.6.

3.3.9 In vivo determination of the Cys Redox Status of NPR1 \(\Delta 513 \)

Due to very low amounts of proteins in the biolistics assays, 80 bombardments were performed with the $\Delta 513$:TA constructs. After a 24-hr incubation period with or without SA, proteins were extracted from Arabidopsis leaves, separated into two aliquots and

processed immediately and in parallel. For detection of reduced cysteines, one aliquot was treated with 0.2mM of 3-(*N*-maleimido-propionyl) biocytin (MPB) for one hour in the dark at room temperature. Proteins were then precipitated four times with acetone. For detection of oxidized cysteines, the second aliquot was first treated with 20mM of *N*-ethylmaleimide (NEM) for one hour, precipitated with acetone and then treated with 20mM dithiothreitol (DTT) for one hour. After another acetone precipitation, proteins were treated with 0.2mM of MPB for one hour in the dark at room temperature followed by two acetone precipitations.

3.3.10 Immunoprecipitation and Detection of Modified Cysteines in NPR1\(\Delta\)513

The cysteine-labeled proteins were incubated for 16 hours at 4°C with anti-VP16 antibody (sc7545 AC, Santa Cruz Biotechnology, CA) coupled to protein A-sepharose beads (Bio-Rad, Mississauga, ON). The VP16 does not contain any cysteines. The beads were subsequently washed three times in IP buffer and the proteins were eluted by boiling in 2X sample buffer (0.12M Tris-Cl pH 6.8, 4% SDS, 20% glycerol, 0.05% bromophenol blue, 10% 2-mercaptoethanol). The supernatant was electrophoresed on an 8% sodium dodecyl sulfate polyacrylamide gel. The proteins were transferred to nitrocellulose and reacted according to the manufacturer's instruction (LI-COR, Lincoln, NE) with a strepavidin:Alexa fluor 680 (Molecular Probes, Eugene, OR) conjugate and detected by infrared imaging using an Odyssey imager (LI-COR, Lincoln, NE).

3.3.11 Statistical Methods

All pooled data are expressed as averages, and error bars represent SD. When data from two independent populations are compared, statistical significance was assessed using a two-tailed Student's t test.

3.4 RESULTS

3.4.1 Recruitment of TGA2 to the PR1 Promoter is Both SA- and NPR1-Independent

SA-induction of the *PR1* gene is positively controlled by a clade of three TGA TFs (TGA2, 5 and 6) with redundant functions. In the triple TGA knock-out plants, the levels of *PR1* transcripts were up to 50-fold higher, when compared to non-stimulated wild-type plants (Zhang et al., 2003). This was interpreted as a loss of TGA factor binding to a negative element in the *PR1* promoter; however, whether this effect was due to direct binding of the TGA TFs to DNA was not addressed. If the interpretation of Zhang et al. (2003) is correct, their results would contradict those of Johnson et al. (2003), who demonstrated using ChIPs that recruitment of TGA2 to the *PR1* promoter is both SA- and NPR1-dependent. These ChIPs were performed on endogenous TGA2 using an anti-TGA2 antibody raised against the N-terminus. However, since ChIPs can generate false negatives when epitopes are inaccessible, we sought to determine whether the apparent lack of interaction between TGA2 and the *PR1* promoter in resting cells observed by Johnson et al. (2003) is due to the absence of antibody recognition, to masking of the epitope or to the absence of TGA2.

As a means of generating an alternative epitope, the *TGA2* coding region was ligated to a 6-histidine tag (His) and the resulting fusion (TGA2:His), under the control of

the *CaMV35S* promoter, was introduced into the *tga2/5/6* knock-out plants. Figure 1A is a diagram of the *PR1* gene which shows the position of the PCR primers used for all the ChIP experiments. Figure 1B shows that a PCR product is present in the lanes corresponding to immunoprecipitations performed with the anti-His antibody (lanes 3 and 7), indicating that TGA2:His interacted with *PR1* in both untreated and SA-treated cells. Immunoprecipitation with pre-immune serum (PI) did not lead to a detectable band (lanes 2 and 6). ChIP performed with the anti-His antibody on the untransformed *tga2/5/6* mutant plant also did not lead to a detectable band (data not shown). The right section of panel B shows the results for a northern blot analysis of *PR1* indicating that TGA2:His restored *PR1* inducibility in the *tga2/5/6* mutant in an SA-dependent fashion and that expression of *PR1* is not constitutive in these plants.

The above results indicate that the lack of interaction previously reported by Johnson et al. (2003) in the absence of SA might have been due to the masking, under certain conditions, of the N-terminal TGA2 epitope chosen by these authors. Therefore, it became relevant to test whether the same phenomenon was responsible for the lack of interaction reported between TGA2 and *PRI* in the *npr1* background (Johnson et al., 2003). To do so, TGA2:His, under the control of the *CaMV35S* promoter, was introduced in the *npr1-3* mutant background and ChIPs were performed using the anti-His antibody. Results of Figure 1C show the presence of a PCR product in the lanes corresponding to immunoprecipitations performed with the anti-His antibody (lanes 3 and 7). This indicates that, in the absence of NPR1, TGA2:His interacted with *PR1* in both untreated and SA-treated cells. Immunoprecipitation with PI did not lead to a detectable band (lanes 2 and 6). ChIP performed with the anti-His antibody on the untransformed *npr1-3*

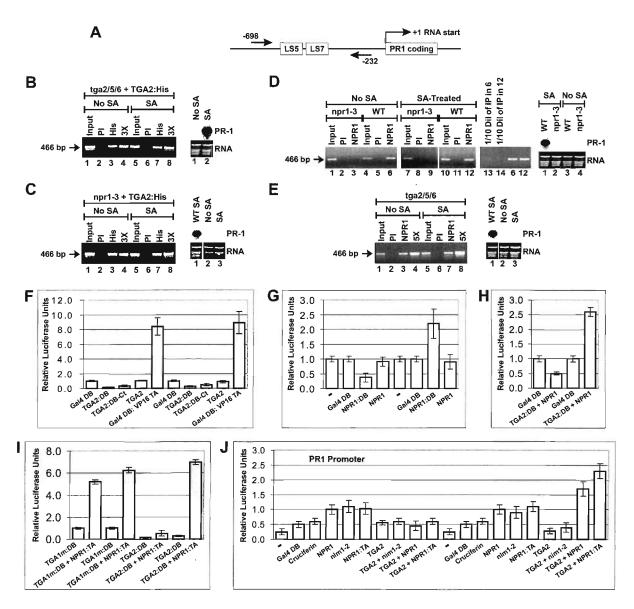


Figure 1. NPR1 Is a Co-Activator Required for Transcriptional Activation by a TGA2-NPR1 Complex in SA-Treated Cells Only.NPR1 Is a Co-Activator Required for Transcriptional Activation by a TGA2-NPR1 Complex in SA-Treated Cells

- (A) Graphic representation of the *PR1* gene. The straight arrows and the numbers indicate the position of the PCR primers used for ChIP experiments. LS5 and LS7 are two DNA regions containing the TGA factors cognate binding sequence TGACG (Lebel et al., 1998).
- (B) Chromatin Immunoprecipitations of TGA2:His expressed in tga2/5/6 knock-out plants. The right section of this panel is a northern blot illustrating that the TGA2:His protein complemented the tga2/5/6 mutation and restored PRI inducibility.
- (C) Chromatin Immunoprecipitations of TGA2:His expressed in *npr1-3* mutant (npr1-3) Arabidopsis plants. The right section of this panel is a northern blot illustrating that the TGA2:His protein expressed in the *npr1-3* mutation did not bring about expression of *PR1* whether tissues were treated or not with SA. Northern blot from wild-type plants

- (WT) treated with SA is shown for comparison. For (B) and (C), ChIPs were conducted with anti-His antibodies conjugated to agarose beads.
- (**D**) Chromatin Immunoprecipitations of NPR1 from wild-type (NPR1) and *npr1-3* mutant (npr1-3). The right section of this panel is a northern blot illustrating that the *PR1* gene is not expressed in the *npr1-3* mutant and expression in the wild-type plant (WT) is dependent upon SA treatment.
- (E) Chromatin Immunoprecipitations of NPR1 from the tga2/5/6 knock-out Arabidopsis plants. The right section of this panel is a northern blot illustrating that the *PR1* gene is not expressed in the *tga2/5/6* mutant whether or not tissues have been treated with SA. A northern blot from wild-type plants (WT) treated with SA is shown for comparison. For (D) and (E), ChIPs were conducted with anti-NPR1 antibodies for which the specificity has been demonstrated previously (Després ate al., 2000).
- In (B), (C), (D), and (E) tissues were untreated (No SA) or treated for 6 hr with 1mM SA. PI indicates that ChIP was performed with pre-immune serum. PCR was conducted with PR1 promoter-specific primers. The arrow indicates the location of the PCR products. The NPR1-3 protein is a deletion version of NPR1 (Cao et al., 1997), which has lost the antigenic region used to raise the anti-NPR1 antibodies used in this study. The inputs represent 2% of the immunoprecipitated material (50-fold dilution). 3X or 5X indicate that the PCR reaction was performed with 3 or 5 times the amount of immunoprecipitated material, respectively, to demonstrate that the PCR reaction was in the linear range. In lanes 13 and 14 of panel (D), 1/10 of the amount of immunoprecipitated material used in lanes 6 and 12, respectively, was used to perform the PCR to demonstrate that the PCR reaction was in the linear range. The right sections of these panels are northern blots using PR1 probes. RNA stained with ethidium bromide is shown for loading comparison. (F) Histograms illustrating the fact that TGA2 tethered to DNA through Gal4 DB fused to the N-terminal (TGA2:DB) or C-terminal (TGA2:DB-Ct) does not activate transcription, while a chimeric transcription activator composed of the Gal4 DB fused to the transactivation domain of viral protein 16 (Gal4 DB:VP16 TA) does. Gal4 DB
- represents the baseline level of transcription.
 (G) Histograms illustrating the transcription activation of NPR1 tethered to DNA through Gal4 DB (NPR1:DB). NPR1 indicates the absence of fusion. (-) indicates that only the reporter and internal standard vectors have been bombarded into the tissues; no effector has been introduced.
- (H) Histograms illustrating the effect of NPR1 on the stranscriptional activity of TGA2:DB. NPR1 indicates that the protein is expressed without a fusion.
- (I) Histograms illustrating the fact that TGA2 tethered to DNA through Gal4 DB (TGA2:DB) interacts very poorly with NPR1:TA in the absence of SA-treatment.
- In (F), (G), (H), and (I) Arabidopsis leaves were left untreated (white bars) or were treated for 24 hr with 1mM salicylic acid (grey bars). The constructs were transfected along with the 5X UAS^{GAL4} :Firefly luciferase reporter and the CaMV35S:Renilla luciferase internal standard vectors. Data are reported as Relative Luciferase Units. The fold-activation represents the Relative Luciferase Units (RLU) obtained with the given protein or protein pair divided by the RLU obtained with the unfused Gal4 DB construct alone (baseline transcription). Values consist of n = 25 samples and represent averages \pm 1 SD. Every bar represents five bombardments repeated five times (n = 25).

(J) Histogram illustrating the effect of NPR1, and nim1-2 on the transcriptional activity of the TGA2-NPR1 complex. All proteins were native (without fusion), with the exception of NPR1:TA (NPR1 fused to the viral protein 16 transactivation domain), which was used to assess the level of interaction between NPR1 and TGA2 in the context of the PRI promoter. The reporter system was the Arabidopsis PRI promoter fused to the firefly luciferase. The CaMV35S promoter:Renilla luciferase fusion was used as an internal standard. (-) indicates that no effector were bombarded along with the reporter and internal standard vectors. Cruciferin is an Arabidopsis storage protein used here to illustrate the background level of this system when expressing an unrelated protein. Gal4 DB served the same purpose. Arabidopsis leaves were left untreated (white bars) or were treated for 24 hr with 1mM salicylic acid (grey bars). Data are reported as Relative Luciferase Units. Values consist of n = 25 samples and represent averages ± 1 SD. Every bar represents five bombardments repeated five times (n = 25).

mutant plant did not lead to a detectable band (data not shown). As indicated by northern blot analysis (right section of panel C), expression of TGA2:His in the *npr1-3* mutant background did not lead to expression of *PR1*, whether or not cells were subjected to SA-treatment.

3.4.2 Recruitment of NPR1 to the PR1 Promoter is both SA- and TGA2/5/6-Independent

Since no experimental evidence exists to indicate that NPR1 can be recruited to the *PR1* promoter, it is unclear whether NPR1 is capable of forming a complex with TGA2 on DNA to modulate transcription. To address this question, we performed ChIP experiments with wild-type and *npr1-3* mutant Arabidopsis plants, before and after SA-treatment (Figure 1D). The *npr1-3* mutant was chosen as a negative control since this allele carries a premature stop codon (Cao et al., 1997), which removes the amino acid region used to raise the anti-NPR1 antibody (Després et al., 2000). The specificity of the anti-NPR1 antibody has been demonstrated previously (Després et al., 2000) and can also be witnessed in Figure 2C, where a band corresponding to NPR1 was detected in the wild-type plant (lane 1) but not in the *npr1-3* mutant plant (lane 2). With the exception of

input lanes (lanes 1 and 7), ChIP performed on the *npr1-3* lines did not yield a band, regardless of whether cells were treated with SA or whether the immunoprecipitation antibodies were from PI or raised against NPR1. Conversely, ChIP performed on wild-type plants indicated that NPR1 interacted with *PR1* in both untreated cells and cells treated with SA (lanes 6 and 12). Immunoprecipitation with PI did not lead to a detectable band (lanes 5 and 11). Northern blot analysis (right section of panel D) confirmed that *PR1* was not expressed in the *npr1-3* mutant nor was it induced in the wild-type plant prior to SA treatment.

Intuitively, knowing that NPR1 and TGA2 can interact with each other and because NPR1 does not contain a known DNA-binding domain, one could expect the recruitment of NPR1 to the *PR1* promoter to be dependent on TGA2. To test this hypothesis, we performed ChIP experiments on the *tga2/5/6* mutant plant using the anti-NPR1 antibody. The presence of a PCR product in the lanes corresponding to immunoprecipitations performed with the anti-NPR1 antibody (Figure 1E, lanes 3 and 7) indicate that NPR1 continues to interact with *PR1* in the absence of TGA2/5/6, in both untreated and SA-treated cells. Immunoprecipitation with PI did not lead to a detectable band (lanes 2 and 6). Note that formaldehyde, the cross-linker used in the ChIP experiments, can cross-link protein to DNA but also protein to protein (Buck and Lieb, 2004). Hence, recruitment of NPR1 to the *PR1* promoter does not indicate that NPR1 binds directly to DNA. The northern blot shown in the right section of panel E confirmed that *PR1* was not expressed in the *tga2/5/6* mutant whether or not cells were subjected to an SA-treatment.

3.4.3 NPR1 Is a Co-Activator Required for Transcriptional Activation by a TGA2-NPR1 Complex in SA-Treated Cells Only

PRI is positively regulated by NPR1 (Cao et al., 1997; Ryals et al., 1997) and by TGA2/5/6 (Zhang et al. 2003). This prompted us to test whether TGA2 can act as a transcriptional activator. To do so, TGA2:DB (N-terminal fusion) and TGA2:DB-Ct (Cterminal fusion) were assayed using an *in vivo* plant transcription assay (Figure 1F). The baseline level of transcription was determined by transfecting leaves with Gal4 DB (not fused to any other protein or protein domain) along with a reporter construct consisting of a firefly luciferase gene under the control of 5 copies of the Gal4 upstream activating sequences (UAS) fused to a minimal promoter. Transfection with TGA2:DB or TGA2:DB-Ct did not result in reporter gene activation beyond the baseline level, regardless of whether cells were treated with SA or not. The same result was obtained with TGA2 that was not fused to Gal4 DB or any other foreign protein domain (TGA2). Transfection with Gal4 DB fused to a strong transactivation domain (Gal4 DB:VP16 TA) led to SA-independent expression of the reporter gene well above the baseline (Figure 1F, white versus grey bars). These results demonstrate that the reporter gene can indeed be activated under our experimental conditions and indicate that TGA2 is not a transcriptional activator, whether or not cells are stimulated with SA. Indeed, we have found that TGA2 is capable of repressing an activated reporter gene prior to and after SA treatment (Supplemental Figure 1). In this system, we used a 3X Gal4:1X LexA:minimal promoter: Firefly luciferase reporter gene which acts as an activated system where repression can be tested. The LexA binding domain present in the promoter is bound by the LexA protein fused to the VP16 transactivation domain and results in reporter gene

expression (Supplemental Figure 1, Gal4DB + LexAVP16 white and grey bars). The empty Gal4DB binds the 3X UAS in this instance but has no affect on reporter gene expression. However, when TGA2 is fused to the Gal4DB in the presence of LexAVP16, reporter gene activation is decreased (Supplemental Figure 1, compare Gal4DB + LexAVP16 to TGA2:DB + LexAVP16, white and grey bars, p<0.05), indicating that TGA2 is not a transcriptional activator but a repressor.

Knowing that NPR1 can be recruited to a promoter *in vivo* (Figure 1D and E), we tested if NPR1 can activate transcription when tethered to DNA. To accomplish this, NPR1 was fused to Gal4 DB (NPR1:DB) and assayed using the *in vivo* plant transcription assay (Figure 1G). In untreated cells (white bars), NPR1:DB did not lead to gene activation beyond the baseline level. However, after SA treatment (grey bars), NPR1:DB activated transcription 2.2-fold above the baseline level. Expression of NPR1 without fusion to Gal4 DB (NPR1) did not lead to gene activation that was significantly different from the baseline level (p<0.05), indicating that transactivation by NPR1:DB observed with SA was dependent on the recruitment of NPR1 to the promoter. The results indicate that NPR1 could potentially act as a transcriptional co-activator if recruited to a promoter via a DNA-binding protein, such as TGA2.

We next addressed whether NPR1 could modulate the transcriptional properties of TGA2. When TGA2:DB was co-expressed with NPR1 (not fused to any foreign transactivation or DNA-binding domain), expression of the reporter gene in untreated cells did not increase beyond the baseline (Figure 1H, white bars). However, transcription rose 2.6-fold above the baseline level after SA treatment (Figure 1H, grey bars). Since neither TGA2:DB nor NPR1 activate transcription of the reporter gene on their own

(Figure 1F and 1G) and NPR1 stimulates transcription when tethered to DNA (Figure 1G, NPR1:DB), results from Figure 1H suggest that the transcriptional activation observed when NPR1 (unfused) is co-expressed with TGA2:DB is likely due to NPR1 being tethered, or recruited, to the DNA through TGA2:DB. Physical interaction between TGA2 and NPR1 at the reporter gene promoter and in the presence of SA was demonstrated using plant two-hybrid assays (Figure 1I, TGA2:DB + NPR1:TA). Together, these observations are consistent with the formation of a ternary complex between DNA, TGA2:DB and NPR1, with NPR1 acting as a co-activator of TGA2 on the Gal4-based promoter.

Using plant two-hybrid assays (Figure 1I), we showed that, in the absence of SA (white bars), NPR1 fused to VP16 TA (NPR1:TA) also interacted with TGA2:DB (significant difference p<0.05 between TGA2:DB and TGA2:DB + NPR1:TA), but very poorly. A similar conclusion was reached based on data from a protein fragment complementation assay (Subramaniam et al., 2001). Thus, in addition to the fact that NPR1:DB (tethered to DNA) does not transactivate in the absence of SA, the very weak interaction between NPR1:TA and TGA2:DB in unstimulated cells may also account for the lack of transcriptional stimulation by NPR1. We also confirmed that, in the absence of SA, NPR1:TA is competent to interact with other proteins as demonstrated by its interaction with a mutant version of TGA1 (Figure 1I, TGA1m:DB), which was previously shown to interact with NPR1 in the presence and absence of SA-treatment (Després et al., 2003).

Next, we tested the transactivation properties of NPR1 and TGA2 in the context of the *PR1* promoter. DNA coding for native (unfused) proteins were delivered by

biolistics as in Figure 1F-1I, except that the reporter consisted of the luciferase gene under the control of the PRI promoter (Figure 1J). Relatively low levels of luciferase activity were detected following transfection of this reporter gene without effector plasmids (-). Since only 20 to 100 cells are effectively transfected under our transient assay conditions and because of the very low endogenous amount of NPR1 and TGA2 protein, modulation of the transiently delivered PRI reporter gene by SA is below baseline level. Transfection of two unrelated effectors, Gal4 DB, which does not bind PR1 (there is no Gal4 binding site in the PR1 promoter), and the seed storage protein Cruciferin, increased reporter gene expression, which most likely represents the unspecific effect of expressing a protein in this system. Thus, expression levels observed with these proteins represent the baseline of this system. Whether cells were treated (grey bars) or not (white bars) with SA, NPR1 led to activation of the PR1 promoter beyond the baseline level. TGA2, on the other hand, had no effect on the baseline activity of the promoter. However, in untreated cells (white bars), when NPR1 was co-expressed with TGA2, transcription values were brought back down to the baseline level, indicating that TGA2 repressed the NPR1-dependent activation of PR1. As observed with NPR1, the protein nim1-2, a variant of NPR1 with a mutation in an ankyrin repeat that does not interact with TGA2 (Després et al., 2000; Ryals et al., 1997), also activated the PRI promoter in the absence of TGA2 in untreated and SA-treated cells. This suggests that the ankyrin repeats are unlikely to be involved in the recruitment of NPR1 to the PR1 promoter. Furthermore, since nim1-2 does not interact with TGA2, this result is also consistent with a TGA2-independent recruitment of NPR1 to the PR1 promoter, as was observed with ChIPs (Figure 1E). Co-expression of nim1-2 with TGA2 also restored

transcription values to the baseline level. Co-expression of TGA2 and NPR1 in SA-treated tissues (grey bars) led to activation of PRI beyond the baseline and significantly beyond what was observed with NPR1 alone (p<0.05), confirming that NPR1 acts as a TGA2-co-activator on the PRI promoter. Also, co-expression of TGA2 with NPR1:TA established that the two proteins interact on the PRI promoter, only in the presence of SA (grey bars), since values observed with TGA2 + NPR1:TA were significantly higher than those obtained with TGA2 + NPR1 (p<0.05) or NPR1:TA alone (p<0.05). Our results indicate that, in untreated cells, TGA2 represses the NPR1-dependent activation of PRI, without the two proteins interacting with each other. However, after SA-treatment, the two proteins interact to form a ternary complex with PRI DNA in which NPR1 acts as a TGA2-co-activator.

3.4.4 The BTB/POZ Domain of NPR1 Is Required for PR1 Activation by SA

To determine the functional importance of the NPR1 BTB/POZ domain, we generated a series of rational mutants based on information available from other model systems. Of the four known structural classes of BTB domains (BTB Zinc Finger, Skp1, ElonginC, and T1), NPR1 is most similar to those associated with zinc fingers, the so-called long-form (Stogios et al., 2005). We thus performed a small scale multiple alignment (Figure 2A) of long-form BTB/POZ domains including the one from human promyelocytic leukemia zinc finger (PLZF), the archetypical BTB/POZ domain (see Aravind and Koonin, 1999 for a more exhaustive alignment of 79 BTB/POZ domains including that of NPR1). Also shown is a representation of the secondary structure of the PLZF BTB/POZ derived from its crystal structure (Ahmad et al., 1998; PDB accession code: 1buo).

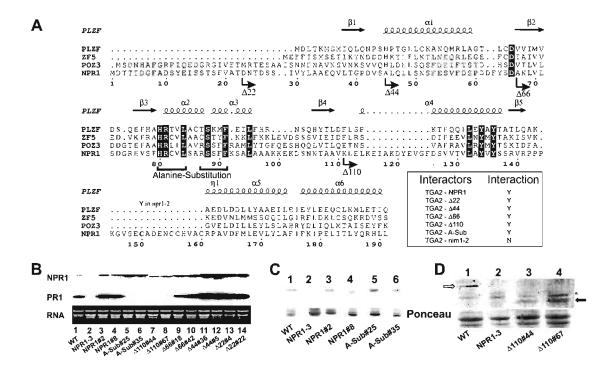


Figure 2. The BTB/POZ Domain of NPR1 Is Required for PR1 Induction

(A) Multiple-alignment of selected BTB/POZ domains. Residues blocked in black are conserved among all sequences. Numbers refer to the amino acid position in NPR1. Straight arrows and coils indicate the position of beta strands and helices in the PLZF crystal structure, respectively. α and η indicates α - and 3_{10} - helices, respectively and β refers to β-strands. The bent arrow indicates the position where the NPR1-deletion proteins begin. The horizontal brackets below the amino acid sequence of $\alpha 2$ and $\alpha 3$ indicate the residues that have been mutated to alanine in the "Alanine-Substitution" mutant. C150 bears a C to Y mutation in the npr1-2 mutant, which abolishes interaction with TGA2, PR gene activation, and deployment of SAR (Cao et al., 1997; Després et al., 2000; Zhang et al., 1999). NPR1, PLZF, POZ3, and ZF5 are from Genbank accession numbers GI:1773295, GI:486933, GI:2291257, and GI:1399185, respectively. The inset represents directed yeast two-hybrid assays using the filter test and the outcome of the experiments. nim1-2 is a mutant version of NPR1 that bears a histidine-to-tyrosine replacement in one of the ankyrin repeats (Ryals et al., 1997), which abolishes interaction with TGA factors (Després et al., 2000 and 2003). Y (yes), indicates an interaction while N (no), indicates an absence of interaction (white color after 24 hr incubation with X-GAL).

(B) Northern blot analysis using *NPR1* or *PR1* probes. RNA stained with ethidium bromide is shown for loading comparison. Lane 1 contains RNA from wild-type Arabidopsis and lane 2 from the *npr1-3* mutant. The remaining lanes contain RNA from *npr1-3* lines expressing the following constructs; wild-type NPR1 (Lanes 3 and 4), the "Alanine-Substitution" mutant (Lanes 5 and 6), and the deletion mutants $\Delta 110$ (Lanes 7 and 8), $\Delta 66$ (Lanes 9 and 10), $\Delta 44$ (Lanes 11 and 12), and $\Delta 22$ (Lanes 13 and 14). Results from two independent transgenic lines are shown per construct. Specific line numbers follow the construct name.

(C-D) Top Panel. Immunoblot analysis of proteins from wild-type Arabidopsis (WT), the npr1-3 mutant (NPR1-3), and the npr1-3 background lines expressing NPR1, the "Alanine-Substitution" mutant and $\Delta 110$ as described in (B). An anti-NPR1 antibody (Després et al., 2000) was used. Bottom Panel. Ponceau staining of the membranes shown in the top panel. In panel (D), the open arrow indicates the position of the full-length NPR1 protein (66 kD), while the black arrow indicates that of the truncated protein $\Delta 110$ (54.4 kD). The asterisk points to a protein interacting non-specifically with the antibody.

Since the N-terminal region of the NPR1 BTB/POZ is longer than that of PLZF, we used the protein secondary structure prediction PSIPRED (Jones, 1999) and identified a potential β-strand formed by residues 19-22 (FVAT). Deletion of this putative structure generated the $\Delta 22$ mutant (Figure 2A). The next deletion, corresponding to the $\Delta 44$ mutant, removed β1, which has been shown to partially destabilize the PLZF dimer (Ahmad et al., 1998). Deletion mutant $\Delta 66$ removed all the structural determinants ($\beta 1$, α 1, and D65) mandatory for BTB/POZ homodimerization (Ahmad et al., 1998). The α 2 and α 3 helices are buried within the BTB/POZ and constitute the monomer core of the domain (Ahmad et al., 1998). Alanine-substitution of the core in PLZF results in disruption of the BTB/POZ fold (Melnick et al., 2000). The core region is well conserved in NPR1, and of note, the sequence "RSSFF", residues 87-91 of NPR1, is identical to the corresponding region in POZ3, and the sequence "HRCVL" residues 80-84, identical to the corresponding region in ZF5. Thus, to permit functional testing of the NPR1 BTB/POZ core without deleting other elements, the conserved residues in α 2 and α 3 were substituted with alanines (Figure 2A, Alanine-Substitution brackets). Finally, since β2, β3, and β4 form a tertiary structure, an N-terminal deletion aimed at removing the core of the BTB/POZ was created after $\beta 4$ but before the next structural element ($\Delta 110$ deletion mutant). The five NPR1 variants mutated in the BTB/POZ (Δ22, Δ44, Δ66,

Δ110, and the alanine-substitution), all interacted with TGA2 in yeast two-hybrid assays (Figure 2A inset). Quantitative yeast two-hybrid assays confirmed that the five NPR1 mutants interacted with TGA2 however, none of the mutants interacted with TGA2 as well as full-length wild-type NPR1 (Supplemental Figure 2).

In order to assess the biological significance of the NPR1 BTB/POZ in controlling PRI expression, we created and tested five cDNA constructs encoding the proteins depicted in Figure 2A. These were introduced, under the control of the CaMV35S promoter, into the *npr1-3* genetic background (Figure 2B). As a control, *npr1-3* plants were transformed with the full-length, wild-type NPR1 coding region fused to the CaMV35S promoter (NPR1, lanes 3 and 4). Wild-type Arabidopsis (WT) accumulated PRI transcripts when treated with 0.5mM SA for 16 hr (lane 1), while npr1-3 plants (NPR1-3) did not (lane 2). PR1 gene expression was restored in 21 of the 25 independent transgenic npr1-3 lines expressing NPR1 (lanes 3 and 4, and data not shown), in all 23 lines expressing $\Delta 22$ (lanes 13 and 14, and data not shown), in 35 of 38 lines expressing $\Delta 44$ (lanes 11 and 12, and data not shown), in 18 of 24 lines expressing $\Delta 66$ (lanes 9 and 10 and, data not shown), but in none of the 31 and 40 independent lines expressing the alanine-substituted BTB/POZ (lanes 5 and 6, and data not shown) or $\Delta 110$ (lanes 7 and 8, and data not shown), respectively. Figure 2C and D indicate that the $\Delta 110$ and alaninesubstitution proteins were expressed in these lines. PRI transcripts were not detected in any of the lines tested in the absence of SA (data not shown).

Altogether, the results of Figure 2 indicate that although alanine-substitution and $\Delta 110$ can interact with TGA2 (Figure 2A, inset and Supplemental Figure 2), their expression cannot complement the *npr1-3* mutation. This demonstrates that the

interaction of NPR1 with TGA2 is in itself not sufficient for biological activity and that the core of the NPR1 BTB/POZ, in the context of the full-length NPR1, is required for *PR1* induction.

3.4.5 The NPR1 BTB/POZ Core Is Required for the TGA2-Co-Activator Function of NPR1 in SA-Treated Cells

To establish a link between the complementation of PR1 expression and the transactivation of the TGA2-NPR1 complex, we determined whether the deletions and the alanine-substitution of the NPR1 BTB/POZ affected the capacity of this protein to act as a TGA2-co-activator. Deletion of the first 22, 44, or 66 amino acids of NPR1 did not substantially affect the capacity of NPR1 to convert TGA2:DB into an activator after treatment with SA (Figure 3A). However, deleting the first 110 amino acids or substituting the BTB/POZ core with alanines abolished transactivation of the coexpressed TGA2:DB (Figure 3A). The nim1-2 protein, which does not interact with TGA2, served as a negative control. In the absence of SA-treatment, none of the mutants significantly altered transactivation of TGA2:DB compared to results obtained with fulllength NPR1 (Figure 1F) and, accordingly, data are not shown. We also tested the alanine-substitution and $\Delta 110$ proteins for their capacity to interact with TGA2 in the plant two-hybrid system (Figure 3B), which evaluates interaction in the context of the promoter. The data indicate that alanine-substitution and $\Delta 110$ fused to VP16 TA interacted with TGA2:DB with no significant differences in the level of interaction (p<0.05) when compared to Δ 22, Δ 44 and Δ 66. However, the interaction of these five mutant proteins with TGA2 was significantly lower than that observed with wild-type NPR1 (p<0.05). These results are consistent with those obtained with quantitative yeast

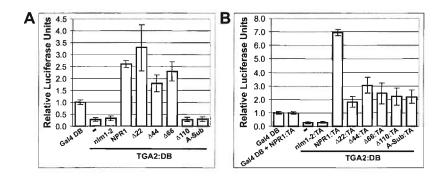


Figure 3. The Core of the NPR1 BTB/POZ Is Required for the TGA2-Dependent Co-Activator Function of NPR1 in SA-Treated Cells

- (A) Histogram illustrating the effect of NPR1 and the mutants described in Figure 2, on the transcriptional activity of the TGA2-NPR1 complex tethered to DNA through Gal4 DB fused to TGA2. Results obtained with TGA2:DB alone (-) are also shown.
- (B) Histogram illustrating the interaction of NPR1 and the mutants described in (A) fused to the VP16 transactivation domain with TGA2 fused to the Gal4 DB. Results obtained with Gal4 DB alone (Gal4 DB), Gal4 DB coexpressed with NPR1:TA (Gal4 DB + NPR1:TA), and TGA2:DB alone (-) are also shown. For (A) and (B) conditions were identical to those described in Figure 1. Data are reported as Relative Luciferase Units. Values consist of n = 25 samples and represent averages ± 1 SD. Every bar represents five bombardments repeated five times (n = 25).

two-hybrid assays (Supplemental Figure 2). Together, the findings shown in Figure 3 indicate that amino acids located between residues 66 and 110 of NPR1, more precisely residues 80 to 84 and/or 87 to 91, which constitute the core of the BTB/POZ, are required for the TGA2-co-activator function of NPR1.

3.4.6 NPR1 Harbors a Cryptic Transactivation Domain in its Last 80 Amino Acids

Given that the core of the NPR1 BTB/POZ is required for transactivation of the TGA2-NPR1 complex, we sought to determine whether this domain harbors autonomous transcriptional regulatory regions. To identify these potential regulatory regions, the NPR1 BTB/POZ (amino acids 1 to 190) was fused to Gal4 DB (POZ:DB) and assayed using the in vivo plant transcription assay (Figure 4A). In the absence (white bars) or presence (grey bars) of SA-treatment, POZ:DB and variants, in which the first 22, 66, and 110 amino acids were deleted (Δ22POZ:DB, Δ66POZ:DB, Δ110POZ:DB) or in which the core of the BTB/POZ was replaced with alanines (A-SubPOZ:DB), did not stimulate transcription beyond the baseline level (Gal4:DB). One of the most salient features of this experiment was the uncovering of a cryptic transactivation domain, revealed when the BTB/POZ was shortened by 44 amino acids at the N-terminus (Δ 44POZ:DB), suggesting that a repressing element is located between amino acids 22 and 44. However, in SAtreated cells, Δ44POZ:DB did not transactivate (Figure 4A; Δ44POZ:DB, grey versus white bars), indicating that the cryptic transactivation domain does not function when cells are induced with SA. Taken together, the results of Figure 4A indicate that the BTB/POZ domain cannot account for the transactivation properties of the full-length NPR1 tethered to DNA through the Gal4 DB.

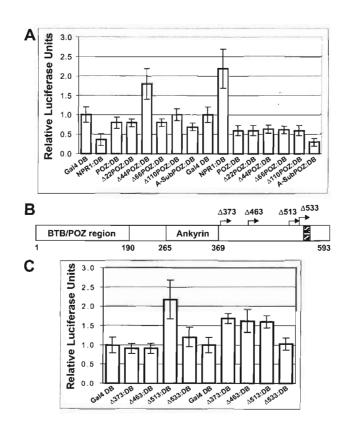


Figure 4. NPR1 Harbors an Autonomous Transactivation Domain in the last 80 Residues

- (A) Histogram illustrating the transcriptional activity of the NPR1 BTB/POZ domain, the deletion mutants of the BTB/POZ, and the Alanine-Substitution mutant tethered to DNA through Gal4 DB. The deletion and the Alanine-Substitution mutants were created starting with the NPR1 BTB/POZ domain. BTBPOZ region represents the first 190 amino acids of NPR1.
- (B) Schematic representation of NPR1 and the deletions analyzed in panel (C). The numbers preceded by Δ indicate the starting amino acid for the particular deletion mutant. NLS indicates the nuclear localization signal. Ankyrin represents the region containing the ankyrin repeats as defined by Pfam and SMART. Diagram is drawn to scale.
- (C) Histogram illustrating the transcriptional activity of the NPR1 deletion mutants described in (B), tethered to DNA through Gal4 DB. For (A) and (C) conditions were identical to those described in Figure 1. Data are reported as Relative Luciferase Units. Values consist of n = 25 samples and represent averages ± 1 SD. Every bar represents five bombardments repeated five times (n = 25).

Having determined that the BTB/POZ domain does not harbor an autonomous transactivation domain active in SA-stimulated cells (Figure 4A), we set out to identify such domains in the C-terminal portion of NPR1. We created additional N-terminal deletions of NPR1 (Figure 4B); one at amino acid 373, which occurs right after the ankyrin repeats as predicted by Pfam (Finn et al., 2006) and SMART (Letunic et al., 2006); one after residue 463, which is the end point of sequence similarity with Drosophila Ankyrin 2 (Genbank accession number: AAN12046.1); one at position 513, which corresponds to the beginning of the last stretch of negatively charged and hydrophobic residues, a known transactivation domain signature (Cress and Triezenberg 1991); and finally, one right before the nuclear localization signal (Kinkema et al., 2000), at amino acid 533. These constructs were fused to Gal4 DB and assayed using the in vivo plant transcription assay (Figure 4C). In unstimulated cells (Figure 4C, white bars), deletion of the first 373 or 463 amino acids of NPR1 (Δ 373:DB and Δ 463:DB), did not show gene activation beyond the baseline level. However, further deletion to residue 513 (Δ 513:DB), resulted in gene activation 2.2-fold above the baseline level, indicating that a repressing region had been deleted, thus exposing a cryptic transactivation domain. Extending the deletion to position 533 (Δ 533:DB) reduced gene activity to the baseline level, emphasizing the importance of residues 513 to 533 for transactivation.

In SA-stimulated cells (Figure 4C, grey bars), deletion of the first 373, 463, or 513 amino acids of NPR1 (Δ373:DB, Δ463:DB, or Δ513:DB) resulted in gene activation 1.6-fold above the baseline level. Extending the deletion to position 533 (Δ533:DB), reduced gene activity to the baseline level, again indicating the importance of residues 513 to 533 for transactivation. The results of Figure 4C demonstrate that, in addition to

amino acids 22-44 in the BTB/POZ, NPR1 possesses a second repression region, located between position 463 and 513 that is also active in unstimulated cells only. Furthermore, a transactivation domain, active in uninduced as well as in SA-stimulated cell, requires residues located between position 513 and 533.

3.4.7 Oxidation of NPR1 Cysteines 521 and 529 Is Required for the Activity of the Transactivation Domain in SA-Treated Cells only

Inspection of the region containing the C-terminal transactivation domain of NPR1 reveals that it contains two cysteine residues (Figure 5A), at position 521 (Cys521) and 529 (Cys529). Since cysteines are susceptible to redox modifications and redox modifications can affect protein function, we first set out to determine whether Cys521 and Cys529 were required for the transactivation of the last 80 amino acids of NPR1 tethered to DNA (Δ 513:DB). Cys521 and Cys529 (the only two NPR1 cysteines found in Δ 513:DB) were individually mutated to a serine, an amino acid similar to cysteine in size and structure, but not reactive to redox modifications. Hence, serine can mimic the reduced form of cysteine and preserve the capacity for hydrogen bonding.

The constructs bearing a mutated cysteine were fused to Gal4 DB and assayed using the *in vivo* plant transcription assay (Figure 5B). In resting cells (Figure 5B, white bars), mutation of the cysteine at positions 521 (Δ513C521S:DB) or 529 (Δ513C529S:DB) had no effect on gene activation, with levels similar to Δ513:DB (no difference, p>0.05), indicating that redox modulation of Cys521 and Cys529 does not play a role in transactivation under non-induced conditions. However, in SA-treated tissue (Figure 5B, grey bars), Δ513C521S:DB and Δ513C529S:DB did not lead to gene activation beyond the baseline level and values were significantly different from

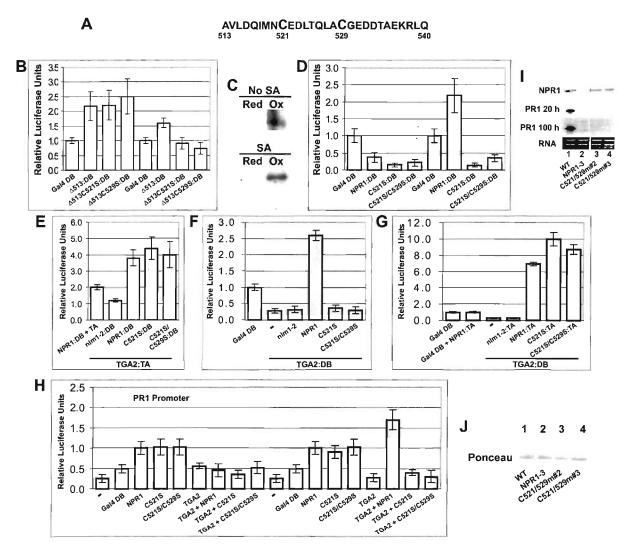


Figure 5. Oxidation of Cys521 and Cys529 Correlates with Transcriptional Activation of the *PR1* Gene by the TGA2-NPR1 Complex.

- (A) Sequence of amino acids located between position 513 and 540.
- (B) Histogram illustrating the transcriptional activity of the $\Delta 513$ deletion mutant of NPR1 and the effect of mutating Cys521 or Cys529, within the context of the $\Delta 513$ protein. Proteins were tethered to DNA through Gal4 DB.
- (C) Blot analysis of NPR1 Δ 513 immunoprecipitate used to assess the *in vivo* redox status of residues Cys521 and Cys529 present in cells of Arabidopsis leaves treated for 24 hr with SA (SA) or left untreated (No SA). Red indicates immunoprecipitates from proteins labeled for reduced Cys residues, while Ox indicates immunoprecipitates from proteins labeled for oxidized Cys (see Experimental Procedures).
- (D) Histogram illustrating the transcriptional activity of the full-length NPR1 and the effect of mutating Cys521 or simultaneously Cys521 and Cys529, within the context of the full-length NPR1. Proteins were tethered to DNA through Gal4 DB.

- (E) Histogram illustrating the interaction of NPR1, the Cys521, or the Cys521 and Cys529 mutants described in (D), with TGA2 fused to the VP16 transactivation domain. nim1-2, which does not interact with TGA2, was also expressed with TGA2:DB as a negative control. NPR1, nim1-2 and the mutants described in (D) were all fused to the Gal4 DB. NPR1:DB was also expressed along with the VP16 transactivation domain (NPR1:DB + TA) as another negative control.
- (F) Histograms illustrating the effect of NPR1 the Cys521, or the Cys521 and Cys529 mutants described in (**D**) on the transcriptional activity of TGA2:DB. All proteins, except TGA2:DB were expressed without a fusion.
- (G) Histogram illustrating the interaction of NPR1, the Cys521, or the Cys521 and Cys529 mutants described in (D) all fused to the VP16 transactivation domain, with TGA2 fused to the Gal4 DB.
- For (B), (D), (E), (F) and (G) conditions were identical to those described in Figure 1. Data are reported as Relative Luciferase Units. Values consist of n = 25 samples and represent averages ± 1 SD. Every bar represents five bombardments repeated five times (n = 25).
- (H) Histogram illustrating the effect of NPR1, nim1-2, and the Cys521, or the Cys521 and Cys529 mutants, on the transcriptional activity of the TGA2-NPR1 complex. All proteins were native (without fusion). The reporter system was the Arabidopsis PRI promoter fused to luciferase. The CaMV 35S promoter:Renilla luciferase fusion was used as an internal standard. (-) indicates that no effector were bombarded along with the reporter and internal standard vectors. Arabidopsis leaves were left untreated (white bars) or were treated for 24 hr with 1mM salicylic acid (grey bars). Data are reported as Relative Luciferase Units. Values consist of = 25 samples and represent averages \pm 1 SD. Every bar represents five bombardments repeated five times (n = 25).
- (I) Northern blot analysis using *NPR1* or *PR1* probes. RNA stained with ethidium bromide is shown for loading comparison. Lane 1 contains RNA from wild-type Arabidopsis and lane 2 from the *npr1-3* mutant. Lanes 3 and 4 contain RNA from two independent *npr1-3* transgenic lines expressing NPR1 bearing cysteine-to-serine mutations at position 521 and 529. Specific line numbers follow the construct name. PR1 20 h and PR1 100h represent a 20-hour and 100-hour autoradiography, respectively. All lanes are from the same gel and blot.
- (J) Top Panel. Immunoblot analysis of proteins from wild-type Arabidopsis (WT), the *npr1-3* mutant (NPR1-3), and the *npr1-3* background lines expressing the mutant described in (I). An anti-NPR1 antibody (Després et al., 2000) was used. Bottom Panel. Ponceau staining of the membranes shown in the top panel.

Δ513:DB (p<0.05). These results indicate that, in the context of the last 80 amino acids of NPR1, Cys521 and Cys529 are required for transactivation only after SA-treatment. To establish the redox status of Cys521 and Cys529, we performed a labeling assay that distinguishes between protein sulfhydryls (reduced Cys residues) and disulfides (oxidized

Cys residues) (see Després et al., 2003 for a flow chart and description of the method). The results (Figure 5C) indicate that cysteine residues in the last 80 amino acids of NPR1 are predominantly oxidized (Ox), whether or not the cells have been treated with SA.

We next tested the effect of their mutations in the context of the full-length NPR1 tethered to DNA by the Gal4 DB (Figure 5D). In unstimulated cells (white bars), NPR1:DB did not lead to transactivation beyond baseline levels whether or not the cysteines were mutated. However, after SA-treatment, in contrast to what is observed with NPR1:DB, mutations of these cysteines abolished transactivation and values were significantly different from wild-type NPR1:DB (p<0.05). Plant two-hybrid experiments confirmed that C521S:DB and C529S:DB were expressed and retained the capacity to interact with TGA2 to an extent comparable to wild-type NPR1:DB (Figure 5E). These results suggest that the TGA2-co-activator function of NPR1 may require Cys521 and Cys529.

3.4.8 Transcriptional Activation of the PR1 gene and TGA2-Co-Activator Function of NPR1 Require Cysteines 521 and 529 of NPR1

Finally, we sought to determine whether mutating Cys521 and Cys529 would affect the TGA2-co-activator function of NPR1. We first tested the role of these cysteines in the context of the *Gal4* promoter and observed that mutation of Cys521 or the double mutation Cys521/Cys529 abolished the ability of the TGA2-NPR1 complex to transactivate (Figure 5F). Plant two-hybrid experiments confirmed that constructs C521S and C521S/C529S retained the ability to interact with TGA2 to an extent comparable to wild-type NPR1 (Figure 5G) in the configuration where TGA2 is fused to the Gal4 DB. Next, we tested the role of these cysteines in the context of the *PR1* promoter. DNA

coding for native (unfused) proteins were delivered by biolistics along with the luciferase reporter gene under the control of the *PRI* promoter (Figure 5H). As observed with NPR1, proteins C521S and C521S/C529S activated the *PRI* promoter in the absence of TGA2 whether or not cells were treated with SA, indicating that these residues of NPR1 are not required for recruitment to the promoter. Under induced (grey bars) and non-induced (white bars) conditions, mutation of these cysteines did not bring about transactivation of the complex with TGA2 as values were not significantly different from those obtained with TGA2 alone (p>0.05). After SA treatment (grey bars), the ability of the TGA2-NPR1 complex to activate the PR1 promoter above TGA2 alone or NPR1 alone (with and without mutated cysteines) is abolished when Cys521 is mutated alone or in the presence of the Cys529 mutation (Figure 5H, compare TGA2 + NPR1 to TGA2 + C521S and TGA2 + C521S/C529S, p<0.05).

To further confirm the biological significance of Cys521 and Cys529 of NPR1 in controlling *PR1* expression, an NPR1 construct harboring the double mutation at cysteines 521 and 529 was introduced, under the control of the *CaMV35S* promoter, into the *npr1-3* genetic background (Figure 5I; Cys521/529m). Wild-type Arabidopsis (WT) expressed *PR1* transcript when treated with 0.5mM SA for 16 hr (lane 1), while *npr1-3* plants (NPR1-3) did not (lane 2). *PR1* gene induction was not restored in any of the 19 independent transgenic *npr1-3* lines expressing the NPR1 double mutant Cys521/529m (lanes 3 and 4, and data not shown). Figure 5J indicates that protein Cys521/529m was expressed at levels similar to those observed with NPR1. None of the lines tested expressed *PR1* in the absence of SA (data not shown).

3.5 DISCUSSION

Our study has demonstrated that TGA2 is not a transcriptional activator whether cells are resting or SA-treated. Furthermore, our data argue that, upon SA-treatment, PR1 is up-regulated by a transactivation complex composed of at least TGA2 and NPR1. First, ChIP in wild-type Arabidopsis confirmed that NPR1 is recruited to the PR1 promoter in both non-treated and SA-treated cells. Second, despite the fact that TGA2 is not a transactivator, NPR1 associates with TGA2 in SA-stimulated cells to form a transcriptional activating complex, both on a heterologous (5X Gal4 UAS) and a native (PRI) promoter. Third, genetic complementation analyses of rationally designed sitedirected and deletion mutants of the NPR1 BTB/POZ establish a role for the core of this domain in activating PR1. This finding is important because it establishes a direct correlation between complementation of PRI expression and transactivation on the heterologous promoter of a complex containing TGA2 and these NPR1 BTB/POZ mutants. Fourth, a cysteine-oxidized transactivation domain in the C-terminus of NPR1 is also required for the activation of PR1 by the TGA2-NPR1 complex. This emphasizes again the correlation between transactivation of the TGA2-NPR1 complex and the activation of PR1. We thus conclude that, in SA-treated cells, NPR1 is a TGA2-coactivator essential for PR1 induction.

3.5.1 TGA2 Is Required for Transcriptional Repression of PR1 in Uninduced Cells

The observation that, under uninduced and INA-induced conditions, the triply knocked-out *tga2/5/6* mutant displayed levels of *PR1* expression 50-fold higher than in the wild-type suggested that TGA2, and members of its clade, could act as transcriptional

repressors (Zhang et al., 2003), which implied that they can bind the PRI promoter independently of treatment with SA. Our ChIP results, using TGA2 fused to a sixhistidine-tag (Figure 1B and C), indeed indicate that recruitment of TGA2 to the PR1 promoter is both SA- and NPR1-independent and thus suggest that the de-repression of PRI observed by Zhang et al. (2003) in the tga2/5/6 knock-out plant is due to the lack of direct binding of these TGA TFs to PRI. However, this contradicts a report in which ChIP indicated that binding of TGA2 to PR1 is both NPR1- and SA-dependent (Johnson et al., 2003). Since the N-terminal region of TGA2 used by Johnson et al. (2003) to raise the anti-TGA2 antibody contains 28% of serine and threonine, two phosphorylatable amino acids, it is plausible that phosphorylation of a number of these residues could contribute to a decrease in the antibody-antigen interaction. To reconcile these apparently incongruous results, we propose that the data of Johnson et al. (2003) together with ours suggest that the N-terminal region of TGA2 is either inaccessible to the antibody or that the epitope is post-translationally modified when cells are unstimulated or in the absence of NPR1.

In an *in vivo* transcription system based on the *PR1* promoter (Figure 1J), we demonstrated that, in uninduced cells, TGA2 represses transcription of the *PR1* promoter activated only by expression of NPR1 (Figure 1J white bars; NPR1 compared to TGA2 + NPR1). However, TGA2 was unable to repress the baseline level of *PR1* expression as defined by expression of the unrelated proteins Gal4 DB and Cruciferin (Figure 1J). Therefore, in the context of *PR1* and in uninduced cells, TGA2 may only serve to repress the activating effect resulting from the recruitment of NPR1 to the promoter. TGA2:DB can also repress transcription from a LexA:VP16-activated synthetic promoter

(Supplemental Figure 1). These data further emphasize the fact that TGA2 is not a transcriptional activator on its own. The repressing effect of TGA2 observed on the *PR1* promoter activated by NPR1 is likely independent of an interaction between TGA2 and NPR1, since there is no detectable interaction between these two proteins in the context of the *PR1* promoter (Figure 1J white bars; TGA2 compared to TGA2 + NPR1:TA). This is further substantiated by the observation that the activating effect resulting from the recruitment of nim1-2 (an NPR1 mutant version that does not interact with TGA2) to *PR1*, is also repressed by TGA2 (Figure 1J white bars; nim1-2 compared to TGA2 + nim1-2).

As determined by northern blot analysis, overexpression of TGA2:His did not lead to constitutive expression of *PRI* in plants used for ChIP experiments (Figure 1). These results are consistent with those reported for native TGA2 (Kim and Delaney, 2002) and a TGA2:Gal4 DB chimeric protein (Fan and Dong, 2002). In contrast, a chemically-inducible TGA2:GFP fusion was shown to lead to the accumulation of *PRI* transcripts in the absence of BTH treatment (Kang and Klessig, 2005). These results are difficult to explain in light of results presented in Supplemental Figure 1, demonstrating that TGA2 is a transcriptional repressor. It is possible that the chemically-inducible nature of the transgene used by Kang and Klessig (2005) resulted in the accumulation of vastly higher amounts of proteins than achieved in our study or those of Fang and Dong (2002) and Kim and Delaney (2002), leading to unspecific effects. Another possibility, not tested by Kang and Klessig (2005), is that the particular TGA2:GFP fusion they created fortuitously generated a transactivation domain leading to SA-independent activation of *PRI*. Finally, these authors did not assess whether the TGA2:GFP fusion is

recruited to *PR1* in planta. Therefore, the possibility that this fusion exerted its effect on *PR1* through an indirect mechanism cannot be ruled out.

3.5.2 NPR1 Is a Transcriptional Co-Activator in SA-Stimulated Cells

When tethered to DNA through the Gal4 DB, NPR1 activates transcription only after cells have been stimulated with SA. However, the finding that expression of NPR1 without fusion to the Gal4 DB does not lead to transcriptional modulation indicates that recruitment to the promoter is required for transcriptional activation. In the absence of a fusion to the Gal4 DB, NPR1 can be recruited to the heterologous Gal4 promoter via TGA2:DB. This recruitment leads to transactivation of the TGA2-NPR1 complex in SAtreated cells (Figure 1H), thus defining NPR1 as a co-activator. Remarkably, the TGA2-NPR1 complex is sufficient to activate the heterologous (Figure 1H) and PR1 promoter (Figure 1J) in an SA-dependent fashion. Therefore, the complex behaves as an SAregulated enhanceosome exposing a unique activating interface (Thanos and Maniatis 1995; Merika and Thanos 2001). Transactivation of the TGA2-NPR1 enhanceosome requires the core of the NPR1 BTB/POZ domain, since deletion beyond it (Δ 110) or its mutation (alanine-substitution), abolishes the function of the enhanceosome both on a transiently-delivered heterologous promoter (Figure 3A) and on the endogenous PRI gene (Figure 2B). Transactivation of the TGA2-NPR1 enhanceosome is also dependent on the oxidation of Cys521 and Cys529 of NPR1 (Figure 5).

Although NPR1 behaves as a transcriptional activator in SA-treated cells when tethered to DNA on the Gal4-based promoter, this may not be the case when NPR1 is recruited to the *PR1* promoter. First, addition of a strong transactivation domain (VP16) to NPR1 (NPR1:TA) did not lead to further activation of the transiently-delivered *PR1*

promoter when compared to unfused NPR1 transfected alone (Figure 1J). Second, mutation of Cys521/Cys529, which abolishes the transactivation properties of NPR1, activated PR1 to the same extent as the wild-type NPR1 (Figure 5H). These results could suggest that in the architectural context of the PR1 promoter, the PR1-activating effect of NPR1 observed over the baseline level, may be de-repression as opposed to activation; that is to say the effect may be due to chromatin structure modification instead of an active recruitment of the basal transcription machinery by NPR1. The discrepancy between the results observed on the Gal4-based and PR1 promoters could arise from dissimilarity in the architecture of the two promoters. It could also arise from dissimilarity in the architecture of protein complexes due to allosteric effects of DNA (Lefstin and Yamamoto 1998), since in one case NPR1 interacts with DNA through a heterologous DB, while in the other, NPR1 is recruited to PR1 through an unidentified DB or through an unknown DNA-binding protein, itself recruited to PR1. Therefore, although ChIPs demonstrated that NPR1 is recruited to PR1 in both resting and SAtreated cells (Figure 1D and E), its role on the uninduced PR1 is unclear. However, it seems reasonable to think that NPR1 interacts with PR1 as a ready-to-go latent coactivator. This is consistent with the fact that overexpression of NPR1 does not lead to constitutive PR1 expression; transcription still requires activation by SA (Cao et al., 1998). The nature of this switch remains elusive.

It has been proposed that the role of NPR1, in a wild-type plant, is to inactivate the repressing effect of SNI1 on PR genes (Li et al., 1999). As such, in the sni1 npr1-1 double mutant, PR gene expression is restored and is inducible. This also led to the proposal that the induction of PR genes requires the activation of TGA TFs in an SA-

dependent, but NPR1-independent fashion. However, it is clear from the results presented here that TGA2, the prototype of the TGA2/5/6 clade, does not display any autonomous transactivation properties whether or not cells are treated with SA, but requires an association with NPR1 to display such activities. Furthermore, the transactivating capacity of the TGA2-NPR1 complex is dependent upon the functionality of a transactivation domain found in the C-terminus of NPR1. Thus, in the case of the PR1 gene, it is unlikely that the role of NPR1 is simply to inactivate SNI1. Instead, we propose that the snil mutation, in the snil-nprl double mutant background, might activate pathways that regulate PR1 in an NPR1- and TGA2/5/6-independent fashion. Indeed, it has been shown that PR1 is regulated in an SA-dependent, but NPR1independent fashion by transcription factor AtWhy1 (Desveaux et al., 2004). In resting cells, AtWhy1 is held inactive by an inhibitor, which prevents it from binding to DNA. Upon SA-treatment, AtWhy1 is released from the effects of this inhibitor, which allows it to be recruited to its cognate DNA (Desveaux et al., 2004). It is thus possible that SNII plays a role in the AtWhy1-dependent pathway leading to PR1 induction as opposed to the TGA2-NPR1-dependent pathway. However, in the absence of ChIP data indicating that SNI1 or AtWhy1 are themselves recruited to the PR1 promoter, it is unclear whether their effects on the PRI promoter are direct or indirect. Furthermore, in the absence of data indicating that SNI1 can physically interact with TGA2 or NPR1, it is very difficult to place, with any confidence, this protein in a model of *PR1* regulation.

3.5.3 Cysteine-Oxidized Transactivation Domain: A New Type of Transactivation Domain

Cysteine residues in eukaryotic transcription factors have been demonstrated to be the target of redox regulation. In most cases cysteines affect DNA-binding activity, which is abolished when these are oxidized (Abate et al., 1990; Lando et al., 2000; Toledano and Leonard 1991). However, in a few instances, oxidation has been shown to control homodimerization (Benezra 1994) and to inhibit nuclear export (Kuge et al., 2001). When one eliminates cases where effects on transactivation are due to modulation of DNA-binding activity (as opposed to modulation of transactivation per se), there is only one example in the literature where a transactivation domain is controlled by cysteine redox. However, in this instance, oxidation abolished transactivation (Morel and Barouki 2000). It thus appears that NPR1 is a rare example of a transactivation domain positively regulated by oxidized cysteines (Cys521 and Cys529). Remarkably, despite the fact that Cys521 and Cys529 are oxidized regardless of whether cells are exposed to SA (Figure 5C), these cysteines only modulate transactivation in SA-stimulated cells (Figure 5B). This suggests that different factors mediating contact between the NPR1 transactivation domain and the basal transcriptional machinery operate in non-induced and SAstimulated cells.

The results reported in this paper constitute a significant advancement of our knowledge on plant disease resistance by elucidating the molecular function of TGA2 as a transcriptional repressor of NPR1-dependent de-repression of *PR-1*. Additionally, we identified NPR1 as a co-activator of TGA2 and established the existence of an SA-

regulated enhanceosome composed of at least TGA2 and NPR1. Figure 6 presents a model that summarizes the results reported in this paper on the regulation of *PR-1*.

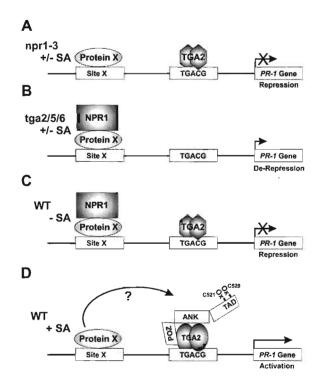
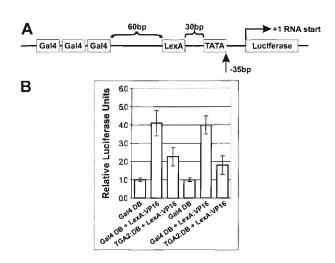


Figure 6. Working Model for Regulation of PR1 by the TGA2-NPR1 Enhanceosome (A) In an npr1 mutant plant such as npr1-3, there is no NPR1-dependent de-repression of PR1 and there is no incorporation of TGA2 into a TGA2-NPR1 enhanceosome. PR1 is repressed. Since NPR1 is recruited to PR1 independently from TGA2 and since NPR1 does not contain a known DNA binding domain, we postulate that in a wild-type plant NPR1 is recruited through an unknown protein (Protein X) binding to an unknown DNA element (Site X). Although TGA3 has been shown to be recruited to PR1, its interaction with the promoter is both NPR1- and SA-dependent (Johnson e al., 2003). Therefore, it is unlikely that NPR1 would be recruited by TGA3 or that TGA3 is the postulated Protein X. Another possible scenario to explain the recruitment of NPR1 to PR1 is that NPR1 interacts directly with DNA using an unidentified DNA-binding domain. Further experimentation is required to distinguish between these two possibilities.

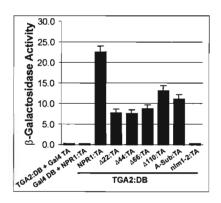
- (B) In the *tga2/5/6* triple knock-out (Zhang et al., 2003), NPR1 is recruited to the *PR1* promoter, which becomes de-repressed. In these plants, the T₆GA2-NPR1 enhanceosome is not recruited to the *PR1* promoter, due to the absence of TGA2, TGA5, and TGA6.
- (C) In a wild-type plant unstimulated with SA, both NPR1 and TGA2 are recruited to the *PR1* promoter independent of each other. However, under resting condition, NPR1 and TGA2 do not interact with each other. Again here, NPR1 is postulated to be recruited through an unknown protein (Protein X).
- (D) In the presence of SA, NPR1 forms an enhanceosome with TGA2. Transactivation of the complex requires the oxidation of Cys521 and Cys529, which are found within the confines of a transactivation domain (TAD) in the C-terminus of NPR1. The BTB/POZ domain of NPR1 is hypothesized to interact with TGA2. NPR1 is postulated to be transferred from the unknown Protein X to TGA2. However, it is possible that NPR1, Protein X, and TGA2 all interact at the same time. The question mark illustrates this fact. The exact nature of the enhanceosome remains undetermined, but it contains at the very least NPR1 and TGA2.

3.6 SUPPLEMENTAL FIGURES



Supplemental Figure 1. TGA2 Represses a LexA:VP16-activated Synthetic Promoter.

- (A) Graphic representation of the synthetic 3X Gal4:1X LexA:minimal promoter:Firefly Luciferase reporter gene. The upward arrow indicate the position of the TATA box relative to the RNA start site. 60bp and 30bp indicate the spacing in base pairs between the most downstream Gal4 element and the LexA element and between the LexA element and the TATA box, respectively. Not shown is an omega translational enhancer in the transcribed region of the Luciferase gene.
- (B) Histograms illustrating the fact that TGA2 tethered to DNA through Gal4 DB (TGA2:DB) represses transcription of a LexA DB fused to the transactivation domain of viral protein 16 (LexA:VP16). Arabidopsis leaves were left untreated (white bars) or were treated for 24 hr with 1mM salicylic acid (grey bars). The constructs were transfected along with the $3X\ UAS^{GAL4}$: $1X\ LexA\ DNA\ element:minimal\ promoter$: Firefly luciferase reporter and the CaMV35S:Renilla luciferase internal standard vectors. Data are reported as Relative Luciferase Units. Values consist of n=25 samples and represent averages \pm 1 SD. Every bar represents five bombardments repeated five times (n = 25).



Supplemental Figure 2. The BTB/POZ Mutants of NPR1 Interact with TGA2 in Yeast Two-Hybrid Assays.

Quantitative yeast two-hybrid assays illustrating the interaction between TGA2 produced as a Gal4DB fusion (TGA2:DB) co-expressed with NPR1, nim1-2, NPR1 lacking the first 22, 44, 66, or 110 amino acids (Δ 22, Δ 44, Δ 66, Δ 110, respectively), or NPR1 mutated in the core of the BTB/POZ domain (A-Sub) expressed as Gal4TA fusions (:TA). TGA2:DB co-expressed with Gal4TA and Gal4DB co-expressed with NPR1:TA served as negative controls. Values represent averages \pm 1 SD.

CHAPTER 4 – The glutathionylation activity of the novel glutaredoxin GRX480 regulates the redox status and activity of the Arabidopsis transcription factor TGA1

Contributions

This manuscript, which is in preparation to be submitted for publication in The Plant Cell, stems from my primary research project and I have had an integral role in deciding the direction of the research. My specific contributions in this manuscript involve experiments, including DNA and protein preparation, performed for Figure 1A through D, Figure 2A through F, Figure 3A through C, Figure 4A through C, Figure 6, Figure 7A and B, Figure 8B, Figure 9, Table 3 (GRX480) and Appendix Figure 1 and Table 2 and 3. I have performed all data analysis and prepared the entire manuscript in conjunction with Dr. Charles Després.

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4.1 ABSTRACT

The protein NPR1 (non-expressor of pathogenesis related gene 1) is the key modulator of systemic acquired resistance (SAR). It is induced by the metabolite SA (salicylic acid) and acts through a class of TGA transcription factors to induce a subset of PR (pathogenesis related) genes resulting in disease resistance. The interaction between TGA1 and NPR1 was previously found to rely on the reduced state of two cysteines, Cys260 and Cys266, in TGA1. As a complement to these results, we found that the ability of TGA1 to activate transcription correlates with the oxidation of Cys260 and Cys266, with differential contribution of each cysteine to transactivation. These data suggests that TGA1 activity is regulated by a novel redox switch in a mutually exclusive manner such that transactivation and interaction with NPR1 do not occur at the same time. Through a yeast two-hybrid screen, TGA1 was found to interact with the novel Arabidopsis glutaredoxin GRX480. Glutaredoxins, which are small oxidoreductases, are part of the thioredoxin superfamily and are capable of reducing disulfide bonds. Although GRX480 has all the attributes required for reduction of a disulfide bond, its preference for binding reduced over glutathionylated TGA1 and lack of activity in a classical glutaredoxin assay suggest it does not have glutaredoxin activity. Instead, we determined that GRX480 is able to glutathionylate TGA1 at Cys260 using GSSG as a substrate. This activity, in the presence of the TGA1-NPR1 complex, was found to abolish transactivation, likely through abrogating the interaction between TGA1 and NPR1 by oxidizing TGA1. This data further defines a role for GRX480 in disease resistance and is the first instance where a protein target and biochemical activity has been defined.

4.2 INTRODUCTION

Systemic acquired resistance (SAR) is a broad-range disease resistance pathway induced in Arabidopsis after attack by avirulent pathogens. Induction of this defense pathway leads to resistance throughout the plant and can be maintained for weeks to months after induction (Ward et al., 1991; Ryals et al., 1996). SAR is characterized by the expression of a subset of pathogenesis related (PR) genes and the corresponding accumulation of PR proteins (Ward et al., 1991). Salicylic acid (SA) is a key metabolite that accumulates during SAR and is required for the successful deployment of this pathway (Métraux et al. 1990; Malamy et al., 1990). Exogenous treatment with SA leads to the induction of SAR and expression of PR genes (Ward et al., 1991), while transgenic Arabidopsis plants deficient in SA accumulation fail in their ability to mount SAR and to accumulate PR gene transcripts (Delaney et al., 1994; Vernooij et al., 1994; Gaffney et al., 1994). The SAR signaling cascade also involves the protein NPR1 (non-expressor of pathogenesis related gene 1), which acts downstream of SA (Cao et al., 1994; Delaney et al., 1995; Shah et al., 1997). Arabidopsis *npr1* mutants are still able to accumulate SA; however, they are non-responsive to it and are unable to accumulate PR transcripts or mount SAR (Delaney et al., 1995; Shah et al., 1997).

Analysis of the NPR1 protein revealed the presence of two protein-protein interaction domains: the BTB/POZ (Broad-Complex, Tramtrack, and Bric-a-brac/Pox virus and Zinc finger) domain and ankyrin repeats (Cao et al., 1997; Aravind and Koonin, 1999). Several of the *npr1* mutants bear single amino acid mutations in either of the protein-protein interaction domains suggesting that NPR1 modulates SAR through interaction with other proteins. NPR1 localizes to the cytoplasm and the nucleus in

unchallenged Arabidopsis (Després et al., 2000 and 2003) while treatment with SA or its analog INA (2,6-dichloroisonicotinic acid) results in nuclear localization which is required for *PR* gene expression (Kinkema et al., 2000; Mou et al., 2003).

NPR1 is able to interact differentially with members of the TGA bZIP (basic domain/leucines zipper) family of transcription factors (Zhang et al., 1999; Després et al., 2000; Zhou et al., 2000). TGA transcription factors, named for their recognition of the cognate DNA motif TGACG, are known to bind the *as-1-like* element, which is a common *cis*-element found in the promoter of many *PR* and SAR-related genes (Strompen et al., 1998; Després et al., 2000). NPR1-TGA interaction has been observed in the nucleus after SAR induction and NPR1 is able to enhance TGA binding to the *as-1* element in response to SA (Després et al., 2000 and 2003).

Of the ten Arabidopsis TGA TFs, only seven have been examined with respect to their ability to interact with NPR1 in yeast. Although the initial yeast two-hybrid assays revealed that TGA1 and 4 were not able to interact with NPR1, Després et al. (2003) showed that TGA1 is able to interact with NPR1 in a plant two-hybrid assay after SA treatment. Examination of a series of TGA1 mutants where segments of TGA1 had been swapped for TGA2 led to the identification of two unique cysteines in TGA1 (Cys260 and Cys266, also conserved in TGA4) that, when mutated to the corresponding amino acids in TGA2, allowed TGA1 to interact with NPR1 in yeast. Furthermore, the identification of the redox status of Cys260 and Cys266 in yeast and Arabidopsis revealed that TGA1 is redox regulated and interaction with NPR1 only occurs when Cys260 and Cys266 are reduced (Després et al., 2003).

Two pathways exist in plants, mammals, yeast and *E. coli* to regulate the redox status of proteins: the thioredoxin and glutaredoxin pathways (Meyer et al., 1991). While these proteins do not share sequence similarity, they do have similar folding (Eklund et al., 1984) to generate a prominent redox active site that reveals partially overlapping functions for reducing disulfide bonds (Meyer et al., 1999). Glutaredoxins, unlike thioredoxins, employ glutathione (GSH) to reduce disulfide bonds, a unique feature that allows them to reduce mixed disulfides between GSH and a protein. Arabidopsis has 26 thioredoxins and 31 glutaredoxins and either pathway could potentially be involved in modulating the redox status of TGA1 (Meyer et al., 2002; Rouhier et al., 2004). In support of this, an Arabidopsis glutaredoxin has been found to interact with TGA2 and this interaction appears to be involved in the negative regulation of *PDF1.2* expression, a jasmonic acid-responsive defense related gene involved in an induced defense pathway different from SAR (Ndamuong et al., 2007).

TGA1 possesses all the structural determinants required for interaction with NPR1, however a redox switch appears to regulate this interaction. The interaction between TGA1 and NPR1, which results from the SA-dependent reduction of Cys260 and Cys266 in TGA1, implies a role for TGA1 in SAR. Here we report a mutually exclusive behavior of TGA1 linked to its redox status: oxidation of Cys260 and Cys266 results in TGA1's ability to transactivate but not interact with NPR1 while reduction of these two cysteines results in a loss of transactivation ability but competence to interact with NPR1. A bipartite transactivation domain was identified in TGA1 that is not involved in the transactivation ability of the TGA1-NPR1 complex. Instead, a novel transactivation domain in NPR1 is responsible for the transactivation of the complex

suggesting the transactivation domain of TGA1 serves another role, likely independent of NPR1. In an attempt to understand TGA1's redox regulation, we performed a yeast two-hybrid screen and identified that TGA1 interacts with GRX480, the same glutaredoxin that interacts with TGA2 (Ndamukong et al., 2007). This interaction also occurs in a plant two-hybrid system and depends on the presence of Cys260 and Cys266. The expression of *GRX480* is induced by SA and the enzyme displays none of the behavior of a classical glutaredoxin, but instead was found to have activity glutathionylating TGA1. Furthermore, we show that GRX480 is involved in shutting down the TGA1-NPR1 complex, most likely through the glutathionylation of TGA1. The data presented here not only suggest two unique roles for TGA1 regulated by a novel redox switch, it also provides a mechanism, through the action of GRX480, for the partial regulation of TGA1's redox status. Additionally, we are the first group to identify a protein target and biochemical activity for a novel plant glutaredoxin.

4.3 METHODS AND MATERIALS

4.3.1 Chemicals

All consumables were purchased from Sigma (St. Louis, MO) unless otherwise stated.

4.3.2 Bacterial Strains and General Cloning Vectors

The *E. coli* strain DH12S (GIBCO-BRL, Grand Island, NY) was used for plasmid maintenance and production. Cloning plasmids used were pBC SK(+) (Stratagene, La Jolla, CA) and pCR2.1 (Invitrogen, Carlsbad, CA). Large-scale plasmid purification was performed using Endo-Free GigaPrep Kits (Qiagen, Mississauga, ON). All constructs used were verified by DNA sequencing.

4.3.3 Plasmid Constructs for Transactivation Assays and Plant Two-Hybrid Assays

The *GAL4 DB*, from the yeast two-hybrid vector pBI880, the *VP16 TA* (provided by Brian Miki, Agriculture and Agri-Food Canada, Ottawa, Ontario, Canada), and the *Renilla luciferase* from plasmid pRL-null (Promega, Madison, WI) were amplified by PCR using appropriate primers and ligated into the expression vector pBI524 (Datla et al., 1993) containing a modified double *Cauliflower mosaic virus 35S* promoter with the *Alfalfa mosaic virus* translational enhancer. The coding sequence for all genes tested was amplified by PCR using gene-specific primers and ligated into pBI524 containing *GAL4 DB* or *VP16 TA* in the appropriate reading frame to permit the expression of N-terminal fusion proteins. The unfused versions (i.e. no DB or TA) were cloned into pBI524 lacking the GAL4DB or VP16 TA. The *5X UASGAL4:luciferase* reporter construct was provided by Masaru Ohme-Takagi National Institute of Advanced Industrial Science and Technology (Tsukuba, Japan; Fujimoto et al., 2000).

4.3.4 Yeast Two-Hybrid Assays

Plasmids pBI880 (DB) and pBI881 (TA) (Kohalmi et al., 1997) in conjunction with the yeast strain YPB2 of *Saccharomyces cerevisiae* were used throughout this study. Yeast cells were transformed using S.c. EasyCompTM Transformation Kit (K505001; Invitrogen, Carlsbad, CA) and grown on synthetic dextrose using the appropriate amino acid drop-out(s). For transactivation assays, where β-galactosidase activity was determined, the desired constructs fused to the Gal4 DNA binding domain were transformed into the yeast and selected for on synthetic dextrose without leucine. For protein-protein interaction assays, where the activity of the *lacZ* reporter gene was

monitored visually by using the X-gal filter assay, yeast cells were co-transformed with the DB and TA plasmids and selected for on synthetic dextrose without leucine or tryptophan.

4.3.5 Plant Two-Hybrid Assays

Arabidopsis thaliana (Columbia) leaves were harvested from four week old plants grown at 21°C (day) and 18°C (night) with a ten hour photoperiod and placed on a medium containing MS salts and micronutrients supplemented with B5 vitamins, 1% sucrose and 0.8% agar at a pH of 5.8. Two leaves were transformed per bombardment. Bombardment and sample processing were as described by Fujimoto et al. (2000). Briefly, sample preparation for bombardments consisted of one microcentrifuge tube containing 48µL DNA-gold mixture (enough for 5 bombardments). With continuous vortexing, 44.4µL of 60mg/mL 1.0 micron gold (165 2263, Bio-Rad, Mississauga, ON) was mixed with 13µL of DNA consisting of the reporter gene, internal standard and the vector(s) for the protein or protein pair being tested. DNA was then precipitated onto the gold particles with 42µL of 3M CaCl₂ and 20µL of 0.1M spermidine. The DNA-gold mixture was then washed once with 70% and 100% EtOH before being resuspended in 48µL of 100% EtOH, 8µL of DNA-gold mixture was pipeted for each bombardment on macrocarriers (165 2335; Bio-Rad, Mississauga, ON) and allowed to dry. The Model PDS-1000/He Biolistic® Particle Delivery System (165 2257; Bio-Rad, Mississauga, ON) was used to deliver the DNA-gold mixture to the Arabidopsis leaves. Rupture disks (156 2329; Bio-Rad, Mississauga, ON) of 1100psi were used in combination with ultra high purity (5.0) helium at 1500psi. When required, filter-sterilized salicylic acid was sprayed on the leaves at a concentration of 1mM for the indicated length of time. Leaves were left for a

period of 24 hours before being assayed. The assay was performed using the Dual-Luciferase® Reporter Assay system (PRE 1960; Fisher Scientific, Ottawa, ON) following the manufacturers instructions. Fluorescence was measured as photon emission (EG and G Berthold Lumat LB9507 Luminometer, Bad Wildbad, Germany) and the value obtained represents the value of the reporter gene divided by the value of the internal standard (as relative luciferase units). Every bar in each graph represents five bombardments repeated five times (n = 25).

4.3.6 In Vivo Determination of Cysteine Redox Status

Proteins extracted from yeast cells, *E. coli* or Arabidopsis leaves were separated into two aliquots and processed immediately and in parallel. For detection of reduced cysteines, one aliquot was treated with 0.2mM of 3-(*N*-maleimido-propionyl) biocytin (MPB) for one hour in the dark at room temperature. Proteins were then precipitated four times with acetone. For detection of oxidized cysteines, the second aliquot was first treated with 20mM of *N*-ethylmaleimide (NEM) for one hour at room temperature (RT), precipitated with acetone and then treated with 20mM dithiothreitol (DTT) for one hour at RT. After another acetone precipitation, proteins were treated with 0.2mM of MPB for one hour in the dark at RT followed by two acetone precipitations. Cysteine labeled proteins were then subjected to an immunoprecipitation to purify TGA1 or GRX480.

4.3.7 Immunoprecipitation and Detection of Modified Cysteines

The cysteine-labeled proteins were incubated for 16 hours at 4°C with the anti-TGA1 antibody (for TGA1C172N/C266S/C287S) or Anti-His H-15 antibody (for GRX480) coupled to protein A-sepharose beads (153-6153; Affi-gel Protein A Gel, Bio-Rad,

Mississauga, ON). The beads were subsequently washed three times in IP buffer (0.1% SDS, 1% Triton X-100, 50mM HEPES pH 7.9, 150mM NaCl, 1mM EDTA) and the proteins were eluted by boiling in 2X sample buffer (0.12M Tris-Cl pH 6.8, 4% SDS, 20% glycerol, 0.05% bromophenol blue, 10% 2-mercaptoethanol). The supernatant was electrophoresed on a 10% (TGA1) or 12% (GRX480) sodium dodecyl sulfate polyacrylamide gel. The proteins were transferred to nitrocellulose and reacted according to the manufacturer's instruction (LI-COR, Lincoln, NE) with a strepavidin: Alexa fluor 680 (Molecular Probes, Eugene, OR) conjugate and detected by infrared imaging using an Odyssey imager (LI-COR, Lincoln, NE). The anti-TGA1 antibody was raised in rabbits against the immunizing peptide "CEDTSHGTAGTPHMFDQEAST" and affinity purified by Alpha Diagnostics International (San Antonio, TX). The specificity of the anti-TGA1 antibody has been demonstrated previously (Després et al., 2003). The Hisprobe (H15) (sc803) was purchased from Santa Cruz Biotechnology, Santa Cruz, CA.

4.3.8 In vivo determination of the Cys Redox Status of TGA1C172N/C266S/C287S

50mL culture of yeast transformed with TGA1C172N/C266S/C287S:TA (Gal4) was used to determine the redox status of Cys260. For Arabidopsis, due to very low amounts of proteins in the biolistics assays, 80 bombardments were performed with the TGA1C172N/C266S/C287S:TA (VP16) construct. After a 24-hr incubation period without SA, proteins were extracted from Arabidopsis leaves or from the overnight 50mL yeast culture, separated into two aliquots and processed immediately and in parallel as described above in Sections 4.3.6 and 4.3.7. Neither TA domain contains cysteines.

4.3.9 Protein Expression

The *E. coli* BL21 derivative strain DE3(+) containing pLys S was used for expression of TGA1, TGA1C172N/C266S/C287S, GRX030 and GRX480. All genes were amplified by PCR and cloned in-frame in pET41a(+) to create N-terminal fusions (with an N-terminal 6X HIS tag). 5mL of an overnight culture, grown at 37°C shaking at 200rpm, was used to inoculate 500mL (TGA1, TGA1C172N/C266S/C287S and GRX030) while 5 × 5mL overnight cultures were used to inoculate 5 × 500mL cultures for GRX480. All cultures were grown at 37°C shaking at 200rpm until they reached an OD600 of 0.4-0.6 at which point they were induced with 1mM IPTG (Isopropyl-β-D-thiogalactopyranoside; IPTG001.5 BioShop, Mississauga, ON) and grown for an additional 2 hours at 37°C shaking at 200rpm. Bacterial pellets were collected by centrifugation at 4°C, 3500rpm for 15 minutes (Sorvall-RC-5C-Plus; Mandel Scientific Company, Ontario, CA). Pellets were washed once with mQH₂O and stored at -20°C.

4.3.10 Protein Extraction

Approximately 1g of TGA1, TGA1C172N/C266S/C287S and GRX030 pellet and approximately 6g of GRX480 pellet was used in each purification. TGA1, TGA1C172N/C266S/C287S and GRX030 pellets were resuspended in 2mL of binding buffer (40mM imidazole, 20mM NaH₂PO₄, 150mM NaCl, pH 7.5) and sonicated (Sonic Dismembrator Model 100; Fisher Scientific, Ottawa, ON) on level 5 for 5 × 10 second bursts with 30 second rests on ice in between. GRX480 pellets (each approximately 1g) were resuspended individually in 4mL of binding buffer and ground in approximately 1g of aluminum oxide Type A-5 (A2039) before being combined. All samples were centrifuged at maximum speed, 4°C for 15 minutes and the supernatant (SN) was

collected and filtered through a 13mm 0.22µm PVDF syringe filter (09-720-3, Fisher Scientific, Ottawa, ON) and used immediately in HIS purification.

4.3.11 FPLC-Adapted HIS Purification

A HisTrap HP 1mL column (17-5247-01, GE Healthcare Bio-Sciences, Baie d'Urfé, QC) was adapted to the ÄKTA Explorer FPLC (GE Healthcare Bio-Sciences, Baie d'Urfé, QC) operated with UNICORN Version 3.00.10 software. The total SN from the protein extraction was loaded onto the column after a 5mL equilibration of the HisTrap column with binding buffer. The column was then washed with 5mL binding buffer followed by elution buffer (1M imidazole, 20mM NaH₂PO₄, 150mM NaCl, pH 7.5) which was collected in 12 × 500µL fractions. Fractions 4 and 5 were shown to contain the purest fraction of protein in all cases by immunoblot (Figure 1 in the Appendix) and were used in subsequent experiments.

4.3.12 GSSG Treatment of TGA1

A desired amount of HIS purified TGA1 (that had been reduced with 20mM DTT before being applied to the HIS column for purification) was subjected to glutathionylation by treatment with 10mM GSSG (prepared in a solution of 10mM Tris-Cl pH 8.0). The mixture was incubated at 37°C, shaking in a benchtop shaker at 1100 rpm (Thermomixer R, Eppendorf, Mississauga, ON) for 1 hour in the dark. Samples were subsequently electrophoresed or subjected to DNA-based pull-downs.

4.3.13 MALDI TOF Mass Spectroscopy

30µL of TGA1 fraction 4 untreated or glutathionylated were electrophoresed on a 10 % SDS-polyacrylamide gel. The gel was reversibly stained with Colloidal Brilliant Blue G

(B2025) for 2 hours prior to being fixed for 1 hour (7% glacial acetic acid in 40% (v/v) MeOH). The gel was destained by a 30 second rinse with 10% glacial acetic acid in 25% (v/v) MeOH and further destained for 2 hours with 25% MeOH. The desired bands were excised and placed in pre-washed (50% acetonitrile (ACN)/0.1% trifloroacetic acid (TFA)) 1.5mL microcentrifuge (MC) tubes. The gel slices were destained 2X with destaining solution (100mM NH₄HCO₃/50% ACN) in a benchtop shaker at 37°C, 1400rpm. After removing the final SN, the gel slices were dehydrated for 10 minutes at RT in 100uL ACN. The ACN was removed and the gel slices were dried in a SPD SpeedVac (SPD111V; Thermo Savant, Calgary, AB). The gel slices, when applicable, were then reduced with 10mM DTT in 25mM NH₄HCO₃ for 1 hour in a 56°C water bath. The SN was discarded and the gel slice allowed to cool before the gel slice was submerged in 55mM iodoacetimide in 25mM NH₄HCO₃ for 45 minutes in the dark at RT with occasional vortexing. The SN was removed and the gel slice was washed 2×10 minutes with 100µL of 25mM NH₄HCO₃ shaking at RT at 1400rpm. The SN was removed and the gel slices were dried in a speed vac. A 20µg/mL solution of Trypsin Gold (V5280, Fisher Scientific, Ottawa, ON) in digestion buffer (40mM NH₄HCO₃/10% ACN) was used to cover the gel slice and left to sit at RT for 1 hour. Enough digestion buffer was then added to cover the gel slice and the mixture was left to shake in the benchtop shaper at 1100rpm and 37°C overnight. The SN was removed and 150µL of mQH₂O was incubated with the gel slice, shaking at 1400rpm, RT for 10 minutes. The SN was kept and stored in a pre-washed 1.5mL MC tube, followed by 2×60 minute extractions of the gel slices with 50µL extraction buffer (50% ACN/5% TFA) shaking at RT, 1400rpm. The two extractions plus the water were pooled and dried through a speed

vac. The dried peptides were them purified using C18 ZipTips (TN-224; Millipore, Billerica, MA) according to the manufacturers instructions. 1μL of the eluted peptides were combined with 1μL of matrix (a saturated solution of α-cyano-4-hydroxycinnamic acid (70990) in a 50/50 ACN/H₂O + 0.1%TFA) and 1μL of this mixture was spotted on a S26/100 AnchorChipTM target (Bruker Daltonics, Billerica, MA) and allowed to dry. Mass spectra were obtained using Autoflex II TOF/TOF (Bruker Daltonics, Billerica, MA) in positive mode and 9% laser power. Spectra were optimized and mass list obtained using FlexAnalysis Version 3.0 (Bruker Daltonics, Billerica, MA). The TGA1 peptide mass fingerprint was confirmed by a significant match in the Mascot database (http://www.matrixscience.com).

4.3.14 HED Assay

Adapted from Holmgren and Áslund (1995). Fraction 4 of GRX480 and GRX030 purified from the HIS column were tested for glutaredoxin activity. A reference cuvette as well as both reaction cuvettes contained 0.5mL 100mM Tris-Cl pH 8.0, 2μL 250mM GSH (in 100mM Tris-Cl pH 8.0), 2μL 100mM NADPH (in 100mM Tris-Cl pH 8.0), 2μL 0.5M EDTA, 1μL 50mg/mL BSA, 1.1μL glutathione reductase and 2μL 0.1672M HED (β-hydroxyethylene disulfide; in 100mM Tris-Cl pH 8.0). This was left to sit for 2-5 minutes to allow for the formation of the mixed disulfide between GSH and HED. Immediately before starting the assay measurements, 5 pmol of GRX480 or GRX030 were added to the reaction cuvettes while nothing was added to the reference cuvette. A decrease in absorbance was measured at 340nm using a UV/Visible spectrophotometer (Ultrospec 3100 pro; Fisher Scientific, Ottawa, ON) every 15 seconds over a 5 minutes

period where the reference absorbance was automatically subtracted from the absorbance of the reactions. One unit of activity is defined as the consumption of 1μ mol of NADPH per minute per mg of protein calculated from the expression:

 $[(\Delta A_{340} \times V)/(\min \times 6.2)]/\text{mg protein}$

Where V is the cuvette volume in mL and 6.2 is the millimolar extinction coefficient for NADPH (Holmgren and Áslund, 1995).

4.3.15 DNA-Coupled Pull-down

500µL of Streptavidin-iron oxide particles from Streptomyces avidini (\$2415) were dispensed in a 1.5mL MC tube and the magnetic particles were pulled to the side of the tube, for 1 minute or until the SN was cleared, with the MPC-E-1 magnet (Dynal, Oslo, Norway). The SN was discarded and the particles were resuspended by pipeting in 500µL binding buffer 1 (20mM HEPES pH 7.9, 2M NaCl) containing 2µL (100pmol) of biotinylated probe. The probe consists of LS5 and biotinylated LS7 DNA, two as-1-like elements in the Arabidopsis PR1 promoter (Lebel et al., 1998) known to bind TGA TFs (Després et al., 2000). The double stranded DNA probe, consisting of the following two 40-mers: 5' CCACATCTATGACGTAAGTAAAATAGTGACGTAGAGAAAC 3', GTTTCTCTACGTCACTATTTTACTTACGTCATAGATGTGG-Biotin 3', was created by resuspending each oligomer to a final concentration of 100pmol/µL in TEN buffer (10mM Tris-Cl pH7.5, 1mM EDTA, 150mM NaCl). Equimolar amounts of each oligomer were annealed to create a 50pmol/µL solution. Particles and probe were incubated on a rotating wheel (Roto-torque, Cole-palmer instrument company, Montreal, QC) for 30 minutes at RT to allow for the streptavidin-biotin interaction to occur. The

particles were magnetically separated as above and the cleared SN discarded. The particles with the bound probe were washed 3X with binding buffer 2 (20mM HEPES pH 7.9, 200mM NaCl) where each wash consisted of magnetically collecting the particles, removing the cleared SN and gently resuspending the particles. Fractions 4 and 5 of TGA1 were combined and then split evenly into aliquots according to the number of pulldowns being performed. Some aliquots were glutathionylated (Section 4.3.13) and the fractions that were not were left untreated but subjected to shaking at 37°C in the dark for 1 hour. The aliquots of TGA1 were topped off to 500μL with binding buffer 2 containing 100mM EDTA and used to resuspend the particles with bound probe. This mixture was incubated for 30 minutes at RT rotating on the wheel. The cleared SN was removed and the particles were washed twice as described above. Combined fractions 4 and 5 of GRX480 were divided into aliquots according to the number of pull-downs being performed. They were topped off to 500µL with binding buffer 2 and applied to the DNA-probe-TGA1 mixture and incubated on the wheel for 30 minutes at RT. The cleared SN was removed and the particles were washed twice as described above. The protein complex samples were eluted with 45uL 1X sample buffer (0.06M Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 0.025% bromophenol blue, 5% 2-mercaptoethanol) heated at 100°C for 5 minutes. The particles were pelleted and the SN was collected. The protein complex samples were electrophoresed on a 12% SDS-polyacrylamide gel and processed as described in Section 4.3.8. His-probe (H15) (sc803, Santa Cruz Biotechnology, Santa Cruz, CA) primary antibody was used in the western detection at 1/1000 and goat antirabbit: Alexa fluor 680 (A-21076; Molecular Probes, Eugene, OR) at 1/15000 was used as

the secondary antibody in coordination with infrared imaging using the Odyssey imager (LI-COR, Lincoln, NE).

4.3.16 Glutathionylation Assay

This assay took place using the DNA-coupled pull-down method with some alterations. In this instance, only TGA1C172N/C266S/C287S was coupled to the DNA-particles. Activity of GRX480 and GRX030 was determined by incubating each of these enzymes with the DNA-coupled TGA1C172N/C266S/C287S with 5mM GSH (in 10mM Tris-Cl, pH 8.0), 5mM GSSG (in 10mM Tris-Cl, pH 8.0) or neither. Additionally, DNA-coupled TGA1C172N/C266S/C287S was also incubated with either 5mM GSH or 5mM GSSG alone. These mixtures were incubated for 30 minutes on the wheel at RT in the dark. The cleared SN was removed and the particles were washed as described. To detect reduced cysteines, the DNA-coupled TGA1C172N/C266S/C287S was treated with 0.2 mM of MPB for 1 hr at RT in the dark. The cleared SN was removed and the particles were washed as described. TGA1C172N/C266S/C287S samples were eluted with 45uL 1X sample buffer by heating at 100°C for 5 minutes. The particles were pelleted and the SN was collected. The protein complex samples were electrophoresed on a 10% SDSpolyacrylamide gel and processed as described in Section 4.3.8 using a strepavidin: Alexa fluor 680 (S-32358; Molecular Probes, Eugene, OR). The loading control was determined by incubating with His-probe (H15) primary antibody and detecting with IR Dye800 conjugated donkey anti-rabbit secondary antibody. The intensity of the bands was determined using the Odyssey Software, Version 1.0.58 (LI-COR, Lincoln, NE) and the relative intensity was determined by dividing the intensity of the reduced cysteines by the intensity of the loading control. TGA1C172N/C266S/C287S and GRX030 were reduced

before purification and equal pmol amounts of GRX030 and GRX480 were incubated with each reaction.

4.3.17 β-Galactosidase Activity

The transactivation ability of the TGA1 and the various mutants was determined from yeast expressing β -galactosidase under the control of the Gal4 UAS. Single colonies were selected and grown for ~ 8 hours at which point the OD600 was determined using a UV/Visible spectrophotometer and aliquots of each culture were taken to induce overnight cultures to get the desired cell density after 16 hours of growth shaking at 30° C according to:

Inoculation volume =
$$\frac{\text{Desired cell density}}{\text{Current cell density}} \times \frac{\text{Culture Volume}}{2^{(\text{# of generations})}}$$

Where the Desired cell density is 0.6 at OD600, 2 hrs is required to obtain each successive generation and the current cell density was determined by measuring the absorbance at OD600. For each construct, two colonies were tested each time at two different time points. Yeast cells were pelleted by centrifugation at 2500rpm, RT for 15 minutes. Pellets were resuspended in 100uL Z-buffer (60.4mM Na₂HPO₄•7H₂O, 39.1mM NaH₂PO₄•H₂O, 10mM KCl, 0.45mM MgSO₄) and subjected to 3 freeze thaw cycles (2 minutes in liquid nitrogen followed by 5 minutes in 42°C water bath) and 5 × 30 seconds vortexing with glass beads (acid-washed; G8772) with 30 second rests on ice in between. Cell debris was pelleted and the SN was divided into 4 equal aliquots. Each aliquot was topped off to 700μL with Z buffer + 0.27% 2-mercaptoethanol. Aliquots were prewarmed in 30°C water bath for 10 minutes at which point 160μL of ONPG (*ortho*-nitrophenol-β-D-galactoside) was added at 4mg/mL in Z buffer to half the aliquots while

160μL of mQH₂O was added to the other half. Half of the water and ONPG aliquots for each construct were incubated for 30 minutes while the remainder were incubated for 60 minutes at 30° C. After the desired time, the reactions were removed from the water bath and stopped by the addition of 400μ L of 1M Na₂CO₃. β-galactosidase cleaves the clear and colorless ONPG to the yellow substrate ONP (*ortho*-nitrophenol) and, as such, the OD of each aliquot was determined at 420nm using a UV/Visible spectrophotometer. β-galactosidase activity was determined as: $\frac{1000 \times (\text{OD}_{420}\text{ONPG-OD}_{420}\text{H}_2\text{O})}{1000 \times (\text{OD}_{420}\text{ONPG-OD}_{420}\text{H}_2\text{O})}$

 $t \times V \times OD600$

Where t represents the length of time incubated at 30° C and V × OD600 represents the volume and cell density of each culture used in each activity measurement.

4.3.18 Expression of GRX480 and PR1

Quantitative PCR was performed on cDNA from Arabidopsis leaves that were either untreated or treated with 1mM SA for 1 or 8 hours. Reactions were performed in duplicate using gene specific primers for *PR1* and *GRX480*. Expression was analyzed by running a sample on an agarose gel where the intensity of PCR bands was determined by densitometry. The amount of Ubiquitin10 transcript was also determined using gene specific primers and used to normalize the level of *PR1* and *GRX480* expression since it is a constitutively expressed gene.

4.3.19 Statistical Methods

All pooled data are expressed as means and error bars represent SD. When data from two independent populations are compared, statistical significance was assessed using a two-tailed Student's t-test.

4.4 RESULTS

4.4.1 Transactivation of TGA1 Inversely Correlates with its Ability to Interact with NPR1 and is Regulated by the Redox Status of Cysteines 260 and 266.

Using an *in vivo* labeling assay, Després et al. (2003) observed that interaction of TGA1 with NPR1 only occurs when Cys260 and Cys266 are reduced. When expressed in yeast, the TGA1 cysteines occur in an oxidized state and when incubated with NPR1, no TGA1-NPR1 interaction is observed However when these two cysteines are mutated, to mimic a reduced state, interaction with NPR1 occurs.

Varying lengths of exposure to SA did not result in completely or predominantly oxidized TGA1 in a transient plant transcription assay (data not shown) making this system inadequate for studying TGA1 transactivation. As a result, we examined the ability of TGA1 to activate transcription in yeast where we observed that TGA1 is capable of auto-activation when fused to a DNA binding domain. The coding sequence of wild-type (Wt) TGA1 and TGA1C260N/C266S were ligated into the yeast vector pBI880 (Kohalmi et al., 1997) which contains the Gal4 DNA binding (DB) domain. Yeast cells were transformed with either construct and transactivation was assessed using the X-Gal filter test, which monitors the activation of the lacZ reporter gene. Transcriptional activation by TGA1 was only observed when Cys260 and Cys266 were oxidized (Table 1, compare Wt TGA1 and TGA1C260N/C266S in yeast). Also shown in Table 1 is data from Després et al. (2003) showing transactivation of TGA1 in Arabidopsis leaves is only observed when some Cys260 and Cys266 are oxidized. Taken together with the NPR1 interaction results (Després et al., 2003), these data suggest a mutually exclusive regulation of TGA1 such that transactivation of TGA1 is only observed when Cys260 and Cys266 are oxidized and interaction with NPR1 only occurs when these cysteines are

Table 1. The Redox Status of TGA1 Dictates Whether TGA1 Transactivates or Interacts with NPR1

	Protein and Treatment	Interaction with NPR1	Transactivation	% Reduced Cysteine (SH) ^c	% Oxidized Cysteine (S-S) ^c
Yeast	Wt TGA1, NT	NOa	YESb	Oa	100ª
Yeast	TGA1C260N/C266S, NT	YESª	NOp	N/A	N/A
Arabidopsis leaves ^a	Wt TGA1, NT	Slight	Slight	~50	~50
Arabidopsis leavesª	Wt TGA1, +SA(24)	YES	NO	100	0

NT = No treatment

N/A = Not applicable

⁺SA(24) = Treatment with 1mM SA for 24 hours

^a Results observed by Despres et al. (2003). In the case of Arabidopsis leaves, all data had been observed previously.

^b Activation of LacZ reporter gene was monitored by X-gal filter assay from colonies grown on medium lacking leucine and tryptophan. YES corresponds to the development of blue colour after approximately 1 hour at 30°C while NO corresponds to white colour observed after 16 hours at 30°C

Reduced and oxidized cysteines in TGA1 were determined using a labeling assay which distinguishes between sulfhydryls (SH) and disulfides (S-S) (Despres et al., 2003). Percentages were based on qualitative observation.

reduced (Després et al., 2003).

4.4.2 Arabidopsis TGA1 Possesses a Bipartite Transactivation Domain

Given that the oxidation of Cys260 and Cys266 is involved in regulating the ability of TGA1 to transactivate, we were interested in determining if other components of TGA1 were involved in transactivation. The coding region of TGA1 and all TGA1 mutants (Figure 1A) were ligated into the yeast vector pBI880 (Kohalmi et al., 1997) as described above. Each of these constructs, along with an empty pBI880 (Gal4DB; no fusion) were introduced into yeast where transactivation was examined through the expression of β -galactosidase.

Figure 1B illustrates that the Gal4 fusion of the Cys260/Cys266 double mutant has a drastic affect on TGA1's ability to activate reporter gene expression (compare TGA1:DB to TGA1C260N/C266S:DB, p<0.001) as expected from the results of Després et al. (2003).

Next we examined the individual effect of Cys260 and Cys266 on TGA1's ability to activate transcription as well as the influence of the other two cysteines, Cys172 and Cys287, in TGA1. Individual cysteine mutants of TGA1 (Figure 1A) were created by site directed mutagenesis and their ability to activate transcription was compared to wild type TGA1. Figure 1B illustrates that mutation of either Cys172 or Cys287 do not affect the ability of TGA1 to transactivate (Figure 1B, compare TGA1:DB to TGA1C172N:DB and TGA1C287S:DB, p>0.05) while the Cys260 mutation significantly reduces TGA1's ability to transactivate. The Cys266 mutation also compromised reporter gene expression (Figure 1B compare TGA1:DB to TGA1C266S, p<0.01; compare TGA1C266S:DB to TGA1C172N:DB or TGA1C287:DB, p<0.05), but not to the extent of Cys260 mutation

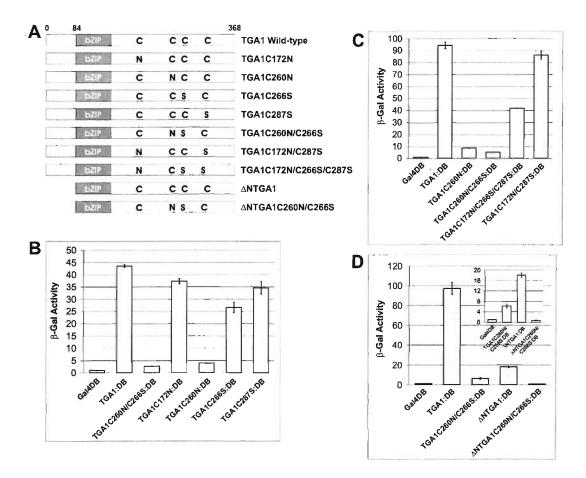


Figure 1. TGA1 has a Bipartite Transactivation Domain

- (A) Schematic representation of the TGA1 mutants that are fused to the Gal4DB in (B), (C) and (D). The amino acid length and position are indicated along the top. The bZIP domain is shaded in grey while each of the four cysteines (C) in TGA1 and their corresponding mutation (asparagine (N) or serine (S)) are indicated. The bZIP domain starts at amino acid 84 and the N-terminal deletion mutant (ΔNTGA1) deletes up to amino acid 83.
- **(B)** Histogram illustrating the effect of individual cysteine mutations on the ability of TGA1 to activate transcription when tethered to DNA through the Gal4DB in yeast.
- (C) Histogram illustrating that the ability of TGA1 to activate transcription in yeast depends on the presence of Cys260 and Cys266 when tethered to DNA through the Gal4DB while Cys172 and Cys287 are not required to active transcription.
- (**D**) Histogram illustrating that the first 83 amino acids of TGA1 are also required for the transcriptional activity of TGA1 in yeast indicating that TGA1 has a bipartite transactivation domain involving Cys260/Cys266 and the N-terminus. Inset represents the histogram illustrated in (**D**) without TGA1:DB included.
- In (B), (C), and (D) yeast were also transfected with an empty Gal4DB with no fusion to establish basal transactivation. Values were divided by the transcriptional activity of the empty Gal4DB. This assay was assessed by the ability to activate β -galactosidase under the control of the Gal4 upstream activating sequence (UAS). Data are recorded as β -galactosidase activity where activity is defined as one nm of ONP (the product of the β -

suggesting that its role in transactivation may be indirect. This is supported by the observation that the Cys260/Cys266 double mutant significantly reduces reporter gene expression below that of the single Cys260 mutant (compare TGA1C260N/C266S:DB to TGA1C260N:DB, p<0.05). Interestingly, none of the individual cysteine mutations nor the double mutant completely abolish TGA1's ability to transactivate (compare Gal4DB to TGA1C260N/C266S and TGA1C260N:DB, p<0.01).

We were interested in determining if Cys260, on its own, would be sufficient for TGA1's ability to activate transcription. Expression of a Gal4DB fusion of a TGA1 all mutant where cysteines, except Cys260, were mutated (TGA1C172N/C266S/C287S:DB, Figure 1A) resulted in reporter gene expression well above baseline (p<0.01) but still significantly lower compared to wild-type TGA1 (Figure 1C, p<0.01). Considering that Cys266 appears to contribute to TGA1's ability to transactivate, we examined a TGA1 mutant where Cys260 and Cys266 were maintained and Cys172 and Cys287 were mutated (TGA1C172N/C287S; Figure 1A). As is illustrated in Figure 1C, Cys266, in the presence of Cys260, restores TGA1's transactivation back to wild-type levels (compare TGA1:DB to TGA1C172N/C287S, p=0.5) suggesting that Cys260 and Cys266 are necessary for the transactivation ability of TGA1.

Given that the mutation of Cys260 and Cys266 do not completely abolish the ability of TGA1 to transactivate, another element in TGA1 must be required. Early work obtained *in planta* by Neuhaus et al. (1994) and Pascuzzi et al. (1998) determined that the

N-terminal domain of tobacco TGA1a, the Arabidopsis TGA1 homolog, is responsible for its transactivation ability. We deleted the first 83 amino acids of TGA1 (ΔNTGA1:DB) and observed a significant decrease in reporter gene activation (Figure 1D, compare to TGA1:DB, p<0.01). However it was not sufficient to abolish TGA1's transactivation ability (Figure 1D (inset), compare Gal4DB to Δ NTGA1:DB, p<0.01), presumably because Cys260 and Cys266 are still present (Figure 1D). In an attempt to completely abolish the transactivation ability of TGA1, we combined the N-terminal deletion TGA1 with Cys260/Cys266 of the mutation (Figure 1A; ΔNTGA1C260N/C266S:DB). The transactivation ability of this mutant was assessed and found to completely abolish TGA1's ability to transactivate since the level of reporter gene activation was no different from baseline (Figure 1 D (inset), compare Gal4DB to Δ NTGA1C260N/C266S:DB, p=0.1).

These results indicate that the N-terminal 83 amino acids and Cys260 and Cys266 are necessary and sufficient for the transactivation ability of TGA1.

4.4.3 The TGA1-NPR1 Complex is Capable of Transactivating and does not Require the Transactivation Domain of TGA1

Once the transactivation domain of TGA1 was identified, we were interested in its role in the TGA1-NPR1 complex (Després et al., 2003). Although interaction with NPR1 occurs prior to SA treatment, we examined interaction following 24 hours SA treatment when Cys260 and Cys266 in TGA1 are in the reduced state (Després et al., 2003). TGA1 and TGA1 transactivation domain mutants were fused to the Gal4 DB while NPR1 was fused to the transactivation domain (TA) of viral particle 16 (VP16) or expressed without a DB or TA domain (Figure 2A).

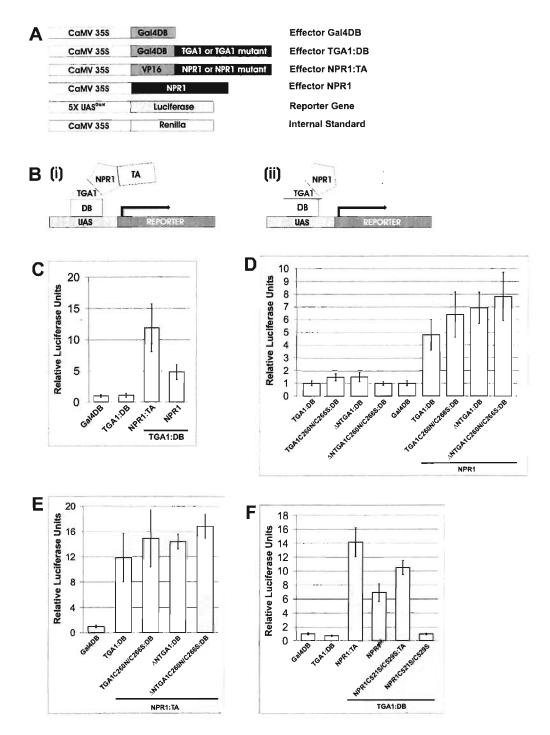


Figure 2. The TGA1-NPR1 Complex is Capable of Activating Transcription Irrespective of the Transactivation Domain in TGA1

(A) Schematic representation of the constructs used for transactivation and interaction with NPR1 in (C) through to (E). Boxed in white are the promoters. CaMV~35S indicates the double CaMV35S:AMV promoter. $5X~UAS^{GALA}$ indicates a promoter composed of a multimerized (5 elements) Gal4 UAS fused to a minimal TATA box and the Ω translational enhancer from the tobacco mosaic virus. Boxed in different shades of grey are the coding sequences. Gal4DB indicates the Gal4 DNA binding domain where the

Gal4DB was fused to wild-type TGA1 (TGA1:DB) or one of three TGA1 mutants (TGA1C260N/C266S:DB, ΔNTGA1:DB and ΔNTGA1C260N/C266S:DB). VP16 indicates the constitutive transactivation domain of viral particle 16 where the VP16 transactivation domain was fused to wild-type NPR1 (NPR1:TA) or the NPR1 mutant NPR1C521S/C529S where cysteines (C) 521 and 529 have been mutated to serines (S) (NPR1C521S/C529S:TA). NPR1 and the mutant were also expressed in a vector without a Gal4DB or VP16 fusion (NPR1 and NPR1C521S/C529S). DB – DNA binding domain, TA – transactivation domain. All constructs possess a 3' terminator signal of the gene for nopaline synthase (not shown). The 35S:Renilla construct is an internal reference to normalize transfection efficiency.

- (B) (i) Schematic representation of a classical two-hybrid depicting how the interacting proteins TGA1 and NPR1, fused to their respective Gal4DB and VP16 TA domains, interact on the *UAS*^{GAL4} to result in reporter gene expression. Illustrated in (ii) is the same scenario without the VP16 fusion to NPR1 (i.e. no artificial transactivation domain is being used). In this case, reporter gene activation results from the transcriptional activation ability of the TGA1-NPR1 complex. Not shown in (i) or (ii) is the contact of the complex with the basal transcriptional machinery which is bound to the TATA box.
- (C) Histogram illustrating the transcriptional activity of the TGA1-NPR1 complex in the absence of an artificial transactivation domain fused to NPR1 where TGA1 is tethered to DNA through the Gal4DB.
- (**D**) Histogram illustrating that none of the TGA1 transactivation domain mutants are able to activate transcription when tethered to DNA through the Gal4DB. Additionally, none of these mutants transfected in the presence of NPR1 without the VP16 fusion affects the ability of the complex to activate reporter gene expression compared to wild-type TGA1.
- (E) Histogram illustrating that interaction between the DB-fused TGA1 transactivation domain mutants and NPR1:TA is not compromised by the mutations in TGA1.
- (F) Histogram illustrating the ability of the TGA1-NPR1 complex to activate transcription is the result of the novel transactivation domain in NPR1 which consists of cysteines 521 and 529. TGA1 is tethered to DNA through the Gal4DB and is able to interact with NPR1:TA and NPR1C521S/C529S:TA. However the ability of TGA1:DB in a complex with NPR1C521S/C529S to activate transcription is abolished.
- In (C), (D), (E) and (F), Arabidopsis leaves were treated for 24 hours with 1mM salicylic acid (SA). The constructs were transfected along with the 5X UAS^{GALA} : firefly luciferase reporter and the CaMV35S:Renilla internal standard. Relative luciferase units were obtained by dividing the value obtained for the reporter gene by the value obtained for the internal standard. Arabidopsis leaves were also transfected with an empty Gal4DB in the presence of the reporter and internal standard and represents the baseline level of transcriptional activation. Data are reported as relative luciferase units where the transcriptional activation of each protein or protein pair is divided by the level of transcription from the empty Gal4DB. Values consist of n = 25 samples and represent averages \pm SD. Every bar represents five bombardments repeated five times.

In the classical two-hybrid experiment, fusion to the DB domain is required to tether the interacting proteins to DNA while the TA domain is required for transactivation of the interacting protein complex and reporter gene expression (Figure 2B(i)). However certain proteins, like TGA1 and NPR1 (Rochon et al., 2006), contain their own transactivation domain, suggesting transactivation of the TGA1-NPR1 complex could occur in the absence of the artificial TA domain (Figure 2B(ii)).

The histogram in Figure 2C illustrates that the co-transfection of TGA1:DB and NPR1 into Arabidopsis leaves treated with 1mM SA for 24 hours is able to activate reporter gene expression well above baseline despite the removal of the artificial TA domain (compare Gal4DB or TGA1:DB to TGA1:DB + NPR1, p<0.01). Reporter gene expression does drop relative to NPR1 fused to an artificial TA domain (compare TGA1:DB + NPR1:TA to TGA1:DB + NPR1, p<0.05) however this can be attributed to a difference in the strength of the transactivation domains.

To determine whether TGA1 or NPR1 is responsible for the transactivation ability of the complex, we repeated the above plant two-hybrid experiment using the three TGA1 transactivation domain mutants expressed as N-terminal Gal4DB fusions: TGA1C260N/C266S:DB, ΔNTGA1:DB and ΔNTGA1C260N/C266S:DB. We first confirmed that none of the TGA1 transactivation domain mutants were capable of auto-activation when fused to the DB (compare Gal4DB to the three TGA1 transactivation domain mutants, p>0.05). Examining the transactivation ability of the TGA1-NPR1 complex in the context of the TGA1 transactivation domain mutants reveals that reporter gene expression is no different than wild-type TGA1 in the presence of NPR1 (Figure 2D, p=0.1). The ability of TGA1 to interact with NPR1:TA is also not compromised by the TGA1 transactivation domain mutants (Figure 2E, compare all to TGA1:DB +

NPR1:TA, p=0.1). This implies that the ability of the TGA1-NPR1 complex to transactivate is not dependent on the transactivation domain of TGA1.

The transactivation domain of NPR1 consists of Cys521 and Cys529 where mutation of either or both cysteines to a serine abolishes the ability of NPR1 to transactivate in a plant transcription assay when fused to the Gal4DB (Rochon et al., 2006). Wild-type NPR1 and NPR1 where Cys521 and Cys529 are mutated to serine were fused to the VP16 TA domain (NPR1:TA and NPR1C521S/C529S:TA) or fused to an empty vector without a DB or TA domain fusion (NPR1 and NPR1C521S/C529S) and transfected with TGA1:DB into Arabidopsis leaves. Figure 2F illustrates that the mutation of Cys521 and Cys529 in NPR1 abolishes the ability of the TGA1-NPR1 complex to transactivate (compare TGA1:DB + NPR1 to TGA1:DB + NPR1C521S/C529S, p<0.01). The fact that the interaction between TGA1 and NPR1 is not affected by the mutation of Cys521 and Cys529 (compare TGA1:DB + NPR1:TA to TGA1:DB + NPR1C521S/C529S:TA, p=0.1) implies that the transactivation domain of NPR1 is directly responsible for the transactivation of the TGA1-NPR1 complex.

4.4.4 TGA1 Interacts with a Novel Glutaredoxin in Plants and is Dependent on the Presence of Cys260 and Cys266

Given that the transactivation domain of TGA1 does not play a role in the transactivation of the TGA1-NPR1 complex, the transcriptional activation ability of TGA1 may be required elsewhere. In an attempt to identify proteins that interact with TGA1, a yeast two-hybrid screen of an Arabidopsis cDNA library was performed using TGA2:DB as bait (data not shown). Using this screen, we identified that TGA2 interacts

with GRX480, a previously identified glutaredoxin (Ndamukong et al., 2007) with an atypical active site.

In the case of TGA1, a directed yeast two-hybrid assay was performed using the classical Arabidopsis glutaredoxin GRX030 (At5g63030) and the novel glutaredoxin GRX480 (At1g28480). The coding region of the two glutaredoxins were cloned into the yeast vector pBI880 (Kohalmi et al., 1997) while the coding region of *TGA1* was cloned into the yeast Gal4TA vector pBI881 (Kohalmi et al., 1997). Interaction was assessed through blue/white selection using an X-gal filter test. Table 2 indicates that an interaction (indicated as YES) was only observed between TGA1 and the novel glutaredoxin GRX480.

In order to begin to understand the TGA1-GRX480 interaction, we examined the nature of this complex in Arabidopsis leaves. *TGA1* was fused to the Gal4 DB (TGA1:DB) and co-transfected into Arabidopsis leaves with either *GRX030* or *GRX480* fused to the VP16 TA (GRX030:TA or GRX480:TA). Reporter gene expression was monitored after 24 hours in the absence and presence of 1mM SA. The histogram in Figure 3A illustrates that TGA1:DB is able to interact with the novel glutaredoxin GRX480:TA before SA treatment (white bars; compare to Gal4DB, p<0.01) and this interaction is significantly enhanced after exposure to SA (grey bars). On the other hand, interaction between TGA1:DB and GRX030:TA, the classical glutaredoxin, is not observed regardless of SA's presence (compare to Gal4DB, p>0.05 before and after SA).

In view of the fact that Cys260 and Cys266 are involved in regulating the ability of TGA1 to transactivate or interact with NPR1, we were interested in determining if interaction with GRX480 was affected by the absence of either or both cysteines. Fusions

Table 2. TGA1 Interacts with the Novel Glutaredoxin GRX480 but not the Classical Glutaredoxin GRX030.

Baita	Active Site ^b	Preyc	Interactiond
Grx030:DB	CGYC (Classical)	TGA1:TA	NO
Grx480:DB	CCMC (Novel)	TGA1:TA	YES

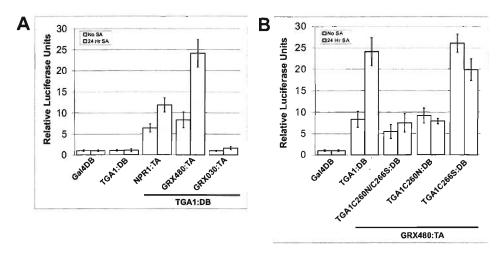
GRX030 (At5g63030) and GRX480 (At1g28480)

^a The bait indicates a Gal4 DNA binding domain fusion (DB)

^bThe Active site represents the active site of the glutaredoxin (classical and novel are defined in the text)

^c The prey indicates a Gal4 transcriptional activation domain fusion (TA)

^d Activation of LacZ reporter gene was monitored by X-gal filter assay from colonies grown on medium lacking leucine and tryptophan. YES corresponds to the development of blue colour after approximately 1 hour at 30°C while NO corresponds to white colour observed after 16 hours at 30°C



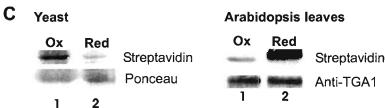


Figure 3. Interaction between TGA1 and the Novel Glutaredoxin GRX480 in Arabidopsis Leaves Differentially Depends on Cys260 and Cys266

- (A) Histogram illustrating that TGA1 tethered to DNA through the Gal4DB (TGA1:DB) and the novel Arabidopsis glutaredoxin GRX480 expressed as a VP16 TA fusion (GRX480:TA) interact to activate transcription. Interaction between TGA1:DB and the classical Arabidopsis glutaredoxin (GRX030:TA) is not observed. Interaction between TGA1:DB and NPR1:TA is used as a positive control while TGA1:DB alone is used as a negative control.
- (B) Histogram illustrating that the mutation of either Cys260, Cys266 or both in TGA1 tethered to DNA through the Gal4DB (TGA1C260N:DB, TGA1C266S:DB and TGA1C260N/C266S:DB respectively) have different affects on the ability of TGA1 to interact with GRX480.
- In (A) and (B), Arabidopsis leaves were untreated (white bars) or treated for 24 hours (grey bars) with 1mM salicylic acid (SA). The constructs were transfected along with the $5X\ UAS^{GAL4}$:firefly luciferase reporter and the CaMV35S:Renilla internal standard. Relative luciferase units were obtained by dividing the value obtained for the reporter gene by the value obtained for the internal standard. Arabidopsis leaves were also transfected with an empty Gal4DB in the presence of the reporter and internal standard and represents the baseline level of transcriptional activation. Data are reported as relative luciferase units where the transcriptional activation of each protein or protein pair is divided by the level of transcription from the empty Gal4DB. Values consist of n = 25 samples and represent averages \pm SD. Every bar represents five bombardments repeated five times.
- (C) Blot analysis of a TGA1C172N/C266S/C287S immunoprecipitate expressed in yeast and Arabidopsis leaves (untreated) to assess the *in vivo* redox status of Cys260. In both instances, the protein was expressed as a transactivation domain fusion (Gal4TA in the

case of yeast and VP16 TA in the case of Arabidopsis leaves) since neither domain contains any cysteines. Ox represents immunoprecipitates from proteins labeled for oxidized cysteine residues (lane 1) while Red indicates immunoprecipitates from proteins labeled for reduced cysteine residues (lane 2). The ponceau (yeast) and anti-TGA1 (Arabidopsis leaves) establish the loading control in each instance.

to the Gal4DB and VP16 TA were performed as described above and reporter gene expression was monitored after 24 hours in the absence (white bars) and presence (grey bars) of 1mM SA (Figure 3B). Mutation of both cysteines in TGA1 abolishes the ability of GRX480 to interact with TGA1 only after SA treatment (grey bars, compare TGA1:DB + GRX480 to TGA1C260N/C266S:DB + GRX480, p<0.01) as interaction before SA treatment was no different (white bars, p=0.1) (Figure 3B). Interaction between TGA1 with Cys260 mutated and GRX480 mimicked the double cysteine mutant before and after SA treatment (grey and white bars, compare TGA1C260N/C266S:DB + GRX480 to TGA1C260N:DB + GRX480, p>0.05). Interestingly, mutation of Cys266 in TGA1 had no affect on interaction with GRX480:TA after SA treatment (grey bars, compare TGA1:DB + GRX480:TA to TGA1C266S:DB + GRX480:TA, p=0.1), however interaction occurred in the absence of SA treatment and was no different from the interaction between wild-type TGA1:DB and GRX480:TA after SA treatment (compare TGA1:DB + GRX480:TA (grey bars) to TGA1C266S:DB + GRX480:TA (white bars), p=0.1) (Figure 3B). Additionally, the interaction of TGA1C266S:DB with GRX480:TA was no different from the level of interaction observed after SA treatment (compare grey and white bars TGA1C266S:DB + GRX480:TA, p=0.1). These results indicate that interaction between TGA1 and GRX480 differentially depends on the presence and redox status of Cys260 and Cys266.

4.4.5 Cys260 Remains Oxidized in the Absence of Cys266

Considering the importance of Cys260 in regulating TGA1's ability to transactivate and interact with GRX480, we examined the redox status of a TGA1 mutant This where cysteines, except Cys260, were mutated. TGA1 mutant, TGA1C172N/C266S/C287S, was fused to either the Gal4TA or VP16 TA and transfected into yeast or plant, respectively and the redox status of Cys260 was determined using a labeling assay described and illustrated in Després et al. (2003). In plants, the redox status was only assessed before SA treatment since all the cysteines in TGA1 are reduced after SA treatment. Figure 3C illustrates that Cys260 is predominately oxidized (Ox) in yeast (lane 1, Streptavidin) while very little reduced (Red) Cys260 is observed (lane 2, Streptavidin) despite approximately equal protein loading (compare yeast lanes 1 and 2, Ponceau). In Arabidopsis leaves, a noticeable amount of Cys260 remains oxidized (lane 1, Streptavidin) considering only 50% of TGA1 is oxidized in the absence of SA (Table 1). While the amount of reduced Cys260 is higher in Arabidopsis leaves (lane 2, Streptavidin) and the protein loading is approximately equal (compare lanes 1 and 2, Anti-TGA1), the observation of any oxidized Cys260, combined with the predominantly oxidized Cys260 in yeast, suggests that Cys260 is oxidized in the absence of Cys266. Given that glutaredoxins are also able to reduce mixed disulfides with glutathione, it seems reasonable to suggest that the observed oxidized Cys260 results from a disulfide bond with glutathione.

4.4.6 GRX480 only Interacts with the Reduced form of TGA1

In an attempt to address the possibility that GRX480 binds TGA1 to reduce a mixed disulfide, we performed a pull-down between E. coli purified GRX480 and reduced and oxidized forms of TGA1. HIS-tagged purified fractions of TGA1 were combined and split in half, one half was treated with 10mM GSSG to glutathionylate TGA1 (TGA1_{Ox}) while the other half was left untreated (TGA1_{Red}). Magnetic beads tagged with streptavidin provide a solid support for the pull-down and were incubated with a DNA probe specifically recognized by TGA TFs fused to biotin (Lebel et al., 1998; Després et al., 2000). Once the probe is coupled to the beads in two separate pulldowns, oxidized and reduced TGA1 are coupled to the DNA. The mixture of beads-DNA-TGA1_{Ox} or -TGA1_{Red} is then incubated with equal aliquots of HIS purified GRX480. As a control, mixtures of beads-DNA-TGA1_{Ox} or -TGA1_{Red} were also incubated with an E. coli extract not expressing GRX480. The pull-downs were eluted and electrophoresed on a 12% SDS-polyacrylamide gel where a 10% input of GRX480 (lane 1, Figure 4A) was included as a point of reference. Figure 4A illustrates that the reduced but not oxidized form of TGA1 (lanes 2 and 3, respectively, open arrow) was able to pull-down GRX480 (solid arrow). This pull-down is specific since incubation of either reduced or oxidized TGA1 with an E. coli extract (lanes 4 and 5, respectively) did not result in any binding.

This result was unexpected as GRX480 was hypothesized to bind the glutathionylated form of TGA1 to reduce it. In order to ensure that glutathionylation of TGA1 was in fact occurring, we subjected a fraction of glutathionylated TGA1 and untreated TGA1 to MALDI TOF mass spectroscopy. After being electrophoresed,

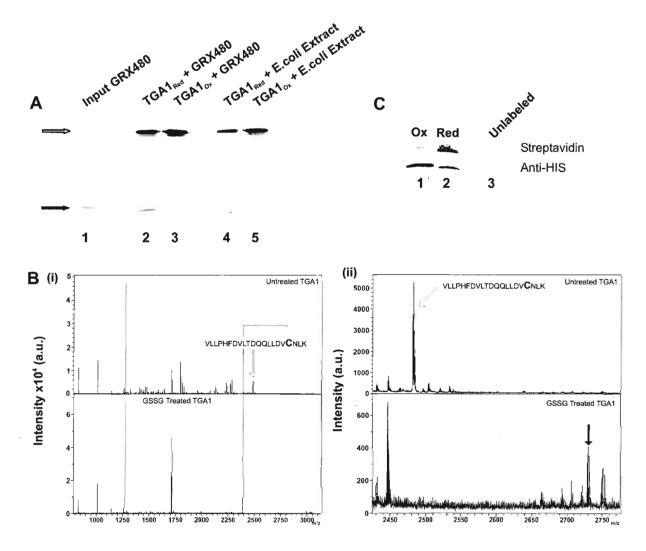


Figure 4. GRX480 only Interacts with the Reduced form of TGA1

(A) Immunoblot of a DNA-based pull-down between E. coli expressed, HIS columnpurified TGA1_{Ox} or TGA1_{Red} and GRX480. The TGA1 HIS purified fractions were collected and divided, one half was treated with 10mM GSSG to glutathionylate the cysteines in TGA1 (TGA1_{0x}) while the other half was left untreated (TGA1_{Red}). In separate reactions, TGA1_{0x} and TGA1_{Red} were coupled to a DNA-bead mixture which contained LS5 and LS7, two DNA regions containing the TGA factors cognate binding sequence TGACG (Lebel et al., 1998). Equal amounts of GRX480 or an E. coli extract were incubated with TGA1_{Ox} and TGA1_{Red}. Protein complexes were then eluted from the DNA beads and electrophoresed on a 12% SDS-polyacrylamide gel. Detection was accomplished using an anti-HIS primary antibody, a secondary antibody coupled to a fluor and infrared imaging using the Odyssey imager. Lane 1 is the GRX480 input and represents 10% of the amount of GRX480 incubated with TGA1_{Red} or TGA1_{Ox} in lanes 2 and 3, respectively. Lanes 4 and 5 are the negative controls where TGA1_{Red} and TGA1_{Ox} coupled to DNA-beads were incubated with an E. coli extract, respectively. The solid arrow indicates the band that corresponds to GRX480 while the open arrow indicates the band that corresponds to TGA1 (either oxidized or reduced).

- (B) MALDI TOF mass spectra of untreated (top panel in (i) and (ii)) and GSSG treated (bottom panel in (i) and (ii)) trypsin-digested TGA1 from *E. coli*. The spectra in (i) represent the entire m/z range for TGA1 while the spectra in (ii) are a close up of the boxed in region in (i) around the Cys260 peptide. The peak corresponding to the peptide containing Cys260, V₄₃ to K₆₃, is indicated by the open arrow (expected m/z = 2480.32, observed m/z = 2482.074) in the top panel in (i) and (ii). The solid arrow in the bottom panel of (ii) indicates the peptide that contains Cys260 has been glutathionylated (expected m/z = 2729.30, observed m/z = 2729.692). The intensity of each peptide is represented along the y-axis in arbitrary units (a.u.) and the charge-to-mass ration of the peptides (m/z) is represented along the x-axis.
- (C) Blot analysis of GRX480 immunoprecipitate from *E. coli* to assess the *in vivo* redox status of all cysteine residues. Ox represents immunoprecipitates from proteins labeled for oxidized cysteine residues (lane 1) while Red indicates immunoprecipitates from proteins labeled for reduced cysteine residues (lane 2). The unlabeled sample (lane 3) represents 25% of the amount in lanes 1 and 2 but was not treated for either Ox or Red labeling and acts as a negative control. The anti-HIS detection acts as a loading control in each lane.

The reduced and glutathionylated forms of TGA1 were subjected to an in-gel tryptic digest and then analyzed in the positive mode by the Autoflex II TOF/TOF mass spectrometer (Bruker Daltonics). Figure 4B(i) illustrates the mass spectra obtained for reduced (untreated; top panel) and glutathionylated (GSSG treated; bottom panel) TGA1. The peptide containing Cys260, which is indicated in the top panel (open arrow; expected m/z = 2480.32, observed m/z = 2482.074), is no longer present in the glutathionylated TGA1 sample (bottom panel). Closer examination of an enlarged version of the spectra, around the Cys260 peptide (Figure 4B(ii)), reveals that the*loss of this peptide in the glutathionylated TGA1 sample (bottom panel) corresponds with the appearance of a peptide with an m/z value equivalent to the Cys260 peptide modified by glutathione (solid arrow; expected m/z = 2729.30, observed m/z = 2729.692). This indicates that glutathionylation of TGA1 produces the desired results. As an interesting side note, we observed that the peptide containing Cys287 (expected m/z = 3044.46, observed m/z = 3044.46, observed m/z = 3044.820, not highlighted in Figure 4B) is unchanged after the GSSG treatment of TGA1

suggesting the reactivity of this cysteine to glutathione is very low or non-existent. The peptides corresponding to Cys172 (expected m/z = 818.42) and Cys266 (expected m/z = 1823.79) did not ionize in untreated or GSSG treated samples. The mass list of the peptide fingerprints for untreated and GSSG treated TGA1 are included in the Appendix in Table 2 and 3, respectively.

The nature of GRX480 purified from *E. coli* is another factor that might account for the unexpected result in Figure 4A. Since GRX480 is a foreign protein being expressed in *E. coli*, it may be non-specifically oxidized or glutathionylated which might affect its activity. In order to establish the redox status of GRX480 isolated from *E. coli*, we performed the same labeling assay described for Cys260 in TGA1 (Figure 3C) to distinguish oxidized and reduced cysteines. Oxidized (Ox) and reduced (Red) labeled fractions were electrophoresed along with a sample of untreated GRX480. Figure 4C reveals that the cysteines in GRX480 are completely reduced (lane 2, Streptavidin). The minor signal observed in the Ox sample (lane 1, Streptavidin) is qualitatively no different from the signal observed in the unlabeled sample (lane 3, Streptavidin) suggesting that there are no oxidized cysteines in GRX480. The loading control, which is based on detection using an anti-HIS antibody, reveals that the loading is biased toward the Ox sample (compare lanes 1 and 2, Anti-HIS).

Therefore, the proper glutathionylation of TGA1 and lack of modification of GRX480 suggest that the observation that only reduced TGA1 is able to pull-down GRX480 is authentic.

4.4.7 GRX480 does not have Classical Glutaredoxin Activity

Since GRX480 only binds reduced TGA1, we questioned whether it had enzymatic activity. In order to test this hypothesis, we used a classical assay, known as the HED assay, to assess the ability of GRX480 to reduce a mixed disulfide (Holmgren and Áslund, 1995). In the HED assay, which is outlined in Figure 5, the substrate HED (β-hydroxyethylene disulfide or XSSX) is reacted with reduced glutathione (GSH) to form the mixed disulfide GSSX. This mixed disulfide is then reduced, by the glutaredoxin being studied, in the presence of GSH to generate the reduced substrate (XSH) and oxidized glutathione (GSSG). The GSSG is then consumed by glutathione reductase in an NADPH dependent manner, generating GSH and NADP⁺ as products. The overall reaction is monitored as a decrease in absorbance at 340nm, due to the loss of NADPH. Comparing the classical Arabidopsis glutaredoxin GRX030 with GRX480 in the HED assay (Table 3) reveals that only the classical glutaredoxin has activity reducing a mixed disulfide.

Glutaredoxins have a GSH binding site that has been identified by NMR (Bushweller et al., 1994) and site-directed mutagenesis (Nikkola et al., 1991). In the glutaredoxin catalytic cycle, GSH binds to oxidized glutaredoxin and reduces it, making the enzyme available for further reduction reactions (Fernandes and Holmgren, 2004). Considering the novel active site in GRX480, other features of the enzyme might also be slightly altered, including the GSH binding site, which might impede the reducing ability of GRX480. A multiple alignment of several glutaredoxins from various organisms is shown in Figure 6 in the region of the GSH binding site where the critical active site glycine, highlighted in grey, is conserved in GRX480 (Nikkola et al., 1991; Xing and

$$GSH + XSSX \longrightarrow GSSX + XSH$$

$$GSH + GSSX \xrightarrow{Glutaredoxin} GSSG + XSH$$

$$GSSG + NADPH + H^{+} \xrightarrow{GSH Reductase} 2 GSH + NADP^{+}$$

$$NADPH + H^{+} + XSSX \longrightarrow NADP^{+} + 2 GSH$$

Figure 5. Schematic of the Reactions that take Place in a HED Assay

The substrate HED (β-hydroxyethylene disulfide; XSSX) is reacted with reduced glutathione (GSH) to produce a mixed disulfide (GSSX). GSSX is then reduced by the glutaredoxin being tested which uses GHS as a substrate. This results in reduced glutathione (GSSG) and the reduced product (XSH). GSSG is then reduced by glutathione reductase (GSH reductase) in the presence of NADPH and H⁺ to give two molecules of GSH and NADP⁺. The overall reaction is also shown. Taken from Holmgren and Åslund (1995).

Table 3. GRX480 Lacks Classical Glutaredoxin Activity in the HED Assay

Enzyme	Signature	Class	Activity (Units/mg)
GRX030	CGYC	Classical	153 ± 14
GRX480	CCMC	Novel	0

Activity was measured in the HED assay as a loss of NADPH followed as a decrease at 340nm.

One unit is defined as the consumption of 1µmol of NADPH per minute.

E.coli Grx1	VPQIFVDQQHIGGYTDFAAWVKEN
Human Grx2	VPRIFVNGTFIGGATDTHRLHKEG
Grx2061	LPQIFIDDQHIGGCDDIYALDGAG
Grx1562	VPQIFVNDQGIGGCDQLYGLDSRG
ROXY1	SPGSLPVVFIGGKLVGAMDRVMAS
ROXY2	SPGALPVVFIGGKMVGAMERVMAS
GRX030	VPNVFIGGNHIGGCDATSNLHKDG
GRX480	LPAVYVGGRLFGGLDRVMATHISG

Figure 6. Multiple Alignment Highlighting the GSH Binding Domain in GRX480 Clustal W alignment of the C-terminal region of *E. coli* Grx 1 (P68688), Human Grx2 (AAK72499), *Synechocystis* Grx 2061 (NP_440852) and Grx1562 (NP_442889) and the Arabidopsis glutaredoxins ROXY1 (NP_186849), ROXY2 (ABY5517), GRX030

(At5g63030) and GRX480 (At1g28480). The underlined region indicates the GSH binding site with the critical glycine (G) residue shaded in dark grey. The C-terminal 99-

123 amino acids are aligned according to the numbering of GRX480.

Zachgo, 2008). ROXY1 and ROXY2 are two novel glutaredoxins in Arabidopsis that contain the same atypical active as GRX480 (Xing and Zachgo, 2008). The importance of a glycine in the GSH binding site of this novel class of glutaredoxins is highlighted by ROXY1, where a mutant with the glycine mutated to an alanine was unable to rescue the phenotype when expressed in *roxy1-5* mutant plants (Xing et al., 2005). This would suggest that GSH binding is critical for the novel class of Arabidopsis glutaredoxins. Therefore, the potential lack of GRX480's ability to bind GSH likely does not account for its lack of activity in the HED assay.

4.4.8 GRX480 Glutathionylates TGA1 at Cys260

One feature of glutaredoxins that has yet to be established as a common activity is the glutathionylation of proteins; this mechanism would involve the addition of glutathione, most likely from GSSG, to a cysteine. This activity has only been characterized for mammalian Grx1 (Starke et al., 2003) and Grx 2 (Beer et al., 2004).

Since GRX480 exhibits none of the behavior associated with a classical glutaredoxin and selectively binds the reduced form of TGA1 over the oxidized form, we postulated that GRX480 acts to glutathionylate TGA1. To address this issue we utilized our DNA-based pull-down assay to tether TGA1 to a solid support in the presence of GRX480 and GSSG. In this instance, used the TGA1 we mutant TGA1C172N/C266S/C287S to specifically address the glutathionylation of Cys260, which appears to be the critical cysteine based on our results thus far. Being aware that simply incubating TGA1 with GSSG is sufficient for chemical glutathionylation, we reduced the concentration of GSSG (5mM instead of 10mM) and decreased the length of exposure to GSSG (30 minutes instead of 1 hour) as well as the conditions (room

temperature instead of 37°C). Once TGA1 was coupled to the DNA-beads, it was incubated with GRX480 alone, GSSG alone or GRX480 in the presence of GSSG. After a 30 minute incubation, the bead-DNA-TGA1C172N/C266S/C287S mixtures were washed and incubated with 0.2mM MPB for 1 hour to label reduced Cys260. The different TGA1C172N/C266S/C287S treatments were then electrophoresed and detection was performed with streptavidin conjugated to the Alexa fluor 680 (Figure 7A). Since TGA1C172N/C266S/C287S was reduced before being HIS purified and applied to the DNA-coupled beads, incubation with GRX480 on its own represents 100% reduced Cys260 (Figure 7A, lane 1, S-H detection). The signal in lane 3 (S-H detection) represents incubation with just GSSG and gives the level of glutathionylation from chemical treatment. The signal in lane 2 (S-H detection), represents the level of glutathionylation by GSSG in the presence of GRX480. The loading control, which was established from an immunoblot of the membrane with anti-HIS antibody, indicates that loading was more or less even between all lanes. The intensity of the bands in each lane was estimated based on the number of pixels (table below each lane) and the relative intensity of each band was determined by dividing the intensity of S-H detection by the anti-HIS detection. Quantitatively, the decrease in relative intensity observed for TGA1 in the presence of GSSG and GRX480 (lane 2) compared to just GSSG (lane 3) indicates that glutathionylation of Cys260 in TGA1 is increased in the presence of GRX480.

Since mammalian Grx1 and Grx 2 are able to glutathionylate and deglutathionylate their target proteins (Starke et al., 2003; Beer et al., 2004), we were interested in examining the specificity of GRX480 for the glutathionylation of TGA1 since it cannot reduce a mixed disulfide. In order to do this, we repeated the experiment

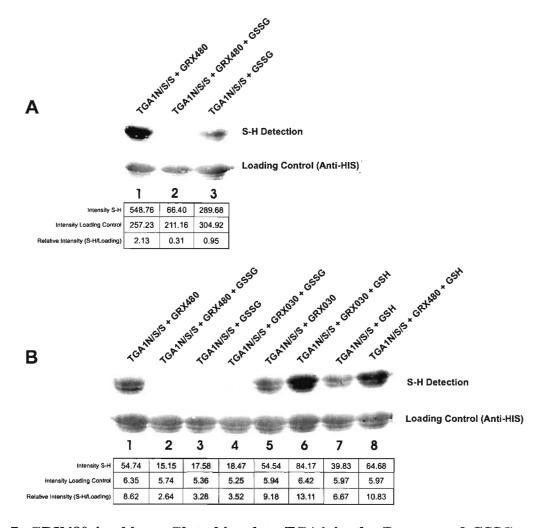


Figure 7. GRX480 is able to Glutathionylate TGA1 in the Presence of GSSG at Cysteines 260

(A) Blot analysis of a TGA1C172N/C266S/C287S (TGA1N/S/S) pull-down to assess the redox status of Cys260 in the presence of GRX480. TGA1C172N/C266S/C287S HIS purified from *E. coli* was coupled to DNA-beads in the same manner described for Figure 4A. An enzyme assay was then performed on TGA1C172N/C266S/C287S by incubating it, in separate reactions, with GRX480 (lane 1), GRX480 and GSSG (lane 2) or GSSG (lane 3). Incubation proceeded for 30 minutes at which point TGA1 was labeled for reduced cysteines by treatment with MPB. TGA1C172N/C266S/C287S was then eluted and electrophoresed on a 10% SDS-polyacrylamide gel and reduced cysteines were detected (S-H detection) using a streptavidin:Alexa fluor conjugate detected by infrared imaging. Loading was assessed with an anti-HIS immunoblot (Loading control).

(B) Blot analysis of a TGA1C172N/C266S/C287S (TGA1N/S/S) pull-down to assess the redox status of Cys260 in the presence of GRX480 and GRX030. The experiment was repeated as described in (A) with the following assays on TGA1C172N/C266S/C287S: GRX480 (lane 1), GRX480 + GSSG (lane 2), GSSG (lane 3), GRX030 + GSSG (lane 4), GRX030 (lane 5), GRX030 + GSH (lane 6), GSH (lane 7) and GRX480 + GSH (lane 8). In (A) and (B), the intensity of each band was assessed with an identical sized box using the Odyssey Imaging software (LICOR). Relative intensity reflects the amount of

in Figure 7A to include the classical glutaredoxin GRX030 and GSH as a substrate. Considering that GRX480 and TGA1C172N/C266S/C287S are both reduced, the addition of GSH, another reduced molecule, by GRX480 to TGA1 is technically not possible. However considering the nature of the novel glutaredoxins and their lack of conformity, we did not want to rule anything out. The results of the expanded experiment can be seen in Figure 7B where the assays with TGA1C172N/C266S/C287S, GSSG and GRX480 were repeated in addition to the same experiment with GSH instead of GSSG and with GRX030 instead of GRX480. What can be surmised from Figure 7B is: (i) GSH is not a substrate for GRX480 glutathionylation of TGA1 (compare lanes 2 and 8 and 1 and 8, S-H detection as well as their corresponding relative intensities) nor is GSH capable of chemically modifying TGA1 (compare lanes 3 and 7, S-H detection and their corresponding relative intensities), (ii) although the qualitative difference between TGA1C172N/C266S/C287S and GSSG versus TGA1C172N/C266S/C287S and GSSG in the presence of GRX480 appears as though there is no difference (compare lanes 2 and 3, S-H detection), the corresponding relative intensities indicate that there is, and (iii) the qualitative difference between glutathionylation of TGA1C172N/C266S/C287S in the presence of GSSG by either GRX480 or GRX030 appears similar (compare lanes 2 and 4, S-H detection) however the corresponding relative intensities indicate that the glutathionylation of TGA1C172N/C266S/C287S in the presence of GSSG and GRX030 is in fact not different from TGA1C172N/C266S/C287S and GSSG alone (compare relative intensity of lanes 3 and 4). These results indicate that GRX480 can

glutathionylate TGA1 at Cys260 using GSSG and not GSH as a substrate and GRX030 cannot.

4.4.9 GRX480 Acts to Shut Down the Transactivation of the TGA1-NPR1 Complex

Since GRX480 is responsible for oxidizing TGA1, we sought to determine what its role could be during SAR. An interesting observation by Ndamukong et al. (2007) was that the expression of GRX480 is SA inducible. This led us to examine the expression profile of GRX480 during SAR to establish timing of expression and possibly relate it to the immediate-early or late timing displayed by many SAR genes (Horvath and Chua, 1996). We performed quantitative PCR using gene specific primers on Arabidopsis leaves that were treated with 1mM SA for 0, 1 or 8 hours (Figure 8A). The level of expression of PR1 and GRX480 were monitored and normalized by the expression of UB10, a constitutively expressed gene. Figure 8A illustrates that expression of GRX480 follows the same profile and responsiveness as PRI to SA suggesting that its expression correlates with late events in SAR. To this end, we examined the effect of GRX480 on the TGA1-NPR1 complex. By transfecting Arabidopsis leaves with TGA1:DB in the presence of NPR1 and GRX480 (both in empty vectors) compared to TGA1:DB with NPR1, we determined that GRX480 almost completely abolishes the ability of the TGA1-NPR1 complex to transactivate in the absence (white bars, compare to Gal4DB, p<0.01) and presence (grey bars, compare to Gal4DB, p<0.01) of SA (Figure 8B). These data suggest that GRX480 acts to glutathionylate TGA1 at Cys260 to impede its interaction with NPR1.

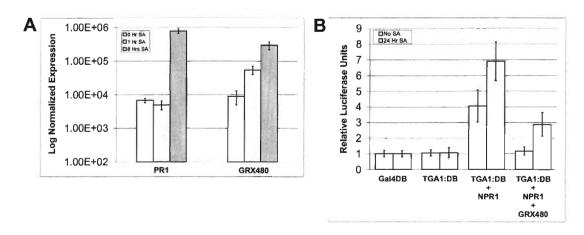


Figure 8. GRX480 acts to Repress the Transactivation of the TGA1-NPR1 Complex (A) Histogram illustrating the expression profile of *PR1* and *GRX480* after 0 (white), 1 (light grey) and 8 (dark grey) hours of 1mM SA treatment in Arabidopsis leaves. Expression was normalized to the expression of the constitutive gene UB10.

(B) Histogram illustrating the negative effect of GRX480 on the transcriptional activation

(B) Histogram illustrating the negative effect of GRX480 on the transcriptional activation of the TGA1-NPR1 complex. TGA1 is tethered to DNA through Gal4DB while NPR1 and GRX480 are expressed without a fusion. Arabidopsis leaves were untreated (white bars) or treated for 24 hours (grey bars) with 1mM SA. The constructs were transfected along with the $5X\ UAS^{GAL4}$: firefly luciferase reporter and the CaMV35S: Renilla internal standard. Relative luciferase units were obtained by dividing the value obtained for the reporter gene by the value obtained for the internal standard. Arabidopsis leaves were also transfected with an empty Gal4DB in the presence of the reporter and internal standard and represent the baseline level of transcriptional activation. Data are reported as relative luciferase units where the transcriptional activation of each protein or protein pair is divided by the level of transcription from the empty Gal4DB. Values consist of n = 25 samples and represent averages \pm SD. Every bar represents five bombardments repeated five times.

4.5 DISCUSSION

Our study has demonstrated that TGA1 has two activities depending on the oxidation state of Cys260 and Cys266. The reduced form of TGA1, which interacts with NPR1 and is predominant after SA treatment, is likely involved in the expression of *PR1* (Després et al., 2003; Zhang et al., 2003; Kesarwani et al., 2007). The function of the oxidized, transactivating form of TGA1 is unclear, although it is believed to be NPR1-independent. Furthermore, our data illustrate that a novel glutaredoxin, which lacks classical glutaredoxin activity, specifically interacts with the reduced form of TGA1 to glutathionylate it, abrogating interaction with NPR1 thereby providing a mechanism by which the TGA1-NPR1 complex is regulated.

4.5.1 The Transactivation Ability that Results from the Oxidation of Cys260 and Cys266 Suggests a Mutually Exclusive Regulation of TGA1 Through a Novel Redox Switch

Després et al. (2003) initially established that the ability of TGA1 to interact with NPR1 is dependent on the redox status of Cys260 and Cys266 in yeast and Arabidopsis leaves. When these two cysteines are oxidized, TGA1 is not able to interact with NPR1. However when these two cysteines are reduced, TGA1 is competent to interact with NPR1 (Table 1). We further expanded on TGA1's behavior by examining its ability to activate transcription in yeast. We observed that transactivation of TGA1 only occurs when Cys260 and Cys266 are oxidized. When these two cysteines are mutated, which mimics a reduced state by abrogating oxidation, transactivation was abolished (Table 1). This correlates with data from Després et al. (2003) who observed, using a transient transcription assay in plants, that TGA1 activates transcription prior to SA treatment

only, which corresponds to a state when Cys260 and Cys266 are oxidized (Table 1). To date, NPR1 is the only other example of a protein whose transactivation domain is positively regulated by cysteine oxidation (Rochon et al., 2006). Interestingly, the transactivation ability of TGA1 is not required for the transactivation of the TGA1-NPR1 complex that forms in Arabidopsis leaves (Figure 2C and D). Instead, a novel transactivation domain identified in the C-terminus of NPR1 (Rochon et al., 2006) is responsible for the transactivation of the complex (Figure 2F, compare TGA1:DB + NPR1 to TGA1:DB + NPR1C521S/C529S).

Based on the above results, the behavior of TGA1 appears to be regulated in a mutually exclusive manner through a novel redox switch. A classical redox switch is normally regulated such that one state, either oxidized or reduced, is the active or ON form of the protein while the other state constitutes the OFF or inactive form (Helmann, 2002; Shelton et al., 2005). Contrastingly, the TGA1 redox switch appears to be unique as the redox status appears to regulate which activity TGA1 participates in rather than if it is ON or OFF; the oxidized form of TGA1 activates transcription while the reduced form interacts with NPR1, neither form seems to constitute an OFF position.

4.5.2 Cys260 is Critical for TGA1 to Activate Transcription and Interact with GRX480 while Cys266 plays a Minor, Albeit Important Role

Although TGA1 has four cysteines, Cys172 and Cys287 are not involved in regulating TGA1's transactivation ability (Figure 1B). A double mutant of Cys260 and Cys266 in TGA1 has already been shown to abolish transactivation (Figure 1B and Després et al., 2003). Examining the contribution of individual cysteines revealed that Cys260 plays a more important role in regulating TGA1's transactivation ability than

Cys266 (Figure 1B, compare TGA1:DB to TGA1C260N:DB and TGA1C266S:DB). However, while a TGA1 mutant where only Cys260 is wild-type is able to activate baseline transcription above (Figure 1C. compare Gal4DB TGA1C172N/C266S/C287S:DB), the presence of Cys266 is required to restore transactivation to the level of wild-type TGA1 (Figure 1C, compare TGA1 C172N/C266S/C287S:DB to TGA1C172N/C287S:DB). Additionally, mutating Cys266 in the presence of Cys260 has a more drastic affect on TGA1's transactivation than just Cys260 mutated on its own (Figure 1B, compare TGA1C260N/C266S:DB to TGA1C260N:DB). Kim et al. (2002) observed a similar phenomenon with the redox regulated transcription factor OxyR. They determined that the mutation of one critical cysteine in OxyR abolished transactivation but optimal transcriptional activity required the presence of the critical cysteine plus a second cysteine nine amino acids away. The Nterminus of TGA1 is also involved in regulating the transactivation ability of TGA1 (Figure 1D) indicating that Cys260, Cys266 and the N-terminal 83 amino acids of TGA1 are necessary and sufficient for transcriptional activation.

The ability of TGA1 to interact with GRX480 depends differentially on the presence and redox status of Cys260 and Cys266 in TGA1. In the plant two-hybrid system, when Cys260 is mutated, interaction with GRX480 prior to SA treatment is not affected whereas the enhanced interaction in response to SA is abolished (Figure 3B, compare TGA1:DB + GRX480:TA to TGA1C260N:DB + GRX480 white and grey bars). Mutation of Cys266 on the other hand, results in a level of interaction between TGA1 and GRX480 before SA treatment comparable to that observed between wild-type TGA1 and GRX480 following SA treatment (Figure 3B, compare TGA1:DB + GRX480:TA (grey

bar) to TGA1C266S:DB + GRX480:TA (white bar)). These data, combined with the results from Table 1, imply that interaction with GRX480 prior to SA treatment is prevented by the oxidation of Cys266. Interaction between TGA1 and GRX480 after SA treatment does not differ upon Cys266 mutation indicating that it only negatively regulates interaction in unstimulated cells while its reduction is required for interaction following SA treatment. The importance of Cys260 in TGA1's interaction with GRX480 is more complex. Up to this point, we have assumed that the mutation of a cysteine is synonymous to a reduced state however this is not sufficient to explain the observations in this instance. If the oxidized versus reduced state of Cys260 was dictating interaction with GRX480, then interaction between GRX480 and wild-type TGA1 or TGA1C260N should be the same after SA treatment. However, this is not the case (Figure 3B, grey bars, compare TGA1:DB + GRX480:TA to TGA1C260N:DB + GRX480:TA). It therefore appears that the presence of a cysteine at position 260, in combination with its redox status, is required for interaction with GRX480. This is further supported by the observation that the enhanced interaction between TGA1 and GRX480 in the absence of SA is abolished when Cys260 is mutated in combination with Cys266 (Figure 3B, white bars, compare TGA1C260N/C266S:DB + GRX480:TA to TGA1C266S:DB + GRX480:TA).

4.5.3 The Novel Glutaredoxin GRX480 Regulates TGA1's Redox Status and Activity through Glutathionylation of Cys260

The selective interaction of the novel glutaredoxin GRX480 with the reduced form of TGA1 over the glutathionylated form (Figure 4A) and the lack of activity in a classical glutaredoxin assay (Table 3) suggests GRX480 does not behave as a classical

glutaredoxin. Instead, our data demonstrates that GRX480 is capable of catalyzing the glutathionylation of Cys260 in TGA1 using GSSG as a substrate (Figure 7A). The fact that we did not observe this activity for the classical glutaredoxin GRX030 (Figure 7B) suggests that the novel class of glutaredoxins has potentially diverged in plants to fulfill the role of glutathionylation while the classical glutaredoxins maintain the function of reducing disulfides.

The reduction of a disulfide by a glutaredoxin, whether between two proteins or with glutathione, involves the N-terminal cysteine of the active site. The pK_a of a free cysteine is approximately 8.3, however the pK_a of the N-terminal cysteine in a glutaredoxin active site is significantly lower due to stabilization by neighboring positively charged amino acids (Hofmann et al., 2002; Jao et al., 2006). This lower pK_a provides the N-terminal cysteine with the reactivity to perform the first step in a reduction which is to attack a cysteine in a disulfide bond or the glutathione in a mixed disulfide (Gravina and Mieyal, 1993; Yang et al., 1998). The inability of GRX480 to reduce a mixed disulfide might be the result of a shift in the redox potential due to the amino acids between the two cysteines (CX_1X_2C) in the glutaredoxin active site (Joelson et al., 1990; Aslund et al., 1997). In support of this, several groups have demonstrated that the redox potential of thioredoxins could be made more oxidizing or more reducing by simply substituting the residues of X_1 and X_2 (Krause et al., 1991; Grauschopf et al., 1995).

The glutathionylation activity and late expression of *GRX480* after SA treatment (Figure 8A) suggest that GRX480 may play a role in regulating the TGA1-NPR1 complex. Indeed, addition of GRX480 in the presence of TGA1 and NPR1, in the

absence or presence of SA, results in a loss of transactivation of the complex (Figure 8B), presumably through GRX480's glutathionylation of TGA1 which abolishes its interaction with NPR1. The loss of transactivation of the TGA1-NPR1 complex in the absence of SA would suggest that the interaction between TGA1 and GRX480 as well as GRX480's glutathionylation of TGA1 is not dependent on SA. More likely, expression of GRX480 to a certain level in response to SA is required before sufficient interaction and glutathionylation of TGA1 can occur. This would also suggest that the effect of SA in enhancing the interaction between TGA1 and GRX480 observed in the plant two-hybrid (Figure 3A) is indirect, resulting from the SA-dependent reduction of TGA1, which is the form that GRX480 interacts with. The low level of interaction before SA treatment can be attributed to the population of reduced TGA1 that exists (Després et al., 2003)

The results presented here report one of the first instances where a novel glutaredoxin target has been identified in plants and the first instance where a biochemical activity of a member of the novel class of plant glutaredoxins has been described. It is also one of the first instances where a redox switch has been defined which regulates two different functions of the same protein. Two roles for TGA1 based on the two different activities regulated by its redox status are supported by tga1-1 Arabidopsis mutants. Kesarwani et al. (2007) observed that these mutants, untreated, were compromised in basal disease resitance, while PR1 gene expression was lower following INA treatment. A model is illustrated in Figure 9 depicting how the two redox active forms of TGA1 behave before and during SAR. The treatment leading to the complete oxidation of Cys260 and Cys266 resulting in the transcriptionally active form of TGA1 is postulated to proceed via a yet identified oxidative stress whereas the exact

mechanism of how GRX480 returns TGA1 to the oxidized form observed in unchallenged Arabidopsis cells is unclear. It is possible that the glutathionylation of Cys260 in TGA1 is transient and only serves to impede interaction between TGA1 and NPR1. This glutathionylation could then collapse to an intramolecular disulfide bond as a consequence of the proximity of Cys266 (Shelton et al., 2005), resulting in the oxidized form of TGA1 believed to exist in resting Arabidopsis cells (Després et al., 2003).

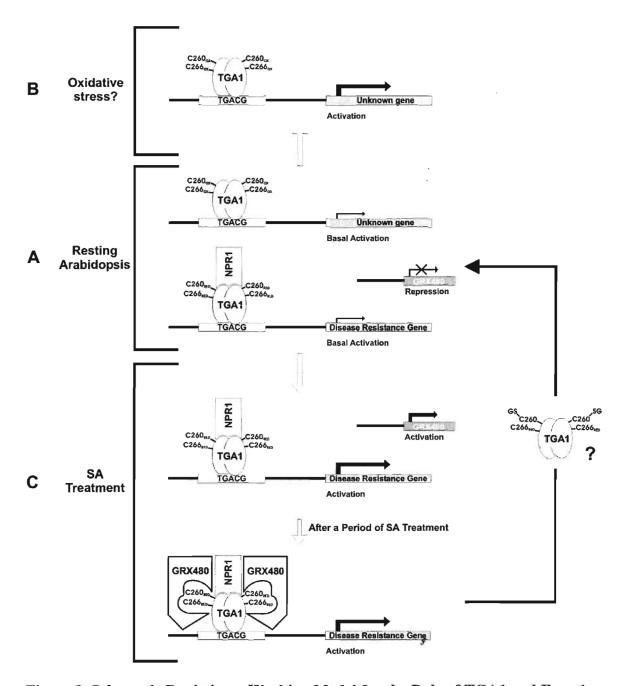


Figure 9. Schematic Depicting a Working Model for the Role of TGA1 and Function of GRX480 in SAR

(A) In resting Arabidopsis cells, TGA1 is present as a dimer in two approximately equal pools; one where Cys260 and Cys266 are oxidized and the other where these two cysteines are reduced. Both forms are able to bind the cognate DNA motif TGACG (Després et al., 2003). The reduced form of TGA interacts with NPR1 and this form is hypothesized to activate transcription of a yet identified disease resistance gene whereas the oxidized form of TGA1, which transactivates on its own, activates transcription of an unknown gene. Since neither form is present in higher than 50%, it is possible that the activation of their respective genes is only at the basal level. Expression of *GRX480* is repressed or expressed at a very low level in resting Arabidopsis cells.

- (B) An oxidative stress, like the oxidative burst that occurs early in SAR, could be responsible for completely oxidizing Cys260 and Cys266 in TGA1. This would result in the activation of the expression of the unknown gene. An immediate-early gene seems like a plausible target for oxidized TGA1 since it is expressed immediately after the onset of SAR (corresponds with the oxidative burst) and its expression is only transient, lasting for several hours at the most (Horvath and Chua, 1996).
- (C) After SA treatment, NPR1 nuclear localizes (Kinkema et al., 2002; Mou et al., 2003) and TGA1 is completely reduced. This results in optimal interaction between the two proteins which in turn results in *PR1* activation. *GRX480* expression is also induced under these conditions and after a period of SA exposure, sufficient levels of GRX480 protein have been translated that it interacts with TGA1, glutathionylates it at cysteine 260 (-SG) resulting in its oxidation which abrogates its interaction with NPR1. This returns the TGA1 pool back to the 50/50 distribution observed in resting cells. The exact mechanism of how Cys260 glutathionylation (indicated by arrow returning to resting Arabidopsis, C260-SG) results in the oxidized state observed for both Cys260 and Cys266 remains unclear. It is possible that glutathionylation of Cys260 is transient and only serves to disrupt the TGA1-NPR1 interaction. Once the interaction has been disrupted, a disulfide is formed between Cys260 and Cys266 which is the form postulated to exist in resting Arabidopsis cells (Després et al., 2003).

CHAPTER 5 – GENERAL DISCUSSION AND CONCLUSIONS

NPR1 is a key modulator of SAR and its overexpression in several plant species results in enhanced resistance with little to no side-effects (Lin et al., 2004; Malnoy et al., 2007). However, little is known about the mechanism by which NPR1 regulates gene expression. TGA TFs are known to bind *as-1* and *as-1-like* elements, the latter of which is over-represented in the promoters of many defense related genes. The fact that NPR1 interacts with these TGA TFs provides a means for NPR1-regulated gene expression in SAR. However the interaction between NPR1 and TGA TFs is complex and studies such as the two presented in this thesis help to unravel the complexities and expand our understanding of the SAR pathway.

5.1 The Behavior of TGA1 and TGA2 in SAR

Arabidopsis has ten TGA TFs, seven of which has been tested with respect to their ability to interact with NPR1. Based on sequence similarity, the seven TGA TFs have been clustered into three clades: (i) TGA1 and 4, (ii) TGA2, 5 and 6, and (iii) TGA3 and 7. In yeast, only the last two clades interact with NPR1 while the first clade, containing TGA1 and 4, does not. However work done by Després et al. (2003) revealed that TGA1 is able to interact with NPR1 in Arabidopsis leaves after SA treatment. This resulted in the discovery that TGA1's interaction with NPR1 is redox regulated such that Cys260 and Cys266 need to be reduced for interaction to occur. The lack of interaction observed for TGA1 and NPR1 in yeast can be attributed to the oxidation of Cys260 and Cys266 in TGA1. Additionally, we discovered that the transcriptional activation ability of TGA1 is controlled by the oxidation of these two cysteines. This led to the conclusion

that the activity of TGA1 is regulated in a mutually exclusive manner such that the oxidation of Cys260 and Cys266 preclude interaction with NPR1 but allow transactivation and vice versa when reduced.

Similar to TGA1, TGA2 interacts with NPR1 following SA treatment in Arabidopsis leaves. Both the TGA1- and TGA2-NPR1 complexes are able to activate transcription and rely on the transactivation domain identified in the C-terminus of NPR1 (Rochon et al., 2006). Interestingly, although TGA1 is able to activate transcription, its transactivation domain is not involved in the transactivation of the complex. Evidence presented in Chapter 3 reveals that the TGA2-NPR1 complex that results following SA treatment is involved in activating *PR1* expression while the role of the TGA1-NPR1 complex has not been directly addressed. We have preliminary evidence that suggests that the TGA1-NPR1 complex is able to activate the expression of a reporter gene under the control of the *PR1* promoter.

Prior to SA treatment, we established that TGA2 acts as a repressor using a modified transient transcription assay in Arabidopsis (Rochon et al., 2006). We then went on to show that TGA2 represses the NPR1-dependent activation of *PR1* supporting evidence from Zhang et al. (2003) who observed an increase in *PR1* expression in untreated TGA2, 5, 6 knock-out Arabidopsis line compared to wild-type. The function of TGA1 before SA treatment is unclear although it appears as though it could be two-fold as TGA1 exists in approximately two equal populations, reduced and oxidized, and these two different forms of TGA1 have different functions (Després et al., 2003 and Chapter 4). In contrast to TGA2, oxidized TGA1, which is only observed before SA treatment, acts as a transcriptional activator. Despite the lack of a defined role before SA treatment,

TGA1 appears to be critical for basal resistance based on results from Kesarwani et al. (2007) who observed that a *tga1-1* Arabidopsis mutant supported a higher level of pathogen growth compared to untreated wild-type plants.

5.2 Redox Regulation as a General Mechanism Controlling Gene Expression during SAR

Activation of certain aspects of the SAR pathway appears to be regulated by a population of pre-existing or latent proteins whose activity is regulated, in part, by PTMs. The redox environment of the cell plays an important role in regulating the PTM of proteins and in SAR. An increase in ROS (Lamb and Dixon, 1997) and a shift in the GSH/GSSG ratio (Mou et al., 2003; Ball et al., 2004) can act to mediate this regulation.

TGA1 and NPR1 are two proteins in the SAR pathway whose activities are modulated by their redox status. Both TGA1 and NPR1 contain a transactivation domain that is activated by an oxidized state. Interestingly, in both instances, the transactivation domain is regulated by two cysteines. In the case of TGA1, Cys260 appears to be more critical for transactivation however Cys266 is required for optimal transactivation. For NPR1, the dependence on each cysteine for transactivation is equal (Rochon et al., 2006). Only one other protein has been identified that contains, a transactivation domain regulated by its redox status, however it functions in the opposite manner such that oxidation prevents transactivation (Morel and Barouki, 2000). Another feature shared by TGA1 and NPR1 is that the reduction of both proteins is required for their interaction inside the nucleus (Després et al., 2003; Mou et al., 2003). These two trends in the redox regulation of TGA1 and NPR1 hint at the possibility that an overall trend of this nature may exist to regulate proteins involved in SAR.

5.3 GRX480 and SAR

There are 31 glutaredoxins in Arabidopsis and 21 of these constitute a novel class only observed in higher plants. This novel glutaredoxin class first appeared in bryophytes and has since expanded during the evolution of land plants suggesting an evolutionary requirement for its divergence (Lemaire, 2004; Xing et al., 2006). Classical glutaredoxins have either a CXXC-type or CXXS-type active site while the active site of the novel class contains a cysteine at the second position and is defined as the CCXX-type. GRX480 is part of the novel class of glutaredoxins with a CCMC active site and interacts with the reduced form of TGA1 to glutathionylate it at Cys260. Mammalian Grx1 (Starke et al., 2003) and Grx2 (Beer et al., 2004) are the only other glutaredoxins know to have glutathionylation activity. Ndamukong et al. (2007) observed that GRX480 is also able to interact with Arabidopsis TGA2, however the significance of this interaction is unclear as there is no evidence that TGA2, although it does contain one cysteine, is redox regulated. If TGA2 is not redox regulated, interaction with GRX480 might be required to recruit GRX480 to NPR1 to maintain the oxidation of Cys521 and Cys529 and therefore the transactivation ability of NPR1 after SA treatment (Rochon et al., 2006). Ndamukong et al. (2007) observed that NPR1 does not directly interact with GRX480 in a yeast twohybrid assay however when TGA2 is included, not fused to any domain, it is able to act as a bridge bringing GRX480 and NPR1 together.

Further intrigue in the GRX480-TGA TF relationship comes from the presence of half an *as-1-like* element in the GRX480 promoter (Ndamukong et al., 2007). The classical *as-1-like* element consists of two imperfect palindromes but GRX480 only has one of these palindromes. Niggeweg et al. (2000) found that there is a preference for

tobacco TGA hetero- and homodimers binding to one or both palindromes of an *as-1-like* element. Tobacco TGA2.1 and TGA2.2 as hetero- or homodimers allow occupation of both palindromes although the preference for single over double occupancy differs depending on the partners in the dimer. TGA1a on the other hand, as a homodimer and as a heterodimer with either TGA2.1 or TGA2.2, only allows single occupancy of the *as-1-like* element (Niggeweg et al., 2000). The presence of part of an *as-1-like* element in the promoter of GRX480 suggests a possible mechanism for auto-regulation, which is supported by the observation that GRX480 expression is abolished in the TGA2, 5, 6 knock-out (Ndamukong et al., 2007).

5.4 Future Experiments

Future experiments should focus on the redox status of proteins involved in SAR as this PTM appears to be an important regulatory principle in plant defense. I would examine the redox status of members of the other two clades of TGA TFs as well as examine different molecules (i.e. 2,4-D, H₂O₂, MeJA and ethylene) that would be responsible for triggering a shift in the redox status. I would also examine the nature of the oxidized state of TGA1 and NPR1 (i.e. glutathionylation versus a disulfide bond or other PTM modification of cysteine including hydroxylation and nitrosylation). It would also be of interest to identify targets of TGA1 in Arabidopsis through ChIP experiments. These experiments would be conducted in the absence and presence of SA. Immediate-early genes would be targeted in the absence of SA treatment based on results from Johnson et al. (2001b) in tobacco and *PR1* and other *PR* genes activated during SAR would be examined prior to and following SA treatment.

Determining enzyme activity for GRX480 is also critical. Initially it would be defined with respect to TGA1 and GSSG as substrates; radioactive glutathione ([³⁵S]-GSH) would be employed, in an oxidized form, to establish the K_m, V_{max} and K_{cat}. Specificity for GSSG as a substrate would also be established as its concentrations are very low and availability may be an issue. Alternative donors would be the GS-thiyl radical (GS•) and S-nitrosoglutathione (GS-NO) (Shelton et al., 2005).

Overall, the data presented in this thesis furthers our understanding of how TGA1, TGA2 and NPR1 behave in SAR. We may have also identified another level of regulation for the SAR defense pathway, redox regulation, which provides an additional target that could be studied and manipulated to enhance disease resistance.

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APPENDIX

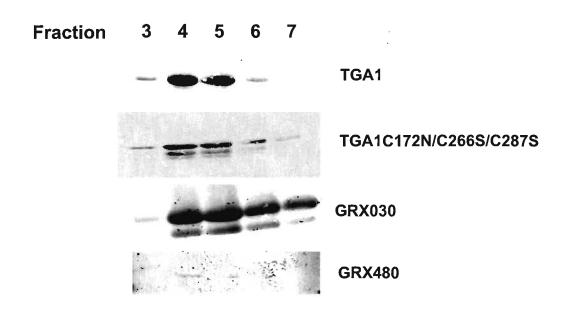


Figure 1: Immunoblots of Proteins Purified by a HIS column.

- (A) Wilt-type TGA1 was expressed in *E. coli* and purified on a HIS column. Approximately 250mL of culture, loaded onto the column as 2mL, was purified and 500µL fractions were collected (see Section 4.3.10 and 4.3.11). 5% of each fraction was electrophoresed on a 10% SDS-polyacrylamide gel along with an equivalent amount of crude, flow through and wash samples. TGA1 was detected with His-probe (H15) primary antibody at 1/1000 and goat anti-rabbit: Alexa fluor 680 at 1/15000 was used as the secondary antibody in coordination with infrared imaging using the Odyssey imager (LI-COR, Lincoln, NE).
- (B) TGA1C172N/C266S/C287S was expressed in E. coli and freated as described in (A).
- (C) GRX030 (At5g63030) was expressed in *E. coli* and treated as described in (A) except proteins were electrophoresed on a 12% gel.
- (D) GRX480 (At1g28480) was expressed in *E. coli* and treated as described in (C) except that 2.5L of culture was loaded onto the column as approximately 30mL. The method of protein isolation also differed (see Section 4.3.10). 10% of each fraction was electrophoresed.
- In (A), (B), (C) and (D) only fractions 3 to 7 are shown. Detection of the band of interest is shown and while detection in fractions other than 4 and 5 is observed, these lanes were contaminated by other proteins detected by coomassie staining (data not shown).

Table 1. The Glutaredoxin Family in Arabidopsis thaliana

Accession Number	Туре	Active Site	Polypeptide Length (amino acids)	Putative localization ^a
At1g03020		CCMS	102	С
At1g03850		CCLG	150	C or M or P
At1g06930		CCLS	99	C
At1g28480		CCMC	137	C
At2g30540		CCMS	103	C
At2g47870		CCMC	103	C
At2g47880		CCLC	102	C
At3g02000		CCMC	136	C
At3g21460		CCMS	102	M
At3g62930	Novel	CCMS	103	C
At3g62950	CCXX	CCMC	102	C
Atg3g62960	Type	CCLC	103	C
At4g15660		CCMS	102	C
At4g15670		CCMS	102	C
At4g15680		CCMS	102	C
At4g15690		CCMS	102	C
At4g15700		CCMS	102	C
At4g33040		CCMC	145	C
At5g11930		CCMC	145	C or M or P
At5g14070		CCMC	140	C
At5g18600		CCMS	102	C
At1g77370		CPYC	130	S or C
At2g20270	Classical	CSYS	179	P
At4g28730	Classical CXXC	CFIS	176	P
At5g20500		CPYC	135	S or C
At5g40370	Type	CPYC	111	C or M or P
At5g63030		CGYC	125	C
At2g38270	Classia 1	CGFS	283	P
At3g15660	Classical	CGFS	169	M or P
At3g54900	CXXS	CGFS	173	M or P
At4g04950	Type	CGFS	488	C

All data was compiled from Rouhier et al. (2004) and Lemaire et al. (2004). In bold are the two Grxs discussed in the thesis.

Abbreviations: C – cytosolic; M – mitochondrial; P – Plastidial; S – secreted protein.

^aPutative localizations are based on several prediction softwares (TargetP, Predator and Psort; see references for details). More than one localization is listed in instances where prediction softwares gave different results.

Table 2. Mass List of Untreated, Trypsin Digested TGA1 from MALDI TOF MS

	Signal to	
m/z ^a	Noise ^b	Intensity ^c
832.637	161	11281.15
833.637	85.3	5976.65
834.630	37.7	2639.68
1011.831	183.7	14373.89
1012.835	109.2	8541.15
1013.823	32.6	2585.43
1140.954	14.7	1251.73
1141.949	10.1	855.83
1142.976	10.5	896.86
1257.064	25.3	2288.25
1258.057	17.2	1556.08
1267.092	513.9	47351.3
1268.103	419.7	38668.09
1269.105	185.9	17129.51
1270.106	53.7	4950.54
1271.090	13.2	1219.79
1289.084	9	829.91
1324.130	20.4	1911.29
1325.135	14.8	1383.44
1410.126	27.2	2605.9
1411.151	21.7	2092.47
1412.153	12.9	1248.64
1426.117	17.2	1654.14
1427.152	15.6	1504.09
1446.118	14.3	1385.79
1447.130	11.4	1101.64
1462.093	30.2	2926.55
1463.102	23.9	2315.89
1464.104	13.1	1265.52
1478.085	27.7	2692.01
1479.094	21.8	2118.68
1480.099	12	1163.34
1517.252	11.2	1090.78
1518.267	10.2	992.6
1538.068	8.9	864.5
1588.292	16.4	1586.63
1589.334	13.1	1266.34
1624.254	7.9	759.48
1625.262	8.5	821.23
1640.251	22.6	2158.22

Table 3. Mass List of GSSG Treated, Trypsin Digested TGA1 from MALDI TOF MS

	Signal to	
m/z^{a}	Noise ^b	Intensity
755.507	7.9	626
755.632	6.7	534
755.702	4.8	382
756.430	4.2	332
772.506	7.9	627
832.559	104.6	. 8343
833.527	53.5	4264
833.605	52.6	4194
834.557	22.5	1792
834.648	19.2	1533
835.455	6.4	509
835.529	6.5	518
835.643	6.1	483
842.644	5.6	444
886.606	4.7	376
1011.697	212.4	17780
1012.711	124.1	10386
1013.697	34.6	2900
1014.716	7.6	634
1033.649	5.8	486
1140.804	34.6	2888
1141.784	20.9	1746
1141.851	19.3	1615
1142.680	4.1	345
1142.797	8	668
1142.912	6	500
1161.293	6.1	506
1161.529	4.3	359
1162.343	4.3	359
1168.836	5.3	438
1169.752	6.1	511
1169.892	5.3	439
1194.811	12.4	1026.5
1195.818	7.9	656.5
1196.812	4.2	350.5
1205.744	6.7	551.5
1221.758	4.6	378
1253.300	4.3	351.5
1256.899	36.6	3018.5
1256.900	38.9	3210.5

1641 054	21.2	2020.21
1641.254	21.2	2030.31
1642.254	12.3	1174.77
1712.432	106.5	10026.18
1713.438	105.1	9897.02
1714.446	50.4	4745.28
1715.442	17.5	1651.38
1796.560	133.8	12404.48
1797.569	145.4	13482.74
1798.582	76.4	7079.81
1799.593	29	2687.67
1800.589	12.8	1190.61
1812.553	47.5	4398.92
1813.562	49.5	4585.19
1814.569	28.5	2639.38
1815.579	9.5	881.61
1828.565	31	2869.87
1829.569	32.8	3038.62
1830.566	18.5	1709.75
1958.681	13.7	1154.06
1959.708	15	1268.13
1960.715	8.6	724.33
2075.754	9.9	763.75
2076.759	13.7	1058.28
2077.778	9.5	734.65
2111.622	12.6	947.53
2112.630	15.2	1144.02
2113.641	11	827.87
2127.624	33.9	2550.85
2128.635	42.9	3122
2129.633	26.3	1919.14
2130.633	11.8	858.48
2144.693	9.9	721.84
2219.778	14.5	990.94
2220.792	14.1	964.91
2231.946	50.5	3448.84
2232.969	69.3	4730.26
2233.933	47.6	3254.11
2234.908	20.4	1391.98
2267.764	45.7	3045.28
2268.776	61.6	4101.28
2269.787	42.8	2848.25
2270.789	21.3	1420.33
	12.2	813.02
2271.765		
2283.794	71	4560.1
2284.796	91.2	5856.27

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1699.461 11.9 814	1697.651	6.5	443
	1698.462	11.6	793
1700.491 5.7 383	1699.461	11.9	814
	1700.491	5.7	383

2285.798	66.3	4262.24
2286.812	31	1989.89
2287.815	12.8	823.44
2288.853	14.6	938.4
2289.850	22.6	1448.82
2290.829	18.4	1180.13
2299.808	14.1	908
2300.812	17.4	1119.66
2301.804	12.2	785.24
2446.946	13.8	747.88
2481.080	68.1	3678.79
2482.074	100	5177.54
2483.073	74.6	3864.4
2484.062	39	2019.35
2485.059	15.3	793.79

Mass spectra were obtained using Autoflex II TOF/TOF (Bruker Daltonics, Billerica, MA) in positive mode and 9% laser power. The mass list was obtained using FlexAnalysis Version 3.0 (Bruker Daltonics).

1712.192	681.4	45716
1713.201	659.8	44265
1714.213	329.8	22125
1715.217	109.2	7324
1716.247	28.9	1941
1717.235	9	606
1720.458	4.4	298
1726.229	5.1	341
1727.228	4.1	278
1734.190	23.5	1544
1735.188	22.3	1467
1736.181	10.4	684
1737.210	5.4	353
1750.176	4.6	305
1751.158	4.5	293
1829.197	4.6	294
1994.265	20.3	1166
1995.265	21.6	1238
1996.263	11.1	635
1997.227	4.9	283
2231.512	11.7	578
2232.540	17.2	853
2233.542	9.5	470
2234.563	5.4	267
2238.487	4.3	213
2267.378	7	341
2268.382	8.5	414
2269.374	8.5	414
2270.374	4.5	218
2283.376	14.1	675
2284.373	20.1	960
2285.383	12.4	594
2286.368	*7	334
2289.409	8.2	392
2290.403	6.3	303
2299.361	6.4	306.5
2300.377	6.6	318
2301.383	5	240.5
2393.651	8.8	406
2394.742	10.7	498
2395.702	8	372
2396.682	4.1	191
2431.491	4	184
2445.495	10	450
2446.485	14.3	646
~ 1 TO: TOD	1-1.0	0+0

^a m/z represents the charge to mass ratio for each peptide.

b The signal to noise ratio is defined as the height of the mass peak above its baseline relative to the standard deviation of the noise.

^c The intensity indicates the relative abundance of each mass peak in the sample

2447.497	9	404
2448.493	7.1	321
2706.437	4.4	172.5
2728.673	7.5	296.5
2729.692	9.7	383.5
2730.686	8.3	327.5
2731.692	5.9	226.5
2749.628	5	194.5
2750.608	6.4	246.5
2751.637	6.5	250.5
2752.641	5.5	213.5
2808.593	5.2	195
2969.681	4.1	139
2969.866	4.2	141.25
2970.702	5.7	192
2971.755	5.1	170.5
2992.916	16.2	546.5
2993.062	18.5	625.5
2994.031	37.4	1231.5
2994.898	26.4	867.5
2995.052	26.8	882.5
2996.022	16.3	536.5
2996.058	15.5	509.5
2996.561	6.2	205.5
2997.013	7.1	233.5
2997.228	5.7	188.5
3005.643	4.2	138.5
3028.820	17.5	575.5
3029.825	32	1052.5
3030.800	27	886.5
3031.814	17.1	561.5
3032.802	9	294.5
3033.765	4.*7	154.5
3033.889	5.2	169.5
3044.838	14.8	474.5
3045.849	30.8	987.5
3046.860	26.4	846.5
3047.856	15.3	490.5
3048.785	7.2	230.5
3049.788	4.7	150.5
3061.855	6.7	214
3062.812	4.1	131
3339.125	8.1	221
3340.129	12.8	347

