TGA2 dual activity: N-terminus regulation of reversible DNA binding influenced by NPR1 and CK2

By

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ABSTRACT

TGA2 is a dual-function Systemic Acquired Resistance (SAR) transcription factor involved in the activation and repression of pathogenesis-related (PR) genes. Recent studies have shown that TGA2 is able to switch from a basal repressor to activator, likely, through regulatory control from its N-terminus. The N-terminus has also been shown to affect DNA binding of the TGA2 bZIP domain when phosphorylated by Casein Kinase II (CK2). The mechanisms involved for directing a switch from basal repressor to activator, and the role of kinase activity, have not previously been looked at in detail. This study provides evidence for the involvement of a CK2-like kinase in the switch of TGA2 activity from repressor to activator, by regulating the DNA-binding activity of TGA2 by phosphorylating residues in the N terminus of the protein.
TABLE OF CONTENTS

ABSTRACT ................................................................................................................................. 2

TABLE OF CONTENTS ............................................................................................................ 3

LIST OF FIGURES .................................................................................................................. 6

LIST OF TABLES .................................................................................................................... 7

LIST OF ABBREVEATIONS ................................................................................................... 8

CHAPTER 1: INTRODUCTION .............................................................................................. 11

CHAPTER 2: LITERATURE REVIEW ..................................................................................... 14

2.1 Systemic Acquired Resistance ......................................................................................... 14

2.1.1 SA required for SAR activation .................................................................................... 14

2.1.2 PR genes ..................................................................................................................... 15

2.2 NPR1/NIM1 .................................................................................................................... 16

2.2.1 NPR1 interacting proteins (TGAs, NIMIN1) ............................................................... 18

2.2.2 SNI1 .......................................................................................................................... 19

2.2.3 NPR1 cross-signaling .................................................................................................. 19

2.3 BZIP transcription factors .............................................................................................. 20

2.3.1 C terminal leucine zipper ........................................................................................... 21

2.3.2 N terminal regulatory domain .................................................................................... 26

2.4 TGA transcription factors ............................................................................................... 26

2.4.1 Group I - TGA1, TGA4 ............................................................................................... 27

2.4.2 Group III - TGA3, TGA7 ............................................................................................ 27

2.4.3 Group II - TGA2, TGA5, TGA6 .................................................................................. 29

2.5 TGA2 ................................................................................................................................ 30

2.5.1 Dual Activity of TGA2 ............................................................................................... 30

2.5.2 Interaction with NPR1 and stoichiometric regulation .................................................. 31

2.5.3 TGA2 interactions with PR-1 promoter elements ....................................................... 33

2.6 CK2 Regulation of TGA2-DNA binding ......................................................................... 36
<table>
<thead>
<tr>
<th>Section</th>
<th>Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.6.1</td>
<td>Phosphorylation of Transcription factors</td>
</tr>
<tr>
<td>2.6.2</td>
<td>CK2 in plants</td>
</tr>
<tr>
<td>2.6.3</td>
<td>CK2 as a regulator of TGA activity</td>
</tr>
<tr>
<td>2.7</td>
<td>Study Objectives</td>
</tr>
</tbody>
</table>

CHAPTER 3: MATERIALS AND METHODS | 41 |
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1 Chemicals and Buffers</td>
<td>41</td>
</tr>
<tr>
<td>3.1.1 Media for <em>E. coli</em></td>
<td>41</td>
</tr>
<tr>
<td>3.1.2 Antibiotics</td>
<td>41</td>
</tr>
<tr>
<td>3.1.3 Buffers</td>
<td>41</td>
</tr>
<tr>
<td>3.2 Plasmid Methods</td>
<td>44</td>
</tr>
<tr>
<td>3.2.1 Bacterial Strain and Plasmids</td>
<td>44</td>
</tr>
<tr>
<td>3.2.2 Plasmid Transformation</td>
<td>44</td>
</tr>
<tr>
<td>3.3 Protein Methods</td>
<td>44</td>
</tr>
<tr>
<td>3.3.1 Protein Expression</td>
<td>44</td>
</tr>
<tr>
<td>3.3.2 His- tag protein purification</td>
<td>45</td>
</tr>
<tr>
<td>3.3.3 Strep- tag protein purification</td>
<td>46</td>
</tr>
<tr>
<td>3.4 Experimental Methods</td>
<td>47</td>
</tr>
<tr>
<td>3.4.1 Gel filtration analysis using S300 column for FPLC</td>
<td>47</td>
</tr>
<tr>
<td>3.4.2 Electrophoretic Mobility Shift Assay (EMSA)</td>
<td>48</td>
</tr>
<tr>
<td>3.4.3 His-tagged NT47 Pull-Down binding assay using nickel charged resin</td>
<td>49</td>
</tr>
</tbody>
</table>

CHAPTER 4: RESULTS | 50 |
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1 TGA2 phosphorylation sites located in the N terminal region may be important for regulation of DNA binding</td>
<td>50</td>
</tr>
<tr>
<td>4.2 N terminus may interact with other TGA2 domains to reduce DNA binding when phosphorylated</td>
<td>54</td>
</tr>
<tr>
<td>4.3 Inhibition of DNA binding by CK2 is partially restored by BTB/FOZ region of NPR1</td>
<td>60</td>
</tr>
<tr>
<td>4.4 NT TGA2 self association is inhibited by CK2</td>
<td>63</td>
</tr>
</tbody>
</table>
CHAPTER 5: DISCUSSION ........................................................................................................ 68

5.1 Phosphorylation of N terminus residues reduces TGA2-DNA binding.................. 68

5.2 Reversible TGA2-DNA binding is influenced by CK2 and NPR1 ......................... 71

5.3 Model of TGA2 dual activity ...................................................................................... 72

CHAPTER 6: CONCLUSIONS ................................................................................................. 74

CHAPTER 7: FUTURE STUDIES ............................................................................................. 75

6.1 CK effects of oligomerization on TGA2 .................................................................... 75

6.2 Interaction between Δ43 and NT47 .......................................................................... 75

REFERENCES ..................................................................................................................... 77

APPENDIX: S300 GEL FILTRATION .................................................................................... 87
LIST OF FIGURES

Figure 2.1 NPR1 is the central regulator of SA and JA responsive genes .......................... 17
Figure 2.2 GCN4 dimer bound to DNA........................................................................... 22
Figure 2.3 Interacting bZIP monomers ........................................................................ 23
Figure 2.4 TGA2.............................................................................................................. 25
Figure 2.5 Dendogram of Arabidopsis TGA factors ...................................................... 28
Figure 2.6 Dual Activity of TGA2.................................................................................. 32
Figure 2.7 From The BTB/POZ domain of NPR1 interacts with the N terminus of TGA2 and Precludes Binding of the TGA2 oligomer to DNA (Boyle et al., 2009). ................................................................. 34
Figure 4.1 CK2 phosphorylation of TGA2..................................................................... 52
Figure 4.2 Δ43 TGA2 and NT47 TGA2 interaction with LS7 probe.............................. 58
Figure 4.3 EMSA showing interaction between TGA2 and BTB/POZ region of NPR1 (POZ) after CK2 phosphorylation ................................................................. 62
Figure 4.4 N terminus association inhibited by CK2...................................................... 66
Figure 5.1 Phosphorylation of bZIP residues causes helical shift and reduced DNA binding, from (Kirchler, 2010)................................................................. 70
Figure 5.2 TGA2 dual activity is influenced by a CK2-like kinase and NPR1.............. 73
Figure A.1 Calibration curve for the S300 Gel Filtration Column............................... 88
LIST OF TABLES

Table A.1 Protein standards for the S300 Gel Filtration Column........................................87
Table A.2 Elution fractions for NT47.........................................................................................89
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>amino acid</td>
</tr>
<tr>
<td>Ala</td>
<td>alanine</td>
</tr>
<tr>
<td>ATP</td>
<td>adenoseine triphosphate</td>
</tr>
<tr>
<td>ARD</td>
<td>ankyrin repeat domain</td>
</tr>
<tr>
<td>as-1</td>
<td>activating sequence 1</td>
</tr>
<tr>
<td>ASF1</td>
<td>as-1-binding factor</td>
</tr>
<tr>
<td>Arg</td>
<td>arginine</td>
</tr>
<tr>
<td>Asn</td>
<td>asparagine</td>
</tr>
<tr>
<td>Avr</td>
<td>avirulence</td>
</tr>
<tr>
<td>BEF</td>
<td>bZIP enhancing factor</td>
</tr>
<tr>
<td>BTB/POZ</td>
<td>Broad-Complex, Tramtrack, and Bric-a-brac/Pox virus and Zinc finger</td>
</tr>
<tr>
<td>BTH</td>
<td>benzothiadiazole S-methylester</td>
</tr>
<tr>
<td>bZIP</td>
<td>basic leucine zipper</td>
</tr>
<tr>
<td>CaMV</td>
<td>cauliflower mosaic virus</td>
</tr>
<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
</tr>
<tr>
<td>CK2</td>
<td>Casein Kinase II</td>
</tr>
<tr>
<td>Cm</td>
<td>chloramphenicol</td>
</tr>
<tr>
<td>CT</td>
<td>C-terminus</td>
</tr>
<tr>
<td>Cys</td>
<td>cysteine</td>
</tr>
<tr>
<td>D</td>
<td>aspartate</td>
</tr>
<tr>
<td>DB</td>
<td>DNA binding domain</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DRB</td>
<td>5,6-Dichloro-1-β-D-ribofuranosyl benzimidazole</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>E</td>
<td>glutamate</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine-tetraacetic acid</td>
</tr>
<tr>
<td>EMSA</td>
<td>electromobility shift assay</td>
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</table>
ET ethylene
EYFP enhanced yellow fluorescent protein
FP free probe
FPLC fast protein liquid chromatography
Gal Galactose
GFP green fluorescent protein
GUS β-glucuronidase
HABA 2-4’-hydroxy-benzeneazobenzoic acid
His Histidine
HR hypersensitive response
INA 2, 6-dichoroisonicotinic acid
ISR induced systemic resistance
JA jasmonic acid
Km kanamycin
Leu leucine
LOX2 lipoxygenase2
LS linker scan
MQHzO Mili-Q HzO
NahG salicylate hydroxylase
Ni-NTA nickel-nitrilotriacetic acid
NIM1 non-inducible immunity 1
NIMIN NIM1-interacting
NLS nuclear localization sequence
NPR1 non-expressor of pathogenesis related genes 1 (also NPR3, NPR4)
NT N-terminus
ODx absorbance at wavelength (x)
PAGE polyacrylamide gel electrophoresis
PK protein kinase
PR Pathogenesis-related
PR-1 pathogenesis-related 1 (also PR-8, PR-13)
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>R</td>
<td>resistance</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>S</td>
<td>serine</td>
</tr>
<tr>
<td>SA</td>
<td>salicylic acid</td>
</tr>
<tr>
<td>SAR</td>
<td>systemic acquired resistance</td>
</tr>
<tr>
<td>SARP</td>
<td>salicylic acid binding protein</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>SNI1</td>
<td>suppressor of NPR1-1, inducible 1</td>
</tr>
<tr>
<td>T</td>
<td>threonine</td>
</tr>
<tr>
<td>Tyr</td>
<td>tyrosine</td>
</tr>
<tr>
<td>TF</td>
<td>transcription factor</td>
</tr>
<tr>
<td>TGA</td>
<td>TGACG motif binding</td>
</tr>
<tr>
<td>TMV</td>
<td>Tobacco Mosaic Virus</td>
</tr>
<tr>
<td>Vir</td>
<td>virulence</td>
</tr>
<tr>
<td>WRKY</td>
<td>WRKYGQR motif binding</td>
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CHAPTER 1: INTRODUCTION

Plants, in their natural environment, can encounter many different types of invaders or attackers such as herbivorous species, or pathogenic diseases and pests. From the outset, plants appear to have limited means of defending themselves against foreign attackers - they are limited in their ability to physically change their location, and do not have specialized mobile defender cells that are integral to many mammalian immune systems defenses (Jones and Dangl, 2006). Nevertheless, plants do have a number of defense mechanisms to ward off a potential attack.

Passive mechanisms such as physical barriers or toxic metabolites may be sufficient to ward off certain threats, by either making cell walls impassable or the ingestion of plant tissues unappealing or harmful. But in the event that these passive barriers fail, plants are able to induce cellular mechanisms at both the site of an invasion and in distant tissues that can either signal for a local hypersensitive response (leading to tissue cell death that kills pathogens and prevents their spread), or the coordination of a more systemic response (Slusarenko et al., 2000).

Launching a systemic response against pathogen invasion causes a long lasting broad defense response throughout the plant called Systemic Acquired Resistance (SAR). This mechanism is activated through chemical signaling processes that involve a complex cascade of effectors (Glazebrook et al., 2005; Jones and Dangl, 2006; Beckers and Spoel, 2006), and is capable of affecting a response both locally and systemically through activation of a series of defense genes called
pathogenesis related (PR) genes (Durrant and Dong, 2004). PR genes, in turn, are able to confer resistance against a wide variety of pathogens (Van Loon et al., 2006). The coordination of these genes involves the organization of a number of molecular transcription factors including positive and negative regulators of cellular systems that divert internal energies and resources towards defense (Durrant and Dong, 2004). PR genes have been linked to a range of antimicrobial activities, however, not all PR gene function has been characterized (Beckers and Spoel, 2006). The activation of specific PR genes may depend on the plant species, and the type of pathogen (Durrant and Dong, 2004).

Large scale array studies have been able to identify transcriptome elements that are upregulated or downregulated during SAR. However, due to the complexity and the number of events occurring at the same time, it is difficult to determine which are directly involved in the plant defense process. In certain cases, differences between susceptibility and resistance are related to timing and magnitude and not differences in gene transcription (Tao et al., 2003). Specific defense mechanisms may be only effective against certain types of invaders. Combinations of defense mechanisms, including transcription of defense genes, have to be deployed in a way that is both efficient and effective (and takes in to consideration the energy cost to the plant); certain plant defense processes will even cause positive and negative feedback to other plant process to optimize signal defense (Beckers and Spoel, 2006; Jones and Dangl, 2006). As a result, to have an understanding of the plant defense process and the molecular events involved, one must take a closer look at the individual regulatory factors.
This thesis considers TGA2, a dual-function transcription factor that is involved in SAR regulation. TGA2 is able to both activate and repress PR gene transcription through interactions with itself and other transcriptional elements (Boyle and Despres, 2010). Detailed investigations into the molecular mechanisms that allow this to happen have only recently started. The purpose of this thesis is to look at a possible interaction between TGA2 and the kinase CK2 that may be involved in regulating this process.
CHAPTER 2: LITERATURE REVIEW

2.1 Systemic Acquired Resistance

The term Systemic Acquired Resistance (SAR) was first used to describe the observation that tobacco plants challenged with tobacco mosaic virus (TMV) showed a non-localized increased resistance to secondary infection (Ross et al., 1961; Durrant & Dong, 2004). Presently, SAR can be described as an induced systemic response, existing in many plant species, in response to avirulent (Avr) genes found in many pathogens (De Wit et al., 1997). SAR causes the activation of localized and systemic defense responses that restricts the growth and spread of pathogens beyond the site of infection. SAR is used to defend against pathogenic bacteria, fungi, oomycetes and viruses (Ryals et al., 1996; Sticher et al., 1997; Durrant and Dong, 2004). The SAR process is triggered by an accumulation of the endogenous signaling molecule Salicylic acid (SA) caused by infection by pathogens, plant elicitors (such as molecules released through cell wall degradation), or stress conditions (Durrant and Dong, 2004; Boyle and Despres, 2010). SAR can also be activated by functional analogs of SA - 2,6-dichloroisonicotinic acid (INA) or benzothiadiazole S-methyl ester (BTH) (Uknes et al., 1992; Ward et al., 1991).

2.1.1 SA required for SAR activation

Some downstream effects of SA accumulation are still elusive; nevertheless, it is known that SA is a requirement for SAR activation (Beckers and Spoel, 2006). In studies in Arabidopsis using a bacterial salicylate hydroxylase transgene, nahG, that inactivates SA by converting it into catechol, plants unable to accumulate SA are
also unable to produce a SAR response. These transgenic planets also have an increased susceptibility to SA responsive pathogens (Gaffney et al., 1993; Delaney et al., 1995).

SA is also involved in signaling other plant processes, either directly or indirectly, such as seed germination, seedling establishment, cell growth, respiration, stomatal closure, senescence-associated gene expression, responses to abiotic stresses, basal thermotol-tolerance, nodulation in legumes and fruit yield (Klessig and Malamy, 1994; Rate et al., 1999; Morris et al., 2000; Rajjou et al., 2006). The involvement of SA in these events may be caused by cross-signaling with other plant hormone or metabolite pathways such as jasmonic acid (JA) and ethylene (ET) (Vlot et al., 2009).

2.1.2 PR genes

Under normal conditions, accumulation of SA causes the transcription of pathogenic related (PR) genes in both local and systemic tissues and is the foundation of the characterization of SAR (Ward et al., 1991; Malek et al., 2000). For this reason, PR genes are often used as biomarkers for the activation of SAR (Durrant & Dong, 2007). In Arabidopsis, SAR induction is usually determined through the activation of the PR gene PR-1.

PR-1 is highly conserved, and is found in every plant species. Homologs for PR-1 also exist in fungi, insects and vertebrates (including humans). PR-1 is associated with defense activity specific for oomycetes, but its biological activity has not been characterized (Van Loon et al., 2006).
There are 17 known families of resistance inducible proteins. While the activity of *PR-1* is not well understood, other PR genes have been better characterized. For instance, *PR-8* encodes a lysozyme that is directed against bacteria, while *PR-13* encodes thionins with antibacterial and antifungal activity (Van Loon et al., 2006).

### 2.2 NPR1/NIM1

The involvement of NONEXPRESSOR OF PR GENES1 (NPR1) (also referred to as NIM1 – NON-INDUCIBLE IMMUNITY1) in SAR was discovered using mutant screens for genes unable to produce a SAR response (indicated using PR-2:GUS reporter gene and SAR-deficient phenotypes) (Cao, 1994). *Npr1* mutant plants are capable of accumulating high levels of SA, however are unable to induce PR gene activity and are more susceptible to pathogen infection (Cao, 1994; Delaney, 1995; Glazebrook, 2003; Shah, 1997).

Under basal conditions, constitutively expressed NPR1 is located in the cytosol and in the nucleus as oligomers held together through intermolecular disulfide bonds (Despres et al., 2000; Despres et al., 2003; Durrant and Dong, 2004). When SAR is induced, either through SA or INA, the plant cell undergoes a change in redox state that ultimately increases the cellular reduction potential (Chen et al., 1993; Vanacker et al., 2000; Klessig et al., 2000). This change causes Cys-82 and Cys-216 residues on the NPR1 oligomers to be reduced, hydrolyzing intermolecular disulfide bridges and monomerizing the NPR1 proteins (Durrant and Dong, 2004). NPR1 monomers are then able to translocate to the nucleus and activate PR gene transcription (Kinkema et al., 2000) (Figure 2.1).
Figure 2.1 NPR1 is the central regulator of SA and JA responsive genes (Beckers and Spoel, 2006).
Cloning of NPR1 reveals that NPR1 contains 2 conserved protein-protein interaction domains: an ankyrin repeat domain (Cao et al., 1997; Ryals et al., 1997; Li et al., 2006) and a Broad-Complex, Tramtrack, Bric-a-brac/Poxvirus, Zinc finger (BTB/POZ) (Arvind and Koonin, 1999; Bardwel and Treisman, 2004) but does not contain a DNA binding sequence; indicating that while NPR1 is required for PR gene activity, it is unable to directly bind to DNA to activate PR gene transcription.

2.2.1 NPR1 interacting proteins (TGAs, NIMIN1)

Yeast 2-hybrid protein-protein interaction studies show that NPR1 interacts with basic leucine zipper (bZIP) transcription factors from the TGA subclass of factors (Despres et al., 2000; Zhou et al., 2000; Zhang et al., 1999). Physical interaction between NPR1 and TGA2 has also been shown to occur in planta (Subramaniam et al., 2001; Fan and Dong, 2002). TGA transcription factors help to induce PR-1 expression by binding to cis-acting PR gene elements with TGA binding motifs (Lebel et al., 1998; Niggeweg et al., 2000b; Rochon et al., 2006). NPR1 is able to bind to TGA factors and stimulate binding activity to SA responsive promoter elements such as PR-1 when stimulated with SA (Johnson et al., 2003; Rochon et al., 2006). (This interaction will be discussed in greater detail in the following sections.)

Yeast 2-hybrid assays have also been used to show that NPR1 interacts with NIMIN (NIM1-interacting) proteins (Weigel et al., 2001). NIMIN1 and NIMIN2 interact with the C terminus of NPR1, while NIMIN3 interact with the N terminus of NPR1 (Weigel et al., 2001). Transgeneic plants that overexpress NIMIN1 have reduced SA-mediated PR gene induction and reduced amounts of NPR1 (Cao et al.,
NIMIN1 also acts as a negative regulator of NPR1 (Fonseca et al., 2010).

### 2.2.2 SNI1

SNI1 (SUPPRESSOR of NPR1, INDUCIBLE) is a negative regulator of NPR1 that was found using genetic screens for suppressors of npr1 (Li et al., 1999). In an npr1 mutant background, sni1 is able to restore SA-inducible PR gene expression. Sni1 mutants show the same level of SA as wild type plants, with marginally higher expression of PR genes without a SAR inducer, indicating that sni1 activity is most likely downstream of NPR1 (Li et al., 1999).

SNI1 is able to synergistically repress PR-I transcription with TGA2 and is responsible for PR gene repression in the absence of NPR1 (Kersawani et al., 2007). In the presence of NPR1, SNI1 counters, and reduces both basal and induced expression of PR genes (Kersawani et al., 2007). SNI1 represses PR-I at an unidentified target site, making it difficult to determine whether or not it directly interacts with NPR1 (Mosher et al., 2006; Boyle et al., 2009). As well, SNI1 has no known DNA binding domain, and probably interacts with other factors instead of binding directly with the promoter (Durrant and Dong, 2004). SIN1 mediated repression likely involves histone modification (Mosher et al., 2006).

### 2.2.3 NPR1 cross-signaling

NPR1 may function outside of the SAR pathway, as a means of cross signaling between SA-induced signaling pathways and JA/ET induced signaling pathways (Beckers and Spoel, 2006).
JA and ET are signal transducers for rhizobacteria-mediated Induced Systemic Resistance (ISR) involved in plant defense against necrotrophic pathogens, and insects (Pieterse et al., 2001b, 2002). Examples of synergism and antagonism between ISR and SAR pathways have both been reported (Pieterse et al., 2001a). NPR1 may interact with mediators of ISR signaling as monomers in the cytosol after an SA-induced redox change (Beckers & Spoel, 2006).

NPR1 has been shown to be required for the SA-mediated suppression of JA-responsive gene expression and may function by limiting the ability to synthesize JA by inhibiting LOX2 gene expression (Spoel et al., 2003)(Figure 2.1). NPR1 may also directly or indirectly inhibit positive regulators of the JA-responsive gene pathways (Spoel et al., 2003; Beckers and Spoel, 2006).

Although ISR and SAR pathways may require or interact with NPR1 downstream of signaling molecules, they result in the activation of different defense genes (Van Wees et al., 1999; Verhagen et al., 2004).

2.3 BZIP transcription factors
TGA factors are part of the bZIP multigene family of transcription factors found in fungi, animals and plants (Deppmann et al., 2006; Amoutzias et al., 2008). They are versatile, and able to interact both with related bZIP factors, and other structurally unrelated proteins. They are integral to the regulation of many different cellular processes. (Chinenov et al., 2001). In plants, bZIP transcription factors are involved in the regulation of light, stress and hormone responses, pathogen defense, seed maturation and flower development (Kirchler et al., 2010). As many as 77 bZIP
proteins are encoded in the *Arabidopsis* genome (Riano-Pachon et al., 2007); they are divided into 10 groups of homologs (Jackoby et al., 2002).

All bZIP transcription factors have 2 highly conserved domains: a leucine zipper domain that forms the structural backbone of the bZIP dimer complex and a N-terminal basic domain that is important for protein regulation and DNA-binding. BZIP Enhancing Factors (BEF, such as NPR1)(Ang et al., 1994) process the leucine zipper and help with dimerization, leading to more stability in the DNA-protein dimer interaction (Hardtke et al., 2000).

### 2.3.1 C terminal leucine zipper

BZIP monomers form a supercoiled alpha helix structure with several 7-amino acid coil motifs in the C terminal end (Vinson et al., 2005) (Figure 2.2). In a typical bZIP monomer, the amino acid located in the 7\textsuperscript{th} position, often referred to as the \(d\) position, is capable of interacting with hydrophobic residues in the \(a\) position to form a complex of two bZIP monomers that lie parallel to each other at their C terminal ends (Vinson et al., 2006) (Figure 2.3). Electrostatic interactions between parallel residues result in the formation of a bZIP dimer with a hydrophobic core.

Variations in the residues at the \(a\) and \(d\) positions, and the number of heptads in the C terminal region are determining factors of the stability of bZIP dimer formation, and may determine whether or not a given bZIP protein will form stable dimers, or alternatively be able to form higher order complexes (Deppmann et al., 2004). Protein complexes with strong, attractive interhelical interactions in the
B-ZIP motif

DNA

major groove

minor groove

N-terminal

heptad 1 2 3

L0 L1 L2 L3

Leucine zipper

Figure 2.2 GCN4 dimer bound to DNA.

The leucines in the $d$ position are shown as grey spheres. The first 3 heptads are numbered 1, 2, 3 respectively (Vinson et al., 2006)
Figure 2.3 Interacting bZIP monomers.

(A) Two bZIP monomers interact to form a supercoiled alpha helix with interacting 
a and d position residues (Sibériol, 2001).

(B) Top view of 2 interacting bZIP monomers, showing hydrophobic interactions 
between a and d position residues (Vinson, 2006).
leucine zipper domain will predominantly form homodimers, while proteins with repulsive interactions will form heterodimers (Deppmann et al., 2004).

Frequently studied human bZIP proteins are typically able to form stable dimers with leucine zippers with 4-7 heptads. Alternatively, Arabidopsis bZIP proteins tend to have longer leucine zipper sequences that are more varied in the number of heptads; 10% of Arabidopsis bZIP proteins have short zipper sequences with only 3 heptads, while >30% have longer sequences with 10 or more heptads (Deppmann et al., 2004). These differences may lead to Arabadopsis leucine zippers having less structural constraints than human leucine zippers (Deppmann et al., 2004). BZIP proteins are able to form heterodimers that have been shown to effect binding specificity and modify interactions with other proteins. The mechanisms involved in forming higher order complexes may be dependant on the function of individual factors.

TGA factors contain short leucine zippers with only 3 heptads, all containing charged amino acid residues in the a position that would be predicted to destabilize the leucine zipper, favouring a heterodimer formation (Deppmann, 2004). However, they have still been shown to be able to form homodimers as well, with the help of bZIP enhancing factors (Niggeweg et al., 2000b; Boyle et al., 2009). TGA2 also has a C terminal region that has been shown to help stabilize dimer formation (Niggeweg et al., 2000b; Boyle et al., 2009) (Figure 2.4).
Figure 2.4 TGA2.
2.3.2 N terminal regulatory domain

Attached to the leucine zipper region by a short sequence called the hinge is the N-terminal basic domain (Siberil et al., 2001). This domain is proline rich and forms a fork that is capable of interacting with a palindromic cognate DNA sequence. The N-terminal binds the major groove of the DNA sequence, typically when the protein dimer is already formed, causing the bZIP complex to lie perpendicular to the DNA sequence (Miller et al., 2009). The DNA binding region of the basic domain contain 5 residues that are highly conserved within given bZIP family, either Asn or Arg, that act as base contacts with the DNA sequence.

While the DNA contact sites are highly conserved, the rest of the basic domain is less conserved, giving it conformational plasticity, and making it possible to promote transient interactions with many other proteins (Miller et al., 2009). Some of these protein interactions are mutually exclusive, allowing for sequential cellular events in response to signals and also allow for posttranslational modifications that help regulate cellular processes (Gardner and Montminy, 2005).

2.4 TGA transcription factors

The TGA family of transcription factors was first characterized through its ability to bind activating sequence 1 (as-1) element of the Cauliflower Mosaic Virus (CaMV) 35S promoter through binding to 2 TGACG motifs (Katagiri et al., 1989). Sequence analysis of TGA factors isolated from tobacco indentified them as bZIP transcription factors (Jackoby et al., 2002; Katagiri et al., 1989).
There are 10 known TGA factors in *Arabidopsis*, of which 7 are able to associate with NPR1 (Jakoby et al., 2002). The seven NPR1-associating TGA factors are divided into 3 subgroups based on sequence similarity (Figure 2.5).

### 2.4.1 Group I – TGA1, TGA4

TGA1 and TGA4 are both positive regulators of basal resistance (Kesarwani et al., 2007), but initially this group of transcription factors was thought to not interact, or have weak interaction to NPR1 (Durrant and Dong, 2004). Studies comparing oxidized and reduced forms of these factors show that they do interact with NPR1, but only in their reduced form (Rochon et al., 2003). When oxidized they form intramolecular disulfide bridges, unique to this clade of factors, which inhibit their interaction with NPR1 (Despres et al., 2003). However, in the reduced form, the disulfide bridges breaks, and NPR1 is able to interact with these factors and stimulate DNA binding (Despres et al., 2003). Using tga1 and tga4 knockout mutants, their activity was found to be partially redundant with TGA1 having a more dominant effect on PR gene activation than TGA4 (Kesarwani et al., 2007).

### 2.4.2 Group III – TGA3, TGA7

Group III transcription factors are capable of interacting constitutively with NPR1 (Despres et al., 2000). Although they are more homologous to Group I factors than Group II factors in their N terminal domain, their ability to interact with NPR1 suggests that their activity more closely resembles Group II TGA factors (Shearer et al., 2009).
Figure 2.5 Dendogram of *Arabidopsis* TGA factors (Chubak, 2006)
TGA3 is the strongest-interactor of NPR1 (Zhou et al., 2003), and may be involved in basal resistance and PR gene expression (Kersawani et al., 2007). Some studies have suggested that TGA7 is less important in terms of activating PR gene transcription, since TGA7 does not alter PR transcription levels (Kersawani et al., 2007). However, the DNA binding activity of TGA7 is enhanced by NPR1. Redundancy between TGA3 and TGA7 makes it difficult to tease out functional properties of TGA7 alone and makes it difficult to characterize its activity (Shearer et al., 2009).

2.4.3 Group II – TGA2, TGA5, TGA6

Group II transcriptional factors are capable of interacting constitutively with NPR1 and are involved in both the NPR1-induced activation and repression of PR gene activation (Despres et al., 2000; Rochon et al., 2006; Boyle et al., 2009).

TGA2 acts as an activator when interacting with NPR1, but switches to a repressor under basal conditions (Boyle et al., 2009). The mechanism that switches TGA2 from a repressor to activator is a process that is not entirely understood, but could be caused by post-translational modification, or interaction with other transcriptional elements, or specific promoter binding and chromatin modification. (The activity of TGA2 will be discussed in greater detail in the following section.)

TGA groups were originally defined by sequence homology, but some studies have indicated that some group function may not be as redundant as was originally thought. While TGA2 and TGA6 are close homologs, TGA6 may not have the same dual function as TGA2. In tga2 mutant plants TGA6 is able to increase PR gene
expression, indicating that it has a role in PR gene activation (Kersawani et al., 2007). But, in the absence of a SAR inducer, TGA6 overexpression does not have a significant effect on PR induction; without the presence of TGA2, in a tga2 mutant, TGA6 is able to increase PR gene transcription (Kersawani et al., 2007). TGA6 may be a constitutive activator with activity that is repressed by TGA2.

TGA3, TGA5 and TGA6 may bind constitutively to the positive element of PR-1 (under basal conditions) and interact with TGA2 and SNI1 that repress their activity (Kersawani et al., 2007). However, when induced with SA, NPR1 can interact with TGA factors that are positive regulators: TGA1, TGA2, TGA3, TGA5, TGA6 (Despres et al., 2003; Johnson et al., 2003; Kerawani et al., 2007; Rochon et al., 2006).

2.5 TGA2

2.5.1 Dual Activity of TGA2

The TGA2-like group II factors were shown to be important in PR-1 gene activation using the triple knockout tga2tga5tga6. These plants when induced with INA or challenged with pathogens have a decreased SAR response when induced, a phenotype similar to the npr1 mutant (Zhang et al., 2003).

However, to complicate a possible transcriptional activating model for TGA2, these same knockout plants also show increased basal levels of PR-1 gene expression, leading to the possibility of a negative or repressive role for TGA2 as
well. *Tga2tga5* mutant plants also show increased *PR-1* expression without SAR induction (Zhang et al., 2003).

The activity of TGA2 may depend on a variety of factors, however, a mechanistic model that details how, and under what conditions, TGA2 activates or represses hat yet to be elucidated. It is possible that multiple factors are involved in TGA2 function. Kerwawani et al. (2007) summarizes some of the types of mechanisms that could cause TGA2 to switch from repressing to activating PR gene transcription as interactions with promoter elements, interactions with other transactivating factors (such as NPR1 and SNII1) and post-translational modifications. Ultimately, the activity of TGA2 may depend on multiple factors.

### 2.5.2 Interaction with NPR1 and stoichiometric regulation

TGA2 acts as a transcriptional activator when forming an enhancesome with NPR1. When induced with SA, TGA2 associates with the BTB/POZ and 2 ankrin repeat domains of NPR1, forming a stable NPR1-TGA2-DNA complex that is most likely in the conformation of a TGA2 dimer bound to 2 NPR1 at each as-1-like element (Boyle et al., 2009). Conversely, while under resting conditions TGA2 is a constitutive repressor, capable of repressing PR gene transcription by binding to the *PR-1* element as an oligomer (Boyle et al., 2009) (Figure 2.6).

To determine which domains are responsible for TGA2 dual activity, Rochon et al. (2006) used a GAL4:DB reporter gene assay system to show that when the N terminal domain is removed, the resulting protein, Δ43 TGA2, is transcriptionally active. Conversely, the N terminus alone is not enough to repress gene activity. This
Figure 2.6 Dual Activity of TGA2 (Boyle et al., 2009).

When TGA2 acts as a repressor it binds to the TGACG sequence as an oligomer of unknown stoichiometry. When TGA2 acts as an activator it binds the sequence with a 2:2 NPR1:TGA2 ratio. NPR1 dimerizes when activated, and interacts with the TGA2 N terminal repression domain.
could imply that the N terminus interacts with other domains to regulate repression by imparting certain structural conformations. To examine how structure may play a role in regulation, Boyle et al. (2009) used EMSA and gel filtration to show that TGA2, with help from its leucine zipper domain, is capable of forming higher order complexes (oligomers, tetramers, dimers) that can bind to an LS7 probe without SA induction. Also, supposing that the N terminal domain can affect how TGA2 binds to DNA, Boyle et al. (2009) performed an EMSA experiment comparing DNA binding of full-length TGA2 and Δ43TGA2 to an LS7+LS5 probe, and was able to show that the two proteins have different binding profiles. The full length protein is capable of forming 2 separate complexes of different sizes, while the TGA2 construct missing the N terminus is only capable of forming a single complex (Figure 2.7). This demonstrates that the N terminal is responsible for the formation of higher order complexes on DNA, and could be responsible for regulating how TGA2 binds to DNA. The NPR1 BTB/POZ domain is also able to interact directly with the N terminus of TGA2 and block its repression activity.

### 2.5.3 TGA2 interactions with PR-1 promoter elements

The PR-1 promoter in *Arabidopsis* has 2 cis-acting elements with TGACG binding motifs, linker scanning 7 (LS7) and LS5. TGA2 binds both elements with equal affinity *in vitro* (Boyle et al., 2009). Mutations in LS7 lead to loss of PR gene induction, while mutations in LS5 increase gene expression, independent of SA or INA induction, demonstrating that each element has a different regulatory activity (Lebel et al., 1998).
Figure 2.7 The BTB/POZ domain of NPR1 interacts with the N terminus of TGA2 (Boyle et al., 2009).

EMSA showing the full length TGA2 protein forming 2 complexes of different sizes when bound to an LS5/LS7 DNA probe (Indicated by grey and black arrows). The BTB/POZ region (POZ) interacts with the N terminus of TGA2. When POZ is added to TGA2 only one complex is formed on the LS5/LS7 probe. The Δ43TGA2 complex is only able to form a complex of one size, with or without POZ.
TGA2 is recruited to the *PR-I* promoter when induced by SA independently of interaction with NPR1 (Johnson et al., 2003), and ChIP experiments have confirmed that TGA2 binds to the *PR-I* promoter constitutively (Rochon et al., 2006). But, low resolution and short distance between the two promoter elements makes it difficult using ChIP to determine which element TGA2 occupies. It’s possible that an oligomeric formation of TGA2-clade transcription factors could bind both elements at the same time (Boyle et al., 2009) physically impeding or protecting the LS7 activating element when not induced by SA (Boyle et al., 2010).

Pape et al. (2010) using transient assays with shortened or mutated TGA-binding site promoter constructs, supports the idea that both binding sites are occupied by TGA2, and that TGA2 binding is involved in transcriptional repression. Promoter constructs with only the LS4 (WRKY binding promoter) and either LS5, LS7 or LS5/LS7 intermediate sequence missing or mutated are unable to support NPR1 mediated promoter activity. This would suggest that LS7 positive element alone cannot support PR gene activation and requires other cis-elements to promote transcription. However Pape et al. (2010) also suggests that the formation of TGA oligomers may not suppress promoter function, but instead, the negative TGA2 activity is controlled through binding to NPR3/4, since like NPR1, NPR3/4 require intact LS5 and LS7 promoter elements for recruitment. No studies have been done to confirm whether NPR3/NPR4 are involved in basal repression of PR transcription.
2.6 CK2 Regulation of TGA2-DNA binding

2.6.1 Phosphorylation of Transcription factors

The negative charge introduced by phosphorylation of transcription factors impacts the amino acid repulsive and attractive forces causing allosteric conformation changes that can directly affect protein stability, localization, DNA-binding, and interaction with other proteins (Holmberg et al., 2002). Indirectly, phosphorylation can affect chromatin structure, affecting the ability of transcriptionally controlled elements (Holmberg et al., 2002). In some circumstances structural changes caused by phosphorylation can generate new, secondary phosphorylatable motifs, that allow a given factor to interact with multiple protein kinases in a context dependent manner, giving added complexity to the transcription factors function (Gardner & Montminy, 2005; Dahan et al., 2009).

2.6.2 CK2 in plants

Casein Kinase II (CK2) kinases are found in all eukaryotic cells, and are predominantly located in the nucleus (Moreno-Romero et al., 2008). CK2 was originally named based in its ability to phosphorylate casein in vitro (Litchfield et al., 2003), but has been found to be able to phosphorylate over 300 substrates, the majority of which are transcription factors, or proteins involved in gene expression or transcription (Moreno-Romero et al., 2008). CK2 has been reported to be involved in many plant functions including plant division, cell cycle control, plant development, seed development photomorphogenesis, and ABA and SA signaling (Moreno-Romero et al., 2008; Ciceri et al., 1997; Hardtke et al., 2000; Klimczak et al.,
1995; Menkens et al., 1995). Unlike many other kinases, CK2 signaling is independent of second messengers such as cyclic nucleotides, phosphatidyl inositolphosphates or calcium (Tuazon and Traugh, 1991). CK2 phosphorylates on Ser/Thr residues in peptide sequences that are followed by a stretch of acidic amino acid residues, commonly in the motif S/TXX D/E (Tuazon and Traugh, 1991; Meggio et al., 1994). In some organisms, CK2 is also able to phosphorylate Tyr in energetically favourable conditions. However, catalytically active Tyr phosphorylating kinases have not been found in plants (Dahan et al., 2009).

CK2 is a holoenzyme composed of two subunits: a catalytic alpha subunit and regulatory beta subunit, that combine to form a 2CKα2CKβ tetramer (Litchfield et al., 2003; Niefind et al., 2001); Arabidopsis has 4 genes coding for each subunit type, making it possible for the existence of several holoenzyme combinations. However, unlike for mammalian CK2, there are limitations to the combinations that are possible due to restrictions caused by either by differences in subcellular location (Selinas et al., 2006) or affinity of alpha and beta subunits (Riera et al., 2001; Moreno-Romero et al., 2008).

2.6.3 CK2 as a regulator of TGA activity

Strange et al. (1997) showed that endogenous protein kinase activity in tobacco plants is able to increase the as-1 element binding activity of nuclear factors, replicating the effects of SA induction. This activity is inhibited by quercetin, a CK2 and protein kinase (PK) inhibitor, but is not inhibited by other inhibitors specific to other kinases (Strange et al., 1997). This would suggest that a CK2 or a CK2-type
kinase might be involved in regulating nuclear factor as-1 binding activity. Another CK2 specific inhibitor, a halogenated benzimidazole derivative, DRB, is also able to inhibit CK2 induced increases as-1 binding activity (Meggo et al., 1999; Szyszeka et al., 1998; Hidalgo et al., 2001).

The as-1 binding nuclear factors in tobacco, ASF-1 (as-1-binding Factor) and SARP (Salicylic Acid Response Protein), both bind to as-1 elements in response to pathogen attack or SA or auxin induction. These protein complexes both contain a TGA2.2 homodimer as a major, functionally important component, as well as a TGA2.1 minor component (Niggeweg et al., 2000a). The DNA binding activity of ASF-1 and SARP complexes can both be suppressed by phosphatase treatment, and ASF-1 binding activity can be stimulated by phosphorylation. This is congruent with studies showing that increased kinase activity, most likely by a CK2-type kinase, is correlated with events downstream of SA induction (Hidalgo et al., 2001).

The TGA2 subgroup of factors (TGA2, TGA5, TGA6) in Arabidopsis has a motif of predicted CK2-phosphorylatable residues in the N terminus that is similar to TGA2.1 and TGA2.2 in tobacco (Kang and Klessig, 2005). Using a series of deletion and Ala mutation TGA2 constructs, Kang and Klessig (2005) determined that CK phosphorylation occurs in the first 20 residues in TGA2, with $^{11}$Ser, $^{12}$Thr and $^{16}$Thr having an integral role. However, contrary to the tobacco nuclear factors that have increased binding to as-1 elements when phosphorylated, TGA2 as-1 and LS7 element binding is reduced when phosphorylated with a putative CK2 kinase (Kang and Klessig, 2005).
Kang and Klessig (2005) suggest that the difference in resulting DNA binding activity induced by CK2 may be caused by the presence of other kinases in plant extracts that inhibit CK2 activity, noting that in Strange et al. (2003) crude extracts of *Arabidopsis* that have been treated with SA, show evidence of phosphorylation of as-1 elements even with the presence of CK2 inhibitor heparin. This could be an indication of other kinase activity. However, differences between TGA2 and tobacco TGA2.2 have been noted before (Thurow et al., 2005). An *Arabidopsis* TGA2 fusion protein construct fused with Enhanced Yellow Fluorescent Protein, EYFP-T2m, is deficient in DNA binding and when transduced into tobacco and has enhance PR1 expression when treated with SA (Pontier et al., 2001); whereas a tobacco TGA2.2 protein construct with mutated DNA-binding function has reduced PR1a expression (Niggeweg et al., 2000b). It could be possible that CK2 interacts differently with tobacco TGA2.2 and *Arabidopsis* TGA2, or causes different downstream effects.

2.7 Study Objectives

The aim of this thesis is to look at the effects of CK2 on TGA2 activity and to determine if phosphorylation may play a role in the ability of TGA2 to switch from forming higher order complexes to forming an enhancesome complex with NPR1. The N terminus of TGA2 has been shown to be a nonautonomous repression domain that when bound to the BTB/POZ region of NPR1 loses its activity (Rochon et al., 2006; Boyle et al., 2009). As well, the N terminus contains a number of phosphorylatable residues that effect TGA2-DNA binding activity (Kang and Klessig, 2005). We use EMSA, gel filtration and pull-down assays to look at the
interaction between TGA2, BTB/POZ when phosphorylated by a CK2 kinase to try and determine some of the underlying mechanisms.
CHAPTER 3: MATERIALS AND METHODS

3.1 Chemicals and Buffers

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

3.1.1 Media for E. coli

2xYT media (per litre)
Tryptone (16g), Yeast Extract (10g), NaCl (5g)

Luria-Bertani (LB) media (per litre)
Tryptone (10g), Yeast Extract (5g), NaCl (10g)

Both media were sterilized by autoclave before use. All components of media were purchased from BioShop.

3.1.2 Antibiotics

Kanamycin (Km) (Km50 = 50mg/ml)
Chloramphenicol (Cm) (Cm25 = 25mg/ml)

Stock solutions of antibiotics are stored at -20°C.

3.1.3 Buffers

Ni-NTA column (1ml) and Ni-NTA Binding Beads

(A) Stripping Buffer (100ml): 0.276g NaH2PO4 (20mM), 2.922g NaCl (500mM), 1.861g EDTA (50mM), pH 7.4
(B) Binding Buffer (100ml): 5ml 1M HEPES (50mM), 3ml 5M NaCl (150mM), 2ml 2M imidazole (40mM), pH 7.4.

(C) Elution Buffer (100ml): 5ml 1M HEPES (50mM), 3ml 5M NaCl (150mM), 6.81g imidazol (40mM), pH 7.4

(D) Sample Buffer (3X): 3.6ml 0.5M Tris (0.06M), pH 6.8, 3.0ml 100% [v/v] glycerol (10%), 0.025% [v/v] Bromophenol blue, 3.0ml [v/v] 20% SDS (2%).

Before use, the Ni-NTA column was stripped of proteins and metals that may have originated from previous experiments using the EDTA containing Stripping Buffer. The column was then recharged with Ni$^{2+}$. The same binding buffer was also used in pull down assays that used Ni-NTA binding beads. Sample Buffer was used in these experiments to elute bound proteins from Ni-NTA binding beads (2X Sample Buffer used, diluted with MQH$_2$O).

Strep-Tactin column (1ml)

(A) Regeneration Buffer (100ml): 0.69g NaH$_2$PO$_4$ (50mM), 1.75g NaCl (300mM), 0.024g HABA (1mM), pH 8.0

(B) Binding Buffer (100ml): 0.69g NaH$_2$PO$_4$(50mM), 1.75g NaCl (300mM), pH 8.0

(C) Elution Buffer (100ml): 0.69g NaH$_2$PO$_4$(50mM), 1.75g NaCl (300mM), 0.054g desthiobiotin (2.5mM), pH 8.0

Before use, the Strep-Tactin column was treated with Regeneration Buffer that contains HABA (2-[4'-hydroxy-benzeneazo] benzoic acid) to get rid of biotin.
(desthiobiotin) that would have originated from the elution buffer from previous experiments. By removing biotin from the column, the matrix components were available for Strep-tag binding.

S300 Gel Filtration

(A) S300 Gel Filtration Buffer (500ml): 25ml 1M HEPES (50mM), 25ml 5M NaCl (250mM), pH 7.4

S300 Gel Filtration Buffer was filtered and degassed.

EMSA

(A) 10XEMSA Buffer (50ml): 10ml 1M HEPES-KOH (20mM), pH 7.9, 25ml 20% [v/v] glycerol, and 2.5ml 0.5% [v/v] Tween 20, 2uL 1M DTT (20mM).
(B) 10XTBE (1L): 121.1g Tris (1M), 61.8g Borate/Boric acid (1M), 3.72g EDTA (10mM).
(C) 10XTE Buffer (100ml): 10ml Tris (1M), 2mL EDTA (0.5M).
(D) TEN Buffer (10ml): 1ml TE Buffer, 0.875g NaCl (150mM).

CK2

(A) CK2 Assay Buffer (10X) (NewEngland Biolabs)
3.2 Plasmid Methods

3.2.1 Bacterial Strain and Plasmids

All experiments used E. coli BL21 strain (DE3) competent cells for transformation; they were stored at -80°C.

PET41a (Novagen) plasmids containing protein constructs were used for transformation. All plasmids contained antibiotic resistance to Km, while wild-type TGA2 also contained antibiotic resistance to Cm. Plasmids were stored at -20°C.

3.2.2 Plasmid Transformation

Electroporation of electro-competent bacterial cells

25μl of electro-competent cells were added to 50-80fmols of plasmid DNA (5μl) and 20μl of MQH2O to a total volume of 50μl. Transferred to a chilled 0.2cm gap electroporation cuvette. Cells were electroporated using Gene Pulsar (Bio-rad) set to capacitance: 25μFD, resistance: 200 ohms, volts: 2.5 kvolts. Cells added to 500μL of 2YT +1 M glucose medium and incubated at 37°C for 1 hr while shaking at 250 rpm. Cells were plated on selective media using Km50 or Km50 and Cm25.

3.3 Protein Methods

3.3.1 Protein Expression

A 5ml culture in 2xYT media containing Km50, or a combination of Km50 and Cm25 was inoculated and grown overnight on a rotary shaker at 250rpm at 37°C. All 5 ml of this saturated culture was inoculated in 1L of LB (Luria-Bertani) media containing either Km50 or a combination of Km50 and Cm25.
When the OD600 of this culture reached 0.4-0.6, it was induced with 0.5M IPTG (Isopropyl-β-D-thiogalactopyranoside; BioShop) and grown for an additional 2hrs on a rotary shaker at 250rpm at 37 °C.

After 2hrs, the cells were harvested by centrifugation at 8000rpm for 10min (Sorvall-RC-5C-Plus, Mandel Scientific Company). The cells were washed with MQ H₂O and centrifuged again at 15000 rpm at 4 °C for 5min. The supernatant was discarded and the pellet was either purified immediately or stored at -20 °C until needed.

Glycerol stocks of proteins for long-term storage were kept at -80°C.

3.3.2 Histag protein purification

The bacterial pellets were resuspended in binding buffer 5ml 1M HEPES (50mM), 3ml 5M NaCl (150mM), 2ml 2M imidazole (40mM), pH7.4, then sonicated (Sonic Dismembrator Model 100; Fisher Scientific) on ice. To separate protein from cellular debris, the sonicated cellular solutions were centrifuged at 15000 rpm at 4°C for 20 minutes. The supernatant was filter sterilized (0.45μm) and applied to the Ni-NTA column pre-equilibrated in 5ml binding buffer.

FPLC

The protein was purified using the ÄKTA Explorer FPLC (GE Healthcare Bio-Sciences) operated with the UNICORN Version 3.00.10 software with a Ni-NTA 1ml column (Healthcare) according to the manufacturer’s instructions.

The filtered sterilized protein was added to the column and the column was subsequently washed with 10ml binding buffer followed by 5ml elution buffer 5ml
1M HEPES (50mM), 3ml 5M NaCl (150mM), 6.81g imidazole (1M), pH7.4. The resulting elution was collected in 500ml fractions. The concentrations of protein in the crude, flow through and wash fractions were determined using the Bio-Rad protein assay reagent (Bio-rad).

**Manual Peristaltic Pump**

Protein was also purified using a manual peristaltic pump. The column was washed with 10ml of binding buffer and the flow through collected. Fractions were eluted using elution buffer. Ten 500ml fractions were collected; their protein concentration was determined using the Bio-Rad protein assay reagent (Bio-Rad).

**3.3.3 Strep-tag protein purification**

The bacterial pellets were resuspended in binding buffer (0.69g NaH₂PO₄ (50mM), 1.75g NaCl (300mM), pH8.0, then sonicated (Sonic Dismembrator Model 100; Fisher Scientific) on ice.

To separate protein from cellular debris, the sonicated cellular solutions were centrifuged at 15000 rpm at 4°C for 20 minutes. The supernatant was filter sterilized and applied to the Strep-Tactin column pre-equilibrated in 5ml binding buffer either on a manual peristaltic pump or using FPLC.

**FPLC**

The protein was purified using the ÄKTA Explorer FPLC (GE Healthcare Bio-Sciences) operated with the UNICORN Version 3.00.10 software with a Strep-Tactin 1ml cartridge (Qiagen) according to the manufacturer's instructions.
The filtered sterilized protein was added to the column and the column was subsequently washed with 10ml binding buffer followed by 5ml elution buffer (0.69g NaH₂PO₄ (50mM), 1.75g NaCl (300mM), 0.054g desthiobiotin (2.5mM), pH8.0) for Strep-Tactin column. The resulting elution was collected in 500ml fractions. The concentrations of protein in the crude, flow through and wash fractions were determined using the Bio-Rad protein assay reagent (Bio-rad).

**Manual Peristaltic Pump**

Protein was also purified using a manual peristaltic pump. The column was washed with 10ml of binding buffer and the flow through collected. Fractions were eluted using elution buffer. Ten 500ml fractions were collected; their protein concentration was determined using the Bio-Rad protein assay reagent (Bio-Rad).

### 3.4 Experimental Methods

#### 3.4.1 Gel filtration analysis using S300 column for FPLC

Strep-tagged purified proteins were diluted to a final volume of 10ml using S300 running buffer (50 mM HEPES, pH 7.4, and 250 mM NaCl) prior to gel filtration analysis on the Sephacryl S300 HR packed in 50-cm-long HR 16 column (GE Health) and equilibrated with S300 running buffer. Elution was collected as 0.5 ml fractions, in S300 running buffer at a flow rate of 0.8 ml/min.

The collected fractions (0.5 ml/fraction) were stored at -20°C in S300 running buffer. Selected fractions were thawed then acetone precipitated overnight, and centrifuged at rpm 15000 rpm at -4°C for 30 minutes. The resulting samples
were analyzed by SDS-PAGE gel (16% gel) and immunoblot with anti-Strep tag antibody (NWSHPQFFEK antibody, GenScript).

3.4.2 Electrophoretic Mobility Shift Assay (EMSA)

The probes used were an LS7 probe labeled with IRDye-700nm (LI-COR) on the 5' end (5’-TATTTTACTTACGTCATAGATGTGGCGGCA-3' annealed to 5’-TGCCGCCACATCTATGACGTAAGTAAAATA-3') (Qiagen) and an LS5-LS7 probe labeled with IRDye-700nm (LI-COR) on the 5’ end (5’-GTTTCTCTACGTCACACTTTTACTTACTACGTCATAGATGTG-3' annealed to 5’-CCACATCTATGACGTAAGTAAAATAGTGACGTAGAGAAAC -3') (Integrated DNA Technologies). 10pmol of stock solution of coding (c) and non-coding (nc) oligos were added with 80ul of TEN buffer (100fmol of probe/ul). The probe solution was boiled overnight at 100°C for 5 minutes, and cooled to room temperature overnight. The probe solution stored in dark at -20°C.

Binding reactions for experiments using samples only containing one recombinant protein construct were performed in the dark at room temperature for 20 min in 50μL of EMSA buffer (20mM HEPES-KOH, pH 7.9, 250mM NaCl, 2mM DTT, 20% [v/v] glycerol, and 0.5% [v/v] Tween 20) with 100fmol of probe. For experiments using two recombinant protein constructs, the 2 protein constructs were combined for 15 minutes at room temperature. The probe was added for the binding reaction, performed in the dark at room temperature for 15 minutes in 50μL of EMSA buffer with 100fmol of probe.
The samples were loaded onto 4% polyacrylamide gel (acrylamide-bisacrylamide in 100mM Tris, 10mM borate, and 10mM EDTA) and ran at 8 V/cm for 70 min. Gels were scanned on the Odyssey infrared scanner (LI-COR).

**Sample Treatment with CK2**

Approximately 100fmols of protein were added to 1μL 10mM ATP (0.2mM), 50mM DTT, 500U CK2 (1μL of 500,000U/ml; New England Biolabs) in 50μl CK2 Buffer (New England Biolabs), then incubated at 30°C for 15 minutes.

### 3.4.3 His-tagged NT47 Pull-Down binding assay using nickel charged resin

His-tagged purified factor Δ43 (500 pmol) in 400 μL of binding buffer (1M HEPES (50mM), 3ml 5M NaCl (150mM), 2ml 2M imidazole (40mM), pH7.4) or NT47 (1700pmol) with 100μl NTA-Ni (beads +buffer), was incubated with the beads for 1 hour at room temperature, with continuous inversion. The beads were washed twice with 500 μL of His-Trap binding buffer. His-tagged purified NT47 (700-1000pmol) treated with CK2 was added to the beads already containing Δ43 and incubated for 1 hour at room temperature, and washed with His-Trap binding buffer as described above. Proteins were eluted by boiling 35μL of 2X SDS-PAGE sample buffer and analyzed by immunoblot.

**Sample Treatment with CK2**

7000pmol of protein were added to 8μL 10mM ATP (0.2mM), 200mM DTT, 2000U CK2 (1μL of 500,000U/ml; New England Biolabs) in 400μl CK2 Buffer (New England Biolabs), then incubated at 30°C for 15 minutes.
CHAPTER 4: RESULTS

4.1 TGA2 phosphorylation sites located in the N terminal region may be important for regulation of DNA binding

Kang and Klessig (2005) suggest that the binding sites important for regulating TGA2 binding to DNA are located in the first 20 residues of the N terminus, with 11Ser, 12Thr and 16Thr being the most efficiently phosphorylated in vitro using rabbit reticulocyte kinase lysate. (Other potential phosphorylatable residues in this region include 4Thr, 5Ser and 8Thr.) These residues also follow a motif that would suggest that they would be able to be phosphorylated by a CK2-like activity (Kang and Klessig).

To confirm the involvement of the N terminus of TGA2 in DNA binding an electrophoretic mobility shift assay (EMSA) was performed to compare the DNA binding ability of a 6xHis tagged recombinant TGA2 construct with a 6xHis tagged TGA2 construct missing the N terminus residues (Δ43) (as shown in Figure 4.1).

EMSA experiments are used to view how a given protein will bind with a small double-stranded section of DNA attached to a fluorescent probe. When proteins interact with the probe, the amount of ‘shift’ in a gel can give a qualitative measure of the DNA-protein interaction and relative size of the complex. For this experiment the probe used was a PR-1 LS7 probe.

In Figure 4.1 (B) the full length TGA2 construct is able to bind to an LS7 element probe. However, when treated with CK2, TGA2-DNA binding is completely
abolished at the lower concentration of TGA2. This shows that CK2 activity is able to interfere with TGA2 binding to the probe. At a higher concentration of TGA2, this effect is less prominent; the binding of TGA2 to the probe is not entirely reduced, likely because of the saturation of the CK2 (or ATP) added.

When Δ43 is treated with CK2, the effects of CK2 on TGA2-DNA binding are reduced. The lanes treated with CK2 show less binding to the probe than the untreated lanes, though the probe binding is not abolished like it is for the full-length TGA2 construct. This would suggest that phosphorylation sites in the N terminus of TGA2 are involved in regulating TGA2 binding. There is still a noticeable difference in probe binding between the CK2-treated and untreated samples, which could be an indication of other regions or factors involved in negating TGA2-DNA binding activity. Similar results were seen by Kang and Klessig (2005) while using a 20 residue N terminal deletion construct (TGA2-ΔN) induced with a recombinant human CK2. In this case it was suggested that the CK2 activity is stronger than the activity that may be seen in vivo, and may be causing the phosphorylation of sites that otherwise may not be phosphorylated.
Figure 4.1 CK2 phosphorylation of TGA2.

(A) Top, first 20 residues of N terminus of TGA2. The predicted phosphorylation sites (Kang and Klessig, 2005; NetPhos) are shown in larger font; 11Ser, 12Thr and 16Thr are more efficiently phosphorylated in vitro and are shown in black. Other potential phosphorylatable residues in this region, 4Thr, 5Ser and 8Thr, are shown in white. Below, diagram of the TGA2 recombinant protein with a 6xHis tag at the C terminus. The start of the Δ43 TGA2 recombinant protein, which is missing the first 43 residues of the N terminal region of TGA2, is indicated above with an arrow.
(B) EMSA using recombinant TGA2 (left) and Δ43 TGA2 (right) with LS7 DNA probe. FP stands for free probe, and indicates a sample where no protein was present. +CK2 indicates that a CK2 reaction was used prior to binding the sample to the DNA probe. All the samples shown, except for FP, also contain ATP as part of the CK2 reaction.
4.2 N terminus may interact with other TGA2 domains to reduce DNA binding when phosphorylated

Studies using Δ43, as well as the results from the previous section, show that TGA2 is able to bind to DNA without the N terminus (Boyle et al., 2009). However, the previous results also show that the N terminus still is able to control TGA2 binding. Phosphorylation of the N terminus reduces DNA binding, which could indicate that while the N terminus may not be integral for DNA binding in vitro, it may still be necessary for invoking regulatory control.

The N terminal region of TGA2 contains a non-autonomous repression domain (Rochon et al., 2006). This repression domain is necessary for TGA2 to form higher order complexes that are capable of binding PR-1 in vitro and in vivo (Boyle et al., 2009). Using qPCR and gel filtration analysis of chromatin cross-linked plants, Boyle et al. (2009) showed that formation of higher order complexes on the PR-1 promoter is dependent on the presence of the TGA2 N terminus. When plants were treated with SA, TGA2 was no longer present in higher order formations on PR-1. Additionally, an EMSA experiment using a PR-1 probe containing two TGA2 binding sites (LS5-LS7) showed that while Δ43 is able to form a single DNA-bound complex, TGA2 is able to form distinct higher and lower order complexes that could represent activating and repressing conformations regulated by the N terminus (Boyle et al., 2009). These experiments, in part, lead to the model that is shown in Figure 2.6.

Based on the results from Rochon et al. (2006) and Boyle et al. (2009), it is predicted that the N terminus interacts with other domains on the TGA2 protein to
impart structural control over how TGA2 binds to PR-1. Phosphorylation may play a role in this regulation by inducing conformational changes in the N terminus, possibly by inducing a helical shift (Kirchler et al., 2010) that causes a change in the interaction between the N terminal domain and the rest of the protein.

For this EMSA experiment, we wanted to consider the possibility that the N terminus of TGA2 is able to interact with its C terminus in a DNA binding scenario, and see if phosphorylation on the N terminus by CK2 could play a role in this interaction. This was done using the same recombinant Δ43 as for the previous experiment with a recombinant 6xHis tagged N terminal construct containing the 47 residues of TGA2 (NT47) (Figure 4.2 A). NT47 was treated with CK2 using the same protocol as for the previous experiment. This experiment also used an LS5-LS7 PR-1 probe to see if the addition of NT47 would alter the size or number of protein-DNA complexes.

Lane 2 shows a sample that only contains the Δ43 construct, and shows the strongest binding to the DNA probe (Figure 4.2 B). This lane also contains 2 distinct bands, with the lower band (black arrow) being noticeably darker. The result of the similar experiment done by Boyle (2009) only showed 1 band with PR-1 probe with Δ43, however, the result of this experiment still shows that a lower order complex is likely favourable.

Lane 3 contains untreated Δ43 and NT47, and shows slightly reduced binding in the lower band (grey arrow) compared with lane 2. The reduced DNA binding by Δ43 could be caused by interaction with NT47, or could also be caused by
interference of Δ43-DNA binding by NT47 - making it more difficult for Δ43 to bind to the probe. However, only the lower order complex appears to be affected by the addition of NT47, which could indicate that NT47 only interferes with the formation of the lower order complex on DNA.

Lane 4 shows a sample where NT47 was phosphorylated with CK2 prior to the probe binding reaction. The binding in both bands is partially reduced when compared with the first two samples. This could be an indication that the N terminus is able to interact with other domains of TGA2, and when phosphorylated reduces Δ43 binding to the probe.

The model of TGA2 activity proposed by Boyle et al. (2009) shows that while TGA2 is in its basal, repressive state (without SA induction or NPR1), such as could be represented in lane 2, TGA2 would favour the formation of higher order complexes that could repress PR gene activity. The model also suggests that the N terminus is involved in regulating the formation of higher order complexes. In lane 1, without the N terminus, lower order complexes are favoured. When CK2 is induced, DNA binding is reduced. CK2 induction may cause a structural change in N terminus affecting other protein domains, including the bZIP domain, allowing TGA2 to reduce binding from DNA. TGA2-DNA binding reduction may make TGA2 available to bind NPR1 to form an enhancedosome complex.

While this experiment allows for a relative examination of how strongly proteins can interact with a DNA probe, it is limited in its ability to determine the
type of interaction as well as the size and makeup of the distinct DNA-protein conformations.
Figure 4.2 Δ43 TGA2 and NT47 TGA2 interaction with LS5-LS7 probe.

(A) Diagram of recombinant Δ43 and NT47 proteins. The Δ43 protein is the same as the construct used in the previous experiment (Figure 4.1). The NT47 recombinant protein construct contains only the N terminal repression domain of TGA2 with a 6xHis tag at the C terminus.

(B) EMSA using recombinant Δ43 and NT47 with LS5-LS7 (PR-1) DNA probe that contains 2 TGA2 binding sites. FP stands for free probe, and indicates a sample where no protein was present. +CK2 indicates that a CK2 reaction was used prior to binding the sample to the DNA probe. All the samples shown, except for FP, also
contain ATP as part of the CK2 reaction. The black and grey arrows represent 2 distinctly sized protein-DNA complexes that were formed when using the LS5-LS7 probe.
4.3 Inhibition of DNA binding by CK2 is partially restored by BTB/POZ region of NPR1

Previous experiments have shown that BTB/POZ region of NPR1 is necessary for the coactivation function of NPR1 and TGA2 (Boyle et al., 2009). Transgenic plants containing a defective BTB/POZ with Δ43 show higher PR transcription than plants with a defective BTB/POZ with full length TGA2. Pull-down binding assays have also shown that BTB/POZ interacts with the N terminal region of TGA2 (Boyle et al., 2009). Because N terminal binding to BTB/POZ has a positive affect on PR gene activation, BTB/POZ binding is predicted to block the repression activity of the N terminus.

Given that CK2 affects TGA2-DNA binding (Kang and Klessig, 2005; previous results), an EMSA was performed to test if BTB/POZ would have an effect on the binding ability of TGA2 that has been treated with CK2.

In this experiment TGA2 was treated with CK2. Recombinant His-tagged NPR1 BTB/POZ (POZ) was added to one sample before inducing the probe binding reaction. Lane 2, shows the sample of TGA2 treated with CK2. Phosphorylation by CK2 reduced the binding of TGA2 to the probe. However, in lane 3, when the POZ is added, the binding interaction of TGA2 with DNA probe is restored.

Because previous studies have shown that N terminus of TGA2 is able to interact with POZ (Boyle et al., 2009), this experiment provides further evidence that there is an N terminus effect of CK2 on TGA2. This experiment also shows that
BTB/POZ is able to negate the effect of CK2 activity on this region *in vitro*, restoring TGA2-DNA binding.

These results could suggest that NPR1 and CK2 (or CK-type) kinase are involved in regulating TGA2-DNA binding, with CK2 reducing TGA2-DNA binding and NPR1 inducing or promoting TGA2-DNA binding.
Figure 4.3 EMSA showing interaction between TGA2 and BTB/POZ region of NPR1 (POZ) after CK2 phosphorylation.

(A) EMSA using recombinant TGA2 and CK2 with LS7 DNA probe. FP stands for free probe, and indicates a sample where no protein was present. +CK2 indicates that a CK2 reaction was used prior to binding the sample to the DNA probe. All the samples shown, except FP, contain ATP as part of the CK2 reaction. +POZ indicates that recombinant POZ was added prior to binding the sample to the DNA probe.

(B) From Model of Dual Activity of TGA2 (Boyle et al., 2009). BTB/POZ domain of NPR1 interacts with the N terminus of TGA2.
4.4 NT TGA2 self association is inhibited by CK2

This experiment was done to further explore the interaction of NT47 with Δ43 using Ni-NTA beads, by investigating whether there is a difference in binding between Δ43 and NT47 when NT47 is phosphorylated.

His-tagged Δ43 was added to Ni-NTA beads (His-tagged proteins bind to immobilized Ni²⁺ ions on the bead surface). Then recombinant strep-tagged NT47 was added. Proteins that remained attached to the beads were eluted and analyzed by SDS-PAGE and immunoblot. A Strep tag antibody was used to detect NT47 bound to Δ43. Samples with NT47 treated with CK2 were compared with samples that were untreated.

In Figure 4.4 (A) there was evidence of binding between NT47 and Δ43 in the untreated sample, but binding was reduced when NT47 was phosphorylated with CK2. This could indicate an interaction between N terminus of TGA2 and the rest of the protein when the N terminus is not phosphorylated. However, these results could contradict results seen previously where it seemed that phosphorylated NT47 was able to influence the DNA binding ability of TGA2, which would have suggested an interaction between NT47 and Δ43 when NT47 was phosphorylated. From this experiment it appears like phosphorylation would reduce interaction between Δ43 and NT47, and NT47 would not be able to influence the DNA binding of Δ43.

A possible reason for these contradictory results could be that CK2 inhibits Δ43 to bind to anything. The amount of CK added to NT might be high enough that residual amounts may phosphorylate sites in Δ43 that may not otherwise be
phosphorylated in vivo with the full length TGA2 protein. CK2 used in these experiments may have a higher activity than putative kinases found in planta (Kang and Klessig, 2005). However, from previous experiments and from Kang and Klessig (2005) CK2 has been shown to minimally affect the ability of N terminal deletion constructs to bind DNA. It could also be possible that interaction between Δ43 and NT47 is transient when NT47 is phosphorylated and washing steps in the protocol could eliminate evidence of interaction.

In Figure 4.4 (B) His-tagged NT47 was added to Ni-NTA beads, then strep tagged NT47 added. The sample untreated with CK2 showed evidence of a much stronger binding reaction than for Δ43 bound with NT47. When the strep-tagged NT47 was treated with CK2, protein binding was reduced.

NT47 is a much smaller protein construct (7.78kDa) than Δ43 (33.21kDa) and could possibly bind better with the Ni-NTA beads; or a possible interaction may have better survived washing steps in the protocol. This could have lead to there being a more noticeable difference in binding after the addition of CK2. Strong binding between NT47 and itself could also be congruent with N terminal activity as a repressor of basal PR gene transcription in uninduced states (Rochon et al., 2006). The greater influence of CK2 on NT47 self-association than NT47 binding with Δ43 may be indication of CK2 activity having more influence in regulating TGA2 function as a repressor more than TGA2 function as an activator. The N terminus could help regulate basal repression by helping form higher order TGA2 complexes that dissociate during SAR when CK2 levels increase. It could also indicate that the
activity that regulates the repression activity of the N terminus is separate from the activity that regulates activation.

Since NT47 is capable of binding with itself, the next step was to look at higher order complex formation of NT47 using S300 gel filtration. Similar experiments using N terminal deletion constructs have already shown that TGA2 is able to form higher order complexes that depend on the bZIP domain (Boyle et al., 2009). Gel filtration analysis however has been unable to predict the size of bZIP containing TGA2 construct oligomers, since the protein complexes are large enough to consistently be eluted in void fractions. (Without the bZIP domain, Δ93 TGA2 construct, is able to form dimers and tetramers.)

Gel filtration shows that NT47 is able to form complexes that would be predicted to be between 2 and 6 units in size, with trimers being predominant. Also monomers are present. While NT47 alone is unable to form complexes as large as the full length TGA2, this result is still significant because it shows that NT47 is still able to form higher order complexes without the presence of either the bZIP domain or the CT stabilizing region. This could indicate that the N terminus of TGA2 could be involved in regulating the formation of higher order complexes.
Figure 4.4 N terminus association inhibited by CK2.

(A) Pull-down of NT47 and Δ43 TGA2 using Ni-NTA beads. Δ43 was incubated for 1hr with Ni NTA beads, then Strep-tagged NT47 phosphorylated with CK2 was added. Input lanes represent samples of purified Strep-tagged NT47 as a positive control. Samples analyzed by SDS-PAGE gel and immunoblot using an anti-strep antibody.

(B) Pull-down of NT47 and NT47 TGA2 using Ni-NTA beads. NT47 was incubated for 1hr with Ni NTA beads, then Strep-tagged NT47 phosphorylated with CK2 was added. Input lanes represent samples of purified Strep-tagged NT47 as a positive control. Samples analyzed by SDS-PAGE gel and immunoblot using an anti-strep antibody.
(C) S300 gel filtration analysis of NT47. Purified NT47 strep was run through a Sephacryl S300 gel filtration column. Fractions representing different oligomer sizes (every 5th 0.5ml fraction of approximately 120 fractions total, labeled Frac) were acetone precipitated. The precipitate was then loaded onto an SDS-PAGE gel and analyzed by immunoblot. Shown are fractions representing oligomer sizes for monomer to octomer (# units), and the volume (vol) they represent from the column. Not shown are larger oligomer sized fractions and void fractions, which when analyzed by immunoblot were determined to contain no protein.
TGA2 is a dual function transcription factor involved in both the activation and repression of PR gene transcription. As a repressor, TGA2 forms higher order complexes bound to the as-1 element of the PR1 promoter, inhibiting activation of PR gene transcription. As an activator, TGA2 binds with ankrin repeat and BTB/POZ domains of NPR1 to form an enhancesome complex on PR1 (Boyle et al., 2009).

The N-terminus of TGA2 is a nonautonomous repression domain that regulates basal PR gene transcription, likely by guiding structural changes to TGA2 (Rochon et al., 2006; Boyle et al., 2009). When SA levels increase and induce the activity of NPR1, the BTB/POZ region binds directly with the N-terminus of TGA2 to curtail its repression activity (Boyle et al., 2009). A CK2 or CK2-like kinase is able to reduce TGA2 binding to DNA through an activity that is located in the N-terminus of TGA2 and therefore may be involved in regulating NPR1-TGA2-DNA interactions (Kang and Klessig, 2005). Nevertheless, how the N-terminal phosphorylation through a putative kinase would affect TGA2 activity has previously not been looked at in detail.

5.1 Phosphorylation of N-terminus residues reduces TGA2-DNA binding

Our results confirm that when TGA2 is phosphorylated by a CK2 kinase in vitro, binding to DNA is reduced, by an activity dependant on the N terminus of TGA2. When Δ43, a TGA2 construct missing the first 43 residues of the N-terminus, is treated with CK2, DNA probe binding is not reduced to the same extent as for the
full length TGA2 protein (Figure 4.1). Kang and Klessig (2005) have suggested that the phosphorylatable residues important in reducing DNA binding are: 11Ser, 12Thr and 16Thr, however, other residues in the N-terminus are potentially phosphorylated as well. It has also been shown that in other Arabidopsis bZIP proteins phosphorylation of residues in basic domain cause helical shifts affecting DNA binding (Kirchler et al., 2010) (Figure 5.1). It is possible that changes in the N-terminus would make available or actively invoke changes in the bZIP domain making residues available for phosphorylation or other modification. While Kirchler (2010) focused on phosphorylatable residues found in Arabidopsis bZIP protein bZIP62, TGA2 contains residues in, or near the bZIP domain that are predicted to be phosphorylated by a CK2 kinase (30Thr, 31Ser) (Blom et al., 1999).

Which residues are phosphorylated may be dependant on the plant kinase activity. While it has been shown that the kinase is likely CK2 (Strange et al., 1997; Hidalgo et al., 2001; Kang and Klessig, 2005), it is possible that the recombinant or rabbit reticulocyte lysate kinase activity used in TGA2-DNA binding experiments may be stronger, or different from the activity that would be seen in vivo. This could make it difficult to pinpoint the exact phosphorylated residues. Regardless, these results and those seen by Kang and Klessig (2005) indicate that phosphorylation of the N-terminus of TGA2 reduces binding on DNA.
Figure 5.1 Phosphorylation of bZIP residues causes helical shift and reduced DNA binding, from (Kirchler, 2010).

(A) Model of Arabidopsis bZIP62 basic/leucine zipper domain and interaction of serine residues with the DNA backbone.

(B) Phosphorylation of serine residues causes a helical shift compared with the original position (transparent) (right image). In some instances for Arabidopsis bZIP proteins, Ser is replaces with Thr. (Kirchler et al., 2010).
5.2 **Reversible TGA2-DNA binding is influenced by CK2 and NPR1**

Our results show that when POZ is added to phosphorylated TGA2, reduced DNA probe binding is restored (Figure 4.3). This could indicate that BTB/POZ domain of NPR1 is able to restore TGA2-DNA binding reduced by CK2 activity. This also confirms that the N-terminus of TGA2 is able to influence DNA binding activity, since BTB/POZ binds exclusively to the N-terminus.

How the N-terminus is able to influence bZIP domain binding to DNA is still unknown. Previous results have suggested that the N-terminus would be able to influence the transcriptional activation activity of TGA2, likely by guiding conformational changes (Figure 4.2; Boyle et al., 2009). The EMSA result experiment showing phosphorylated NT47 affecting the ability of Δ43 to bind to DNA would confirm this suggestion. But, results from the pull-down assay using Δ43 and NT47 seem to indicate that Δ43 binds to NT47 preferentially when NT47 is not phosphorylated (Figure 4.4A). It is possible that wash steps in the protocol may have eliminated evidence of an interaction if the interaction is transient; or using two separate protein constructs causes stress or instability during conformational changes that wouldn't occur in the full length protein. This is an area that could be further explored.

NTA-Ni pull-down assay and gel filtration analysis showed strong N-terminal self-association and higher order complex formation without the TGA2 bZIP domain and C-terminal region (Figure 4.4). When NT47 is treated with CK2, self-association is reduced, and NT47 likely exists primarily as a monomer. This could suggest that
phosphorylation of the N-terminus is involved in regulating higher-order complexes of TGA2. A higher-order complex may block activating elements in the PR promoter, and may make TGA2 residues involved in bZIP helical shift and PR activation with NPR1 unavailable.

5.3 Model of TGA2 dual activity

Under basal conditions, TGA2 forms higher-order complexes on DNA, influenced by electrostatic interactions of $a$ and $d$ position residues in the bZIP domain (Vinson, 2005; Deppmann, 2004). TGA transcription factors contain charged residues in these positions that destabilize the leucine zipper and favour the formation of heterodimer over homodimer complexes. Higher-order complexes in TGA2 are also stabilized by a C-terminal stabilizing region (Niggeweg et al., 2000b; Boyle et al., 2009); and based on our results, the N-terminus of TGA2 may also be involved in stabilizing the formation of higher-order complexes in basal conditions.

SAR induction causes a rise in SA as well as an increase in kinase activity. Phosphorylation of the N-terminus of TGA2 reduces binding from the as-1 element in PR1, likely by causing a helical shift in the bZIP domain.

SAR induction also causes increased nuclear NPR1. NPR1 is able to bind to TGA2 with the POZ region binding specifically to the N-terminus of TGA2. This likely inactivates the N-terminus of TGA2 while stabilizing TGA2 homodimers. The NPR1-TGA2 complex is then able to bind to the as-1 activating element (LS7) to promote PR gene transcription.
Figure 5.2 TGA2 dual activity is influenced by a CK2-like kinase and NPR1.

(A) Under basal conditions (when not induced by SA) TGA2 forms higher-order complexes that are promoted by N-terminal binding. Higher-order complexes bind to DNA to repress PR transcription.

(B) When induced by SA, kinase levels increase and cause N-terminal conformational changes, reducing N-terminal domain binding to itself and basic domain binding to DNA. NPR1 promotes TGA2 homodimer formation and DNA binding to activate PR transcription.
TGA2 binding to DNA is reversible, and influenced by interactions with NPR1 and a putative kinase. These interactions could be involved in regulating the dual activation and repression of PR-1 activity by TGA2 by guiding structural modification in the N-terminus of TGA2.

The N-terminus of TGA2 acts as a basal repression domain, by influencing the formation of higher-order complexes that block PR-1 gene transcription. Phosphorylation of the N-terminus by a CK2-like kinase likely causes a reduction in TGA2-DNA binding as an oligomer, and promotes NPR1-TGA2-DNA binding as a TGA2 homodimer bound to a NPR1 dimer, activating PR-1 transcription.

It is still unknown how the N-terminus is able to influence TGA2 bZIP domain DNA binding, or how the N-terminus influences the formation of higher order complexes. These are areas that could be further explored.
CHAPTER 7: FUTURE STUDIES

6.1 CK effects of oligomerization on TGA2

The results shown in Chapter 4 provide some evidence of CK2 involvement in regulating TGA2 complex formation by phosphorlating residues in the N-terminus, which could favour the formation of homodimer stabilized by NPR1. Our studies focused on how the CK2 affected complexes formed by the N-terminus, however it is still not known how this would translate in the context of the whole protein.

Gel filtration experiments could be done comparing CK2-treated wild-type TGA2 with CK2-treated Ser/Thr to Ala TGA2 mutant constructs to determine if certain residues or domains are involved in regulating higher-order complex formation. This type of experiment can also be done using an LS7 probe and may be able to provide information regarding phosphorylation of residues affecting TGA2-DNA binding, since CK2-phosphorylatable residues exist in the bZIP domain and C-terminal region as well.

6.2 Interaction between Δ43 and NT47

Some results in Chapter 4 showed evidence of an interaction between Δ43 and NT47 when NT47 is phosphorylated, however, other results would suggest that Δ43 only interacts with NT47 prior to phosphorylation. Dual Polarization Interferometry (DPI) is a technique that would test for real-time interaction, by using dual waveguides to detect changes in protein mass and structure (Ronan, 2004). A protein sample, such as Δ43, can be immobilized onto a chip/slide and then
challenged with a second, smaller protein, such as NT47. If the smaller protein interacts, even transiently, with the larger protein it would cause an optical interference pattern that could be detected. This is a technique that could be used to determine if there are changes in protein interaction between Δ43 and NT47 when NT47 is phosphorylated.
REFERENCES


function of the salicylic acid signaling pathway in controlling cell death, defenses, and cell growth. Plant Cell 11, 1695-1708.


APPENDIX: S300 GEL FILTRATION

Parameters for the S300 Gel Filtration Column:

Vt (Total bed volume of the column) = 100.5 ml

Vo (Void volume of the column evaluated with Blue Dextran 2000) = 38 ml

Kav = (ve - Vo)/(Vt - Vo)

Table A.1 Protein standards for the S300 Gel Filtration Column

<table>
<thead>
<tr>
<th>MW (kDa)</th>
<th>Elution vol (ml)</th>
<th>logMW</th>
<th>Kav</th>
</tr>
</thead>
<tbody>
<tr>
<td>67</td>
<td>41.89</td>
<td>1.826074803</td>
<td>0.124900264</td>
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<td>43</td>
<td>46.18</td>
<td>1.633468456</td>
<td>0.190726079</td>
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<td>1.397940009</td>
<td>0.335880439</td>
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<td>13.7</td>
<td>62.12</td>
<td>1.136720567</td>
<td>0.435309642</td>
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</tbody>
</table>
Figure A.1 Calibration curve for the S300 Gel Filtration Column.

Four protein standards shown in Table A.1 were used to establish a standard curve.
Table A.2 Elution fractions for NT47.
Predicted elution volumes for Step-tagged NT47 species for the S300 Gel Filtration column shown in Figure 4.4 (C) and corresponding elution fractions based on the calibration curve in Figure A.1. No protein was present in Vo fractions*.

<table>
<thead>
<tr>
<th>NT47-Strep species</th>
<th>MW (kDa)</th>
<th>logMW</th>
<th>Predicted Kav</th>
<th>Predicted Ve (ml)</th>
<th>Column Fractions Peak</th>
</tr>
</thead>
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<tr>
<td>monomer</td>
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<td>0.820857989</td>
<td>0.634339497</td>
<td>77.64621854</td>
<td>98-100</td>
</tr>
<tr>
<td>dimer</td>
<td>13.24</td>
<td>1.121887985</td>
<td>0.556974788</td>
<td>72.81092424</td>
<td>89-91</td>
</tr>
<tr>
<td>trimer</td>
<td>19.86</td>
<td>1.297979244</td>
<td>0.511719334</td>
<td>69.98245839</td>
<td>83-85</td>
</tr>
<tr>
<td>tetramer</td>
<td>26.48</td>
<td>1.422917981</td>
<td>0.479610079</td>
<td>67.97562993</td>
<td>79-81</td>
</tr>
<tr>
<td>6-mer</td>
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<td>1.59900924</td>
<td>0.434354625</td>
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<td>8-mer</td>
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<tr>
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<td>0.377339497</td>
<td>61.58371854</td>
<td>66-67</td>
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</tbody>
</table>

*Void volume (Vo) has a predicted Ve of 38ml and a S300 gel filtration peak would be represented by column fractions 22-21. [The full-length TGA2 protein, has been shown by gel filtration to elute in Vo fractions, and therefore a represent large oligomer species of unknown size (Boyle et al., 2009).]