Isolation and Characterization of Proteins Interacting with Tobacco Transcription Factor TGA2.2

Dissertation

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In the name of Allah, the Compassionate, the Merciful

They are asking you concerning the Spirit. Say: The Spirit is by command of my Lord, and you are not given aught of knowledge but a little.

The Noble Qur´an 17:85

Table of Contents

1. Abstract

In plants, the *as-1 cis*-element and its *trans*-acting factors, known as TGA transcription factors, play an important role in the transcriptional control of salicylic acid- and auxin-inducible gene expression. Previous experiments have established that TGA2.2 is the major component of the nuclear *as-1*-binding factor (ASF1) and cellular salicylic acid response protein (SARP) that bind to the *as-1* element. Using TGA2.2 as bait in a modified yeast hybrid screen, several cDNA TGA2.2-interacting proteins were isolated.

TGA2.2 interacts specifically in yeast and *in vitro* with two closely related proteins, At1g50570 and At5g55530 that contain a C2 domain in their amino acid sequences. At1g50570 interacts with class-II members of tobacco TGA factors. The At1g50570 and At5g55530 proteins function as transcriptional activators in yeast. At1g50570 protein activated the transcription of the *β-glucuronidase* reporter gene driven by the *as-1* element in BY-2 protoplasts. At1g50570-green fluorescent protein fusion protein localized mainly to the nuclear envelope and endoplasmic reticulum in BY-2 protoplasts. Expression of the *At1g50570* gene was induced in response to pathogen infection. Overexpression or repression of the *At1g50570* gene in transgenic Arabidopsis plants did not alter the expression of the *PR-1* gene under unstressed or salicylic acid -induced conditions. The role of At1g50570–TGA2.2 interaction in *as-1*-mediated gene activation has yet to be examined.

TGA2.2 interacts specifically with At1g28480, a glutaredoxin protein, which is known to catalyze reductions of disulfides. At1g28480 interacts specifically with all identified members of the tobacco TGA transcription factors. The At1g28480-TGA2.2 interaction was neither affected by oxidative stress conditions nor by site-directed mutagenesis of the TGA2.2 and At1g28480 encoded conserved cysteine residues. At1g28480 enhances indirectly the TGA2.2 dimerization and *in vitro* DNA binding activity by increasing steady state TGA2.2 protein levels in yeast. The interaction between At1g28480 and TGA2.2 was confirmed *in planta* by a protoplast two-hybrid system. In addition, At1g28480 interacts via TGA2.2 with NPR1, a key regulator of systemic acquired resistance, in a yeast three-hybrid system. The *At1g28480* gene is expressed in Arabidopsis plants in response to SA and pathogen infection. Overexpression or repression of the *At1g28480* gene in transgenic Arabidopsis plants did not alter the salicylic acid-inducible gene expression. In contrast, overexpression of the *At1g28480* repressed the auxin-induced expression of an *as-1::GUS* reporter construct in Arabidopsis transgenic plants. These results indicate that At1g28480 protein might function in signal transduction pathways involving TGA transcription factors.

2. Introduction

2.1 Plant Pathogen Interaction

During their life span, plants encounter unusual or adverse conditions that have a significant impact on their physiology and development. For instance, plants are constantly exposed to many enemies such as pathogenic microorganisms including fungi, bacteria, nematodes and viruses. Successful pathogen invasion ensues from the disability of the plant to recognize the pathogen. Alternatively, the activated defense mechanism might be ineffective or pathogen virulence factors might suppress host defenses (Hammond-Kosack and Jones, 1996).

When a pathogen invades a host plant there are three main strategies to attack: necrotrophy, biotrophy, or hemibiotrophy (Hammond-Kosack and Jones, 1997). Necrotrophic pathogens have a broad host range and are characterized by killing the host cells first before metabolizing their contents. The biotrophic have a narrow host range and they invade living organisms and exploit their metabolism without killing them. A hemibiotrophic pathogen acts similarly as a biotrophic pathogen but its invasion will lead eventually to host cell death. When a pathogen successfully invades the host and causes disease, the pathogen is said to be virulent, the host to be susceptible and the interaction to be compatible (Glazebrook *et al.*, 1997a). In the contrary, if the host responds by activating defense responses that suppress pathogen colonization, the pathogen is said to be avirulent, the host is resistant and the interaction is incompatible.

Plants show resistance to most pathogens. This phenomenon of general resistance is due to a constitutive arsenal of powerful weapons that can be used against pathogen invasions in a nonspecific manner (Ryals *et al.*, 1996). Plants show other types of resistances, which are inducible (Bent, 1996). For instance, the inducible pathogen resistance, known as race or cultivar specific resistance, is activated upon the recognition of a specific pathogen by the host.

2.2 Gene-for-Gene Resistance

Harold Flor, working with flax and flax rust fungus, proposed a genetic definition for the plant pathogen interactions, known as the gene-for-gene hypothesis (Flor, 1971). Flor postulated that the host resistance occurs, when a particular *avirulence* gene (*Avr* gene) product carried by a pathogen is recognized by a particular host *resistance* gene (*R* gene) product, *i.e., R* gene confers resistance (incompatibility) only against pathogens carrying a correspondent *Avr* gene and as a consequence the race specific resistance is deployed (Table 2.1; Bent, 1996).

Table 2.1. Types of plant and pathogen genetic interactions in the gene for gene resistance.

		Host plant genotype	
		R	*r*
Pathogen genotype	*Avr*	***Incompatible***	Compatible
	avr	Compatible	Compatible

Several plant *R* genes have been isolated and were categorized upon their structural motifs into five different classes: class I- intracellular proteins with a nucleotide-binding site (NBS), a leucine-zipper motif and a leucine-rich repeat (LRR) domain and a coiled-coil (CC) domain, class II- intracellular NBS-LRR proteins with a region of similarity to the cytoplasmic domain of mammalian IL-1 receptor and the *Drosophila* Toll proteins (TIR domain), class III- intracellular protein kinases, class IV- proteins with an LRR domain that encodes membrane-bound extracellular proteins and class V receptor-like kinases with an extracellular LRR domain (Dangl and Jones, 2001; Martin, 1999; Martin *et al.*, 2003; McDowell and Woffenden 2003). The model plant Arabidopsis genome contains around 200 *R* genes that encode proteins with similarity to the nucleotide-binding site and other domains characteristic of many identified plant resistance proteins (Meyers *et al.*, 2003).

The pathogen *Avr* genes are only present in certain strains of a given pathogen and show little similarity to each other (although myristoylation encoding sites is a

common motif found in many Avr proteins). In additional to their role as avirulence factors, they are beneficial for the pathogen through their interaction with host virulence targets that are involved in metabolism or in plant defense (Bonas and van den Ackerveken, 1999; Luderer and Joosten, 2001).

The incompatible interaction between the host and pathogen triggers a series of defense responses at the site of infection that includes synthesis of antmicrobial compounds (such as glutathione S-transferases (GSTs), phytoalexins and pathogenesis related (PR) proteins), programmed cell death, production of reactive oxygen species (ROS; Reviewed in: Abel and Hirt, 2004; Lamb and Dixon, 1997), ion fluxes, cell wall fortification, activation of protein kinases and the accumulation of signaling molecules such as salicylic acid (SA), jasmonic acid (JA), ethylene and nitric oxide (Bent, 1996; Cohn *et al.*, 2001; Hammond-Kosack and Jones, 1996).

Gene-for-gene mediated resistance against avirulent necrotizing pathogens triggers a localized programmed cell death around the infection site, a phenomenon known as the Hypersensitive Response (HR) (Figure 2.1; Heath, 2000; Lam *et al.*, 2001; Lamb and Dixon 1997). The HR results in the production of ROS and the formation of lesions around the infection site, which will restrict the spreading of the pathogen into the surrounding tissue.

Figure 2.1. A classic HR from tobacco in response to tobacco music virus infection (Lam *et al.*, 2001).

2.3 Systemic Acquired Resistance

Concomitant with the appearance of the HR, an inducible long lasting resistance appears systemically. This resistance, known as the systemic acquired resistance (SAR), is directed to a broad spectrum of different pathogens and is characterized by a reduction in disease symptoms after subsequent pathogen infections (Reviewed in: Durrant and Dong 2004; Ryals *et al.*, 1996; Sticher *et al.*, 1997). The SAR is associated with the local and systemic accumulation of PR proteins and has been well characterized in different plant species (Sticher *et al.*, 1997).

It was postulated that a systemic signal produced at the infection site is translocated to the uninfected tissues (Ross, 1966). White (1979) showed that tobacco treatment with acetyl salicylic acid (aspirin) induced the accumulation of PR proteins and decreased disease symptom development. Van Loon (1983) found that SAR is induced by the synthesis of plant secondary metabolites such as 2,6-dihydroxybenzoic acid, which mimics the action of SA. In support of these findings, increased SA levels were observed in the phloem of tobacco mosaic virus (TMV)-infected tobacco and tobacco necrosis virus infected cucumber plants (Malamy *et al.*, 1990; Métraux *et al.*, 1990). These findings led to the suggestion that SA might be the systemic signal involved in SAR.

Conclusively, the importance of SA in SAR was further documented using Arabidopsis and tobacco transgenic plants expressing the *NahG* gene that encodes a *Pseudomonas putida* salicylate hydroxylase enzyme that converts SA to catechol (Gaffney *et al.*, 1993; Delaney *et al.*, 1994). In *NahG* plants, SA accumulation is reduced and a subsequent breakdown in SAR occurs. However, recent genetic analysis of Arabidopsis *NahG* plants demonstrated that the SAR breakdown is not only related to the absence of SA, but the *NahG* expression and the resulting levels of catechol have also a pleotrophic effect on the plant defense response (Heck *et al.*, 2003; van Wees *et al.*, 2003). Based on these observations, many of the results based solely on phenotypes of *NahG* plants remain to be reconciled.

SA synthesis *in planta* is proposed to be as a product of the phenylpropanoid metabolism pathway via the decarboxylation of trans-cinnamic acid to benzoic acid and its subsequent 2-hydroxylation to SA (Figure 2.2; Lee *et al.,* 1995; Shah 2003). In some bacteria species, SA is synthesized from chorismate via isochorismate through two important enzymes: the isochorismate synthase (ICS) and isochorismate pyruvate lyase (IPL) (Serino *et al.*, 1995). Recent studies have shown that the expression of the bacteria ICS and IPL lyase enzymes in plants led to enhanced SA accumulation and conferred disease resistance against pathogens (Mauch *et al.*, 2001; Verberne *et al.*, 2000). Functional analysis of an Arabidopsis gene encoding an ICS revealed that SA is synthesized through the isochorismate pathway in the chloroplast compartment (Figure 2.2; Wildermuth *et al.*, 2001).

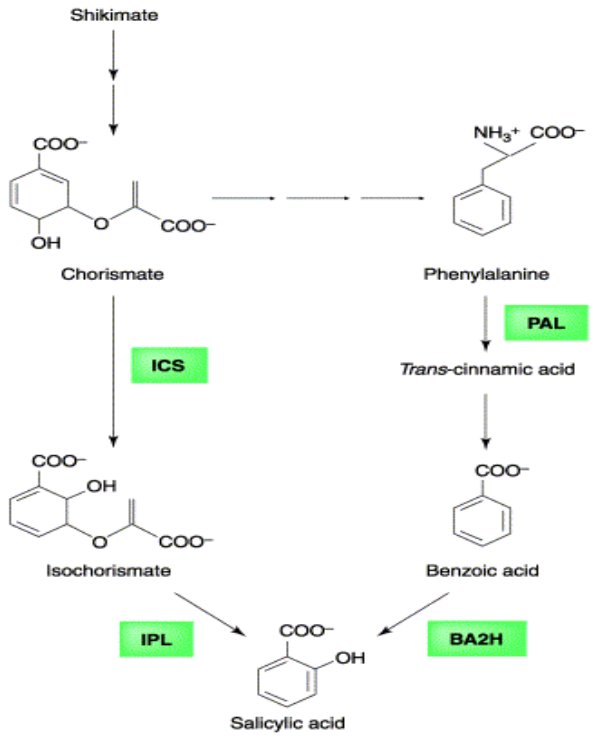


Figure 2.2. Proposed SA biosynthetic pathways in plants (Shah 2003).

Abbreviations: ICS, isochorismate synthase; PAL, phenylalanine ammonia-lyase; BA2H, benzoic acid-2 hydroxylase; IPL, isochorismate pyruvate lyase.

However, some studies had suggested that SA is not the primary mobile signal. For instance, grafting experiments using transgenic tobacco plants expressing the *NahG* gene showed that the *NahG* tobacco rootstocks were capable of delivering a signal that make nontransgenic scions resistant to further pathogen infection (Vernooij *et al.*, 1994). In contrast, other experiments with labeled SA showed that SA was originally synthesized in and exported from the infected leaf (Mölders *et al.*, 1996; Shulaev *et al.*, 1995).

The molecular markers for SAR, the *PR* genes, were first described in tobacco leaves infected with TMV and were classified as basic or acidic PR forms (Ryals *et al.*, 1996; Ward *et al.*, 1991). An abbreviated definition of PR proteins is: a group of proteins detected and defined as being absent in healthy plants but accumulating in large amounts after pathogen infection (van Loon and van Kammen, 1970). The *PR* genes are found in different plant species and several cloned genes such as *PR-1*, *β-1,3-glucanases* (*PR-2*), *chitinasases* (*PR-3*), *PR-4* and *osmotin* (*PR-5*) showed *in vitro* antmicrobial activities (Sticher *et al.*, 1997; van Loon and van Strien, 1999). SA induces the expression of many *PR* genes, for instance, exogenous application of SA or its analogs such as 2,6-dichloroisonicotinic acid (INA) or benzo(l,2,3)thiadiazole-7-carbothioic acid S-methyl ester (BTH) induced the expression of many *PR* genes (Friedrich *et al.*, 1996; Vernooij *et al.*, 1995; Ward *et al.*, 1991).

2.4 Other Forms of Plant Resistance

In addition to gene-for-gene resistance and SAR, other plant responses against pathogens were identified. The wound response, which mediates resistance against insect feeding, plays an important role in plant defense against specific fungal pathogens (Diaz *et al.*, 2002; Vijayan *et al.*, 1998). The wound response requires JA and ethylene as signaling molecules and both molecules work synergistically on the induction of many plant resistance genes including the basic form of *PR-1*, *osmotin*, *thionin2.1* and *plant defensin1.2* (*PDF1.2*) (Penninckx *et al.*, 1998; Xu *et al.*, 1994). The *PDF1.2* gene from Arabidopsis, which encodes a protein with demonstrated antifungal

activity, is induced strongly by either pathogen challenge or JA but not by SA (Penninckx *et al.*, 1996).

Nitric oxide, which acts as a signaling molecule in the animal immune responses, exerts signaling properties that regulate the activation of plant defense responses after pathogen attacks (Delledonne *et al.*, 1998; Klessig *et al.*, 2000; Chandok *et al.*, 2003). Nitric oxide induces hypersensitive cell death in soybean and moreover, inhibitors of nitric oxide synthesis compromise Arabidopsis disease resistance against *Pseudomonas syringae* (*P. syringae*), promoting disease and bacterial growth (Delledonne *et al.*, 1998).

Another form of plant inducible resistance, known as the induced systemic resistance (ISR), was discovered after the colonization of Arabidopsis roots with the plant growth promoting nonpathogenic rhizobacteria *Pseudomonas fluorescens* (Pieterse *et al.*, 2002). ISR confers systemic resistance against many pathogens in a SA-independent manner but it requires an intact JA and ethylene pathway (Pieterse *et al.*, 1998).

2.5 SA-Dependent Signal Transduction Pathways

To understand the mechanisms through which SA regulates SAR, biochemical approaches were used to define SA binding proteins. The first SA binding protein identified was the SA binding protein 1 (SABP1), a tobacco catalase that converts hydrogen peroxide (H_2O_2) to H_2O and O_2 (Chen *et al.*, 1993). A second tobacco SA-binding protein (known as SABP2), a lipase, was isolated and showed higher binding specificity for SA and SA analogs (Du and Klessig, 1997; Kumar and Klessig, 2003). The silencing of *SABP2* expression suppressed local resistance to TMV, SA induction of *PR-1* gene expression and SAR development, indicating that SABP2 is a SA receptor that is required for the plant immune response (Kumar and Klessig, 2003). A third SA binding protein, termed SABP3 and identified as a chloroplastic carbonic anhydrase protein, was isolated and the silencing of *SABP3* gene expression in

tobacco leaves suppressed the HR in a well-known plant pathogen incompatible interaction (Slaymaker *et al.*, 2002).

The SA inhibitory role on SABP1 catalase activity leads to elevate the levels of H_2O_2, which is known to induce the expression of *PR-1* gene (Klessig *et al.*, 2000). However, many reports questioned this catalase inhibition hypothesis as some findings indicate that H_2O_2 might function upstream of SA rather than downstream (Chamnongpol *et al.*, 1998; Neuenschwander *et al.*, 1995). Another postulated hypothesis for SA mode of action is based on the generation of SA free radicals, which are produced through the SA-mediated inhibition of catalases (Durner and Klessig, 1995).

To analyze the SA-dependent signal transduction pathways controlling the defense responses against pathogens, the *Arabidopsis thaliana* model plant was used extensively to isolate and characterize genes that are involved in these processes (Reviewed in: Glazebrook *et al.*, 1997a). Arabidopsis plants possess favorable traits for experimental work, such as short life cycle, small size, self-pollination capability, small genome size and its feasibility for molecular and genetic methods (Meyerowitz, 1987).

To elucidate the SAR signaling pathways in Arabidopsis, many genetic screens were conducted to identify genes that are involved in SA synthesis and/or SAR establishment. The correlation of HR and lesion formation with the development of SAR stimulated the search for mutant lines that are able to form spontaneous lesions; such mutant lines were isolated and termed the *lesion mimic mutants* (*lsm*) (Reviewed in: Lorrain *et al.*, 2003). The *lsm* are characterized with constitutive high SA levels and elevated defense gene expression in the absence of pathogen infection, suggesting that these mutants mimic the HR response that might stimulate SAR signaling (Glazebrook, 2001). About 37 *lsm* were identified in Arabidopsis and were classified into two groups: initiation or propagation mutants. Initiation mutants form lesions of determinate size whereas propagation mutants have uncontrolled lesion size (Lorrain *et al.*, 2003).

To analysis the function of those mutants regarding SA signaling pathways many crosses between *NahG* transgenic plants and *lsm* mutants were conducted. In some crosses, an important role of SA in lesion formation was found, for instance a suppression of spontaneous lesion formation was found when the *lesion simulating disease resistance response 6* (*lsd6*), *lsd7* and *constitutive expresser of PR 22* (*cpr22*) mutants were crossed to *NahG* plants (Weymann *et al.*, 1995; Yoshioka *et al.*, 2001). Other lesion mimic mutants were not affected by the SA absence. For instance, the lesion formation in *lsd2*, *lsd4* and *lsd5* mutants was not suppressed in the *NahG* background (Hunt *et al.*, 1997).

The identification of *defense, no death 1* (*dnd1*) mutant, encoding a cyclic nucleotide-gated ion channel, questioned the assumption that HR development and lesion formation are associated with SAR establishment (Yu *et al.*, 1998). The *dnd1* exhibited elevated SA levels and pathogen resistance with an apparent reduced HR formation phenotype (Clough *et al.*, 2000). Other Arabidopsis mutants, such as *cpr1* and *cpr6*, exhibit increased concentrations of SA, constitutive *PR* gene expression and enhanced resistance to pathogen without any lesion formation (Bowling *et al.*, 1994: Clarke *et al.*, 1998). So caution should be considered dealing with *lsm*, as they might be evolved from other stresses that might influence indirectly SA accumulation (Dong, 2001).

Besides *NahG* plants, Arabidopsis mutants that accumulate low SA levels and *PR* transcripts after pathogen inoculation were isolated. The *SA induction-deficient 1* (*sid1*; also known as *enhanced disease susceptibility 5* (*eds5*) (Rogers and Ausubel, 1997)), *sid2* (also known as *eds16* (Dewdney *et al.*, 2000)), and *eds4* mutants did not accumulate SA after pathogen inoculation and showed enhanced susceptible to pathogens (Gupta *et al.*, 2000; Nawrath and Métraux, 1999). The *SID1*/*EDS5* encodes a protein homologous to members of the multidrug and toxin extrusion (MATE) transporter family (Nawrath *et al.*, 2002). SID1 might be involved in transportation of phenolic compounds transportation involved in SA biosynthesis (Shah, 2003). The *SID2* encodes the ICS described above (Wildermuth *et al.*, 2001). SA application

complemented the *sid2* phenotype and restored *PR-1* expression indicating that *SID2* is involved only in SA synthesis (Nawrath and Métraux, 1999).

The *EDS1* and *phytoalexin deficient 4* (*PAD4*) Arabidopsis genes, encode lipase-like proteins that can interact physically with each other and act upstream of SA accumulation (Falk *et al.*, 1999; Feys *et al.*, 2001; Jirage, *et al.*, 1999). The *pad4* and *eds1* mutant plants are compromised in SA synthesis after pathogen infection (Feys *et al.*, 2001; Zhou, *et al.*, 1998). The *cpr1*, *cpr6* and *enhanced disease resistance 1* (*edr1*) mutants with high SA levels, *PR* gene expression and resistance phenotypes are completely dependent on *EDS1* and *PAD4* function and their role was placed upstream of SA accumulation (Clarke *et al.*, 2000; Clarke *et al.*, 2001; Frye *et al.*, 2001; Jirage *et al.*, 2001). The high levels of SA, found in the lesion mimic mutant *cpr5*, were reduced in the *eds1* and *pad4* mutants and completely blocked in the *eds5* mutant, indicating that the SA accumulation does not relay absolutely on *EDS1* and *PAD4*, whereas *EDS5* seems to be crucial (Clarke *et al.*, 2000; Clarke *et al.*, 2001; Jirage *et al.*, 2001). The elevated SA levels in the *dnd1* mutant were unaffected by PAD4, suggesting the presence of PAD4-independent pathway for SA accumulation, however, a reduction of the *dnd1* enhanced resistance was observed in *pad4* plants suggesting that some resistance responses required PAD4 regardless its SA accumulation function (Jirage *et al.*, 2001).

Lipid signaling seems to modulate SA mediated responses in plants. The fact that, *SABP2*, *EDS1* and *PAD4* genes encode proteins with lipase domains suggests that a lipid derivative might act as a signal that modulates SA accumulation and function after pathogen infection (Dong, 2001; Kumar and Klessig 2003). The Arabidopsis α-dioxygenase enzyme that oxidizes 16- and 18-C fatty acids and results in 2-hydroperoxy fatty acids production was strongly induced after infection with *P. syringae* (de Léon *et al.*, 2002). The levels of hydroperoxy, hydroxy and keto fatty acids were elevated during pathogenesis and especially during the intiation of HR (Rustérucci *et al.*, 1999). Exogenous application of lipid peroxides (the products of lipid peroxidation) induces *PR* gene expression in tobacco cell cultures (Anderson *et al.*,

1998). The *defective in induced resistance 1* (*dir1*) mutant is unable to develop SAR or express *PR* genes, although it has a normal local resistance to pathogens (Maldonado *et al.*, 2002). The wild-type *DIR1*, which has sequence similarity to lipid transfer proteins (LTP), have a wild-type SA metabolism and a normal response to SA and INA. However, petiole exudate experiments showed that the phloem sap from *dir1* is deficient in the SAR mobile signal, while *dir1* plants still respond to a signal contained in the sap from wild-type petiole exudates. These data suggest that DIR1 might function in the generation or transmission of a lipid mobile signal that's is involved in SAR establishment.

Using activation tagging, the *constitutive disease resistance 1* (*CDR1*) gene encoding an apoplastic aspartic protease was identified (Xia *et al.*, 2004). The overexpression of *CDR1* causes enhanced resistance to virulent *P. syringae* and it seems that CDR1 mediates a peptide derivative signal that is involved in SA accumulation. SA is speculated to generate feedback loops that modulate SA upstream signals components (Shah, 2003). SA could be a part of an amplifying mechanism that lead to the activation of a broad spectrum plant resistance, for instance, the application of SA activates the expression of some *R*, *EDS1*, *PAD4*, *EDS5* and *SID2* genes.

The involvement of mitogen activated protein kinase (MAPK) cascades in SAR signaling pathways were also described (Reviewed in: Innes, 2001; Zhang and Klessig, 2001). In tobacco, the TMV-infection of tobacco plants that carry the *N* resistance gene against TMV leads to the activation of two tobacco MAPKs: a SA-induced protein kinase (SIPK) and a wound-induced protein kinase (WIPK) (Zhang and Klessig, 1998). Both proteins are speculated to be positive signaling components acting downstream of SA in both local and systemic defense responses. Strikingly, SIPK protein regulates *WIPK* gene expression, demonstrating that both proteins are involved in the positive regulation of the same disease resistance-signaling pathway (Liu *et al.*, 2003).

In Arabidopsis, a MAPK gene known as *MPK4* negatively regulates SA-mediated defense responses (Petersen *et al.*, 2000). A mutation in *MPK4* causes the accumulation of high levels of SA, constitutive *PR-1* expression and enhanced disease

resistance. The *mpk4* phenotype was blocked when it was crossed to the *NahG* transgenic plants, indicating that MPK4 exerts its function upstream of SA. The *EDR1* gene, belongs to the group of Raf-like MAPKKs, has a regulatory function in SA singaling pathways (Frye and Innes, 1998). The *EDR1* exerts a negative regulatory function in defense responses as *edr1* mutant showed enhanced resistance and had rapid expression of *PR* genes. The *NahG* transgene suppresses *edr1* phenotype, indicating that *EDR1* function upstream of SA accumulation (Frye *et al.*, 2001).

A complete plant MAP kinase cascade that may function downstream of the flagellin receptor involves MEKK1, MKK4/MKK5 and MPK3/MPK6) and WRKY22/ WRKY29 transcription factors was identified using an Arabidopsis leaf cell system (Asai *et al.*, 2002). The BWMK1, a MAPK from rice, can phosphorylate the rice ethylene-responsive element-binding protein 1 (OsEREBP1) transcription factor, which is known to bind to *GCC* box located in the promoter region of several *PR* genes (Cheong *et al.*, 2003).

2.6 NPR1, a Key Regulator in Controlling SAR Gene Expression

Several different genetic screens were employed to identify SAR signaling components acting downstream of SA accumulation. A key regulator in controlling *PR* gene expression and SAR deployment is the non-expressor of *PR-1* (NPR1) protein, also known as non-inducible immunity 1 (NIM1) (Cao *et al.*, 1994; Delaney *et al.*, 1995; Glazebrook, *et al.*, 1997b). The Arabidopsis *npr1* mutant displays a compromised resistance against avirulent pathogens, non-responsiveness to different SAR-inducing agents (SA, INA), low expression levels of *PR* genes, hyper-susceptibility to bacterial and fungal infections, hyper-sensitivity to SA (indicating that NPR1 protein is not only required for SA signal transduction but also for a negative feedback regulation mechanism of SA accumulation) and it does not mount an effective SAR (Cao *et al.*, 1994; Cao *et al.*, 1997; Delaney *et al.*, 1995).

The *NPR1* gene was cloned and found to encode a novel 65 kDa protein with no obvious biochemical function except the presence of a nuclear localization signal at its

C-terminus end part and two domains involved in protein-protein interaction, an ankyrin repeat domain located to the central region and a BTB/POZ (broad-complex, tramtrack, and bricà-brac/poxvirus, zinc finger) domain at the N- terminus end (Cao *et al.*, 1997; Ryals *et al.*, 1997). The presence of protein-protein interaction domains in NPR1 suggests that NPR1 may function as a regulatory protein that might associate with other proteins such as transcription factors (Cao *et al.*, 1997; Ryals *et al.*, 1997). Indeed, screening for NPR1 interacting proteins using the yeast two-hybrid system (Y2HS) identified basic leucine zipper (bZIP) transcription factors from the TGA family (also known as *ocs* element binding factors (OBFs)) as putative candidates (Després *et al.*, 2000; Zhang *et al.*, 1999; Zhou *et al.*, 2000). The *activation sequence-1* (*as-1*) element, which is the binding site for TGA transcription factors, is present in the *PR-1* promoter region and is required for the activation or repression of this gene (Lebel *et al.*, 1998). The significance of NPR1-TGA factors interaction will be discussed in the next sections.

The function of NPR1 and its regulatory role in SAR was further analyzed using both molecular and genetic approaches. *NPR1* gene expression is constitutive under normal condition and is slightly induced upon treatment with SAR chemical inducers or after *Pernospora parasitica* infection (Cao *et al.*, 1997; Ryals *et al.*, 1997; Weigel *et al.*, 2001). The overexpression of the *NPR1* gene in Arabidopsis and rice plants enhanced disease resistance to both bacterial and fungal pathogens after treatment with lower does of SAR inducers, *i.e.,* overexpression of *NPR1* did not result in constitutive *PR* gene expression, indicating that the NPR1 protein requires an activation step modulated directly or indirectly by SA, which subsequently will lead to the activation of *PR* genes. (Cao *et al.*, 1998; Chern *et al.*, 2001; Friedrich *et al.*, 2001).

In a previous study, Kinkema *et al.* (2000) showed that the bipartite nuclear localization signal found at the C-terminus end of NPR1 mediates its nuclear localization in response to SAR inducers. A functional NPR1-green fluorescent protein (GFP) accumulates in the nucleus in response to SAR inducers (Figure 2.3).

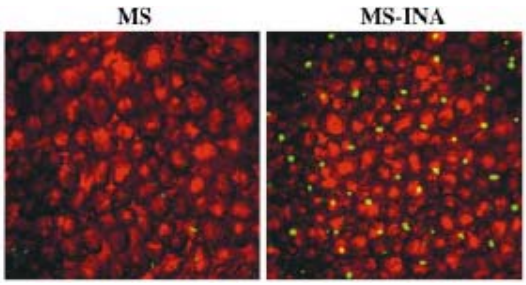


Figure 2.3. Nuclear localization of NPR1-GFP in response to INA induction (Kinkema *et al.*, 2000).

An NPR1 activation step modulated by SA was recently discovered removing some of the ambiguity covering the nature of the SAR signal. The Arabidopsis NPR1 undergoes a conformational change from an inactive oligomer form to the active monomer form as a result of an alerted cellular redox status modulated by SA accumulation during SAR (Mou *et al.*, 2003). SA accumulation seems to prompt a boost of oxidant production at early stage and subsequently the cells will overcompensate this with antioxidants production, converting the oxidizing environment into a reducing one that unhinges NPR1 from an oligomeric complex allowing it to accumulate in the nucleus and to activate gene expression. *NPR1* mutants in the cysteine 82 or 216 are constitutively monomeric, nuclear localized and activate *PR* genes expression.

Additional proteins functioning downstream of NPR1 had been isolated. Screening for suppressors of *npr1* phenotype identify the *suppressor of npr1-1, inducible 1* (*sni1*) mutant, which has restored inducible levels of *PR-1* gene expression and resistance to pathogens, indicating that the wild-type *SNI1* gene product, which encodes a novel protein sharing limited homology with the mammalian tumour suppressor retinoblastoma gene product, may function as a negative regulator of SAR (Li *et al.*, 1999). SNI1 is suggested to exert its function through its recruitment to the *PR-1* promoter and the interaction with WRKY transcription factors (Durrant and Dong, 2004). In a screen for *suppressors of sni1* (*ssn*), three mutants were identified, *ssn1*, *ssn2*, and *ssn3*, which have alleviated the background *PR-1* gene expression of *sni1* (Durrant and Dong, 2004 and references therein). It is speculated that the *SSN* genes are involved in controlling both basal and SA-inducible *PR-1* gene expression by

regulating the same transcription factor(s) that is probably responsible for the background and SA-inducible NPR1-independent *PR-1* gene expression.

The identification of the *detachment 9* (*dth9*) mutant in Arabidopsis established a novel component in the SAR signalling pathways as the *DTH9* gene product appears to act downstream of SA in the development of SAR (Mayda *et al.*, 2000). The expression of *PR* genes in *dth9* mutant was unaffected although the pathogen resistance was compromised.

Using the Y2HY, three novel genes, *NIMIN-1*, *NIMIN-2* and *NIMIN-3* (*NIMIN* for *NIM1-interacting*) that encode structurally related proteins interacting physically with NPR1 were identified (Weigel *et al.*, 2001). All NIMIN proteins share a short LXL repeat near the C-terminus that is highly homologous to the ethylene responsive element binding factors (ERF) -associated amphiphilic repression motif found in some ERF transcription factors (Ohta *et al.*, 2001).

Genetic analysis of previously identified mutants acting upstream of SA in the *npr1* mutant background and the identification of new mutants acting downstream of SA revealed the presence of a SA-dependent but NPR1-independent resistance pathway (Dong, 2001; Shah, 2003). Crossing the *cpr6* mutant to *eds5* blocks the enhanced disease resistance against *P. syringae* and *Pernospora parasitica*, whereas in an *npr1* mutant background the resistance to *P. syringae* was only compromised leaving *Pernospora parasitica* unaffected, hence a SA-dependent but NPR1-independent resistance pathway was postulated (Clarke *et al.*, 1998; Clarke *et al.*, 2000). The existence of such pathway was further supported when the Arabidopsis resistance to turnip crinkle virus conferred by the *HR to turnip crinkle virus (HRT*) *R*-gene was compromised in the *NahG* plants but not in the *npr1* mutant background (Kachroo *et al.*, 2000).

The identification of mutants that suppress *npr1* mutant phenotype uncovered some of the mechanisms mediating the NPR1-independent resistance pathway. For instance, the discovery of *suppressor of SAI 2* (*ssi2*) that encodes a stearoyl-acyl

carrier protein desaturase (which desaturate stearic acid to oleic acid) in a genetic screen for suppressors of *npr1-5* suggests a role for lipid-derived signal in the NPR1-independent resistance pathway (Kachroo *et al.*, 2001; Shah *et al.*, 2001). The isolation of *suppressor of the fatty-acid-desaturase deficiency 1* (*SFD1*) that encodes a glycerol-3-phosphate dehydrogenase, which is required for the glycerolipid biosynthesis, strengthens those observations as *sfd1* suppress the *ssi2*-conferred NPR1-independent expression of the *PR-1* gene and resistance to *P. syringae* by lowering the content of the hexadectrienoic acid (16:3), which was elevated in the *ssi2* mutant (Nandi *et al.*, 2003).

2.7 SAR Gene Expression Mechanisms

An important step in controlling plant defense responses appears to be the transcriptional activation or repression of the defense responsive genes. Up to 20% of transcriptional changes in the Arabidopsis genome are associated with *R* gene-mediated and basal pathogen resistance (Nimchuk *et al.*, 2003).

Studying gene expression changes associated with compatible and incompatible local resistance using large-scale gene expression profiling technologies showed no changes in gene expression patterns between both interactions. However, the R-dependent responses had higher amplitudes and rapid induction kinetics of specific subsets of defense genes compared with the compatible interaction (Maleck *et al.*, 2000; Tao *et al.*, 2003). Strikingly, the expression levels of all genes upregulated during SAR establishment (represented by 132 ESTs) were compromised in the *npr1* mutant (Maleck *et al.*, 2000).

Transcriptional regulation of gene expression is largely mediated by the specific recognition of *cis*-acting promoter elements by *trans*-acting sequence specific DNA-binding transcription factors. The Arabidopsis genome encodes about 1500 transcription factors that are capable of activating and/or repressing transcription through binding to short specific DNA motifs (Riechmann *et al.*, 2000). In Arabidopsis, a number of families of transcription factors such as ERF, TGAs, Myb, and WRKY

proteins, each containing a distinct type of DNA binding domain (BD), have been implicated in plant defense responses (Chen *et al.*, 2002; Singh *et al.*, 2002). For instance, ERF proteins are a subfamily of the APETALA2 (AP2)/EREBP transcription factors family that can bind to the *GCC* box, which is found in several *PR* genes promoter regions, and involved in ethylene signalling against necrotrophic pathogens (Singh *et al.*, 2002). The *ERF1* transcript was induced after *Botrytis cinerea* infection, and its overexpression in Arabidopsis leads to enhance resistance to *Botrytis cinerea* and *Plectosphaerella cucumerina* (Berrocal-Lobo *et al.*, 2002).

WRKY proteins are a class of DNA-binding proteins and comprise 74 members in Arabidopsis that recognize the *TTGAC(C/T) W-box* elements found in the promoters of a large number of plant defense-related genes such as *PR-1* (Eulgem *et al.*, 2000). In the *PR-1* gene promoter region, a negative promoter element containing a *W-box* was identified suggesting the involvement of WRKY transcription factors in SAR-related gene expression (Lebel *et al.*, 1998). The expression of *NPR1* gene is under the control of WRKY transcription factors (Yu *et al.*, 2001). The overexpression of WRKY70, a SA-inducible transcription factor, increases resistance to virulent pathogens and results in constitutive expression of SA-induced pathogenesis-related genes (Li *et al.*, 2004).

The AtWhirly1 (WHY1) protein, a SA inducible single strand DNA binding transcription factor, is required for both SA-dependent disease resistance and SA-inducible expression of PR-1 protein (Desveaux *et al.*, 2004).

2.8 The *As-1* Element and TGA Transcription Factors Regulate SA Mediated Gene Activation

The bZIP transcription factors are a large family of transcription factors in plants with 75 members present in Arabidopsis alone (Figure 2.4; Jakoby *et al.*, 2002). A well-characterized class of the bZIP proteins is the TGA family the members of which bind to the *as-1* element and regulate the expression of many stress-responsive genes such as *PR-1* and *GST6* (Chen and Singh, 1999; Lebel *et al.*, 1998).

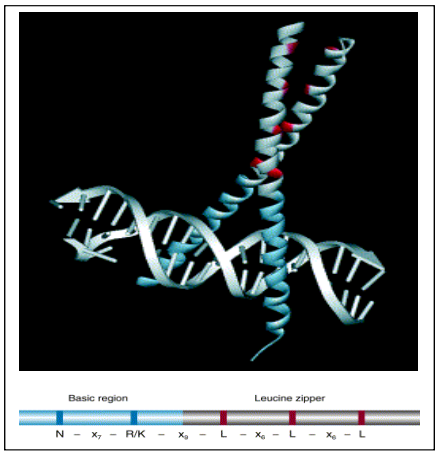


Figure 2.4. Three-dimensional structure of the bZIP domain bound to DNA (Jakoby *et al.*, 2002).

The *as-1* element consists of two *TGACGTCA* imperfect palindrome motifs and was originally identified in the -90 region of the *35S* promoter from cauliflower mosaic virus (CaMV) using promoter deletion analysis (Figure 2.5; Lam *et al.*, 1989). In plant leaves, the expression of genes containing *as-1* or *as-1* like elements in their promoter regions was induced after treatment with SA (Qin *et al.*, 1994) or auxin (Liu and Lam, 1994) or oxidative stress (Garreton *et al.*, 2002).

```
5´ TGACGTAA |ggga| TGACGCAC 3´
3´ ACTGCATT |ccct| GATGCGTG 5´
```

Figure 2.5. Sequence of *as-1* element from the *CaMV* promoter.

As-1-like elements are found in promoter regions of genes controlling early defense responses (transiently induced after 1-2 hours after SA or auxin treatment) such as *GST6* from Arabidopsis (Chen and Singh, 1999) and in promoter regions of genes controlling late defense responses (long lasting gene induction after 10-12 hours after treatment and closely associated with SAR) such as *PR-1* from Arabidopsis (Zhang *et al.*, 1999). Interestingly, the SAR regulatory protein NPR1 controls only late defense genes such as *PR-1*, while the activation of immediate early genes by SA proceeds through an NPR1-independent pathway, indicating that the activation by SA

of immediate early and late genes occurs by at least two different mechanisms (Uquillas *et al.*, 2004).

Using the electrophoretic mobility shift assay (EMSA), which is used to study the interaction between transcription factors and their DNA target sequences, a nuclear protein complex from tobacco that was able to bind to the *as-1* element was identified and was termed the activation sequence factor 1 (ASF1) (Lam *et al.*, 1989). A similar *as-1* binding activity was also observed using a total cellular protein extracts from SA treated leaves and was termed the SA response protein (SARP) (Jupin and Chua, 1996).

The first *as-1* binding protein was isolated from a tobacco cDNA library using a labeled *TGACG*-containing DNA probe and was termed TGA1a (Katagiri *et al.*, 1989). Sequence analysis of the TGA1a bZIP transcription factors revealed that it consists of a basic DNA-binding region, a leucine zipper region important for dimer formation, an acidic N-terminus domain for activation, a glutamine-rich with transcriptional activation potential and dimer-stabilizing region in the C-terminus, respectively (Figure 2.6; Katagiri *et al.*, 1992; Neuhaus *et al.*, 1994; Pascuzzi *et al.*, 1998).

Figure 2.6. Structural features of the TGA1a transcription factor.

Subsequently several proteins homologous to TGA1a had been isolated from different plant species. In Arabidopsis, ten members of this group were described and six were grouped into three classes based on homologies within their amino acid sequences: class I- TGA1 and TGA4, class II- TGA2, TGA5 and TGA6, and class III- TGA3 (Jakoby *et al.*, 2002). In tobacco, five TGA factors have been identified and were grouped according to their homology with the TGA factors from Arabidopsis into three

classes: class I- TGA1a and PG13, class II- TGA2.1 and TGA2.2 (Niggeweg *et al.*, 2000b) and class IV- TGA10 (Schiermeyer *et al.*, 2003).

Different TGA members are involved in different stress responses such as xenobiotic- or SA-mediated responses. For instance, chromatin immunoprecipitation (ChIP) studies using a cultured cell line showed that tobacco TGA1a binds *in vivo* to *as-1* elements found in xenobiotic-responsive promoters but not to *as-1* elements found in *PR-1* promoters (Johnson *et al.*, 2001a). The Arabidopsis TGA2 protein is only responsive to SA but not to any other biotic or abiotic stress (Fan and Dong, 2002).

As described above, the *as-1* element contains two binding motifs that can be occupied with either one or two TGA dimers. Using EMSA, two complexes are identified representing single (lower complex) and double occupation (upper complex) of the *as-1* element (Tokuhisa *et al.*, 1990). Heterodimerization and homodimerization between different *in vitro* translated TGA factors from different classes was observed (Niggeweg *et al.*, 2000b).

Analysis of the ASF1 composition in leaves of Arabidopsis using super-shift assays with specific antibodies revealed that TGA class-II factors contribute significantly to ASF1 activity. About 50% of the ASF1 activity was related to the TGA2 factor (Lam and Lam, 1995), whereas in tobacco, ASF1 consist of ca. 80% TGA2.2 and 20% TGA2.1 (Niggeweg *et al.*, 2000a). These results demonstrate the importance of TGA class-II factors in relation to the SA-inducible gene expression.

The importance of the TGA class-II factors regarding the SA-inducible transcriptional activity from *as-1*-like elements was further confirmed using transgenic plants overexpressing *TGA* wild type and/or mutated forms. Transgenic tobacco plant with mutant derivative of the tobacco *TGA2.2* that lacks DNA-binding activity had suppressed SA-activated *PR-1* and *GSTs* gene expression (Niggeweg *et al.*, 2000a). However, the overexpression of a dominant-negative Arabidopsis *TGA2* mutant in tobacco, which lacks DNA-binding activity and suppresses all *as-1*-element-binding activity, exhibited heightened induction of *PR* genes after SA application and pathogen

challenge (Pontier *et al.*, 2001). In another study, using a trans-dominant approach that targeted just *TGA2* in Arabidopsis plants, the expression of *PR-1* gene following SA treatment was suppressed (Fan and Dong, 2002). Examination of an Arabidopsis triple knockout mutant in the *TGA2*, *TGA5*, and *TGA6* genes revealed that they are functionally redundant and are essential for both the induction of SAR genes and the negative regulation of the basal *PR-1* gene expression (Zhang *et al.*, 2003b). Overexpression of *TGA2* sense and antisense transcripts in Arabidopsis did not affect resistance to *Peronospora parasitica* and *PR-1* gene expression. However, transgenic plants overexpressing *TGA5* displayed enhanced resistance to this pathogen without altering the *PR-1* expression levels (Kim and Delaney, 2002). Using leaf transfection assays, other TGA factors from Arabidopsis belonging to class-I and class-III had activated reporter gene expression in a SA-dependent manner (Després *et al.*, 2003; Johnson *et al.*, 2003).

The contradiction in these results suggests that the TGA factors can function as both positive and negative regulators of SA-dependent gene expression (Shah, 2003). Indeed, the *PR-1* promoter contains two *as-1*-like elements known as *LS5* and *LS7* that act as weak silencer element and activator element, respectively (Lebel *et al.*, 1998). This makes it difficult to define the function of any specific TGA factor in relation to *PR-1* gene expression mechanism.

Functional analysis of TGA transcription factors revealed that there activity must be regulated *in vivo*. Consolidation of this assumption is excogitated from the fact that their DNA-binding activity and transactivation potential increase only upon SA induction (Fan and Dong 2002; Jupin and Chua, 1996; Stange *et al.*, 1997). The steady state concentration of TGA factors present in leaf nuclear extracts was not affected after SA induction (Johnson *et al.*, 2003). Transgenic plants encoding a fusion protein between TGA2.2 and the activation domain (AD) of the adenovirus viral particle 16 (VP16) (TGA2.2-VP16) showed higher inducible expression of target genes as compared to wild-type plants, however, transcription was not elevated in the non-induced state, indicating that no constitutive DNA-binding occurs (Lenk, 2001). In conclusion, it seems

that specific TGA members are sequestered from binding to the *as-1* element under non-inducing conditions through unknown mechanisms.

The enhancement of TGA DNA binding after SA treatment was reversed when the total protein extracts were treated with alkaline phosphatase (Jupin and Chua, 1996), while treatment of nuclear protein extracts from non-induced leaves with ATP led to enhance the DNA binding activity to the *as-1* element (Stange *et al.*, 1997) hinting that phosphorylation events could play a role in manipulating the DNA binding activities of ASF1/SARP. However, in neither case, direct phosphorylation or dephosphorylation of the TGA factors was shown. In consistence with these results, a nuclear casein kinase 2 protein is speculated to regulate the SA-activated binding of ASF1 to the *as-1* sequence (Hidalgo *et al.*, 2001). The DNA binding activity of HBP-1a, a wheat bZIP transcription factor homologous to TGA2, is suggested to be under the control of Ca^{2+}-stimulated phosphorylation events (Meshi *et al.*, 1998).

Treatment of protein extracts from non-induced leaves with formamide and other detergents resulted in stronger binding of ASF1/SARP to the *as-1* element. This could be interpreted probably by the dissociation of an inhibitor protein from SARP complex (Jupin and Chua, 1996). A speculative model describing mechanism leading to SA-inducible gene expression was based on the presence of a hypothetical repressor protein (named SA-Inhibitor, (SAI)) that associates with SARP under non-inducing hindering TGAs to bind the *as-1* element and dissociates after SA induction.

Besides NPR1, the TGA transcription factors were found to interact with other plant proteins. OBP1 (for OBF binding protein 1), a DNA binding protein from Arabidopsis, interacts with TGA factors and enhances their DNA binding activity (Zhang *et al.*, 1995). A TGA1a-interacting protein of apparent molecular size of 120 kDa (p120) which under non-induced conditions hindered the binding of TGA1a to the *as-1* element was identified using a coimmunoprecipitation assay with ^{35}S methionine radio-labeled extracts from tobacco cell suspension culture (Johnson *et al.*, 2001b). Is the p120 identity related to SAI? It's still an open question.

Besides SA, the *as-1* element is also regulated via oxidative stress and it seems that the SA activating effect on the *as-1* element seems to be mediated through the production of certain oxidative species (Garreton *et al.*, 2002). TGA1 and TGA3 proteins are regulated in their protein stability during development by a post-transcriptional mechanism that likely involves specific targeting to the proteasome (Pontier *et al.*, 2002).

A conserved spacing of 12 bp between the two centers of the palindromes of the *as-1* element was observed in the promoter region of the auxin inducible genes and the 'immediate early' SA inducible genes, whereas the spacing in the 'late´ SA inducible genes is less conserved (Krawczyk *et al.*, 2002). TGA-DNA binding was not affected when the two palindromes were not correctly spaced (the *as-1* lacked two bp between the two palindromes), whereas a lack in TGA factors transcriptional activity was observed. The tobacco TGA2.1 and TGA1a, but not TGA2.2 (the main factor of the ASF1 and SARP), function as transcriptional activators in yeast, indicating that TGA2.2 might require a post transcriptional modification or to interact with a plant encoded coactivator to exert its function (Niggeweg *et al.*, 2000b).

2.9 Interaction between NPR1 and TGA Transcription Factors Leads to SA-Mediated Gene Activation

The ample evidence for the direct physical interactions between NPR1 and the TGA factors and the fact that NPR1-TGA interaction is not constitutive and enhanced by SA, notarize the positive regulatory role of NPR1 in the activation mechanism of SAR genes (Després *et al.*, 2000; Fan and Dong, 2002; Subramaniam *et al.*, 2001; Zhang *et al.*, 1999). Previous studies have indicated that NPR1 interacts with Arabidopsis TGA2, TGA3, TGA5, TGA6, and TGA7 factors in yeast, whereas no interaction with TGA1 and TGA4 could be observed (Zhang *et al.*, 1999; Després *et al.*, 2000; Zhou *et al.*, 2000). The enhancement of the TGA-DNA binding in response to SA requires a functional NPR1 protein as the TGA-DNA binding activity in the *npr1* mutant

background was severely suppressed (Després *et al.*, 2000; Fan and Dong, 2002; Johnson *et al.*, 2003).

In elegant experiments, Fan and Dong (2002) showed that Arabidopsis TGA2 is a transcription activator and is involved in NPR1-mediated SA signaling. They showed that overexpression of a truncated form of TGA2, which lacks DNA binding activity but is able to interact with NPR1, has a dominant-negative effect and confers a phenotype similar to that of the *npr1* mutant. A chimeric TGA2 protein, generated by replacing the TGA2 bZIP domain with the Galactose utilization 4 (GAL4) DNA-BD, expressed in transgenic plants harboring a *β-glucuronidase* (*GUS*) reporter construct under the control of *GAL4 upstream activation sequence* (UAS^{GAL4}), was able to bind to the UAS^{GAL4} and transactivate reporter gene in an INA- and NPR1-dependent manner.

Using a leaf ChIP assay, Johnson *et al.* (2003) studied the interactions of Arabidopsis TGA2 and TGA3 with the *PR-1* promoter *in planta* and their results clearly demonstrate that both factors are recruited to this promoter in response to a stimulus induction pathway involving SA and NPR1. Després *et al.* (2003) suggest doubt regarding TGA class-I no interaction with NPR1 as they showed that TGA1 and TGA4 possess all the structural elements required for the interaction with NPR1. To interact with NPR1, the TGA1 and TGA4 factors should undergo conformational changes that are modulated by SA. SA accumulation leads to the reduction of two critical cysteine residues within the TGA1 and TGA4 that are involving in the formation of intramolecular disulfide bridge that inhibits their interaction with NPR1. Although SA mediates TGA1-NPR1 interaction (Després *et al.*, 2003) and TGA3-dependent reporter gene activation (Johnson *et al.*, 2003), the role of TGA class-I and -III factors in SA inducible gene expression is still questionable as Arabidopsis triple knockout mutant in TGA class-II factors was still impaired in SA-inducible *PR-1* gene expression indicating that TGA class-I and -III could not complement this loss of function phenotypes (Zhang *et al.*, 2003b).

The NIMIN proteins can form a ternary complex with NPR1 and TGA factors in a Yeast Three-Hybrid System (Y3HS), speculating that an interaction between NPR1-

NIMINs with TGA could take place may be at the *as-1* level (Weigel *et al.*, 2001). Indeed overexpression of the *NIMIN-1* gene in Arabidopsis suppresses SAR phenotypes and *PR-1* expression (R. Weigel, unpublished data). The repression effects of NIMIN-1 might be related to the presence of the ERF-associated amphiphilic repression motif.

In conclusion, the signal transduction pathway during SAR will lead to SA accumulation that modulate conformational changes in NPR1 from the inactive oligomer to the active monomer form that enables it to accumulate in the nucleus and activates *PR-1* gene expression through increasing the affinity of some TGA factors to their DNA binding sites or sequestering some of them from binding and/or probably by replacing the SAI repressor from the SARP/ASF1 complex (Figure 2.7).

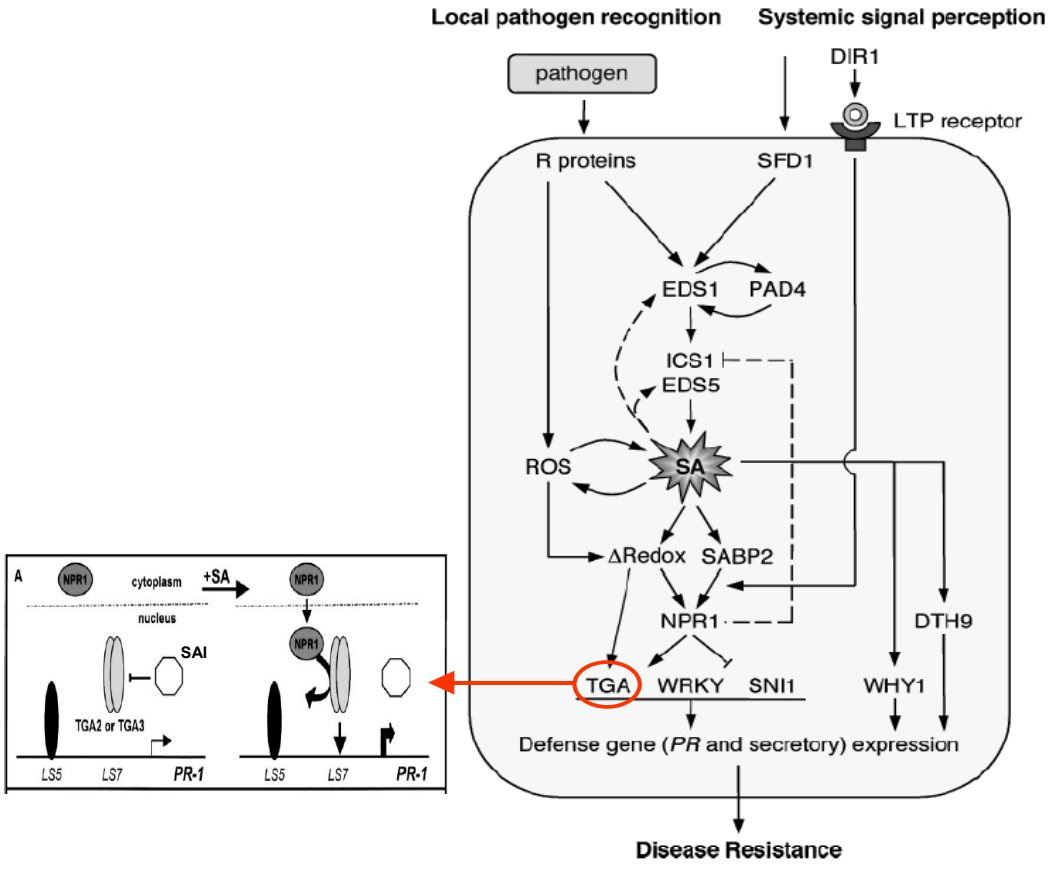


Figure 2.7. Model of PR-1 transcriptional regulation (Durrant and Dong 2004; Johnson *et al.*, 2003).

2.10 Yeast Hybrid Systems as a Tool to Study Protein Interactions

A useful tool for the identification and analysis of protein-protein interactions is the Y2HS. The Y2HS is a sensitive *in vivo* assay used for the detection of specific protein-protein interactions (Fields and Song, 1989). The Y2HS utilizes two separable modules or domains of a transcription factor: the DNA-BD that mediates binding of the transcription factor to gene promoters by sequence specific DNA recognition and AD that recruits the transcriptional apparatus to the gene for mRNA production (Coates and Hall, 2003).

In the Y2HS, two fusion proteins are generated: the bait protein, which is constructed to have the DNA BD that enables it to bind to its corresponding DNA binding elements found upstream of a reporter gene integrate in the yeast genome, and its potential interacting partner (the fish or prey), which is fused to an AD (Figure 2.8; Fields and Sternglanz, 1994). If the bait protein interacts with the prey protein, the interaction of both proteins will form an intact and functional transcriptional activator, which will lead to reporter gene activation.

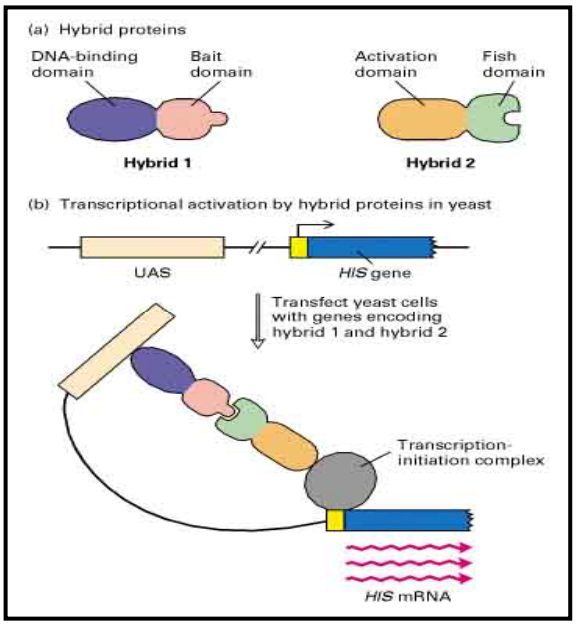


Figure 2.8. Y2HS for detecting proteins that interact.

In addition to the fusion proteins, plasmids contain selection or marker genes (such as *TRP1* and *LEU2* that encode enzymes involved in tryptophan and leucine biosynthesis, respectively) that contribute to the cell survival under a particular environment and enable the selection for transfected cells (Fields and Sternglanz, 1994).

There are broad applications of the Y2HS system for the investigation of the protein network of a cell, but the most important use is to identify potential regulatory interacting partners through screening a cDNA library encoding many proteins from a selected organism (Drees, 1999).

In the last few years, several new modifications of Y2HS have been established recommending the system for several different approaches (Brachmann and Boeke, 1997; Serebriiskii *et al.*, 2001). For instance, the Y3HS was generated involving a third component such as a nucleic acid or a protein and was developed to detect factors mediating the interaction of two proteins (Tirode *et al.*, 1997). The Y3HS makes it possible to investigate ternary protein complex formation by allowing the expression of three proteins together: A DNA-BD fusion, a bridge protein and a DNA-AD fusion protein. The Yeast One Hybrid System (Y1HS) is another derivative from the Y2HS, which is used for isolating of novel genes encoding proteins that bind to a target, *cis*-acting regulatory element (Brachmann and Boeke, 1997).

Limitations in applying the two-hybrid system have been reported by several scientists working with proteins from different organisms (Serebriiskii *et al.*, 2001). A major difficulty in the Y2HS screens is the elimination of false positives. These are clones that cause the activation of reporter genes but not as a result of a specific interaction with the bait (Bartel *et al.*, 1993).

3 Objectives

The evidence presented in the introduction points to a crucial function of TGA transcription factors binding to the *as-1* element with respect to immediate early gene expression in response to SA and auxin and with respect to systemic acquired resistance.

As TGA transcription factors are constitutively expressed even in the uninduced cell, their activity has to be regulated at the protein level. Evidence presented by various groups (Jupin and Chua, 1996; Stange *et al.*, 1997) indicates the existence of a protein that interferes with DNA binding capacity of TGA factors. Our data suggest the existence of a co-activator that has to be recruited to TGA factors bound to the *as-1* element (Krawczyk *et al.*, 2002).

To isolate putative interacting proteins, a modified yeast one-hybrid system was established using TGA2.2 from tobacco as a bait. We focused on this protein for several reasons:

1. TGA2.2 represents a large fraction of the *as-1* binding complex isolated from tobacco nuclear or cellular extracts.

2. Plants overexpressing TGA2.2 showed increased expression of the *Nt103* after SA induction.

3. Plants with reduced levels of TGA2.2 show reduced levels of *Nt103* after SA and auxin induction.

Here, I describe the identification and characterization of several proteins that interact with the tobacco TGA2.2 factor. Molecular and genetic analysis of TGA2.2 interacting partners will be presented and discussed. I hope the data presented here will help in better understanding of the signaling networks that orchestrate the TGA function in plants and to establish a solid foundation for further work to understand the physiological role for TGA factors in plants.

4 Materials

4.1 Organisms

4.1.1 Bacteria

Bacteria Strain	Description	Reference
Agrobacterium tumefaciens GV3101 (pMP90)	pMP90RK; *Rif^r, Gm^r*.	Koncz and Schell, 1986
Escherichia coli BL21	F-; *ompT hsdSB* (*rB mB) gal dcm*.	Studier and Moffat, 1986
Escherichia coli DH5α	F-; *gyrA96 (Nalr), recA1, relA1, endA1, thi-1, hsdR17 (rk-mk+), glnV44, deoR, D (lacZYA-argF) U169 p80dD (lacZ) M15]*.	Hanahan, 1983
Escherichia coli W3110	F´, *lacIq L8, hsdR-, hsdM+*.	Brent and Ptashne, 1981
Escherichia coli XL1Blue	F´, *Tn10 (tetr), proA+B+, lacIq, glnV44, recA1, D(lacZ)M15, relA1, endA1, (Nalr), hsdR17 (rk-mk+) gyrA96 thi-1*.	Bullock *et al.*, 1987
Pseudomonas syringae pv. maculicola	With or without *avrRpt2* gene ES4326.	Whalen *et al.*, 1991

4.1.1 Yeast (*Saccharomyces cerevisiae*)

Yeast Strain	Description	Reference
HF7c	MATα; *ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3, 112, GAL4-542, gal80-538, LYS 2-GAL1UAS-GAL1TATA-HIS3, URA3-(GAL 17mers) 3-Cyc1 TATA-lacZ*.	Clontech
YRWH2	An YM4271-derivative: *ura3-52, lys2-801, ade2-101, ade5, trp1- 901, leu2-3, 112, tyr1-501, GAL4-D512, gal80-D538, ade5::hisG. The HIS3* reporter gene is under the control of *3x as-1* elements.	R. Weigel, unpublished
YRWZ2	An YM4271-derivative: *ura3-52, lys2-801, ade2-101, ade5, trp1- 901, leu2-3, 112, tyr1-501, GAL4-D512, gal80-D538, ade5::hisG. The Lac-Z* reporter gene is under the control of *3x as-1* elements.	R. Weigel, unpublished

4.1.3 Plants

Plant	Description	Reference
-90-GUS	*Arabidopsis thaliana* Columbia-0 transgenic plant contains an *as-1* element upstream of the *uidA* (*GUS*) reporter gene, *km* ^{r}.	Redman *et al.*, 2002
At1g28480 antisense	-90-GUS plant contains pFGC5941/At1g28480 binary vector with an *At1g28480* RNAi cassette under the control of *CaMV 35S* promoter, *km* ^{r} *PPT* ^{r}.	This work
At1g28480 overexpressors	-90-GUS plant contains the Alligator2/At1g28480 plasmid with a *3x HA-At1g28480* construct under the control of *2x CaMV 35S* promoter, *strp* ^{r}.	This work
At1g50570 antisense	-90-GUS plant contains pFGC5941/At1g50570 binary vector with an *At1g50570* RNAi cassette under the control of *35S* promoter, *km* ^{r} *PPT* ^{r}.	This work
At1g50570 overexpressors	-90-GUS plant contains the Alligator2/At1g50570 binary vector with a *3x HA-At1g50570* construct under the control of *2x CaMV 35S* promoter, *strp* ^{r}.	This work

4.2 Media and Additives

4.2.1 Bacterial Media

Medium	Components and Concentrations
Agrobacterium tumefaciens inoculation medium	50 g/l Sucrose 500 µl/l Silwet L-77
dYT	16 g/l Tryptone 10 g/l Yeast Extract 5 g/l NaCl Adjust pH to 7.0 with NaOH
King's B	10 g/l Proteose-Peptone 8.6 mM NaH_2PO_4 15 g/l Glycerin Adjust pH 7.0 with HCl Autoclave 5 ml of 1 M Magnesium sulfate were added

Medium	Components and Concentrations
LB medium	10 g/l Tryptone 5 g/l Yeast Extract 10 g/l NaCl Adjust pH to 7.0 with NaOH
YEB	10 g/l Beef Extract 2 g/l Yeast Extract 5 g/l Peptone 5 g/l Sucrose 2 mM $MgSO_4$ Adjust pH to 7.0 with NaOH

4.2.2 Yeast Media

Medium	Components and Concentrations
YPD-Medium + adenine	20 g/l Tryptone / Peptone 10-20 g Agar (for plates only) 10 g/l Select yeast extract 20 g/l Glucose 20 mg/l Adenine Adjust pH to 5.8 with HCl
SD-Medium	6.7 g Yeast nitrogen base without amino acids 10-20 g Agar (for plates only) 850 ml H_2O Adjust pH to 5.8 with KOH, Autoclave 100 ml of 10x Drop-in Solution 5-30 mM 3-Amino-1,2,4 Triazole (3-AT) was added when necessary

4.2.3 BY-2 Protoplasts Media

Medium	Components and Concentrations
MS for Bright Yellow-2 (BY-2) protoplast preparations	1x MS salts 100 mg/l Myo-inositol 1 mg/l Thiamine 0.2 mg/l 2,4-D 255 mg/l KH_2PO4 30 g/l Sucrose Adjust pH to 5.0 with KOH

4.2.4 Additives

Substance	Working concentration	Stock Solution and Solvent
Agar	15-20 g/l	
Ampicillin	100 mg/l	25 mg/ml H_2O
Gentamycin	25 mg/l	23.8 mg/ml in DMF
IPTG	60 mg/l	60 mg/ml
500x Histidine	1 mg/l	1 g Histidine in 100 ml H_2O
Kanamycin	50 mg/l	50 mg/ml H_2O
100x Leucine	1 mg/l	1 g Leucine in 100 ml H_2O
Methionine	1 mM	1 M in H_2O
Rifampicin	100 mg/l	20 mg/ml Methanol
Streptomycin	20 mg/l	20 mg/ml in H_2O
Tetracycline	10 mg/l	10 mg/ml
500x Tryptophan	1 mg/l	1 g Tryptophan in 100 ml H_2O
X-Gal	60 mg/l	20 mg/ml in DMF

4.3 Nucleic Acids

4.2.5 Plasmids

Plasmid	Description	Reference
Alligator2	Gateway binary vector for constitutive expression of a 3x HA tagged protein under the control of *2x 35S* promoter; *GFP* marker gene under the control of *At2S3 promoter*; *strp* ^{r}.	http://www.isv.cnrs-gif.fr/jg/alligator/
Alligator2/At1g28480	Alligator2 derivative for constitutive expression of a 3x HA-At1g28480 fusion protein; *strp* ^{r}.	This work
Alligator2/At1g50570	Alligator2 derivative vector for constitutive expression of a 3x HA-At1g50570 fusion protein; *strp* ^{r}.	This work
as-1-GUS	pUC18 derivative contains the *uidA* reporter gene coding sequence under the control of the *-90 CaMV 35S* promoter; *amp* ^{r}.	Thurow, 2002
HBT-2.2VPs	HBT derivative contains the *TGA2.2-VP16* coding sequence under the control of the chimeric *HBT* promoter; *amp* ^{r}.	Thurow, 2002
HBT-L	pUC18 derivative contains the chimeric *HBT* promoter and a *nos* termination signal; *amp* ^{r}.	Nickolov, 2003

Plasmid	Description	Reference
HBT-L-GFP	pUC18 derivative contains the coding sequence of a synthetic and optimized codon-usage *GFP* (S65T) under the control of the chimeric *HBT* promoter and with *Nos* termination signal; *amp* ^{r}.	Nickolov, 2003
HBTL/At1g28480	HBT-L derivative contains the *At1g28480* coding sequence under the chimeric *HBT* promoter; *amp* ^{r}.	This work
HBTL/At1g28480-GFP	HBT-L-GFP derivative contains the *At1g28480-GFP* coding sequence under the control of the chimeric *HBT* promoter; *amp* ^{r}.	This work
HBTL/At1g50570	HBT-L derivative contains the *At1g50570* coding sequence under the control of the chimeric *HBT* promoter; *amp* ^{r}.	This work
HBTL/At1g50570-GFP	HBT-L-GFP derivative contains the *At1g50570-GFP* coding sequence under the control of the chimeric *HBT* promoter; *amp* ^{r}.	This work
HBTL/GAL4BD-At1g28480	HBT-L derivative contains the *GAL4BD-At1g28480* coding sequence under the control of the chimeric *HBT* promoter; *amp* ^{r}.	This work
HBTL/NLS-At1g50570-GFP	HBT-L-GFP derivative contains the *NLS-At1g50570-GFP* coding sequence under the control of the chimeric *HBT* promoter; *amp* ^{r}.	This work
pBD	A pBridge derivative, yeast vector for the expression of GAL4BD-fusion protein under the control of the *ADH1* promoter and the expression of HA-NLS-fusion protein under the control of the *Met25* promoter; *TRP1*, *amp* ^{r}.	R. Weigel, unpublished
pBD-/At1g28480	pBD derivative, contains *HA-NLS-At1g28480* coding sequence under the control of the *Met25* promoter; *TRP1*, *amp* ^{r}.	This work
pBD-/At1g50570 P	pBD derivative, contains the *At1g50570* isolated cDNA in-frame with *HA* and *NLS* under the control of the *Met25* promoter; *TRP1*, *amp* ^{r}.	This work
pBL	A pBridge derivative, vector for the expression of GAL4BD-fusion protein under the control of the *ADH1* promoter and the expression of proteins under the control of the *Met25* promoter; *TRP1*, *amp* ^{r}.	This work
pBL-/TGA1	pBL derivative contains the *TGA1* coding sequence under the control of the *Met25* promoter; *TRP1*, *amp* ^{r}.	This work
pBL-/TGA2.2	pBL derivative contains the *TGA2.2* coding sequence under the control of the *Met25* promoter; *TRP1*, *amp* ^{r}.	This work

Plasmid	Description	Reference
pBL-/TGA2.2-VP16	pBL derivative contains the *TGA2.2-VP16* coding sequence under the control of the *Met25* promoter; *TRP1*, *amp*^{r}.	This work
pDONR207	The Gateway donor vector; *Gm*^{r}, *Cm*^{r}.	GIBCOBRL
pDONR207/At1g28480	pDONER207™ derivative contains the *At1g28480* full-length coding sequence as a gateway construct; *Gm*^{r}.	This work
pDONR207/At1g50570	pDONER207™ derivative contains the *At1g50570* full-length coding sequence as a gateway construct; *Gm*^{r}.	This work
pET28a	Vector for 6x His-fusion protein expression under the control of the IPTG-inducible *T7* promoter and *lac*-operator; *km*^{r}.	Novagen
pET28a/TGA2.2^{Cys181Ser}	pET28a derivative contains the *6x His-TGA2.2*^{Cys181Ser}*-StripTag* coding sequence; *km*^{r}.	This work
pET28a/TGA2.2	pET28a derivative contains the *6x His-TGA2.2-StripTag* coding sequence; *km*^{r}.	R. Weigel, unpublished
pFGC5941	Gateway binary vector for RNAi constructs under the control of the *35S* promoter; *km*^{r}, *PPT*^{r}.	http://ag.arizona.edu/chromatin/fgc5941.html
pFGC5941/At1g28480	pFGC5941 derivative contains an *At1g28480* RNAi construct; *km*^{r}, *PPT*^{r}.	This work
pFGC5941/At1g50570	pFGC5941 derivative contains an *At1g50570* RNAi construct; *km*^{r}, *PPT*^{r}.	This work
pGAD10/A.th cDNA	pGAD10 derivative contains an Arabidopsis cDNA library in-frame with *GAL4AD* coding sequence; *LEU2, amp*^{r}.	Clontech
pGAD10/At1g28480	pGAD10 derivative contains the *At1g28480* isolated cDNA fused in-frame with *GAL4AD*; *LEU2, amp*^{r}.	This work
pGAD10/At1g50570 P	pGAD10 derivative contains the *At1g50570* isolated cDNA insert; *LEU2*, *amp*^{r}.	This work
pGAD10/At4g00270	pGAD10 derivative contains the *At4g00270* isolated cDNA fused in-frame with *GAL4AD; LEU2*, *amp*^{r}.	This work
pGAD10/At5g55530 P	pGAD10 derivative contains the *At5g55530* isolated cDNA in-frame with *GAL4AD*; *LEU2*, *amp*^{r}.	This work
pGAD424	Yeast vector for the expression of GAL4AD-fusion protein under the control of the *ADH1* promote; *LEU2*, *amp*^{r}.	Clontech
pGAD424/At1g28480	pGAD424 derivative contains the *At1g28480* coding sequence in-frame with *GAL4AD*; *LEU2*, *amp*^{r}.	This work
pGAD424/At1g50570 F	pGAD424 derivative contains the *At1g50570* coding sequence in-frame with *GAL4AD*; *LEU2*, *amp*^{r}.	This work

Plasmid	Description	Reference
pGAD424/At5g55530 F	pGAD424 derivative contains the *At5g55530* coding sequence in-frame with *GAL4AD*; *LEU2*, *amp* ^{r}.	This work
pGAD424/GDM	pGAD424 derivative contains the *At1g28480 cysteine double mutant* (*GDM*) coding sequence in-frame with *GAL4AD*; *LEU2*, *amp* ^{r}.	This work
pGAD424/GSM	pGAD424 derivative contains the *At1g28480 cysteine single mutant* (*GSM*) coding sequence in-frame with *GAL4AD*; *LEU2*, *amp* ^{r}.	This work
pGAD424/N.t cDNA	pGAD424 derivative contains an tobacco cDNA library in-frame with *GAL4AD*; *LEU2*, *amp* ^{r}.	Strathmann *et al.*, 2001
pGAD424/NPR1	pGAD424 derivative contains the *NPR1* coding sequence in-frame with *GAL4AD*; *LEU2*, *amp* ^{r}.	R. Weigel, unpublished
pGAD424/TGA1a	pGAD424 derivative contains the *TGA1a* coding sequence in-frame with *GAL4AD*; *LEU2*, *amp* ^{r}.	Thurow, 2002
pGAD424/TGA2.1	pGAD424 derivative contains the *TGA2.1* coding sequence in-frame with *GAL4AD*; *LEU2*, *amp* ^{r}.	Thurow, 2002
pGAD424/TGA2.2	pGAD424 derivative contains the *TGA2.2* coding sequence in-frame with *GAL4AD*; *LEU2*, *amp* ^{r}.	Thurow, 2002
pGAD424/TGA10	pGAD424 derivative contains the *TGA10* coding sequence in-frame with *GAL4AD*; *LEU2*, *amp* ^{r}.	This work
pBDAt1g28480/Met25::TGA1	pBD derivative contains the *At1g28480* coding sequence in-frame with *GAL4BD* under the control of the *ADH1* promoter and the *HA-NLS-TGA1* coding sequence under the control of *Met25* promoter; *TRP1*, *amp* ^{r}.	This work
pBDAt1g28480/Met25::TGA2.2	pBD derivative contains the *At1g28480* coding sequence in-frame with *GAL4BD* under the control of the *ADH1* promoter and the *TGA2.2* coding sequence under the control of *Met25* promoter; *TRP1*, *amp* ^{r}.	This work
pBDGDM/Met25::TGA2.2	pBD derivative contains the *GDM* coding sequence in-frame with *GAL4BD* under the control of the *ADH1* promoter and the *HA-NLS-TGA2.2* coding sequence under the control of *Met25* promoter; *TRP1*, *amp* ^{r}.	This work
pBDTGA2.2/Met25::At1g28480	pBD derivative contains the *TGA2.2* coding sequence in-frame with *GAL4BD* under the control of the *ADH1* promoter and the *HA-NLS-At1g28480* coding sequence under the control of *Met25* promoter; *TRP1*, *amp* ^{r}.	This work
pGBT9/At1g28480	pGBT9 derivative contains the *At1g28480* coding sequence in-frame with *GAL4BD*; *TRP1*, *amp* ^{r}.	This work

Plasmid	Description	Reference
pGBT9/At1g50570 F	pGBT9 derivative contains the *At1g50570* coding sequence in-frame with *GAL4BD*, *TRP1 amp*^{r}.	This work
pGBT9/At1g50570 P	pGBT9 derivative contains the *At1g50570* isolated cDNA in-frame with *GAL4BD*; *TRP1*, *amp*^{r}.	This work
pGBT9/At5g20500	pGBT9 derivative contains the *At5g20500* coding sequence in-frame with *GAL4BD*, *TRP1 amp*^{r}.	This work
pGBT9/At5g40370	pGBT9 derivative contains the *At5g40370* coding sequence in-frame with *GAL4BD*, *TRP1 amp*^{r}.	This work
pGBT9/At5g55530 F	pGBT9 derivative contains the *At5g55530* coding sequence in-frame with *GAL4BD*, *TRP1 amp*^{r}.	This work
pGBT9/GDM	pGBT9 derivative contains the *GDM* coding sequence in-frame with *GAL4BD*; *TRP1*, *amp*^{r}.	Thurow, 2002
pGBT9/TGA2	pGBT9 derivative contains the *TGA2* coding sequence in-frame with *GAL4BD*; *TRP1*, *amp*^{r}.	R. Weigel, unpublished
pGBT9/TGA2.2	pGBT9 derivative contains the *TGA2.2* coding sequence in-frame with *GAL4BD*; *TRP1*, *amp*^{r}.	Thurow, 2002
pGBT9/TGA2.2 C-term	pGBT9 derivative contains the *TGA2.2* C-terminus coding sequence in-frame with *GAL4AD*; *TRP1*, *amp*^{r}.	This work
pGBT9/TGA5	pGBT9 derivative contains the *TGA5* coding sequence in-frame with *GAL4BD*; *TRP1*, *amp*^{r}.	R. Weigel, unpublished
pGBT9/TGA6	pGBT9 derivative contains the *TGA6* coding sequence in-frame with *GAL4BD*; *TRP1*, *amp*^{r}.	R. Weigel, unpublished
pGBT9/TGA2.2^{Cys181Ser}	pGBT9 derivative contains the *TGA2.2*^{Cys181Ser} coding sequence in-frame with *GAL4BD*; *TRP1*, *amp*^{r}.	This work
pGEM-T/At1g28480	pGEM-T derivative contains *At1g28480* PCR product; *amp*^{r}.	This work
pGEM-T/At1g50570	pGEM-T derivative contains *At1g50570* PCR product; *amp*^{r}.	This work
pGEM-T/At5g55530	pGEM-T derivative contains *At5g55530* PCR product; *amp*^{r}.	This work
pGEM-T/GDM	pGEM-T derivative contains *GDM* PCR product; *amp*^{r}.	This work
pGEM-T/GSM	pGEM-T derivative contains *GSM* PCR product; *amp*^{r}.	This work
pGEX-4T-1	Expression vector for GST-protein fusion under the control of the IPTG-inducible *tac* promoter; *lacI*^{q}, *amp*^{r}.	Amersham Pharmacia
pGEX-4T-1/At1g50570	pGEX-4T derivative contains the *GST-At1g50570* fusion protein coding sequence; *amp*^{r}.	This work
pLEU-/Met25::TGA2.2	pGAD424 derivative contains the *TGA2.2* coding sequence under the control of *Met25* promoter; *LEU2*, *amp*^{r}.	This work

4.2.6 Primers

Primer	Sequence 5´→ 3´
At1g28480 C63 C	AGG ATG TTG CAT GTC TCA TGT GGT GAG G
At1g28480 C63 N	TCA CCA CAT GAG ACA TGC AAC ATC CTC TC
At1g28480 C60-C63 C	GGA GAG GAT CTT GCA TGT CTC ATG TGG
At1g28480 C60-C63 N	TCA CCA CAT GAG ACA TGC AAG ATC CTC TC
At1g28480 gateway bck	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC ATT AAT TTA CAA TCA CAA CC
At1g28480 gateway fwd	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC CAT GCA AGG GAA CGA TTT CTT G
At1g28480 GFP bck	GGT ACC CAA CCA CAG AGA GCC CCA ACT TC
At1g28480 transient bck	CTG CAG TCA CAA CCA CAG AGC CCC AA
At1g28480 transient fwd	GGT ACC ATG CAA GGA ACG GAT TTC TTG
At1g50570 full bck	GGA GAT CTT CGC TAC TTC TGA TGC CAA AC
At1g50570 full fwd	AGA TCT TTA TGG AAT CTC CAC ATT CT
At1g50570 GFP bck	GGT ACC GAA GAA GGC TCT ACT CCC GT
At1g50570 gateway bck	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC TCG CTA TTC TGA TGC CAA A
At1g50570 gateway fwd	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC CAT GAA ATC TCC ACA TTC TGA
At1g50570 transient bck	GCG GCC GCT CAG AAG AAG GCT CTA CTC C
At1g50570 transient fwd	GGT ACC TCT CCA TGG AAT CTC CAC ATT CTG A
At5g55530 full bck	AGA TCT CAA ACA TGA TTA GGC AGC AGA AGT TC
At5g55530 full fwd	AAA GAT CTA CAT GTC TCT TCT GGA TGT GCA AGT TT
Actin18 sense	AGG TCT CCA TCT CTT GCT CG
Actin18 antisense	GCT GGA TTC GCT GGA GAT GA
GAD lower	GAT GCA CAG TTG AAG TGA ACT TGC GGG G
GAD upper	TTC GAT GAT GAA GAT ACC CCA CCA AAC CC
pBridge seq bck	TCC AAA GAA GCA CCA CCA CCA GTA
pBridge seq fwd	TAC AGG GTC GTC AGA TAC ATA
TGA2.2 C	GCG AAT TCC AAC GAG CTC CAC AAC AGG GCA TAT TTA
TGA2.2 ^{Cys181Ser} C	GAG ATC CTT CTT GTG GCT TGG TGG
TGA2.2 ^{Cys181Ser} N	CCA AGC CAC AAG AAG GAT CTC

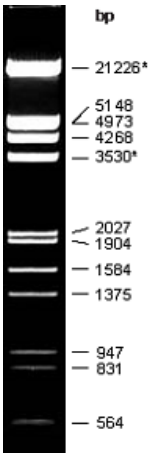
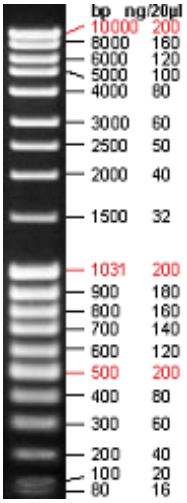
4.2.7 Oligonucleotides and DNA Fragments

Oligonucleotide or Fragment	Sequence 5´→ 3´
As-1 element (fragment from pUC18/as-1)	nnnnnTGACGTAAgggaTGACGCACnnnnn
pBridge linker (oligonucleotide from MBI)	CAT ATG GGG GCC ATA CCA TGG GCG AGC TCA CTA GTA GAT C

4.2.8 Hybridization Probes

Probe	Source	Reference
At1g28480	A 500 kb *At1g28480* PCR product	This work
At1g50570	An 1130 kb *At1g50570* PCR product	This work
GST6 from Arabidopsis	A 600 bp *Sal*I fragment from pGEM-GST6	Brenner, 2002
PR-1 from Arabidopsis	A 500 bp *Eco*RI fragment from pSK-AtPR1	Brenner, 2002

4.2.9 DNA Standards

DNA Standard	Manufacture	Ladder
λ *Eco*RI, *Hind*III	Self preparation	
MassRuler™ DNA Ladder Mix	MBI Fermentas	

4.4 Proteins

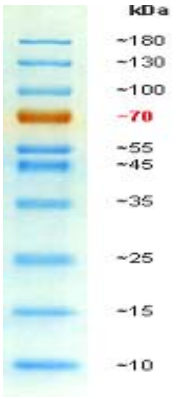
4.2.10 Antibodies

Antibody	Specificity	Properties	Reference
α-rabbit IgG	Rabbit immunoglobulin G	Donkey polyclonal	Amersham
α-HA	HA-Epitoptag	Rabbit polyclonal	Santa Cruz
α-His	6x His	Rabbit polyclonal	BioScience
α-GST	GST	Rabbit polyclonal	Amersham
α -TGA2.2N-term	N-terminus of TGA2.2	Rabbit polyclonal	R. Weigel, unpublished

4.2.11 Enzymes

Enzyme	Manufacture
Advantage™ DNA Polymerase Mix	Clontech
Calf intestine alkaline phosphatase	MBI Fermentas
Cellulase “Onozuka R-10”	Serva
DNase (RNase-free)	Promega
DNA restriction enzymes type II MBI	Fermentas Gibco BRL New England Biolabs
Klenow fragment exo$^-$	MBI Fermentas
Lyticase	Sigma
Lysozym	Serva
Macerozyme R-10	Serva
Pectinase	Fluka
Pfu DNA-Polymerase	Stratagene
RNase A (DNase-free)	MBI Fermentas
T4 DNA ligase	MBI Fermentas
Taq DNA polymerase	MBI Fermentas
Ribonuclease Inhibitor	MBI Fermentas

4.2.12 Protein Standard

Protein Standard	Manufacture	Ladder
Prestained Protein Ladder	MBI Fermentas	

4.3 Chemicals

Chemical	Manufacture
30% (w/v) acrylamide: N,N-methylene-bisacrylamide (37.5:1)	Roth
40% (w/v) acrylamide: N,N-methylene-bisacrylamide (19:1)	Roth
3-Amino-1,2,4 Triazole (3-AT)	Sigma
4-Methylumbelliferyl (4-MU)	Sigma
4-Methylumbelliferyl-β-D-Glucuronid (4-MUG)	Roth; Duchefa
2-[N-morpholino]-ethanesulfonic acid (MES)	Sigma
3-[N-morpholino]-propanesulfonic acid (MOPS)	Roth
[α-32P]-dATP (800 Ci/mmol)	Hartmann Analytic
Acetic Acid	Roth
Adenine	Sigma
Agar Bacteriological	GIBCO BRL
Agarose SeaKem LE	Biozym
Ammonium persulfate (APS)	Biometra
Ampicillin (Amp)	AGS
ß-mercaptoethanol (ß-ME)	Roth
Basata®	AgrEvo
Bicine	Serva
Boric acid	Serva
Bovine Serum Albumin (BSA)	Serva
Bradford reagent	Roth

Chemical	Manufacture
Bromphenolblue	Roth
Calcium carbonate	Merck
Calcium chloride ($CaCl_2$)	Merck
Chloroform	Merck
Coomassie brilliant blue R-250	Merck
N,N-dimethylformamide (DMF)	J.T. Baker Chemicals
Dimethylsulfoxide (DMSO)	Roth
Diamide	Sigma
Dithiotreitol (DTT)	Sigma
DNA carrier	Sigma
DNTPs	MBI
Ethylenediaminetetraacetic (EDTA)	Roth
Ethanol	Merck
Ethidium Bromide (EtBr)	Roth
Formaldehyde (37%)	Roth
Formamid	Fluka
Gentamycin (Gm)	Duchefa
Glacial acetic acid	Merck
Glucose	Sigma
Glutathione	Sigma
Glycerol	Roth, Merck
Glycine	Roth
Hepes	Roth
Histidine	Sigma
Hydrochloric acid (HCl)	Roth
Imidazol	Sigma
Isoamyl alcohol	Roth
Isopropanol	Roth
Isopropylthiogalactoside (IPTG)	BioTech Grade
Leucin	Sigma
Lycine	Sigma
Kanamycin (Km)	Sigma
Magnesium acetate	Merck
Magnesium Chloride ($MgCl_2$)	AppliChem
Magnesium sulfat-Heptahydrat ($MgSO_4.7H_2O$)	AppliChem

Chemical	Manufacture
Manitol	Roth
Methanol	Roth
Methionine	Sigma
Non-fat dried milk SUCOFIN®	TSI
o-Nitrophenyle-β-D-Galactopyranosid (ONPG)	Sigma
Orange G	Sigma
Peptone	Roth
Phenol	AppliChem
Phenylmethanesulfonyl fluoride (PMSF)	Fluka
Polydesoxyinosin-desoxycitidine (poly dI/dC)	Sigma
Polyethylene glycol (PEG) diverse	Roth, Sigma
Potassium acetate	Merck
Potassium ferricyanide	Merck
Potassium ferrocyanide	Merck
Potassium chloride (KCl)	Roth
Potassium glutamate	Merck
Lithium acetate	Sigma
SA	Sigma
Select Agar	Life Technologies
Select Sarcosyl	Sigma
Selected yeast Extract	GIBCO BRL
Sephadex G-50	Pharmacia
SDS (Sodiumlsulfat)	Roth
Silwet L77	Sigma
Sodium acetate	Roth
Sodium chloride (NaCl)	Roth
Sodium hydroxide (NaOH)	Merck
Sodium carbonate	Merck
Sodium citrate	Merck
Sodium phosphate, dibasic (Na_2HPO_4)	Merck
Sodium phosphate, monobasic (NaH_2PO_4)	Merck
Sorbitol	Sigma
Streptomycin (Strp)	Duchefa
Sucrose	Roth
TEMED	Roth

Chemical	Manufacture
Tetracycline	Sigma
Tryptophan	Sigma
Tris (hydroxymethyl) aminomethane	Roth
Triton X-100	Roth
Tween® 20	Sigma
Tyrosine	Sigma
Uracil	Sigma
Urea	Roth
X-Gluc	Roth
X-ray film developer LX24	Kodak
X-ray film fixator AL4	Kodak
Xylenecyanol FF	Roth
Yeast nitrogen base without amino acids	Difco

4.4 Consumables

Product	Manufacture
3MM paper	Whatman
Aluminum folia	Roth
Electroporation cuvettes	BioRad
Micro Spin™ G25 Column	Pharmacia
Glass material	Brand, Schott
Glutathione Sepharose 4B	Amersham Pharmacia
Microscopic slides	Roth
Microtiter plates	Roth
Nickel Nitrilotriacetic Acid (NiNTA®) Resin	Qiagen
Nylon membranes Hybond-N+	Amersham Pharmacia
Parafilm® M	American National Can TM
Protran® Nitrocellulose Sheets	Schleicher BioScience
Single-use plastic ware	Sarstedt, Eppendorf
Greiner, Biozym	Roth
X-ray films Cronex 10T, Cronex 4	DuPont

4.5 Devices

Device	Model	Manufacture
Autoclave	3870 ELV	Tuttnauer
Automatic pipettes		Gilson
Balance	SPO51, SAC62	Scaltec
	1207 MP2	Sartorius
Cold chamber (4 °C)		ILKA Zell
Cooling centrifuge	Sorvall RC 5B Plus	DuPont
CytofFluorII plate reader		PerSpetive
Digital camera	Coolpix	Nikon
Electroporator	GenePulser II	BioRad
Fluorescent microscope	BX 51	Olympus
Gel documentation station		MWG Biotech
Gel electrophoresis chamber, horizontal		Institute's workshop
Gel electrophoresis chamber, vertical	Joey™ #JGC-4	AGS
	V 15-17	Gibco BRL
Handheld radiation monitor	Contamat	Eberline
Heating blocks	HB-130	Unitek
Heating shaker	Thermo mixer 5436	Eppendorf
Heating stirrer	RCT basic	IKA Labortechnik
Hybridization ovens		Bachhofer
Hybridization shaking water bath	The Belly Dancer®	Stovall
Ice machine	AF-20	Scotman
Incubation chambers		WTC binder Memmert
PCR thermocycler	ProGene	Techne
Microtiter plate Spectrophotometer	MRX Plate Reader	Dynex
MiniCycler	PTC-150	MJ Research
Peristaltic pump	Cyclo1	Roth
pH-meter	HI 9321	Hanna Instruments
Phosphoimager	BAS-1000	Fuji
Photometer	Unikon 720 LC	Kontron
Plant growth chambers		Weiss Technik
	I-37L4VL	Percival Scientific
Polyacrylamide Gel Chamber		AGS
RNA-/DNA-calculator	GeneQuant II	Pharmacia
Shaker	ST 5	M. Zipperer GmbH

Device	Model	Manufacture
Scales	A 120 S	Sartorius Analytic
Scanner	ScanJet 4c	Hewlett Packard
Sequencing station	ABI PRISM™ 310	Perkin Elmer
Spectrophotometer	Novaspek Biochrom	LKB
Sterile benches	Microflow Laminar	Nunc
Table-top centrifuges	Biofuge Pico	Heraeus
Table-top cooling centrifuges	5403, 5415R	Eppendorf
Ultra-centrifuge	Centricon T-1065	Kontron
Ultra-low freezers (-80 °C)	C54285	New Brusnwick Scientific
Ultra-sound sonicator	SoniPrep 150	Scientific Instr.
UV-transilluminator	FLX 20 M	Vilber Lourmat
Vacuum evaporator	SpeedVac L 05	WKF
Vacuum gel dryer	Phero-Temp	Biotec-Fischer
Vortex	L46	Labinco BV
Water deionization system	Option 4, Maxima	ELGA
Water bath	1086	GFL
Wet Blotting Apparatus		Institute's workshop

4.6 Kits

Kit	Manufacture
Advantage™ PCR Enzyme Systems	Clontech
BigDye™ Enhanced Terminators (ET) Cycle Sequencing Kit	Perkin-Elmer
Enhanced Chemiluminescence Plus™ (ECL+) Kit	Amersham Biosciences
Gateway® Technology Kit	Invitrogen
Invisorb® Spin Plant RNA Mini Kit I	Invitek
Megaprime. DNA Labeling Systems	Amersham Pharmacia
pGEM®-T vector system Kit	Promega
QIAGEN® QIAfilter™ Midi-/Maxi- Mega Kits	QIAGEN
QIAprep Spin Miniprep® Kit	QIAGEN
QIAquick® Gel Extraction Kit	QIAGEN
RevertAid™ Minus First Strand cDNA Synthesis Kit	MBI Fermentas
RNeasy Plant Mini® Kit	QIAGEN

4.7 Buffers and Solution

Buffer or Solution	Components and Concentrations
5x Binding buffer for EMSA	125 mM Hepes, adjust pH to 7.5 with KOH 50 mM $MgCl_2$ 1 mM $CaCl_2$ 50% (v/v) Glycerol 5 mM DTT (fresh) 2 mM PMSF (fresh)
Blocking buffer for Protran® membranes	1x PBS-T 5% (w/v) Non-fat dried milk
Blotting buffer for Protran® membrane	192 mM Glycine 25 mM Tris 20% (v/v) Methanol
Buffer 1 for small-scale plasmid DNA isolation	50 mM Tris-HCl, pH 8.0 10 mM EDTA
Buffer 2 for small-scale plasmid DNA isolation	0.2 M NaOH 1% SDS
Buffer 3 for small-scale plasmid DNA isolation	29.4 g Potassium acetate 5 ml Glacial acetic acid H_2O up to 100 ml
Cracking buffer	8 M Urea 5% w/v SDS 40 mM Tris-HCl, pH 6.8 0.1 mM EDTA 0.4 mg/ml Bromophenol blue
Church hybridization solution	250 mM Sodium phosphate, pH 7.2 1 mM EDTA, pH 8.0 7% (w/v) SDS
Coomassie fixing solution	25% (v/v) Isopropanol 10% (v/v) Glacial acetic acid
Coomassie staining solution	0.01% (w/v) Coomassie brilliant blue G-250 10% (v/v) Glacial acetic acid
Coomassie destain solution	10% (v/v) Glacial acetic acid
10x Drop-in -Leucine -Tryptophan -Histindin solution	200 mg/l L-Adenine /Hemisulfat 300 mg/l L-Lysine 300 mg/l L-Tyrosine

Buffer or Solution	Components and Concentrations
DNA loading buffer	67% (w/v) Sucrose 50 mM EDTA pH 8.0 0.42% (w/v) Bromphenolblue 0.42% (w/v) Xylenecyanol 0.42% (w/v) Orange G
DNA extraction buffer	0.1 M NaCl 0.01 M Tris-HCl, pH 7.5 1 mM EDTA 1% SDS
Denaturation extraction buffer for total protein	4 M Urea 16.6% (v/v) Glycerol 5% (v/v) ß-ME (fresh) 5% (w/v) SDS 0.5% (w/v) Bromphenolblue
Fix prot extraction buffer	50 mM Hepes, pH 7.5 with KOH 20 mM KCl 2.5 mM DTT (fresh) Protein inhibitors mix
Glutathione elution buffer	100 mM Glutathione 50 mM Tris-HCl, pH 8.0
GUS extraction buffer	50 mM Sodium phosphate, pH 7.0 10 mM EDTA 0.1% Triton X-100 0.1% Sarcosyl 10 mM ß-ME (fresh)
GUS staining solution	50 mM Sodium phosphate buffer, pH 7.0 500 µM Potassium ferrocyanide 500 µM Potassium ferricyanide 2 mM X-Gluc
H Buffer for total protein extraction from yeast	25 mM HEPES, pH 7.5 with KOH 10% glycerol 1 mM EDTA 50 mM NaCl 5 mM $MgCl_2$ 0.5% ß-ME
10x KGB buffer	1 M Potassium glutamate 250 mM Tris-HCl, pH 7.5 100 mM Magnesium acetate 5 mM ß-ME or DTT 0.5 mg/ml BSA

Buffer or Solution	Components and Concentrations
10x Klenow buffer	500 mM Tris-HCl, pH 8.0 50 mM $MgCl_2$ 10 mM DTT
Lac-Z buffer	16.1 g/l $Na_2HPO_4.7H_2O$ 5.50 g/l $NaH_2PO_4.H_2O$ 0.75 g/l KCl 0.246 g/l $MgSO_4.7H_2O$ Adjust pH to 7.0 with KOH
Ligation buffer	40 mM Tris-HCl 10 mM $MgCl_2$ 10 mM DTT
Loading buffer for EMSA	42% (v/v) 5x Binding buffer for EMSA 58% (v/v) Glycerol
MMM solution for BY-2 protoplast preparations	0.5 mM Manitol 0.1% (w/v) MES 15 M $MgCl_2$
Nickel nitrilotriacetic acid elution buffer	50 mM NaH_2PO_4 300 mM NaCl 250-1000 mM Imidazole Adjust pH to pH 8.0
Nickel Nitrilotriacetic Acid Lysis Buffer	50 mM NaH_2PO_4 300 mM NaCl 10 mM Imidazole Adjust pH to 8.0
Nickel nitrilotriacetic acid wash buffer	50 mM NaH_2PO_4 300 mM NaCl 20 mM Imidazole Adjust pH to 8.0
Osmoticum (for BY-2 protoplast preparation)	10 mM Sodium acetate pH 5.8 250 mM Manitol 50 mM $CaCl_2$ 0.01% (v/v) ß-ME 0.5% (w/v) BSA
1x PBS buffer	68 mM NaCl 58 mM Na_2HPO_4 17 mM NaH_2PO_4 pH 7.4 (NaOH)
1x PBS-T	1x PBS 0.1% (v/v) Tween 20

Buffer or Solution	Components and Concentrations
PCI mix	25 volumes Phenol 24 volumes Chloroform 1 volume Isoamylalcohol
PEG solution for BY-2 protoplast preparations	40% (v/v) PEG-4000 0.4 M Manitol 0.1 M Calcium carbonate Adjust pH around 8.0-9.0 with KOH Autoclave, stabilize pH at 5.0-6.0
Pseudomonas syringae infection solution	10 mM $MgCl_2$ 0.01% Silwet L-77
RIPA buffer	150 mM NaCl 10 mM Tris, pH 7.2 0.1% SDS 1% Triton X-100 1% Deoxycholate 4 mM EDTA
3x RNA loading buffer	50% Formamid 10% 10x MOPS 0.45% Formaldehyd 7% Glycerin 0.5% Bromphenolblau
10x SDS-PAGE running buffer	25 mM Tris-HCl, pH 8.3 200 mM Glycine 0.1% (w/v) SDS
Solution 2 for BY-2 protoplasts	250 mM Manitol 250 mM sorbitol 50 mM $CaCl_2$ 1 mM MES Adjust pH to 5.8 with KOH
Solution A for yeast	10 mM Bicine 1 M Sorbitol 3% Ethylenglycol (v/v) pH 8.35 (KOH)
Solution B for yeast	200 mM Bicine 40% PEG 1000 (w/v) Adjust pH to 8.35 with KOH
Solution C for yeast	10 mM Bicine 150 mM NaCl Adjust pH to 8.35 with KOH

Buffer or Solution	Components and Concentrations
20x SSC	2 M NaCl 0.3 M Sodium citrate Adjust pH to 7.0 with HCl
20x TAE	800 mM Tris 20 mM EDTA 2.3% (v/v) Glacial acetic acid
5x TBE	450 mM Tris 450 mM Boric acid 1 mM EDTA Adjust pH to 8.0
100x TE	1 M Tris-HCl, pH 8.0 0.1 M EDTA
W5 solution for BY-2 protoplast	154 mM NaCl 125 mM $CaCl_2$ 5 mM KCl 5 mM Glucose Adjust pH around 5.8-6.0 with KOH
Wall-digestion solution for BY-2 protoplasts	Osmoticum 1% (w/v) Cellulase Onozuka R10 0.5% (w/v) Macerozyme Onozuka R10 0.1% (w/v) Pectinase

4.8 Software

Program	Company
Acrobat Reader 6.0	Adobe
BLAST and Bioinformatics	NCBI, MIPS, TAIR and TIGER
Color View Soft Imaging System	Olympus
CorelDRAW	Corel
Office	Microsoft
PCBAS® Reader 2.09	Raytest GmbH
PhotoPaint	Corel
PhotoShop	Adobe
TINA® 2.0	Raytest GmbH

5 Methods

5.1 Cultivation of Microorganisms

5.1.1 Cultivation of Bacteria

A single colony of *E. coli* cells was cultured overnight at 37 °C in LB or dYT liquid or solid media in the presence of selective antibiotics. The liquid culture was grown under continuous shacking at 250 rpm in a 37 °C shaker, while solid cultures were grown in a 37 °C incubator. Number of cells in a liquid culture was determined by measuring the Optical Density at 600 nm (OD_{600}). A liquid culture with OD_{600} equal to 0.1 corresponds to 2 x 10^7 *E. coli* cells/ml.

Agrobacterium tumefaciens cells were grown in YEB solid or liquid media at 28 °C for two or three days. The GV3101 strain requires the presence of rifampicin and gentamycin antibiotics in the media.

P. syringae pv. *maculicola* ES4326 cells were grown on King's B medium at 28 °C for two days. Virulent strain required the presence of streptomycin antibiotic in the media, while the avirulent strain carrying the *avrRpt2* gene requires the presence of streptomycin and tetracycline.

5.1.2 Cultivation of Yeast (*Saccharomyces cerevisiae*)

Untransformed yeast cells were grown in YPD rich medium in 30 °C incubators. The yeast YPD liquid culture needs 16-18 hours to grow, while YPD solid culture needs from 2-5 days. The addition of adenine to YPD was crucial to enhance the growth of yeast strains that contain the *ade2-101* mutation (all the strains in this study carry this mutation). Transformed yeast strains were grown on SD drop-in medium (lacks specific nutrients) in order to keep selective pressure on transformed plasmids. The 3-amino-1,2,4-triazole (3-AT), a competitive inhibitor of the *HIS3* gene product, was added in order to suppress background growth on SD medium lacking Histindin, which is resulted from the autonomous *HIS3* gene expression. Cells number of the liquid culture was determined by measuring the OD_{600}. A liquid culture with OD_{600} equal to 1 corresponds to 1 x 10^6 cells/ml.

5.2 Cultivation and Manipulation of Plants

5.2.1 Cultivation and Manipulation of Arabidopsis

5.2.1.1 Soil Culture

The Arabidopsis plants were grown in soil (Mini-Tray type) in 6 cm in diameter and 6 cm height pots. Soil was autoclaved for 30 minutes at 100 °C before usage. Before sowing the seeds, pots were

irrigated with water containing a starter fertilizer and an insectside. Sowed seeds were vernalized at 4 °C for two days before transferring them to an Arabidopsis growth chamber. The Arabidopsis growth chamber conditions were set at 24/18 °C (day/night temperature), 65-75% relative humidity, 150 $\mu E/m^2/S^2$ brightness and 14 hours photoperiod. After one week of germination, seeds were transplanted into new pots with a planting density of four plants/pots, watering of the pots took place once or twice every week. For seeds collection, plants were left to set seeds and then were transferred to greenhouse. The plants were covered with bags and left until completely dried. The seeds were sieved, collected and stored at room temperature.

5.2.1.2 The *Agrobacterium tumefaciens*-Mediated Transformation of Arabidopsis

The transformation of Arabidopsis was done using the floral dip method (Clough and Bent, 1998). Plants were grown at a density of four plants/pot, and when they reached the primary bolts (at a height of 5 to 10 cm) stage, a clipping procedure was conducted. The secondary bolts were allowed to grow until reached about 2-10 cm (with few open flowers). *Agrobacterium tumefaciens* strain GV3101 (pMP90) carrying the corresponding binary plasmid were grown on 5 ml YEB liquid media supplemented with corresponding antibiotics at 28 °C for two days under continuous shacking at 250 rpm. This starter culture was used to inoculate a 500 ml YEB culture that was allowed to grow overnight. Next day, the cells were harvested by centrifugation for 5 minutes at 5000 rpm. The cells pellet was washed twice in water, collected by centrifugation and resuspended in 400 ml infiltration medium to a final OD_{600} of approximately 2.0 prior to use. For the floral dip procedure, the inoculum was filled in a 500 ml beaker. The pots were inverted into the inoculum such that all aboveground tissues were submerged, and plants were then removed after 30 seconds of gentle agitation. Dipped plants were removed from the beaker, placed in a plastic tray and covered with a tall clear-plastic dome to maintain humidity for two days. Plants were grown for a further 3-5 weeks until siliques were mature and dry. Seeds were harvested as described above.

5.2.1.3 Inoculation with Bacterial Pathogen

P. syringae pv. *maculicola* ES4326 was grown as described above. Cells were harvested by centrifugation for 5 minutes at 5000 rpm and diluted in a *P. syringae* infection solution to a final OD_{600} of 0.002. The bacterial suspension was pressure-infiltrated on the abaxial side of 3-4 weeks old leaves using a 1 ml syringe.

5.2.1.4 Chemical Treatment of Plants

For SA treatments, an aqueous solution of 1 mM SA was sprayed directly on the leaves until run off. Leaves were harvested at the indicated time points after treatment, flash frozen in liquid nitrogen and

stored at -80 °C. Transgenic plants carrying the gene resistance for Basta® herbicide were grown on soil. After one week from seeds germination an aqueous solution of 1 mM Basta® was sprayed directly on the seedlings until they were completely wetted. After one week from the treatment the Basta® resistance plants were transferred to new pots.

For studying the salicylic acid (SA) or auxin (2,4-D) *GUS* reporter gene–inducible gene expression for the GUS staining of Arabidopsis, plants were grown in soil for 2 weeks. Plants were floated on 20 ml of 20 mM potassium phosphate buffer pH 5.8 and incubated in a climate chamber at 24°C. The inductions were performed with 1 mM SA or 100 µM 2,4-D. The plants were collected after 24 hours and analysed for GUS activity.

5.2.2 Cultivation and Manipulation of BY-2 Protoplasts

BY-2 cells suspension culture was grown in a modified MS medium supplemented with 3% sucrose, 0.9 g/l of myo-Inositol and 0.9 g/l of thiamine in a 100 ml flask. Cells were cultivated at 26 °C, complete darkness and continuous shacking at 120 rpm. Every week a 3 ml culture was transferred to 27 ml medium for subculturing purpose. The preparation of protoplasts from the BY-2 tobacco suspension cells took place after 3 days of subculturing. 20 ml culture were pelleted using centrifugation at 1440 rpm (soft start option, room temperature and 5 minutes duration were used in all centrifugation steps). The cells were resuspended in 20 ml osmoticum, collected and resuspended in 45 ml wall-digestion solution. The 45 ml volume was divided into 3 plates, which were incubated overnight at 26 °C in darkness.

5.3 Standard Molecular Biology Methods

5.3.1 Isolation of Plasmid DNA from Bacteria

5.3.1.1 Isolation of Plasmid DNA from *E. coli*

Isolation of small amounts of plasmid DNA from *E. coli* for analytical purposes was done using a modification of the alkaline lysis method (Le Gouill *et al.*, 1994). 1.5 ml of *E. coli* overnight culture were collected by centrifugation at 13000 for 1 minute. The supernatant was removed using a vacuum pump and the cells were resuspended in 100 µl of buffer 1 for plasmid DNA isolation. The cell suspension was lysed for 5 minutes on ice using 200 µl of buffer 2 for small-scale plasmid DNA isolation. The suspension was neutralized with 150 µl of buffer 3 for small-scale plasmid DNA isolation. The solution was mixed well by inverting 5-6 times and the suspension was centrifuged for 10 minutes at 13000 rpm at room temperature. The aqueous solution (~450 µl) was transferred into a new eppendorf tube containing 1 ml of 96% (v/v) ethanol. The DNA was left to precipitate for 1 hour at -20 °C. Plasmid DNA was collected by centrifugation for 10 minutes at 13000 rpm and 4 °C. The pellet was washed with 70% (v/v) ethanol and dried for 5 minutes using a speedvac. The DNA was dissolved in 100 µl of TE buffer.

For sequencing and yeast transformation purposes, high-purity plasmid DNA was isolated using QIAprep® Spin Mini kit following the manufacturer's instructions. For the isolation of large amounts of high-purity plasmid DNA, the QIAfilter® Midi-, Maxi- and Mega- kits from QIAGEN were used following the manufacturer's instructions.

5.3.1.2 Large-scale Preparation of pGAD424/N.t cDNA library Plasmid DNA from *E. coli*

The amplification of the pGAD424/N.t cDNA library plasmid was done following a protocol described by Parchaliuk *et al.* 1999. In brief, ~1 x 10^7 of pGAD424/N.t cDNA transformed bacterial cells were grown overnight on large LB media plates. Bacterial cells were harvested by flooding a plate with 10 ml of 150 mM NaCl and scarping the colonies from the agar surface using a glass rod, the suspension was dump onto another plate and this step was repeated with another plates using the same suspension. Each 10 ml were used to scarp up to 5 plates. All aliquots of bacteria were mixed together and added to 1 liter LB media and the culture was grown further for 2 hours under continuous shacking. Plasmid DNA was isolated using QIAfilter® Mega kit from QIAGEN following the manufacturer's instructions.

5.3.2 Isolation of Plasmid DNA from Yeast

For plasmid isolation from yeast, a single colony was used to inoculate a 5 ml overnight SD liquid culture (lacking the nutritional marker to keep selective pressure on the plasmid) at 30 °C. Next day, cells were vortexed briefly and a 1.5 ml aliquot was centrifuged for 5 minutes at 13000 rpm to harvest the cells. Pelleted cells were suspended in 30 µl of Lyticase solution, an enzyme that hydrolyzed the β-1, 3-glucose polymer and facilitate cell wall degradation. The cells suspension was incubated to digest for 1.5 hour at 37 °C. The suspension was used then to isolate the plasmid DNA using the QIAprep® Spin Mini kit following the manufacturer's instructions. The isolated yeast DNA plasmid amount considered low, so plasmids were amplified in *E. coli* and DNA isolation was done as described above.

5.3.3 Isolation of Genomic DNA from Arabidopsis

For Arabidopsis genomic DNA isolation, 100 mg vegetative tissues were ground in liquid nitrogen into fine powder. The powder was resuspended in 1 ml DNA extraction buffer. To the suspension, 1 ml of PCI mix was added and extraction mixture was vortexed strongly. The mixture was incubated for 5 minutes at room temperature and then centrifuged for 20 minutes at 13000 rpm. The upper aqueous supernatant was transferred into new eppendorf tube and the DNA was precipitated by the addition of 300 µl of sodium acetate and 1 ml of isopropanol. Genomic DNA was collected by centrifugation for 10 minutes at 13000 rpm and 4 °C. The pellet was washed twice with 70% (v/v) ethanol and dried for 5 minutes using a speedvac. The DNA was dissolved in 50 µl TE buffer and stored at -20 °C.

5.3.4 Total RNA Isolation from Arabidopsis

Tissue samples were collected from treated plants at indicated time points. Samples were flash frozen in liquid nitrogen and the total RNA was isolated using the Invisorb® Spin Plant RNA Mini kit I. In brief, 100-200 mg tissues were grounded in liquid nitrogen and collected in a safe lock eppendorf tube. Grounded tissues were extracted in 900 µl of lysis solution and incubated for 30 minutes in a thermomixer at 25 °C under continuous shaking. Samples were centrifuged for 1 minute at 13000 rpm and the supernatant was transferred into a prefilter tube and centrifuged for 1 minute at 10000 rpm. 500 µl of absolute ethanol were added to the filtrate (approximately 800 µl) and the suspension was mixed thoroughly. Approximately 750 µl of the mixture were transferred into a RNA binding spin filter placed into a new receiver tube. The mixture was incubated for 1 minute and then centrifuged at 10000 rpm for 1 minute, and this step was repeated with the rest. The RNA binding spin filter was washed once with 500 µl of R1 wash buffer and twice with 600 µl of R2 wash buffer. To eliminate any traces of ethanol, a centrifugation at 13000 rpm for three minutes was conducted. To elute RNA, the RNA binding spin filter was transferred into an RNase-free Elution tube and 40 µl of elution buffer R was added. A 5-10 minutes incubation period took place and the RNA was collected by centrifugation for 1 minute at 13000 rpm.

5.3.5 Estimation of Nucleic Acids Concentration and Purity

The concentration of nucleic acids was estimated by measuring their absorption in a spectrophotometer at a wavelength of 260 nm (maximum nucleic acid absorption value; due to the π-electron systems of the heterocycles of the nucleotides). An OD_{260} equal to 1 in case of a 1 cm cuvette corresponds to 50 µg/ml double stranded DNA and to 40 µg/ml RNA. Absorption at 280 nm (for the presence of aromatic rings from amino acids and phenol compounds) was used to give information about the purity of the DNA or RNA sample, an optimal ratio OD_{260}/OD_{280} is in the range of 1.9-2.0 for RNA and 1.8 for DNA. DNA concentrations lower than 100 ng were measured on an agarose gel using the MassRuler™ DNA Ladder Mix.

5.3.6 Nucleic Acids Gel Electrophoresis

5.3.6.1 Separation of DNA on Agarose Gels

DNA samples were mixed with 1/10 volume of 10x DNA loading buffer and then separated on horizontal agarose gels (10 x 7 x 0.3 cm) containing 1x TAE buffer. The gel was prepared by dissolving Agarose in 1x TAE and the concentration of the gel ranged between 1-2% depending on the size of the expected DNA fragment, shorter the fragment higher agarose concentration. Electric current of 3 V/cm was used for 1-2 hours to run the gel, and the gel was ended depending on the distance between the migrated bands of the dyes present in the DNA loading buffer. Ethidium bromide solution (0.1% w/v) was

used to satin the DNA fragments. The DNA detection was done under UV light. Before exposure to the UV light, the gel was rinsed briefly in H_2O to reduce background staining. In a gel-documentation station, gels were visualized on a UV-transilluminator and documented. The sizes and amount of the DNA fragments were determined using DNA standards.

5.3.6.2 Separation of RNA on Denaturing Agarose Gels

Total RNA isolated from plants was separated by electrophoresis using denaturing agarose gels. The gel composed of 1.5 g agarose dissolved in 105 ml H_2O before adding 15 ml of 10x MEN buffer followed by 22.5 ml of formaldehyde. The agarose mixture was poured into a gel box and left to polymerise for 30 minutes. The polymerised gel was placed into a vertical gel electrophoresis chambers containing 1x MEN buffer. RNA samples, with a concentration range between 7.5-10 ug, were equalized with RNase free water and a defined volume of 3x RNA loading buffer (containing 1 µg Ethidium Bromide/sample). Samples were denatured for 10 minutes at 65 °C and incubated on ice before loading them into the gel. The electrophoresis was run at 300 V, 500 mA, 12 W/gel and 0.20 kVh until the bromphenolblue dye was 2-3 cm from the end of the gel. The separation of the RNAs was visualized on a UV transilluminator and photographed.

5.3.6.3 Elution of DNA Fragment from Agarose Gel

The elution of DNA fragments from agarose gel was done using the QIAquick® Gel Extraction kit following the manufacturer's instructions. The eluted fragments were verified by electrophoresis as described above.

5.3.7 Restriction Digestion of DNA Molecules

The restriction enzymes of endonucleases type II were used to digest a double stranded DNA molecule for analytical and cloning purposes. The enzymes cut the DNA either as 5´ or 3´ "sticky" overhangs or as blunt ends. The digestion reactions were incubated in a buffer system optimized for the used enzyme and in the case of double digestion a universal buffer system was used. The activity of the restriction enzymes was estimated in units (U), where 1 U stands for the amount of enzyme cutting completely at optimal conditions 1 µg of λ DNA for 60 minutes. The minimal amount of enzyme necessary for each restriction was determined according to the following formula:

$$U_{min} = \frac{\textbf{[bp (λ) . recognition sites (DNA)]}}{\textbf{[Recognition sites (λ) . bp (DNA)]}}$$

Where bp (λ) = 48500

The digest reaction mixture was incubated for 2 hours at 37 °C. The digestion was confirmed by running the reaction on agarose gel.

5.3.8 Dephosphorylation of DNA Fragments

When a plasmid DNA (used as a vector for cloning a donor DNA fragment) linearized using a single restriction enzyme, a hydrolyization of the 5´-terminal phosphate residue (dephosphorylation) was performed using calf intestine alkaline phosphatase (CIAP) enzyme in order to prevent self-enclosure of the vector in a subsequent ligation reaction. The linearized vector was incubated with the CIAP enzyme for 30 minutes at 37 °C before DNA separation on agarose gel.

5.3.9 Fill-in of 3´-Overhangs with Klenow Fragment of DNA Polymerase I

The *E. coli* klenow fragment of DNA-Polymerase I has a 5´-3´ exonuclease activity. Using the Klenow fragment, it is possible to incorporate nucleotides in a free 3´ OH group of DNA fragments. A reaction mix used for the filling the 3´ overhangs contains a 10x klenow buffer and 0.25 mM dNTPs mix. The reaction was incubated for 1 hour at 37 °C and stopped by the precipitation of the DNA fragments.

5.3.10 Cloning

5.3.10.1 Ligation of DNA Fragments

The conventional cloning of a DNA fragment into a selected plasmid was performed using the T4-DNA ligase enzyme, which is able to catalyze the formation of a phosphodiesther chemical bond between free 5´-phosphate and 3´-OH groups of double-stranded DNA fragments and vectors. The donor DNA fragment (10x accesses to the vector) was incubated with the vector DNA, 2 µl of ligation buffer and 1 µl of T4-DNA ligase for 2 hours at room temperature. The ligation of DNA fragments with blunt ends was performed in the presence of 5% (w/v) PEG 4000 with the ligation mix described above.

5.3.10.2 Gateway Cloning

Gateway® Technology is a new universal cloning technology based on the site-specific recombination properties of the bacteriophage lambda. The Gateway® Technology kit provides a rapid and highly efficient way to move DNA sequences into multiple vector systems for functional analysis and protein expression. The first step in this cloning is the amplification of an attB-PCR then the cloning of the attB-PCR product into a pDONR207 plasmid. This cloning step was called the BP recombination reaction and was catalyzed by the BP Clonase enzyme mix. The final product of this reaction is known as the pDONER207/entery clone plasmid. The pDONER207/entery clone plasmid is used for the recombination

of an entry clone (the attB-PCR product) into a destination vector to create the pENTR/expression clone plasmid using the LR Clonase enzyme mix.

The BP recombination reaction contained a 40-100 fmol of the attB-PCR product, 300 ng pDONER207™ vector (linearized before use), 4 µl 5x BP Clonase reaction buffer and TE buffer, pH 8.0, up to 16 µl before adding 4 µl of BP Clonase enzyme mix. The reaction was incubated at 25 °C for 2 hours. The reaction was concluded by adding 2 µl of 2 µg/µl of proteinase K solution and was incubated at 37 °C for 10 minutes. The reaction mix was used to transform *E. coli* competent cells. The LR recombination reaction was performed similarly except that the PCR product and pDONER207™ were replaced by the pDONER207/entery clone (linearized before use) and pDONER207/entery clone plasmid, respectively.

5.3.11 Radioactive Labeling of DNA Fragments

The radioactive labelling of DNA fragments for Northern blot analysis was done using the random prime labelling method (Feinberg and Vogelstein, 1982). A reaction mixture composed of 50 ng of purified DNA fragment, 1.4 µl random primers and H_2O up to 33 µl were denatured at 95 °C for 5 minutes and then left to cool down at room temperature. To the denatured mixture, 5 µl of 10x Klenow buffer (MBI Fermentas), 6 µl of dNTP-A mix (dCTP, dGTP, dTTP, 500 µM each), 5 µl of [α-^{32}P]-dATP (800 Ci/mmol) and 1 Unit of klenow exo$^-$ were added. The reaction mix was incubated for 2 hours at 37 °C. The unlabeled nucleotides and primers were separated from the mixture using a Sephadex G50 gel filtration columns. The columns were prepared from a 1 ml blue pipit tip closed with a filter paper and filled with Sephadex G50. The elution of the radioactive labelled DNA from the Sephadex G50 gel filtration columns was done by centrifugation for 5 minutes at 1500 rpm.

The radioactive labeling of DNA fragment for EMSA was done using the following reaction: 3 ug digested DNA (pUC-as-1 plasmid (containing a *BpiI* cloned *as-1* fragment) digested with *BpiI* restriction enzyme), 2.0 µl 10x KGB buffer, 0.8 µl klenow exo$^-$, 5.0 µl [^{32}P]-α-ATP and H_2O up to 20 µl. The reaction was incubated for 2 hours at 37 °C. Radioactive labeled DNA was separated from dNTPs by a MicroSpin G25 column (Pharmacia) following the manufacturer's instructions. The eluted sample was mixed with 5 µl of BOX dye and the mixture was applied to a native 5% polyacrylamide gel. The gel was run for 2 hours at 180 V, and then exposed to an X-ray film for 10 minutes. After the development of the X-ray screen, two bands were detected, where the lower one corresponded to the *as-1* fragment. The *as-1* fragment was excided from the predicted position and the gel piece was transferred into a new screw-cup tube. The sample was homogenized in 400 µl TE buffer and incubated overnight at 37 °C. Next day the tube was centrifuged at 13000 rpm for 20 minutes and the supernatant was transferred into a new screw-cup and was stored at -20 °C.

5.3.12 Polymerase Chain Reaction (PCR)

Specific DNA fragments were amplified using the polymerase chain reaction (PCR) (Mullis and Faloona, 1987). The reaction started with the denaturation of two strands of a DNA template. The 5´ complementary strands of the denatured DNA was recognized and hybridized with specific primers (annealing). A *Taq*-polymerase enzyme catalyzes elongation of a newly synthesized chain and the complementary polymerization of nucleotides to the free 3´-OH group of the primer. Repeating the previous steps (denaturation, annealing and elongation) for x cycles (usually from 25 to 35) will exponentially enrich the reaction with the primer-flanked DNA sequence. In some cases a suitable synthetic restriction sites were incorporated to the 5´-end of the primer for cloning purposes. The PCR reaction was carried out in a 20 µl reaction volume with the following constituents: 10-50 ng template DNA, 2 µl of 10 pmole sense primer, 10 pmole antisense primer, 0.2 mM dNTPs, 2 µl of 10x *Taq*-buffer, 2 U *Taq*-polymerase and H_2O up to 20 µl. The amplification reaction was done in a PCR thermocycler using the following program:

	Initial denaturation	**4 minutes 94 °C**
	Denaturation	**1 minutes 94 °C**
25-35 x Cycle	**Annealing**	**30 seconds 50-65 °C**
	Elongation	**1 minute/kb 72 °C**
	Final elongation	**10 minutes 72 °C**

5.3.12.1 Cloning of PCR Products

The DNA molecule amplified using the *Taq*- Polymerase is characterized by the presence of additional deoxyadenosine nucleotides (dA) at the 3´-end of the PCR product, which is due to the terminal deoxy-nucleotidiltransferase activity nature of the *Taq*-polymerase enzyme. PCR product with the 3´-dA overhangs can be used to clone a vector having a complementary 3´-deoxytimidine (dT). For this purpose the pGEM®-T vector system kit (Promega) was used. The ligation reaction was performed following the manufacturer's instructions.

5.3.12.2 PCR Site Directed Mutagenesis

The principle of the PCR site-directed mutagenesis is that a mismatched oligonucleotide is extended and incorporating a "mutation" into a strand of a selected DNA that can be cloned (Ho *et al.*, 1989). In this study, the PCR site-directed mutagenesis was used to create point mutations to help define the role of individual amino acid residues in a protein.

In this study the point mutation was introduce using the PCR overlap extension method (Figure 5.1). In the overlap extension method two separate PCRs were conducted to amplify two PCR fragments (Figure 5.1; PCR1 and PCR2). Each PCR reaction uses one flanking primer that hybridizes at one end of the target sequence (primers A or D) and one internal primer that hybridizes at the site of the mutation and contains the mismatched nucleotide bases (primers C and D). Finally, a third PCR was conducted using primers A and D and the generated DNA fragments from PCR1 and PCR2 as template (Figure 5.1; PCR3).

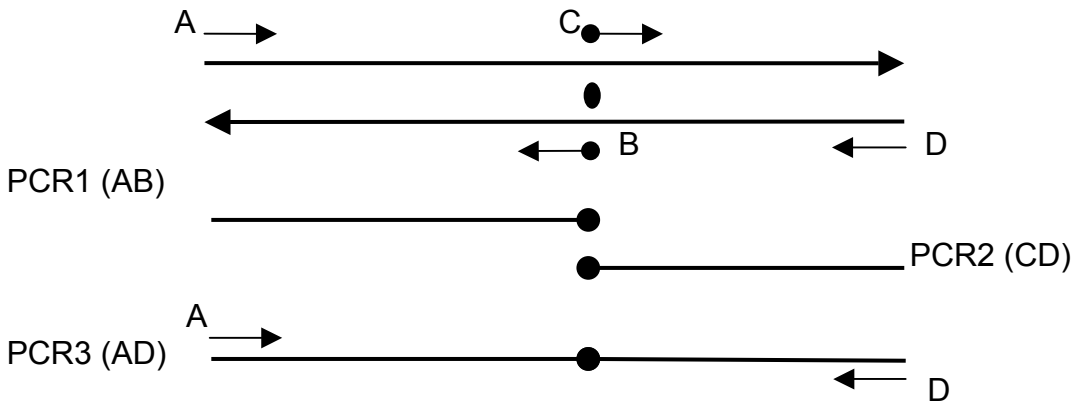


Figure 5.1. Schematic diagram of site directed mutagenesis by overlap extension

5.3.12.3 Screening Bacterial Colonies Using PCR

To verify the presence of a certain DNA molecule in bacterial colonies (*E. coli* or *A. tumefaciens*), a PCR reaction was conducted using the cell lysate as a DNA template. Bacteria cells were picked up with yellow tips from a single colony that was grown on master plate. The tip was vortexed in 10 µl of H_2O and the cells suspension was boiled for 5 minutes at 95 °C. The lysate was centrifuged for 5 second to pellet cell debris. A PCR mixture was added to the cell lysate (represents DNA template) and a PCR reaction was run as described above.

5.3.12.4 Reverse Transcription PCR (RT-PCR)

The reverse transcription PCR (RT-PCR) is a technique used for mRNA detection and quantification. The technique consists of two parts: the synthesis of cDNA from RNA by reverse transcription and the amplification of a specific cDNA by PCR. The RT-PCR reaction was conducted using the RevertAid™ Minus First Strand cDNA Synthesis kit following the manufacturer's instructions. In brief, a reaction mixture containing 500 ng isolated total RNA, 0.2 µg/µl random hexamer primers and RNase free H_2O up to 11 µl was prepared. The reaction was incubated for 10 minutes at 70 °C then chilled on ice. To this mixture 4 µl of 5x Reaction Buffer, 20 units of RNase inhibitor and 2 µl of 10 mM

dNTPs were added and the reaction was incubated for 5 minutes at 25 °C. To the mixture, 1 µl of RevertAid™ H Minus M-MuLV Reverse Transcriptase enzyme (200u/µl) was added and the mixture was incubated at 25 °C for 10 minutes followed by 42 °C for 60 minutes. Finally, the reaction was heated at 70 °C for 10 minutes. A PCR reaction using the Advantage™ PCR Enzyme Systems kit was carried out using 2 µl of the synthesized cDNA reaction.

5.3.13 DNA Sequencing

The DNA sequencing was done using the BigDye™ Terminator Enhanced Terminators Cycle Sequencing kit. The principle of DNA sequencing is based on the chain-termination method (Sanger *et al.*, 1977). In the chain-termination method, dideoxynucleotides (terminators) are incorporated into a newly synthesized complementary chain that will lead to stop its elongation in a PCR reaction. Each of dideoxynucleotides is labeled with a specific fluorescent dyes and the terminated chains can be specifically detected using an ABI Prism 310 Capillary Sequencer. The PCR sequencing reaction was prepared using 300-1000 ng plasmid DNA, 5 pmol primer, 2 µl ET sequencing mix and H_2O up to 10 µl. The samples were subjected to 25 cycles of: 10 seconds at 95 °C, 5 seconds at 50 °C, 4 minutes at 60 °C in a thermocycler. The DNA product was precipitated using 1 µl sodium acetate and 41 µl of absolute ethanol and left on ice for 1 hour. The DNA was collected by centrifugation for 15 minutes at 13000 rpm. The pellet was washed using 200 µl 70% ethanol and then centrifuged for 15 minuets at 13000 rpm. The pellet was dried at 95 °C for one minute and resuspended in 20 µl of template-suppression reagent (TSR). A denaturation step at 95 °C for 2 minutes took place and the tube was immediate placed on ice. The reaction was transferred into a special sequencing tube and then loaded on an ABI-Prism™ 310 capillary electrophoresis sequencing station (Perkin- Elmer) for analysis.

5.3.14 Gene Transfer in Bacteria

The *E. coli* and *A. tumefaciens* have no competence nature, *i.e.,* they are not able to accept naked DNA molecules from the environment. To enable the bacterial cells to take up circular vector DNA they have to be made competent using special treatments. Two transformation methods were used to transform bacteria cells: the heat shock and the electroporation. The heat shock method was used only to transform *E. coli* chemical competent cells. The transformation procedure was done after Hanahan (1983). In brief, 50-100 µl competent *E. coli* cells were thawed on ice slowly before adding 2-30 µl of plasmid DNA and the mixture was briefly vortexed. The mixture was incubated on ice for 30 minutes. The cells were heat shocked for 90 seconds at 42 °C and were placed immediately on ice for at least 2 minutes. 1 ml of dYT medium was added to the tube and the suspension was agitated for 1 hour at 37 °C. Different volumes of the culture were plated on plates containing LB medium supplemented with antibiotics. The plates were incubated overnight at 37 °C.

The transformation using electroporation was done for *E. coli* and *A. tumefaciens* cells after (Dower *et al.*, 1988). The electroporation was done using a Gene Pulser® II. Bacteria competent cells were thawed on ice slowly before adding 2 µl of plasmid DNA. The mixture was transferred into an ice-cooled electroporation cuvette (2 mm electrode distance). The cuvette was subjected to electroporation at 25 µF, 2.5 kV, 200 Ω. The cells were suspended immediately with 1 ml dYT medium and incubated for 1-2 hours at selected temperature depending on the bacteria type. Different volumes of the culture were plated on LB media supplemented with selective solid medium (depending on bacteria type) and incubated at selected temperatures for 1-2 days depending on the bacteria type.

5.3.15 Gene Transfer in Yeast

The yeast cells were transformed with plasmid DNA using two different methods. The first method was after Dohmen *et al.* (1991) and was conducting using yeast competent cells prepared as follows. A 5 ml culture (YPD or SD) inoculated with a single yeast colony and grew overnight at 30 °C. The culture was used to inoculate a 100 ml YPD media. Cells were allowed to grow under continuous shacking at 200 rpm at 30 °C until an OD_{600} of 0.6 reached. The cells were collected and washed in 20 ml of solution A, pelleted at 5000 rpm and resuspended in 5 ml of solution A. 100 µl from the suspension were pipetted as aliquots in eppendorf tube and stored at -80 °C. For the yeast transformation, a mixture consisting from 5 µl of DNA carrier (2 mg/ml) (denatured at 90 °C for 5 minutes and cooled on ice) and 1 µg of DNA plasmids were added to the frozen cells. The mixture was incubated for 3 minutes at 37 °C and the thawed cells were vortexed briefly before adding 1 ml of solution B. the suspension was mixed thoroughly and incubated for 1 hour at 30 °C. Cells were harvested by centrifugation for 10 seconds at 13000 rpm, washed with 800 µl solution C, collected by centrifugation again, plated on yeast media and grown for 3-4 days at 30 °C.

The second transformation method used was after Gietz and Woods (2002). About 30 µl of yeast cells (from liquid or solid culture) were resuspended in 1 ml sterile H_2O. The cells were collected by centrifugation and resuspended in 1 ml of 100 mM lithium acetate. The cell suspension was incubated for 5 minutes at 30 °C. The following transformation mixture was added to the cells pellet:

240	**µl**	**PEG (50% W/V)**
36	**µl**	**1 M Lithium acetate**
50	**µl**	**DNA carrier**
x	**µl**	**Plasmid-DNA (0.1-10 µg)**
34-x	**µl**	**H_2O**

The transformation mixture was vigoursley vortexed for at least 1 minute. The mixture was incubated for 40 minutes at 42 °C. The cells were harvested by centrifugation and the supernatant was removed and the pellet were resuspended in 150 µl H_2O and plated on a proper media.

5.3.16 Transient Transfection of Tobacco BY-2 Protoplasts

BY-2 protoplasts for the transient assays were prepared using three days old sub-cultivated BY-2 cells as described above. The cell suspensions dispensed into 3 petri dishes (~15 ml each) were collected separately in 50 ml tubes by centrifugation (soft start) at 780 rpm for 5 minutes. 10 ml of the supernatant were removed and the protoplasts were resuspended gently in the rest. The protoplasts were washed with 20 ml osmoticum and then collected by centrifugation at 780 rpm. The supernatant was removed and the protoplasts were resuspended in the rest. To the protoplasts, 10 ml of W5 solution were added (stepwise application of 1, 2, 3, 4 ml) and the suspension was mixed gently. The protoplasts were collected and resuspended in the rest of the supernatant. Stepwise application of 5 ml (1, 2, 2 ml) W5 took place and the protoplasts suspensions from the three tubes were collected in one of them. The tube was incubated at 4 °C for 1 hour and meanwhile protoplast number was determined. After the incubation, protoplasts were collected and the supernatant were completely removed and the pellet was resuspended in an appropriate volume of MMM solution to a protoplasts density of 2 x 10^6 protoplasts/ml.

For the transfection of BY-2 protoplasts, DNA plasmids were added to a defined point in a red cap glass tubes. 300 µl protoplasts were pipetted directly to the defined point and the suspension was mixed thoroughly. To the mixture, 300 µl of PEG solution were added drop-wisely and the suspension was mixed gently. The samples were incubated for 20 minutes at room temperature. 1 ml of W5 solution was added to stop the reaction and the suspension was mixed gently. A stepwise addition of (2, 3, 4 ml) W5 took place and the suspension was gently mixed after each step. The protoplasts were collected, the supernatant was removed and the pellet was resuspended in 700 µl of MS sucrose medium. The samples were incubated overnight at 24 °C in darkness.

5.3.17 Northern Blot Analysis

5.3.17.1 Transfer of RNA into Nylon Membranes

After separation of RNA on denaturing agarose gels, a blotting of the gel onto nylon membranes (Hybond N^+, Amersham) using the capillary blotting method was conducted. The blot was constructed in a plastic tray filled with 500 ml of 10x SSC buffer. A flat surfaced plastic stand was placed above the buffer level and covered with 2 pre-wetted 30 x 15 cm^2 3MM paper stripes with their ends dipped in the buffer (avoiding air bubbles was considered in all steps). The upper side of the RNA gel (10 x 15 cm^2) was placed downwards to the plastic stand. The areas around the gel were surrounded by plastic foil stripes to prevent any drying-out. A 10 x 15 cm^2 Hybond N^+ membrane piece, pre-wetted in 10x SSC and

3 layers of pre-wet 3MM papers (10 x 15 cm^2) were placed on the lower surface of the gel. A thick layer of paper towels (10 cm) was placed upon the 3MM papers and an equally distributed weight of approximately. About 1 kg weight (thick catalogue, etc.) was placed upon the paper towels. The blotting duration was for at least 14 hours and after that the construction was dismantled and the transfer of RNA on the nylon membrane was controlled under UV light and photographed. The covalent binding of the RNA molecules to the membrane surface was performed by incubation at 80 °C for 2 hours.

5.3.17.2 Hybridization of Northern Blot

The membrane was wetted with 2x SSC and placed with the RNA-bound sides inwards in a hybridization glass tube and pre-hybridized at 65 °C in 10 ml of church solution for 1 hour. This step was important to ensure that free non-specific binding sites on the membrane are saturated. 100 µl of DNA carrier were added to the radioactively labeled probe and the mixture was denatured on a boiling water bath for 5 minutes. The probe was immediately transferred to ice and left there for 5 minutes and was then added to the pre-hybridized membrane by pipitting it directly to the church solution in the hybridization tube. The hybridization of the labelled single stranded probe with its complementary RNAs sequences took place overnight at 65 °C under continuous rotation. Next day, the solution containing the probe was collected in a falcon tube and stored at -20 °C for multiple usages. The membrane was subjected to serial washing procedure with buffers containing increasing percentage of SSC (2%; 1%; 0.5%; 0.1% - each containing 0.1% SDS) in order to remove unspecific-bound probe. The washing was concluded when the radioactivity of the membrane was fewer than 30 cpm. The membrane was sealed in plastic foil and exposed to an Imager Plate (IP) overnight. The signals were detected with a Bioimager and processed and quantified with the PCBAS® 2.09 and TINA® 2.0 programs. For normalization, signals were compared with the signals from the Ethidium Bromide staining of the RNAs.

5.3.17.3 Stripping and Re-Probing of Northern Membranes

RNA blotted membrane can be reused again for hybridization with other probes. The hybridized probes need to be removed from the membrane. This was achieved by 2 times for 30 minutes washing with 250 ml of a pre-warmed 0.1% (w/v) SDS at 75 °C on The Belly Dancer®, Stovall.

5.3.18 Yeast Hybrid System Screen (Agatep *et al.*, 1998)

A large single yeast colony transformed with the bait plasmid was used to inoculate a 5 ml SD-tryptophan liquid culture that was allowed to grow at 30 °C overnight. Next day, cells were vortexed briefly and the culture was poured into 200 ml SD-tryptophan media and left to grow overnight at 30 °C. The cells were vortexed briefly and the culture was poured into 500 ml volume of YPD medium and the cells left to grow for 4 hours at 30 °C until the OD_{600} of 1 ml was >1.5. The cells were harvested by

centrifugation at 5000 rpm for 5 minutes. The cells pellet was washed in 1/2 volume of sdd water and was collected again. The cells pellet was resuspended in an appropriate volume of 100 mM lithium acetate. The cells were transferred to an appropriate centrifuge tube and were incubated for 15 minutes at 30 °C. The cells were collected again by centrifugation at 3000 rpm and the supernatant were discarded.

To the cells pellet the following transformation mixture was added:

24	**ml**	**PEG (50% W/V)**
3.6	**ml**	**1 M Lithium acetate**
100	**µl**	**DNA carrier (2mg/ml)**
100	**µl**	**Library plasmid DNA**
6.5	**ml**	**H_2O**

The transformation mixture was vigoursley vortexed for at least 1 minute and the suspension was incubated for 30 minutes at 30 °C. The cell suspension was incubated at 42 °C for 60 minutes and the culture tube was inverted several times every 5 minutes to equilibrate the temperature in the tube. The cells were harvested by centrifugation and the pellet was resuspended in 10 ml H_2O. The cells suspension was plated into large plates with SD -tryptophan, leucine and histidine medium, supplemented with 30 mM 3-AT.

5.4 Construction of Plasmids

5.4.1 Gateway Plasmids

5.4.1.1 pDONR207/At1g28480

An *At1g28480* attB-PCR was amplified using an Arabidopsis cDNA library with primers designed with site-specific recombination sites. The cloning of *attB-At1g28480* PCR product into the pDONR207 vector was done using the gateway BP recombination reaction following the manufacturer's instructions.

5.4.1.2 pDONR207/At1g50570

An *At1g50570* attB-PCR was amplified from an Arabidopsis cDNA library with primers designed with site-specific recombination sites. The cloning of *attB-At1g50570* PCR product into the pDONR207 vector was done using the gateway BP recombination reaction following the manufacturer's instructions.

5.4.2 Binary Plasmids for Stable Transformation of Arabidopsis

5.4.2.1 Alligator2/At1g28480

A binary plasmid generated for the overexpression of 3x HA-tagged At1g28480 protein in plant. The strong *2x CaMV 35S* promoter drove the constitutive expression of *3x HA-At1g28480* construct. The cloning of *At1g28480* into the Alligator2 vector was done using the gateway LR recombination reaction following the manufacturer's instructions.

5.4.2.2 Alligator2/At1g50570

A binary plasmid generated for the overexpression of *3x* HA-tagged At1g50570 protein in plant. The strong *2x CaMV 35S* promoter drove the constitutive expression of *3x HA-At1g50570* construct. The cloning of *At1g50570* into the Alligator2 vector was done using the gateway LR recombination reaction following the manufacturer's instructions.

5.4.2.3 pFGC5941/At1g28480

A binary plasmid generated for the expression of a RNAi *At1g28480* construct in plant in order to reduce the endogenous expression of *At1g28480* in plants. The *CaMV* 35S promoter drove the constitutive expression of the RNAi At1g28480 construct. The cloning of *At1g28480* into pFGC5941 was done using the gateway LR recombination reaction following the manufacturer's instructions.

5.4.2.4 pFGC5941/At1g50570

A binary plasmid generated for the expression of a RNAi *At1g50570* construct in plant in order to reduce the endogenous expression of *At1g50570* in plants. The *CaMV 35S* promoter drove the constitutive expression of the RNAi *At1g50570* construct. The cloning of *At1g50570* into pFGC5941 was done using the gateway LR recombination reaction following the manufacturer's instructions.

5.4.3 Plasmids for Protein Expression in Yeast

5.4.3.1 pBD-/At1g28480

The isolated *At1g28480* cDNA clone was excised from pGAD10/At1g28480 as 550 bp *Bgl*II fragment and ligated into pBD opened with the same enzymes. The *At1g28480* cDNA was under the control of *Met25* promoter.

5.4.3.2 pBD-/At1g50570 P

The isolated *At1g50570* partial cDNA clone was excised from pGAD10/At1g50570 as 950 bp *Bam*H1 fragment and ligated into pBD opened with *Bgl*II. The *At1g50570* cDNA was under the control of *Met25* promoter.

5.4.3.3 pBL-/TGA1

The *TGA1* full-length coding sequence was amplified using PCR from an Arabidopsis cDNA library with primers designed with artificial restriction sites. The PCR product was cloned into pGEM-T vector following the manufacturer's instructions. An 1100 bp *Bgl*II excised fragment from the pGEM-T/TGA1 was ligated into pBL opened with the same enzymes. The *TGA1* coding sequence was under the control of *Met25* promoter.

5.4.3.4 pBL-/TGA2.2

Two-step cloning procedure was employed in order to clone the pBL-/TGA2.2. A pGBT9/TGA2.2 plasmid was digested with *Nco*I and *Bam*HI. The digestion produces two fragments: a 800 bp, a *Nco*I fragment that begins from the *TGA2.2* start codon, and a 200 bp that represents a *Nco*I and *Bam*HI fragment or the rest of *TGA2.2* coding sequence. The 200 bp *Nco*I and *Bam*HI fragment was ligated first into pBL vector opened with *Nco*I and *Bgl*II and the cloning product was termed pBL/200bpTGA2.2. The 800 bp *Nco*I fragment was inserted into pBL/200bpTGA2.2 opened with the same enzyme. The *TGA2*.2 coding sequence was under the control of *Met25* promoter.

5.4.3.5 pBL-/TGA2.2-VP16

Two-step cloning procedure was employed in order to clone the pBL-/TGA2.2-VP16. A pSK2.2VP16 (Lenk, 2001) plasmid was digested with *Nco*I and *Spe*I. The digestion produces two fragments: a 800 bp, a *Nco*I fragment that begins from the *TGA2.2* start codon and a 650 bp that represents a *Nco*I and *Bam*HI fragment or the rest of *TGA2.2* and the *VP16* coding sequences. The 650 bp *Nco*I and *Spe*I fragment was ligated first into the pBL vector opened with *Nco*I and *Spe*I and the cloning product was termed pBL/650bpTGA2.2-VP16. The 800 bp *Nco*I fragment was inserted into pBL/650bpTGA2.2-VP16 opened with the same enzyme. The *TGA2.2-VP16* coding sequence was under the control of *Met25* promoter.

5.4.3.6 pGAD424/At1g28480 and pGBT9/At1g28480

The isolated *At1g28480* cDNA insert was excised from pGAD10/At1g28480 as 550 bp *Bgl*II fragment and ligated into pGAD424 or pGBT9 opened with *Bam*HI. The *At1g28480* cDNA was under the control of *ADH1* promoter and fused in-frame to the *GAL4AD* or *GAL4BD*.

5.4.3.7 pGAD424/At1g50570 F and pGBT9/At1g50570 F

The *At1g50570* full-length coding sequence was amplified using PCR from an Arabidopsis cDNA library with primers designed with artificial restriction sites. The PCR product was cloned into pGEM-T vector following the manufacturer's instructions. An 1100 bp *Bgl*II excised fragment from the pGEM-T/At1g50570 was ligated into pGAD424 opened with *Bam*HI. The *At1g50570* coding sequence was under the control of *ADH1* promoter and fused in-frame to the *GAL4AD* or *GAL4BD*.

5.4.3.8 pGAD424/At5g55530 F and pGBT9/At5g55530 F

The *At5g55530* full-length coding sequence was amplified using PCR from an Arabidopsis cDNA library with primers designed with artificial restriction sites. The PCR product was cloned into pGEM-T vector following the manufacturer's instructions. A 1450 bp *Bgl*II excised fragment from the pGEM-T/At5g55530 was ligated into pGAD424 or pGBT9 opened with *Bam*HI. The *At5g55530* coding sequence was under the control of *ADH1* promoter and fused in-frame to the *GAL4AD* or *GAL4BD*.

5.4.3.9 pGAD424/GDM and pGBT9/GDM

The *GDM* full-length coding sequence (An *At1g28480* cysteine double mutant generated by PCR site directed mutagenesis) was amplified using overlap extension PCR method with primers designed with artificial restriction sites and base pair mismatching sequence. The PCR product was cloned into pGEM-T vector following the manufacturer's instructions. A 450 bp *Bgl*II excised fragment from the pGEM-T/GDM was ligated into pGAD424 or pGBT9 opened with *BamH*I. The *GDM* coding sequence was under the control of *ADH1* promoter and fused in-frame to the *GAL4AD*.

5.4.3.10 pGAD424/GSM

The *GSM* full-length coding sequence (An *At1g28480* cysteine single mutant generated by PCR site directed mutagenesis) was amplified using overlap extension PCR method with primers designed with artificial restriction sites and base pair mismatching sequence. The PCR product was cloned into pGEM-T vector following the manufacturer's instructions. A 450 bp *Bgl*II excised fragment from the pGEM-T/GSM was ligated into pGAD424 opened with *BamH*I. The *GSM* coding sequence was under the control of *ADH1* promoter and fused in-frame to the *GAL4AD*.

5.4.3.11 pBDAt1g28480/Met25::TGA1

The DNA sequence of the *Met25*::*HA-NLS-TGA1*::*PGK* expression cassette was cut out from the pBL/TGA2.2 plasmid as *Stu*I fragment (~1950 bp) and ligated into pGBT9/At1g28480 opened with *Pvu*II.

5.4.3.12 pGBDAt1g28480/Met25::TGA2.2

The DNA sequence of the *Met25*::*TGA2.2*::*PGK* expression cassette was cut out from the pBL/TGA2.2 plasmid as *Stu*I fragment (~1950 bp) and ligated into pGBT9/At1g28480 opened with *Pvu*II.

5.4.3.13 pGBDGDM/Met25::TGA2.2

The DNA sequence of the *Met25*::*TGA2.2*::*PGK* expression cassette was cut out from the pBL/TGA2.2 plasmid as *Stu*I fragment (~1950 bp) and ligated into pGBT9/GDM opened with *Pvu*II.

5.4.3.14 pGBT9/At1g50570 P

The isolated *At1g50570* cDNA insert was excised from pGAD10/At1g50570 as 950 bp *Bgl*II fragment and ligated into pGBT9 opened with *Bam*HI. The *At1g50570* cDNA insert was under the control of *ADH1* promoter and fused in-frame to the *GAL4BD*.

5.4.3.15 pGBT9/At5g20500 and pGBT9/At5g40370

The *At5g20500* and *At5g40370* full-length coding sequences were amplified using PCR from an Arabidopsis cDNA library with primers designed with artificial restriction sites. The PCR product was cloned into pGEM-T vector following the manufacturer's instructions. A ~400 bp and 500 bp *Eco*RI excised fragments from the pGEM-T/At5g20500 pGEM-T/At5g40370 were ligated into pGBT9 opened with *Eco*RI, respectively. The *At5g20500* and *At5g40370* coding sequences were under the control of *ADH1* promoter and fused in-frame to the *GAL4BD*.

5.4.3.16 pGBT9/TGA2.2 C-term

The *TGA2.2 C-terminus* coding sequence was amplified using PCR method with primers designed with artificial restriction sites. The PCR product was cloned into pGEM-T vector following the manufacturer's instructions. A 735 bp *Eco*RI and *Bgl*II excised fragment from the pGEM-T/TGA2.2 C-term was ligated into pGBT9 opened with *Eco*RI and *Bam*HI. The *TGA2.2 C-terminus* coding sequence was under the control of *ADH1* promoter and fused in-frame to the *GAL4BD*.

5.4.3.17 pGBT9/TGA2.2^{Cys181Ser}

The *TGA2.2*^{Cys181Ser} full-length coding sequence (A *TGA2.2* cysteine single mutant generated by PCR site directed mutagenesis) was amplified using overlap extension PCR method with primers designed with artificial restriction sites and base pair mismatching sequence. The PCR product was cloned into pGEM-T vector following the manufacturer's instructions. A 1000 bp *Eco*RI and *Bgl*II excised fragment from the pGEM-T/TGA2.2^{Cys181Ser} was ligated into pGBT9 opened with *Eco*RI and *Bam*HI. The *TGA2.2*^{Cys181Ser} coding sequence was under the control of *ADH1* promoter and fused in-frame to the *GAL4AD*.

5.4.3.18 pLEU-/Met25::TGA2.2

The DNA sequence of the *Met25*::*TGA2.2*::*PGK* expression cassette was cut out from the pBL/TGA2.2 plasmid as *Stu*I fragment (1950 bp) and ligated into pGAD424 opened with *Pvu*II.

5.4.4 Plasmids for Transient Expression in BY-2 Protoplasts

5.4.4.1 HBTL/At1g28480

The *At1g28480* full-length coding sequence was amplified using PCR from the *At1g28480* isolated cDNA insert with primers designed with artificial restriction sites. The PCR product was cloned into pGEM-T vector following the manufacturer's instructions. A 450 bp *Acc*651 and *Pst*I excised fragment from the pGEM-T/At1g28480 was ligated into HBTL opened with *Acc*651 *and Pst*I. The *At1g28480* coding sequence was under the control of the chimeric *HBT* promoter.

5.4.4.2 HBTL/At1g28480-GFP

The *At1g28480* full-length coding sequence was amplified using PCR from the *At1g28480* isolated cDNA insert with primers designed with artificial restriction sites. The PCR product was cloned into pGEM-T vector following the manufacturer's instructions. A 450 bp *Acc*651 excised fragment from the pGEM-T/At1g28480 was ligated into HBTL-GFP opened with *Acc*651. The *At1g28480* coding sequence was under the control of the chimeric *HBT* promoter and has a C-terminus in-frame fusion with *GFP* coding sequence.

5.4.4.3 HBTL/GAL4BD-At1g28480

The *GAL4BD-At1g28480* coding sequence was amplified using PCR from the pGAD424/*At1g28480* plasmid with primers designed with artificial restriction sites. The PCR product was cloned into pGEM-T vector following the manufacturer's instructions. A 450 bp *Acc*651 *and Pst*I excised fragment from the pGEM-T/At1g28480 was ligated into HBTL-GFP opened with *Acc*651 and *Pst*I. The *At1g28480* coding sequence was under the control of the chimeric *HBT.*

5.4.4.4 HBTL/At1g50570

The *At1g50570* full-length coding sequence was amplified using PCR from the pGEM-T/At1g50570 with primers designed with artificial restriction sites. The PCR product was cloned into pGEM-T vector following the manufacturer's instructions. An 1100 bp *Acc*651 and *Not*I excised fragment from the pGEM-T/At1g50570 was ligated into HBTL opened with *Acc*651 and *Not*I. The *At1g50570* coding sequence was under the control of the chimeric *HBT* promoter.

5.4.4.5 HBTL/At1g50570-GFP

The *At1g50570* full-length coding sequence was amplified using PCR from the pGEM-T/At1g50570 with primers designed with artificial restriction sites. The PCR product was cloned into pGEM-T vector following the manufacturer's instructions. An 1100 bp *Acc*651 excided fragment from the pGEM-T/At1g50570 was ligated into HBTL opened with *Acc*651. The *At1g50570* coding sequence was under the control of the chimeric *HBT* promoter and has a C-terminus in-frame fusion with *GFP* coding sequence.

5.4.5 Plasmids for Protein Expression in *E. coli*

5.4.5.1 pGEX-4T-1/At1g50570

The *At1g50570* full-length coding sequence was excised from pGAD424/At1g50570 F as 1100 bp *Bgl*II fragment and ligated into pGEX-4T-1 opened with *Bam*HI. The *At1g50570* has a N-terminus in-frame fusion with the *GST* coding sequence and was under the control of the IPTG-inducible *TAC* promoter.

5.4.5.2 pET28a/ TGA2.2^{Cys181Ser}

The *TGA2.2*^{Cys181Ser} full-length coding sequence was excide from pGBT9/TCM181 as 1100 bp *Eco*RI and *Sac*I fragment and ligated into pET28a opened with *Eco*RI *and Sac*I. The *TGA2.2*^{Cys181Ser} has a N-terminus in-frame fusion with the *6x His* coding sequence and was under the control of the IPTG-inducible *T7* promoter.

5.5 Standard Protein Biochemical Methods

5.5.1 Preparation of Total Protein Extract

5.5.1.1 Total Native Cellular Protein Extracts from Arabidopsis

Total native cellular protein extracts from Arabidopsis were prepared using vegetative tissues. About 50-100 mg leaf material were grinded in liquid nitrogen to fine powder. The powder was collected in eppendorf tube and 300 µl of GUS extraction buffer or fix prot extraction buffer were added. The mixtures were homogenized and centrifuged for 20 minutes at 4 °C and 13000 rpm. The supernatant was transferred into a new eppendorf tube and was stored at -80 °C.

5.5.1.2 Total Native Cellular Protein Extracts from BY-2 Protoplasts

Total native cellular protein extracts from BY-2 protoplasts was used to estimate the GUS activity in a MUG assay. Transfected BY-2 protoplasts were resuspended in 700 µl of solution 2 for BY-2 protoplasts and the mixture was harvested by centrifugation at 1000 rpm (soft start) for 5 minutes at room

temperature. About 1 ml of the supernatant was discarded and the protoplasts (~500 µl) were transferred into an eppendorf tube. 1 ml of solution 2 for BY-2 protoplasts was added and the mixture was centrifuged for 10 minutes at 5000 rpm at room temperature. The supernatant was aspirated and the pellet was resuspended vigoursley (in order to destroy the protoplasts) in 100-200 µl GUS extraction buffer. The mixture was dipped in liquid nitrogen and then thawed at 37 °C and this step was repeated twice. The mixture was centrifuged for 10 minutes at 13000 rpm at 4 °C and the supernatant was transferred into new tube and stored -80 °C.

5.5.1.3 Total Native Cellular Protein Extracts from Yeast

For protein extraction, cells from a 50 ml liquid culture grown to an OD_{600} of 1 were resuspended in H buffer and lysed by sonication. After addition of NaCl to 400 mM and Nonidet P40 to 0.2% the homogenate was kept on ice for 20 minutes, centrifuged for 15 minutes at 13000 rpm and the supernatant (native protein extract) was stored at -80 °C.

5.5.1.4 Total Denatured Cellular Protein Extracts from Arabidopsis

The preparation of total denatured cellular protein extracts from Arabidopsis was done using the Urea/SDS extraction method. 100 mg leaf martial was grinded in liquid nitrogen into fine powder. 300 µl of denaturation extraction buffer for total protein (5% β-ME and 0.2 mM PMSF were added prior to use) were added to the powder in an eppendorf tube and the mixture was homogenized. The mixture was incubated 10 minutes at 65 °C for 10 minutes under continues shacking. The cell debris and unbroken cells were pelleted in a centrifuge at 13000 rpm and 4 °C for 10 minutes. About 200 µl of the supernatant were transferred into a new eppendorf tube and stored at -80 °C.

5.5.1.5 Total Denatured Cellular Protein Extracts from Yeast

The preparation of total cellular protein extracts from yeast was done using the Urea/SDS extraction method. A 5 ml culture was used to inoculate 50 ml YPD medium and the culture was incubate at 30 °C with continuous shaking (220-250 rpm) until the OD_{600} for 1 ml reaches 0.4-0.6. The OD_{600} unties for the entire culture was calculated by the multiplication of the OD_{600} with the total culture volume. The culture was poured into a prechilled 100 ml centrifuge tube halfway filled with ice and the tube was placed in a prechilled rotor and centrifuged at 3000 rpm for 5 minutes at 4 °C. The supernatant was removed and the cell pellet was resuspended in 50 ml of ice-cold H_2O. The cells were pelleted by centrifugation at 3000 rpm for 5 minutes at 4 °C. The cells pellet was transferred into new eppendorf tube and immediately frozen in liquid nitrogen. The cells were quickly thawed and resuspended in prewarmed cracking buffer. The cell suspension was transferred to a 1.5-ml safe cap microcentrifuge tube containing 80 µl of glass beads/7.5 OD_{600} units of cells and the samples were heated at 70 °C for 10 minutes. The

samples were vortexed vigoursley for 1 minute. The cells debris and unbroken cells were pelleted in a microcentrifuge at 13000 rpm for 5 minutes at 4 °C. The supernatants was transferred to fresh 1.5 ml screw-cap tubes and place on ice or stored at -20 °C until use.

5.5.2 Protein Concentrations Determination

Protein concentration was estimated by a colorimetric assay after Bradford (1976). The assay was conducted by pipitting equal amounts of protein extract into a microtiter plate containing 200 µl of 5-fold diluted Bradford reagent. The OD_{595} was measured with a MRX plate reader (Dynex). Protein concentrations were calculated with the help of a standard curve derived from different BSA protein amounts (1 µg, 4 µg, 8 µg) on the same plate.

5.5.3 Expression and Purification of Recombinant Proteins in *E. coli*

5.5.3.1 Expression and Purification of Recombinant GST Fusion Proteins in *E. coli*

5.5.3.1.1 Screening Recombinants for GST-Fusion Protein Expression in *E. coli*

The cloned plasmids for the expression of GST fused protein were transformed into BL21 or W3110 *E. coli* strains using the electroporation. The pGEX recombinants were screened for fusion protein expression as follows. Several colonies of *E. coli* transformed with the pGEX recombinants were picked and transferred into separate tubes containing 2 ml of dYT medium. The liquid cultures were grown to an OD_{600} of 0.6-0.8 (3-5 hours) with vigorous agitation at 30-37 °C. The fusion protein expression was induced in all cultures, except for one (uninduced control) with 1 mM IPTG. The cultures were incubated for 2 hours with vigorous agitation at 30-37 °C. After 2 hours, 1.5 ml of the culture were harvested in eppendorf tubes by centrifugation for 1 minute at 13000 rpm. The cells pellet was resuspended in 300 µl of ice-cold 1x PBS supplemented with 1 mM PMSF. The cell suspension was disrupted using an ultra-sound sonicator on ice 6 times for 10 seconds. 10 µl of the lysate (crude lysate) were analyzed on SDS gel and stained with Coomassie brilliant blue stain. The positive lysate was centrifuged at 13000 rpm for 10 minutes at 4 °C and the supernatant (cleared lysate) was transferred into new tube. 10 µl of the cleared lysate were saved for SDS gel analysis. To the rest of the cleared lysate and 20 µl of 50% slurry of glutathione sepharose 4B (prepared as described by the manufactured) were added and the tubes were mixed gently for 5 minutes at room temperature. The mixture was washed three times with 100 µl of ice-cold 1x PBS and the beads were collected by centrifugation. The supernatant was discarded and the beads were resuspended in 10 µl of protein loading buffer. The purified GST fusion protein in positive lysate was analyzed on SDS gel and stained with Coomassie brilliant blue stain.

5.5.3.1.2 Purification of Recombinant GST-Fusion Proteins

For the purification of the GST-fusion proteins, an affinity chromatography on glutathione-coupled sepharose 4B resin (Amersham Pharmacia) was performed according to the manufacturer's instructions. A large-scale bacterial sonicate was prepared as follows. A single colony of *E. coli* cells containing a positive recombinant pGEX plasmid was used to inoculate 100 ml of dYT media and the culture was incubated for 12-15 hours at 37 °C with vigorous shaking. The culture was induced with 1 mM IPTG and the incubation continued for an additional 6 hours. The cells were harvested by centrifugation at 5000 rpm for 5 minutes at 4 °C and the supernatant was discarded. The pellet was completely resuspended in 2.5 ml of ice-cold 1x PBS supplemented with 1 mM PMSF, 0.1% Tween-20 and 1% Lysozym. The suspension was incubated on ice for 30 minutes. The suspended cells were disrupted using an ultra-sound sonicator on ice 6 times for 10 seconds each. The crude lysate was centrifuged for 10 minutes at 13000 rpm for 10 minutes at 4 °C. The supernatant was transferred to a fresh tube and incubated with 100 µl of the 50% slurry of glutathione sepharose 4B and the suspension was mixed gently for 30 minutes at room temperature. The mixture was washed three times with 1 ml of ice-cold 1x PBS and the beads were collected by centrifugation. The supernatant was discarded and the beads were resuspended in 50 µl of glutathione elution buffer and incubated for 15 minutes at room temperature. The suspension was centrifuged for 1 minute at 13000 rpm and the supernatant (the eluate) was transferred into a new tube, and the elution step was repeated twice with a yield averaged between 0.5-10 µg/µl. The eluates were stored at -20 °C till use.

5.5.3.2 Expression and Purification of Recombinant 6x His Fusion Proteins in *E. coli*

5.5.3.2.1 Screening Recombinants for 6x His-Fusion Protein Expression in *E. coli*

The cloned plasmids for the expression of 6x His-fused protein were transformed into BL21 or W3110 *E. coli* strains by electroporation. The pET28a recombinants were screened for positive clones. Several colonies of *E. coli* transformed with the pET recombinants were picked and transferred several into separate tubes containing 2 ml of dYT medium. The fusion protein expression was induced in all cultures except for one (uninduced control) with 1mM IPTG. The cultures were incubated for 2 hours with vigorous agitation at 30-37 °C. After 2 hours, 1.5 ml of the culture were harvested in eppendorf tubes by centrifugation for 1 minute at 13000 rpm. The cells pellet was resuspended in 300 µl of nickel nitrilotriacetic acid lysis buffer. The cell suspension was disrupted using an ultra-sound sonicator on ice 6 times for 10 seconds. 10 µl of the lysate (crude lysate) were analyzed on SDS gel and stained with Coomassie brilliant blue stain. The positive lysate was centrifuged at 13000 rpm for 10 minutes at 4 °C and the supernatant (cleared lysate) was transferred into new tube. 10 µl of the cleared lysate were saved for SDS gel analysis. To the rest of the cleared lysate, 20 µl of 50% NiNTA®-matrix resin (prepared as described by the manufactured) were added. The tubes were mixed gently for 5 minutes at room temperature. The mixture was washed three times with nickel nitrilotriacetic acid wash buffer and

the beads were collected by centrifugation. The supernatant was discarded and the beads were resuspended in 10 µl of protein loading buffer. The purified 6x His-fusion protein in positive lysate was analyzed on SDS gel and stained with Coomassie brilliant blue stain.

5.5.3.2.2 Purification of Recombinant 6x His-Fusion Proteins

For the purification of the 6x His fusion proteins, an affinity chromatography on NiNTA®-matrix resin (Qiagen) was performed according to the manufacturer's instructions. A large-scale bacterial sonicate was prepared. A single colony of *E. coli* cells containing a positive recombinant pET plasmid was used to inoculate 100 ml of dYT medium and the culture was incubated for 12-15 hours at 37 °C with vigorous shaking. The culture was induced with 1 mM IPTG and the incubation continued for an additional 6 hours. The cells were harvested by centrifugation at 5000 rpm for 5 minutes at 4 °C and the supernatant was discarded. The pellet was completely resuspended in 5 ml of nickel nitrilotriacetic acid lysis buffer supplemented with 1% Lysozym and the suspension was incubated on ice for 30 minutes. The suspended cells were disrupted using an ultra-sound sonicator on ice 6 times for 10 seconds each. The crude lysate was centrifuged for 10 minutes at 13000 rpm for 10 minutes at 4 °C. The supernatant was transferred to a fresh tube and incubated with 1 ml of the 50% NiNTA®-matrix resin and the suspension was mixed gently for 1 hour at 4 °C. The mixture was washed three times with 250 µl of nickel nitrilotriacetic acid wash buffer and the beads were collected by centrifugation. The supernatant was discarded and the beads were resuspended in 250 µl Nickel nitrilotriacetic acid elution buffer and incubated for 30 minutes at 4 °C. The suspension was centrifuged for 1 minute at 13000 rpm and the supernatant was transferred into new tube, and the elution step was repeated twice with a yield averaged between 0.5-1 µg/µl. The eluates were stored at -20 °C till use.

5.5.4 Denaturing SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

In SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE), proteins are separated largely on the basis of polypeptide length. The electrophoresis of the protein was done using a discontinuous buffer system, in which a non-restrictive large pore gel, called a stacking gel, is layered on top of a separating gel called a resolving gel (Laemmli, 1970). The recipe for the resolving gel was consisting of: 10-12% (w/v) acrylamide/bisacrylamide (19:1), 400 mM Tris-HCl pH 8.8, 0.1% (w/v) SDS, 0.1% (w/v) TEMED and 0.1% (w/v) ammonium persulfate. The stacking gel was consisting of: 4% (w/v) acrylamide/bisacrylamide (37.5:1), 125 mM Tris-HCl pH 6.8, 0.1% (w/v) SDS, 0.2% (w/v) TEMED and 0.1% (w/v) ammonium persulfate. The denatured protein extract samples were boiled at 95 °C for 5 minutes then cooled on ice and loaded into the gel. The native extracted protein samples were mixed with 10 µl of protein loading buffer (in some cases the ß-ME was omitted) and denatured at 95 °C for 5 minutes, cooled on ice and then loaded on the gel. The electrophoresis was performed at 120 V in 1x SDS-PAGE running buffer until

the bromphenolblue band run out of the gel. 6 µl prestained protein ladder was loaded on each gel for the estimation of the size of the separated proteins.

5.5.5 Coomassie Staining of Proteins Separated on SDS-PAGE

The Coomassie Brilliant Blue G-250 dye was used to detect proteins separated on SDS-PAGE (Ausubel *et al.*, 1988). The gels were fixed in a Coomassie fixing solution for 10 minutes under continuous agitation then the fixing solution was removed. The fixed gel was incubated with Coomassie staining solution for at least 2 hours at room temperature. The gel was destained using Coomassie destain solution for 2 hours.

5.5.6 Western Blot Analysis

5.5.6.1 Wet Transfer of Proteins onto a Protran® Membrane

The transfer of proteins from the SDS-PAGE gel onto Protran® membranes was performed using the tank electroblotting method. After the electrophoresis, the stacking gel was removed and the resolving gel was wetted with the blotting buffer for Protran® membranes. A blotting sandwich was constructed, in which the gel was placed on three wetted filter papers (similar to gel size). Then a wet Protran® membrane (similar to gel size) was placed above the gel. A second sheet of three moistened filter papers where placed on top of the Protran® membrane and any trapped air bubbles were removed by rolling a pipit on the sandwich. The sandwich was placed between two sponges. The construct was placed between a porous foam plastic sheet on top and a metal sheet to ensure uniform membrane contact with the gel. The assembled cassette was inserted into the blotting apparatus that was filled with 2 liters of blotting buffer for Protran® membranes and the proteins were transferred overnight from the SDS-PAGE gel to the Protran® membrane using a constant electric current of 120 mA.

5.5.6.2 Immuno-detection of the Proteins with Specific Antibodies

The efficiency of the blotting was determined by visualization of the prestained protein ladder on the membrane. The membrane was washed 3 times for 5 minutes in 1x PBS-T buffer. The non-specific protein-binding capacity of the Protran® membrane was blocked by the incubation with 5% (w/v in 1x PBS-T) non- at dried milk for 2 hours under continuous shacking. The blocked membrane was washed three times for 5 minutes with 1x PBS-T buffer. The membrane was incubated with the primary antibody (diluted appropriately in 5% (w/v in 1x PBS-T) non-fat dried milk) for 2 hours under continuous gentle agitation. The membrane was washed 3 times for 5 minutes with 1x PBS-T buffer. The membrane was incubated for 2 hours with the secondary antibody (anti-rabbit IgG antibody peroxidase conjugate diluted

1:20000 in 5% (w/v in 1 x PBS-T). The membrane was washed twice for 5 minutes with 1 x PBS-T followed by 3 times washing for 5 minutes in 1 x PBS buffer. The detection of proteins on the membrane was done using the Enhanced Chemiluminescence Plus™ (ECL^+) kit (Amersham) following the manufacturer's instructions. The membrane was incubated with the enzyme substrate for 5 minutes at room temperature and the chemiluminescence was detected by an exposition to a Cronex 10T X-ray film (DuPont).

5.5.7 Far Western Analysis

Far western blotting is an *in vitro* method for detecting protein-protein interactions. The protein sample (1 µg) was fractionated on an SDS-PAGE gel by electrophoresis as described above. The proteins were transferred from the gels onto a Protran® membrane by electroblotting as described above. The non-specific protein-binding capacity of the Protran® membrane was blocked by the incubation with 5% (w/v in 1x PBS-T) non-fat dried milk for 2 hours under continuous agitation. The membrane was washed three times with 1x PBS-T for 5 minutes and then the membrane was incubated for 2 hours with estimated 10-fold excess (~10 µg) of purified 6x His-fusion proteins. The membrane was washed three times for 5 minutes with 1x PBS-T buffer to risen it, and then the membrane was incubated with the primary antibody (diluted appropriately in 5% (w/v in 1x PBS-T) non-fat dried milk) for 2 hours under continuous gentle agitation. The membrane was washed 3 times for 5 minutes with 1x PBS-T buffer and then was incubated for 2 hours with the secondary antibody (anti-rabbit IgG-antibody-peroxidase conjugate; diluted 1:20000 in 5% non-fat dried milk. The detection of proteins on the membrane was done as described above.

5.5.8 GST Pull-Down Assay

The GST pull-down assay is used to detect protein-protein interaction *in vitro*. In this method 1 ml of cleared lysate containing bacterial overexpressed GST fusion proteins was incubated with 1 ml of cleared lysate containing bacterial overexpressing the 6x His-fusion protein for 1 hour at room temperature under continuous agitation. 200 µl of 50% glutathione slurry matrix was added to the mixture and the reaction was incubated for 1 hour at room temperature. The beads were collected by centrifugation (1 minute at 13000 rpm) and were washed three times with 1 ml of ice-cold 1x PBS. The beads were collected by centrifugation and the supernatant was discarded. The beads were resuspended in 100 µl of glutathione elution buffer and incubated for 15 minutes at room temperature. The suspension was centrifuged for 1 minute at 13000 rpm and the supernatant was transferred into new tube. To the eluate, 400 µl of cold methanol, 100 µl of cold chloroform and 400 µl cold H_2O were added. The mixture was mixed gently and centrifuged for 1 minute at 13000 rpm for 2 minutes. The aqueous upper phase was carefully discarded and 300 µl of cold methanol were added to the pellet. The proteins

were then precipitated by centrifugation at maximum speed for 2 minutes. To the dried pellet, 20 µl of 1x PBS were added. A western blot analysis was carried out as described above.

5.5.9 Immunoprecipitation

Immunoprecipitation involves the interaction between a protein and its specific antibody, the separation of these immune complexes with proteinA beads, and the subsequent analysis by western blotting. Native protein extract from Arabidopsis leaves (1 mg) was thawed on ice before adding 2 µl of HA antibody. The mixture was incubated on ice for 1 hour. A volume of 50 µl of proteinA beads (prepared in 20% RIPA buffer according to manufacture recommendations) was added to the mixture. The mixture was then incubated for 1 hour at 4 °C under continuous agitation. The proteinA beads were collected by centrifugation at 13000 rpm for 2 minutes and the supernatant was removed completely by aspiration. The beads were washed three times in 1 ml of 20% RIPA buffer and the supernatant was discarded. The beads were resuspended in 50 µl of loading buffer for proteins. 30 µl of Immunoprecipitated eluates were used for western blot analysis as described above.

5.5.10 Determination of GUS Activity

The activity of *GUS* reporter gene was done using a modified GUS assay (Jefferson *et al.*, 1987). The GUS assay allows the quantification of β-Glucuronidase enzyme activity using Methylumbelliferyl-ß-Glucuronid (4-MUG) as a substrate. The catalectic activity of GUS will convert the 4-MUG substrate into Methylumbelliferon and the fluorescent of Methylumbelliferon can be estimated using a Spectrophotometer for microtiter plate. 5-20 µg total protein extract were diluted in 100 µl GUS extraction buffer in a microtiter plate. The reaction was started by the addition 100 µl of 4 mM MUG to the protein extract in a microtiter plate well and the plate was incubated at 37 °C. After 15 minutes incubation (time A) half of the reaction was transferred into new well containing 100 µl of 200 mM sodium carbonate (to stop the reaction), the rest of the reaction was run between 45 minutes to 2 hours (time B). The rest of the reaction was stopped by adding 100 µl of 200 mM sodium carbonate. The quantification of Methylumbelliferon fluorescent was done using CytoFluorII plate reader (excitation wavelength 360 nm; emission measurement wavelength 460 nm) and 50 pmol Methylumbelliferon was used as standard.

The GUS activity was measured using the following formula:

$$A_{GUS} = \frac{[\Delta F * 50 \text{ pmol Methylumbelliferon}]}{[\text{min} * \text{mg Protein} * F_{50\ \text{pmol}}\ \text{Methylumbelliferon}]}$$

Where A_{GUS} = GUS activity (pmol Methylumbelliferon min^{-1} mg^{-1} Protein).

ΔF = difference in fluorescent between time B and time A.

min = time B - time A in minutes.

5.5.11 Determination of β-Galactosidase Activity (ONPG Assay)

The activity of *β-Galactosidase* reporter gene was done using ONPG (o-Nitrophenyle-β-D-Galactopyranosid) assay. The β-Gal catalyzed the colorless substrate ONPG into the fluorescent o-Nitrophenyle substance. 2 ml of yeast culture were grown on selective medium overnight. In the next day, 8 ml of YPD medium were added to the overnight culture and the culture was further incubated for 3-5 hours at 30 °C. After 3-5 hours 1 ml of the culture was used to record the OD_{600}. When the culture reached an OD_{600} between 0.5-0.8, 1.5 ml of the culture was placed into three 1.5-ml microcentrifuge tubes and the tubes were centrifuged at 13000 rpm for 30 seconds. The supernatant was removed and the cell pellet was resuspended in 1.5 ml of Lac-Z buffer. The cells suspension was centrifuged and the supernatant was removed. The cell pellet was resuspended in 300 µl Lac-Z buffer (the concentration factor is 1.5/0.3 = 5-fold) and 100 µl of the cell suspension were transferred into a fresh microcentrifuge tube (safe lock). The tubes were placed into liquid nitrogen until the cells were frozen and then they were allowed to thaw at 42 °C. This step was repeated twice to ensure that the cells have broken.

For the ONPG assay, a blank tube with 100 µl of Lac-Z buffer was set, then 700 µl of Lac-Z buffer (+ β-ME) were added to the samples and to the blank tubes. 160 µl of ONPG (4 mg/ml in Z buffer) were added immediately to the reaction tubes and the reactions were placed at 30 °C until a yellow color develops. 400 µl of 1 M Na_2CO_3 were added to stop the reaction and the elapsed time was recorded in minutes. The reaction tubes were centrifuged for 10 minutes at 13000 rpm to pellet cell debris and the supernatants was carefully transferred into clean cuvettes. The spectrophotometer was adjusted against the blank at OD_{420} and the OD_{420} of the reactions was measured relative to the blank.

The β-Gal activity was measured using the following formula:

$$\beta\text{-Gal}_{units} = \frac{1{,}000 \times OD_{420}}{(T \times V \times OD_{600})}$$

Where
- $\beta\text{-Gal}_{units}$ = β-Gal activity in units
- T = Elapsed time (in minutes) of incubation.
- V = 100 µl x concentration factor.
- OD_{600} = OD_{600} of 1 ml of culture.

5.5.12 Electrophoretic Mobility Shift Assay (EMSA)

The EMSA is a simple and rapid method for detecting DNA-protein interactions that is used widely in the study of sequence-specific DNA binding proteins such as transcription factors. The assay is based on the observation of DNA-protein complexes migration through a native polyacrylamide gel, which is slower than the free DNA migration. The EMSA were performed using a labeled *as-1* fragment.

The components for the preparation of 5% (w/v) non-denaturing gels were 28 ml H_2O, 10 ml of 5x TBE buffer, 5.75 ml of 86% glycerol, 6.25 ml of 40% acrylamide, 200 µl of 15% APS and 20 µl TEMED. After gel polymerization, the gel was mounted in an EMSA electrophoresis chamber and was pre-run at 100 V for 30 minutes. Protein samples were mixed with 6 µl of 5x binding buffer, 3 µl of 1 µg/µl poly-dIdC (a non-specific DNA competitor) in a total volume of 28 µl and the mixture was incubated on ice for 10 minutes. 4 µl of the radioactively labeled *as-1* fragment were added and the samples were incubated for at least 10 minutes at room temperature. For loading of the binding reactions on the gel, 10 µl of loading buffer were added. The samples were loaded and the gel was run overnight at 4 °C at 60 V. The next day, the gel was dried under vacuum at 80 °C for 2 hours. The dried gel was exposed for radiography to a PhosphoImager plate overnight.

5.6 GUS Staining of Arabidopsis

Two-week-old soil grown plants were stained for GUS activity using a solution containing 2 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) in 0.1 M Na_2HPO_4, pH 7.0, 10 mM EDTA, 0.5 mM potassium ferricyanide/ferrocyanide, and 0.06% Triton X-100 (Jefferson *et al.*, 1987) at 37°C for 16 hours. The samples were cleared of chlorophyll by sequential washing in 70% ethanol. Microscopy

5.7 Microscopy

Transfected BY-2 Protoplasts were mounted in MSF and viewed with a BX 51 fluorescent microscope (Olympus) with filter sets for UV light (excitation filter EF 395-440; beam splitter FT-460; band-pass filter LP-470) and for blue light (excitation filter EF 450-490; beam splitter FT-510; band-pass filter LP-520). Water-immersion objectives with up to 40x magnifications were used for observations. The microscopy was performed using 50 µl of protoplast suspension that was pipetted with a clipped yellow tip on a microscopic slide. The slides were covered carefully with a covering glass to avoid any mechanical shearing of the protoplasts. Screening for protoplasts expressing the transfected GFP-fusion proteins was performed using the blue filter set on a dark background to minimize photo-damages and bleaching of the chromophore of GFP. The photographs were taken with a mounted photo camera at blue and visible light fields.

6 Results

6.1 Establishing the Modified Yeast-One Hybrid System

6.1.1 The Modified Yeast-One Hybrid System Screening Strategy

After having shown that TGA2.2 is involved in SA-mediated gene expression, our group addresses the question: how is the activity of TGA2.2 regulated? As no direct posttranscriptional modifications have been detected yet, it is likely that protein-protein interactions mediate the TGA2.2 regulation. For instance, a protein inhibiting DNA binding in the absence of SA was postulated (Jupin and Chua, 1996). As TGA2.2 does not activate transcription in yeast, a coactivator might be recruited. Thus, TGA2.2 may be a good bait to isolate putative regulatory proteins using yeast hybrid systems.

Accordingly, a yeast hybrid system strategy was established based on the Y1HS principles. In the classical Y1HS, a cDNA expression library is constructed that encodes hybrid proteins fused to the C-terminus of an AD. The cDNA expression library is transformed into a yeast strain carrying reporter genes under the control of specific *cis*-acting regulatory elements. Hybrid proteins that can recognize these *cis*-elements can act as transcriptional activators of the reporter genes. A modified Y1HS (MY1HS) that utilizes TGA2.2 binding to the *as-1* element and its disability of reporter gene transactivation was used to screen a cDNA library fused to the end of a transcriptional AD of the yeast GAL4 (GAL4AD) to isolate putative TGA2.2-interacting proteins (Figure 6.1).

A parental yeast strain carrying as reporter gene an integrated copy of *HIS3* with three-times tandemly repeated *as-1* elements upstream of the *TATA* element was constructed (R. Weigel, unpublished data). The resulting yeast strain, termed YRWH2, transcribes the *HIS3* reporter gene at basal levels and is not able to grow on media lacking histidine in the presence of 30 mM 3-AT, a competitive inhibitor of the *HIS3* gene product. The YRWH2 yeast cells were transformed with a construct encoding TGA2.2 as a bait protein. The YRWH2 yeast cells harboring TGA2.2 were used to

screen for TGA2.2 interacting proteins using different types of cDNA libraries prepared from tobacco or Arabidopsis vegetative tissue.

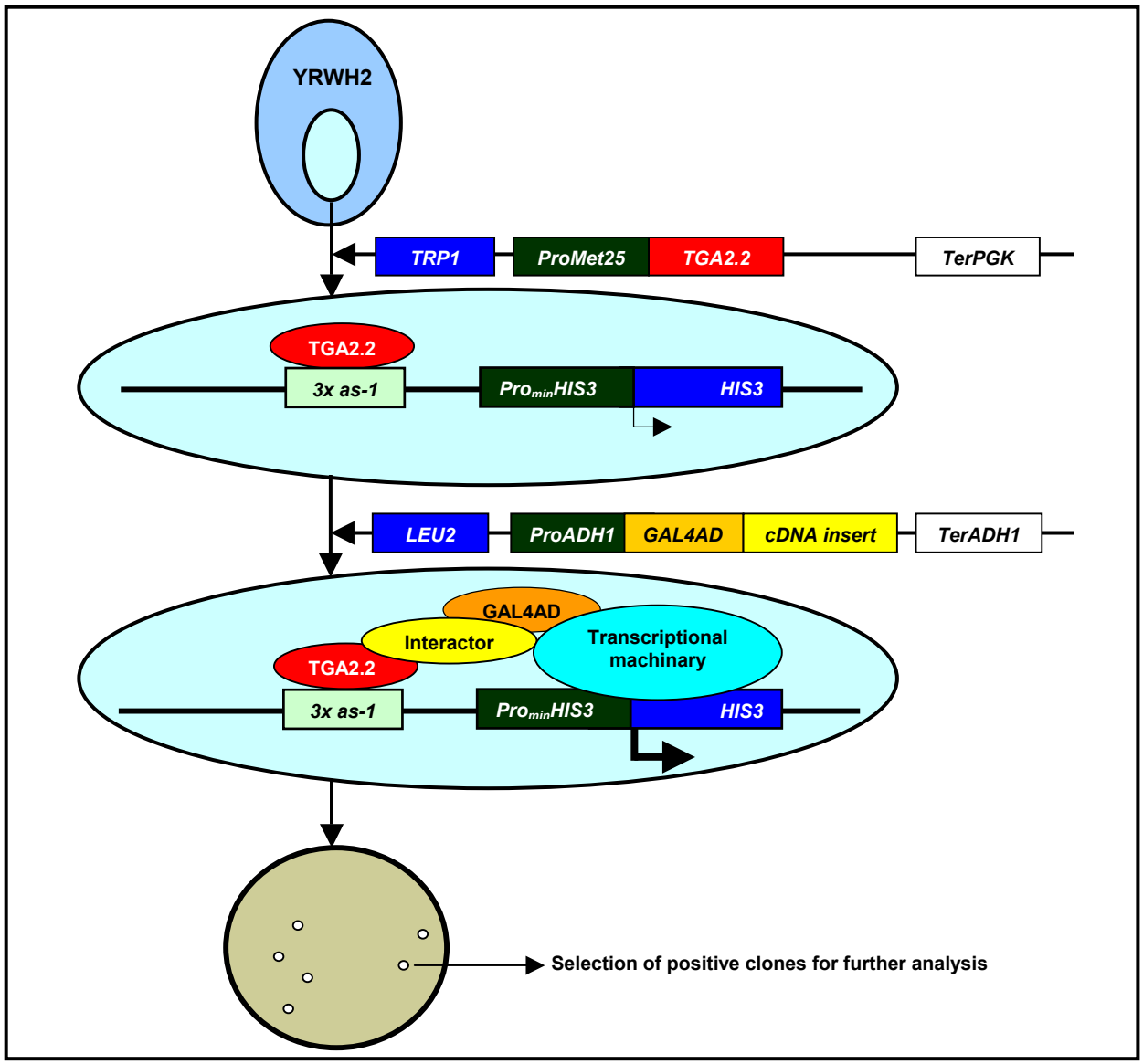


Figure 6.1. Strategy for the isolation of TGA2.2-interacting proteins by selection in yeast.

An expression library of hybrid proteins was transformed into the YRWH2 strain carrying the *HIS3* reporter gene under the control of *3x as-1* element. The *TGA2.2* bait protein coding sequences was first transformed into YRWH2. Hybrid proteins fused to the C-terminus of the GAL4AD were used for TGA2.2 interacting proteins screening. Cells grown on SD medium lacking histidine in the presence of 30 mM 3-AT were considered to be putative positive and selected for further analysis. *Pro$_{min}$HIS3* indicates the minimal promoter of the *HIS3* gene, *TerPGK* indicates the terminator of the *phosphoglycerokinase* (*PGK*) gene, *ProADH1* indicates the promoter of the *alcohol dehydrogenase1* (*ADH1*) gene, and *TerADH1* indicates the terminator of the *ADH1* gene.

6.1.2 Construction of Yeast Strains and Plasmids for the MY1HS

The establishment of the MY1HS started with the construction of yeast strains carrying reporter genes under the control of three-times tandemly repeated *as-1* elements upstream of a *TATA* box. In addition to the YRWH2 strain described above, another yeast strain carrying an integrated copy of *lac-Z* reporter gene with three-times tandemly repeated *as-1* elements upstream of the *TATA* element was constructed (R. Weigel, unpublished data). The resulting yeast strain, termed YRWZ2, was used for ONPG assays. The YRWH2 strain was used to screen cDNA inserts encoding TGA2.2-interacting proteins, while YRWZ2 was used to quantify the interactions. The YRWH2 strain transcribes the *HIS3* gene at basal levels and was not able to grow on medium lacking histidine in the presence of 30 mM of 3-AT, indicating that the *as-1* elements in YRWH2 are transcriptionally inactive.

The TGA2.2 bait protein was expressed in yeast cells using the pBL vector (a pBridge derivative from CLONTECH). TGA2.2 was expressed as a native protein, *i.e.,* TGA2.2 was not fused to any other domains. The pBL-/TGA2.2 plasmid harbors the *Met25::TGA2.2::PGK* cassette, which allows the conditional expression of *TGA2.2* from the *Met25* promoter in response to methionine levels in the yeast medium (Tirode *et al.*, 1997). *TGA2.2* expression is repressed in the presence of methionine and it is induced if the methionine is omitted from the media.

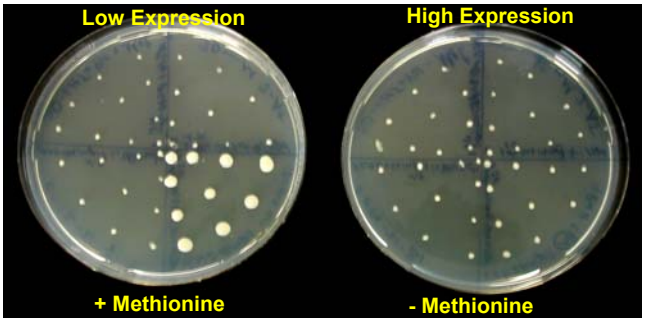
Before performing the MY1HS, the binding of the TGA2.2 bait protein to the *as-1* element was tested. However, as described above, TGA2.2 does not transactivate reporter genes in yeast (Niggeweg *et al.*, 2000b). To overcome this deficit, the *TGA2.2-VP16* coding sequence, encoding a TGA2.2 protein fused in-frame to the VP16 AD, was cloned into the pBL vector under the control of the *Met25* promoter. The *TGA2.2-VP16* plasmid was designated as pBL-/TGA2.2-VP16. The presence of the *TRP1* gene as a selectable auxotrophic marker in the pBL vector allows the selection of transformed yeast cells on SD medium lacking tryptophan.

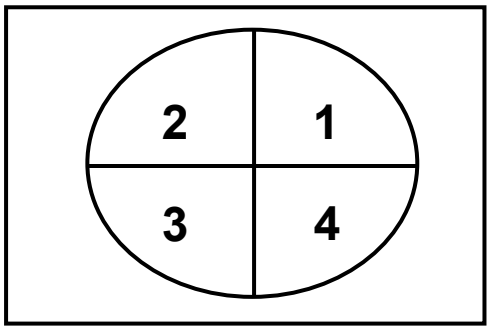
6.1.3 Testing and Optimizing the MY1HS Screening Conditions

Before embarking on the MY1HS screens for TGA2.2-interacting proteins, several control experiments were conducted to test the MY1HS feasibility, credibility and reliability.

To test the effect of TGA2.2 and TGA2.2-VP16 overexpression or repression on the yeast transformation efficiency, the pBL-/TGA2.2 and pBL-/TGA2.2-VP16 plasmids were transformed into the YRWH2 strain and the transformants were plated on selective SD media lacking tryptophan, supplemented with or without methionine. Surprisingly, when TGA2.2 and TGA2.2-VP16 proteins were overexpressed in the YRWH2 cells, *i.e.,* on SD medium lacking methionine, a transformation efficiency value of null was obtained. These results indicate that TGA2.2 overexpression is toxic to the YRWH2 yeast cells. Adding 1 mM of methionine to the transformation medium repressed TGA2.2 expression and thereby their toxicity, enabling YRWH2 transformants to grow.

In order to familiarize the expected results from the M1YHS screens and in order to test the effect of TGA2.2 on reporter gene expression, TGA2.2 and TGA2.2-VP16 proteins were analyzed for *as-1* element binding and auto-activation in YRWH2 strain. For this purpose, *TGA2.2* and *TGA2.2-VP16* transformants were grown on SD medium lacking tryptophan and histidine, supplemented with 30 mM 3-AT and with or without methionine. The TGA2.2 bait alone did not activate the expression of the *HIS3* reporter gene (Figure 6.2, sector 2). The restoration of histidine prototrophy in *TGA2.2-VP16* transformants ensures that the TGA2.2 is able to bind to *as-1* element (Figure 6.2, sector 4). The TGA2.2-VP16-dependent *HIS3* gene activation was dependent on the presence of methionine, verifying that the TGA2.2 overexpression was toxic to the YRWH2 cells. Similar results were obtained using the YRWZ2 strain harboring the *lac-Z* reporter gene (data not shown).



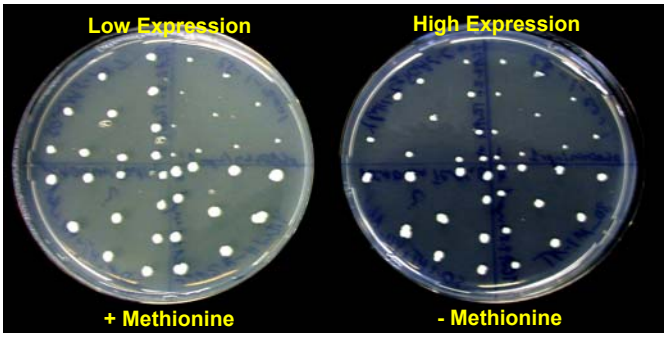


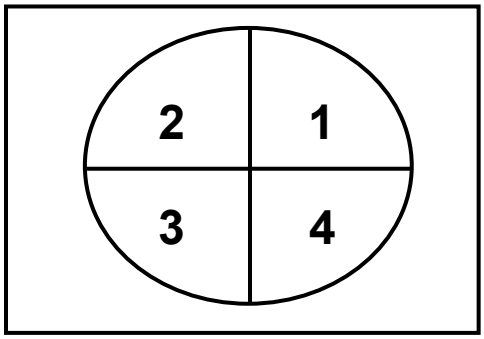
		Growth on SD lacking histidine	
Sector	**Bait (*TRP1* marker)**	**- Methionine**	**+ Methionine**
1	pBL	-	-
2	TGA2.2	-	-
3	pBL	-	-
4	TGA2.2-VP16	-	+++

Figure 6.2. M1HYS assays of TGA2.2 and TGA2.2-VP16 binding to the *as-1* elements in YRWH2.

YRWH2 cells containing pBL (1 and 3), pBL-/TGA2.2 (2) and pBL-/TGA2.2-VP16 (4) plasmids were grown for 4 days at 30 °C on selective SD medium lacking tryptophan and histidine, supplemented with 30 mM 3-AT and with or without methionine.

The well-studied interactions between NPR1 protein fused to the GAL4AD (GAL4AD-NPR1) and TGA2.2 were tested in the MY1HS as a positive control. The GAL4AD-NPR1 was found to interact with TGA2.2 in the MY1HS (Figure 6.3, sector 2). This result shows the interaction between NPR1 and TGA2.2 at the *as-1* element. Intriguingly, the GAL4AD-NPR1 protein partially reduces the toxic effect of TGA2.2 overexpression on the YRWH2 cells; YRWH2 cells containing TGA2.2-VP16 and GAL4AD-NPR1 proteins were able to grow on SD media lacking methionine (Figure 6.3, sector 4). YRWH2 cells expressing the GAL4AD-TGA1a protein were used as a control (Figure 6.3, sector 3).





			Growth on SD lacking histidine	
Sector	**Bait (*TRP1* marker)**	**Prey (*LEU2* marker)**	**- Methionine**	**+ Methionine**
1	pBL	GAL4AD-NPR1	-	-
2	TGA2.2	GAL4AD-NPR1	-	++
3	pBL	GAL4AD-TGA1a	+++	+++
4	TGA2.2-VP16	GAL4AD-NPR1	++	+++

Figure 6.3. M1HYS assays of interactions between NPR1 and TGA2.2 proteins.

YRWH2 cells containing pBL + pGAD424/NPR1 (1), pBL-/TGA2.2 + pGAD424/NPR1 (2), pBL-/TGA2.2-VP16 + pGAD424/NPR1 (3) and pBL + pGAD424/TGA1a (4) plasmids were grown for 4 days at 30 °C on selective SD medium lacking leucine, tryptophan and histidine, supplemented with 30 mM 3-AT and with or without methionine.

In order to optimize the MY1HS screening conditions, several pre-screen tests were conducted to measure the cDNA library plasmid transformation efficiency, the background growth rate and the optimal methionine and 3-AT concentrations in the media. In addition to that, several miniscreens using different concentrations of NPR1 and TGA1a (as positive controls) were conducted to have a general picture about the MY1HS screens outcome. In conclusion, the MY1HS screening conditions were calibrated so that YRWH2 yeast cells already containing pBL/TGA2.2 plasmid were used directly for transforming 100 µg of cDNA library plasmid and the resulted transformants were plated on SD media lacking tryptophan, leucine and histidine, supplemented with 30 mM 3-AT and 1 mM methionine in order to avoid excessive background growth and to reduce TGA2.2 toxicity, respectively.

The yeast cells were separately transformed with two expression libraries of cDNA fragments of mRNAs prepared from Arabidopsis and tobacco plants. The cDNA fragments from both libraries were fused to the yeast GAL4AD. The Arabidopsis cDNA

library was cloned into the pGAD10 vector (Weigel *et al.*, 2001), while the tobacco cDNA library was cloned into the pGAD424 vector (Strathmann *et al.*, 2001). The presence of *LEU2* as selectable auxotrophic marker in the pGAD10 and pGAD424 plasmids allows the selection of transformed yeast cells on SD medium lacking the amino acid leucine.

6.2 Isolation of cDNAs Encoding *As-1* Binding and TGA2.2-Interacting Proteins Using the MY1HS

To isolate cDNAs encoding TGA2.2 interacting proteins, the MY1HS screening system was deployed. The YRWH2 cells harboring the *TGA2.2* coding sequence were separately transformed with the expression cDNA libraries prepared from Arabidopsis and tobacco plants. Out of ~1 x 10^6 yeast transformants/screen, several colonies were selected on the basis of histidine prototrophy and 3-AT resistance. These primary positives were further analyzed by growing them successively on SD media lacking tryptophan, leucine and histidine, supplemented with 30 mM 3-AT and 1 mM methionine. From these primary positives, 45 positive clones (28 and 17 from Arabidopsis and tobacco cDNAs, respectively) were able to continue to grow on the selective media. Restriction enzyme digestion and DNA sequencing analysis of cDNA fragments of the isolated plasmids led to the classification of 45 cDNA clones into four distinct cDNA groups (Table 6.1). Among the four groups, clone 5 from group 4 was the most abundant.

Table 6.1. General characteristics of positive cDNA clones isolated in this work.

Group	Clone	Insert size (bp)	Frequency	Source of cDNA library
1	13	2000	12	Tobacco
	54	1300	5	Tobacco
2	16	1000	1	Arabidopsis
3	44	950	2	Arabidopsis
	82	1450	3	Arabidopsis
4	5	550	22	Arabidopsis

To confirm the interactions with TGA2.2, the recovered prey plasmids were retransformed into YRWH2 cells containing either pBL or pBL-/TGA2.2 plasmids. The transformants were assayed for histidine prototrophy. Remarkably, the tobacco cDNA library positive clones conferred auto-activation of the *HIS3* reporter gene, *i.e.,* they do not need to interact with TGA2.2 to activate reporter gene expression. This result hints at either false positive clones or *as-1* binding proteins. Strikingly, sequence analysis revealed that both cDNA inserts represent two closely related genes encoding TGA transcription factors (Table 6.2). Clone 13 contained the full-length sequence of a TGA10 cDNA. The clone 54 contained an open reading frame encoding truncated TGA2.1 protein. As both proteins were extensively analyzed in our group, they were excluded (Niggeweg *et al.*, 2000b; Schiermeyer *et al.*, 2003).

In contrast to tobacco results, the restoration of histidine prototrophy using the Arabidopsis library was dependent on the presence of TGA2.2, indicating that the expression of *HIS3* reporter gene driven by the *as-1* elements required an interaction between both proteins. Sequence analysis of isolated plasmids revealed that the Arabidopsis cDNA clones encode different polypeptides, identifying four independent genes (Table 6.2). The structural features of the Arabidopsis cDNA clones will be discussed in more details in the next sections.

Table 6.2. Identity of positive cDNA clones isolated in this work.

Group	Clone	NCBI Accession Number	Gene Name	Product Description
1	13		*TGA10*	TGA10 bZIP factor
	54	U90214	*TGA2.1*	Truncated TGA2.1 bZIP factor
2	16	NM_116248	*At4g00270*	*S. cerevisiae* ADR1 like protein
3	44	NM_103938	*At1g50570*	Unknown protein
	82	NM_186999	*At5g55530*	Unknown protein
4	5	NM_102616	*At1g28480*	Putative glutaredoxin

6.3 Functional Analysis of the *At4g00270* Isolated cDNA Clone

6.3.1 Structural Analysis of *At4g00270* cDNA Clone

To examine the structure of the Arabidopsis cDNA clone 16, the sequenced inserted DNA fragment was analyzed using the vector NTi program and by searching the DNA databases for corresponding genes. The DNA sequence analysis revealed that the clone 16 contains a full-length cDNA, encoding 302 amino acids that represents a putative protein with a predicted molecular mass of 34.05 kDa (Figure 6.4).

The cDNA sequence was identified in the Arabidopsis sequence database and was corresponding to the *At4g00270* designated gene (http://arabidopsis.org). The full-cDNA coding sequence of clone 16 was fused in-frame to the GAL4AD of the prey plasmid and contained a translation stop codon, 3´-untranslated region and a poly (A) tail of adenosine residues.

```
                                                                ggtcgtgaagattgggaatcactgctctgcatgcgccctagaatcagcaagtctaaagcaagcaataag

M  V  T  P  K  Q  I  D  F  S  S  C  V  G  A  D  N  S  N  G  T  L  S  H  R  R  S  P  R  N  I  P
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S  S  K  R  A  A  S  V  A  E  E  E  T  M  K  K  K  M  K  M  K  K  K  K  K  L  D  P  P  L  I
TCCTCAAAGCGTGCTGCTTCCGTCGCAGAAGAAGAGACTATGAAGAAGAAGATGAAGATGAAGAAGAAGAAGAAATTGGATCCGCCGTTGATC

V  R  I  W  N  E  E  D  E  L  S  I  L  K  G  L  V  D  Y  R  A  K  T  G  F  N  P  K  I  D  W  D
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A  F  C  S  F  L  G  S  S  I  V  E  R  F  S  K  D  Q  V  L  S  K  I  R  K  L  K  R  R  F  H  V
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H  S  E  K  I  N  Q  G  N  D  P  K  F  T  R  S  S  D  S  E  A  F  G  F  S  S  M  I  W  G  Q  G
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D  D  D  G  M  D  K  E  H  E  V  N  G  N  G  A  A  E  N  R  T  N  E  S  G  E  E  M  L  K  E  H
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E  E  E  V  A  N  T  E  L  L  N  E  N  G  A  A  K  T  T  E  N  G  T  S  S  G  K  E  R  H  D  E
GAGGAGGAAGTGGCTAATACTGAACTTTTAAATGAGAATGGGGCAGCCAAAACAACAGAGAATGGGACTAGTAGTGGAAAAGAGAGACATGATGAG

D  N  D  D  D  D  E  L  C  A  V  Q  D  A  F  E  A  V  M  S  Q  G  L  S  G  Y  Q  K  K  L  Q  L
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E  K  L  M  N  L  G  N  G  K  R  R  E  L  S  D  E  W  K  A  L  C  V  E  E  T  R  F  N  I  K  K
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L  R  F  S  A  K  L  A  E  A  A  N  D  S  *
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aaaaaaaaa
```

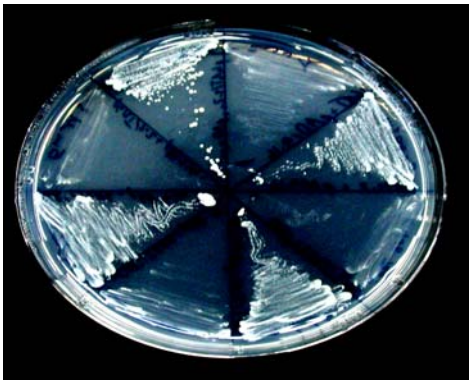
Figure 6.4. Full-length sequence of *At4g00270* cDNA.

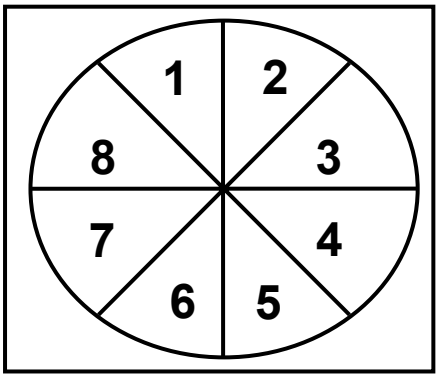
The protein databases analysis predicted that the *At4g00270* gene encodes an unknown protein that contains low similarity to the *S. cerevisiae ADR1* gene and to storekeeper DNA-binding protein (*Solanum tuberosum*) (Bemis and Denis, 1988; Zourelidou *et al.*, 2002). The complete sequence of *At4g00270* gene is found on bacterial artificial chromosome (BAC) clone designated F5I10 (GenBank Accession Number: AF013293).

6.3.2 At4g00270 Interacts with TGA2.2 in the Y2HS

To determine whether Arabidopsis At4g00270 protein interacts with the Arabidopsis homologs of TGA2.2 and to verify the observed interaction between TGA2.2 and At4g00270 in the MY1HS, a classical Y2HS assay was conducted. DNA fragments encoding TGA2.2, TGA2 and TGA6 were cloned downstream the GAL4BD domain into the pGBT9 vector. The resulted constructs were cotransformed with the pGAD10/At4g00270 plasmid (encoding GAL4AD-At4g00270) into the Y2HS HF7c strain, carrying the *HIS3* reporter gene under the control of UAS^{GAL4} element, and were then assayed for histidine prototrophy in the presence of 5 mM 3-AT. As observed for MY1HS, TGA2.2 interacts with At4g00270 in the Y2HS (Figure 6.5, sector 3). The interaction affinity between both proteins is similar to that of the NPR1-TGA2.2 interaction (positive control). The At4g00270 protein was also able to interact with the Arabidopsis TGA2 and TGA6 proteins (Figure 6.5, sectors 6 and 8).

In domain swap experiments, in which *GAL4BD-At4g00270* and the *GAL4AD-TGA2.2* coding sequences were cotransformed into HF7c strain and assayed for histidine prototrophy, a loss of TGA2.2-At4g00270 interaction was observed (data not shown). This loss of TGA2.2-At4g00270 interaction could be attributed to changes in interaction affinity between both proteins in the swap orientation, the At4g00270 protein is not well expressed or it does not fold properly in yeast. As the TGA2.2- At4g00270 interaction was lost in the domain swap experiment, the At4g00270 protein was excluded from further analysis.





Sector	Bait (*TRP1* marker)	Prey (*LEU2* marker)	Growth on SD lacking histidine
1	GAL4BD-TGA2.2	GAL4AD-NPR1	++
2	GAL4BD	GAL4AD-At4g00270	-
3	GAL4BD-TGA2.2	GAL4AD-At4g00270	++
4	GAL4BD-TGA2.2	GAL4AD	-
5	GAL4BD-TGA2	GAL4AD-At4g00270	++
6	GAL4BD-TGA2	GAL4AD	-
7	GAL4BD-TGA6	GAL4AD-At4g00270	++
8	GAL4BD-TGA6	GAL4AD	-

Figure 6.5. Y2HS assays of interactions between GAL4AD-At4g00270 and TGA proteins.

HF7c cells containing pGBT9/TGA2.2 + pGAD424/NPR1 (1), pGBT9 + pGAD424/At4g00270 (2), pGBT9/TGA2.2 + pGAD424/At4g00270 (3), pGBT9/TGA2.2 + pGAD424 (4), pGBT9/TGA2 + pGAD424/At4g00270 (5), pGBT9/TGA2 + pGAD424 (6), pGBT9/TGA6 + pGAD424/At4g00270 (7) and pGBT9/TGA6 + pGAD424 (8) plasmids were grown for 3 days at 30 °C on selective SD medium lacking leucine, tryptophan and histidine, supplemented with 5 mM 3-AT.

6.4 Functional Analysis of The *At1g50570* and *At5g55530* Isolated cDNA Clones

6.4.1 Structural Analysis of *At1g50570* and *At5g55530* cDNA Clones

DNA sequencing of the cDNA inserts of clone 44 and clone 82 showed that they encode for two homologues proteins. The cDNA sequence of clone 82 contains a partial cDNA coding sequence with an in-frame fusion to the GAL4AD, a translation stop codon, a 3´-untranslated region and a poly (A) tail of adenosine residues. A BLAST search of the Arabidopsis databases showed sequence similarity with the

At5g55530 gene that encodes a 405 amino acid protein with a predicted molecular mass of 47.97 kDa and shows low similarity to cold-regulated gene *SRC2* from soybean (Figure 6.6; Takahashi and Shimosaka 1997). The sequence of the *At5g55530* gene was found on the MTE17 BAC clone (GenBank Accession No. AB015479).

At5g55530 protein sequence analysis uncovered a conserved region at residues 40 to 111 that resembles a C2 domain (calcium dependent phospholipid binding domain (Figure 6.6, the red background). The C2 domain is a region containing approximately 130 residues involved in binding phospholipids in a calcium dependent or independent manner. It is present in a large number of proteins, including protein kinase C, phospholipases and synaptogamin (Reviewed in: Nalefski and Falke, 1996; Rizo and Südhof, 1998). C2 domains have been identified in a growing number of eukaryotic signalling proteins that mediate a broad array of critical intracellular processes, including membrane trafficking, the generation of lipid-second messengers, activation of GTPases, and the control of protein phosphorylation and protein-protein interactions (Nalefski and Falke, 1996). According to Kopka *et al.*, 1998, the C2 domain can be divided into three subdomains, called A, B and C; subdomain A, generally consisting of DPYVK, is located on the N-terminal side, subdomain B is a variable polybasic core region, which in most cases contains a KXK(R)-T motif; subdomain C, represented by the segment LNPXWN(X)-EXFXF, is positioned C-terminus to the basic core region.

Many genes encoding proteins with C2 domains are found in higher plants and as in animal cells these proteins are most likely involved in a variety of signal transduction pathways. For instance, a mutant in the *copin* gene, which belongs to a class of copin proteins that are characterized by their unique combination of two C2 domains, exhibits morphological abnormalities, increased resistance to virulent strains of *P. syringae* and *Peronospora parasitica*, and constitutive expression of *PR* genes under low-humidity conditions (Jambunathan *et al.*, 2001).

gattgtttgttaggttttaca

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GGCATAAATTCCAATGGGAAAGATTCTAAAAGTTGTGGCCGGCAAGACCTTGTTGGTGCTCTTGAGGTTTATGTCCATCAGGCAAGGGATATCCAT

N I C I Y H K Q D V Y A K L C L T S D P D K S V S T K I I N G G
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G R N P V F D D N V K L D V R V L D T S L K C E I Y M M S R V K
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E N H V T N S V T S I L K Q D S P E S S N A T N G A A S P H A S
GAGAATCATGTGACTAATTCTGTCACTTCTATACTGAAGCAAGATTCACCTGAGAGCAGCAATGCAACAAATGGAGCTGCTTCTCCTCACGCTTCA

A H S A T E T P N H E H L S V V N S K A S S Q E S E S E A S G E
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T S E E K T V K S V L T V K V E P E S K V V Q Q D I V D M Y M K
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Figure 6.6. Full-length sequence of *At5g55530* cDNA.

The red background represents the C2 domain region and the yellow box points for the beginning of the isolated cDNA clone.

DNA sequence analysis of clone 44 was an eccentric issue. The cDNA sequence of clone 44 contains a partial cDNA coding sequence that is inversely inserted into the pGAD10 prey plasmid, *i.e.,* no in-frame fusion between GAL4AD and the 44 cDNA coding sequence. Astoundingly, the clone 44 did not confer any auto-activation of the *HIS3* reporter gene (data not shown), *i.e.,* the presence of the TGA2.2 bait protein was crucial to activate the reporter gene expression in the presence of clone 44. Mining the Arabidopsis databases revealed that the clone 44 represents the Arabidopsis *At1g50570* gene that encodes a 388 amino acids protein with a predicted molecular mass of 42.1 kDa (Figure 6.7). The sequence of the *At1g50570* gene was found on the F11F12 BAC clone (GenBank accession No.: AC012561).

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GATTCAGACAGTTTCATCGGTGTGCTTGAGGTTTTTGTTCACCAAGCTAGGGACATCCACAACATCTGTATCTATCATAAGCAAGATGTGTATGCT

K L C L T N D P E N S L S T K I I N G G G Q N P V F D D T L Q F
AAGCTTTGTCTCACAAATGATCCCGAAAACTCCTTGTCCACAAAGATCATCAATGGTGGAGGGCAAAACCCTGTCTTCGACGATACCCTTCAGTTC

D V K N L D C S L K C E I F M M S R V K N Y L E D Q L L G F S L
GACGTTAAGAACCTGGATTGTTCGCTTAAGTGTGAGATATTTATGATGAGCCGCGTGAAGAATTATCTTGAGGATCAGTTACTTGGATTCTCTCTT

V P L S E V I V R N G K L E K E F S L S S T D L Y H S P A G F V
GTACCTTTGTCTGAAGTGATTGTGAGGAATGGGAAATTGGAGAAAGAGTTCTCCCTTTCTTCAACCGATTTGTATCACTCTCCTGCAGGTTTTGTC

E L S L S Y A G D S P D V M H I P A V P T A D E T E L A P I E F
GAGTTGTCTCTCTCGTACGCAGGAGATTCGCCTGACGTGATGCATATTCCTGCGGTTCCAACTGCGGATGAGACCGAGTTGGCTCCTATCGAGTTT

D E S E F L D P K I V C E N N Q M V S K Y F S T T C S D S D D F
GATGAGAGTGAGTTTTTGGATCCAAAGATTGTCTGTGAAAACAATCAAATGGTGTCTAAGTACTTCTCCACTACGTGTTCTGATTCTGATGATTTT

A S S E T G F V E V N S I L S A V V E T A V D E A A P A N S V S
GCAAGCTCTGAAACTGGCTTTGTGGAAGTAAACAGCATCCTGTCTGCAGTTGTTGAAACTGCTGTTGACGAAGCAGCACCGGCCAATTCTGTCTCA

T N G I S S P S T A V S S G S S G T H D V S K Q S S E G N N S D
ACAAATGGAATCTCATCTCCATCAACTGCAGTTAGCTCTGGCTCCTCGGGAACTCATGATGTTTCAAAGCAATCTAGTGAAGGAAACAACTCAGAT

S E Q E A K K P T D I I K S G D L D K T D E E A V V K P V L T V
TCAGAACAAGAAGCGAAGAAGCCAACAGATATCATCAAGAGTGGTGATTTAGATAAGACTGATGAAGAAGCAGTTGTGAAACCGGTTCTGACAGTG

N I E P E Q K V V Q Q D I V D M Y T K S L Q Q F T E S L A K M K
AACATTGAACCAGAGCAAAAGGTAGTGCAACAAGATATTGTTGACATGTACACAAAAAGCTTGCAGCAATTCACTGAGTCACTGGCTAAGATGAAA

L P L D I D S P T Q S E N S S S S Q Q T P K S A S S R V F Y G S
CTTCCTTTGGACATCGATAGCCCAACCCAATCAGAGAACTCAAGCTCCTCCCAGCAGACGCCAAAGAGTGCTAGCTCCCGTGTTTTCTACGGGAGT

R A F F *
AGAGCCTTCTTCTGAagctcagaaatcatctcaagtttggcatcagaagtagcgagcatgtagtgaaagtgtaataagttaaaagtagacactatctaaaatgtgtaactatcttcctttgaag
cctttagcttttcttgttatctgcaacttttccaatttagtactaaagtttcataatctcttcccttctcatataacagatagacataaagacaagaagtttgtgtttgaagaagaaaacaatacaaag
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ctag

Figure 6.7. Full-length sequence of *At1g50570* cDNA.

The red background represents the C2 domain region and the yellow box points for the beginning of the isolated cDNA clone.

The striking issue of the At1g50570 protein, as revealed by BLAST searches and sequence alignments, was its sequence homology with the At5g55530 protein. At1g50570 and At5g55530 proteins deduced amino acids showed 54% identity (Figure 6.8). The deduced amino acid sequences of At1g50570 and At5g55530 showed significant high sequence identity in the C2 domain. Besides the C2 domain, the two proteins share a conserved region at the C-terminus end. Based on this observation, both clones were considered for further analysis.

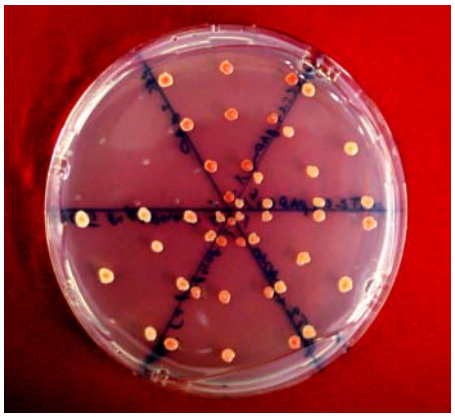
```
At1g50570                      MESPHSEASIVNGSIHLNGSGETKTKNIVMSSDSDSFIGVLEVF 44
At5g55530  MDSPQSVVSPFKIGESENENSNSVQSSGNQSNGINSNGKDSK-----SCGRQDLVGALEVY 56

At1g50570  VHQARDIHNICIYHKQDVYAKLCLTNDPENSLSTKIINGGGQNPVFDDTLQFDQLLGFSLV 129
At5g55530  VHQARDIHNICIYHKQDVYAKLCLTSDPDKSVSTKIINGGGRNPVFDDNVKLDQLLGFTLV 141

At1g50570  PLSEVIVRNGKLEKEFSLSSTDLYHSPAGFVELSLSYAGDSPDVMHIPAVPTA---DET-- 185
At5g55530  PMSELLFKNGKLEKEFSLSSTDLYHSPAGFVQLSLSYYGSYPDVMAIPSMPSSVSIDETTK 202

At1g50570  -----ELAPIEFDESEFLDPKIVCENNQMVSKYFSTTCSDSDDFASSETGFVEVNSILSAV 226
At5g55530  DPEGSESVPGELDKIEFPDPNVANENEKMVSEYFGISCSTIDSETSDSLVTSDAENHVTNS 263

At1g50570  VETAVDEAAPANTVSTNGISSP-STAVSSGSSRTHD-VSKQSSEGNNSDSEQEAKKPTDII 241
At5g55530  VTSILKQDSPESSNATNGAASPHASAHSATETPNHEHLSVVNSKASSQESESEA------- 317

At1g50570  IKSGDLDKTDEEAVVKPVLTVHIEPEQKVVQQDIVDMYTKSLQQFTESLAKMKLPLDIDSP 360
At5g55530  --SGE---TSEEKTVKSVLTVKVEPESKVVQQDIVDMYMKSMQQFTDSLAKMKLPLDIDSP 373

At1g50570  T---QSENSSSSQQTPKSAS-SRVFYGSRAFF 388
At5g55530  TKSENSSSDSQKLPTPKSNNGSRVFYGSRPFF 405
```

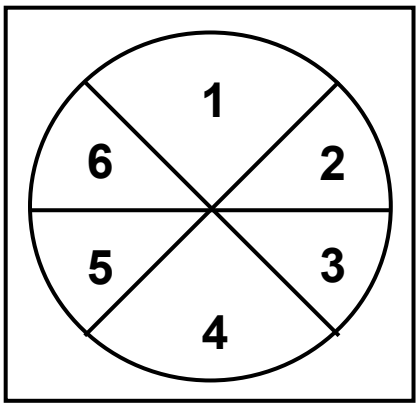
Figure 6.8. Comparison of deduced amino acid sequences of the At1g50570 and At5g55530 proteins.

Red letters represent perfectly conserved amino acid residues, and dashes indicate gaps introduced to maximize alignment. The light blue background represents the C2 domain amino acids.

6.4.2 At1g50570 and At5g55530 Interact with TGA2.2 Factor in the Y2HS

To assess whether TGA2.2 interacts with full lengths At1g50570 and At5g55530 proteins in the classical Y2HS, DNA fragments containing the full-length *At1g50570* and *At5g55530* genes were amplified and cloned in-frame with the GAL4AD into the pGAD424 plasmid. Interactions between At1g50570 and At5g55530 and TGA2.2 were tested for histidine prototrophy in the HF7c Y2HS strain in the presence of 5 mM 3-AT. As observed for MY1HS, the GAL4BD-TGA2.2 protein interacts with GAL4AD-At1g50570 (Figure 5.9, sector 2) and GALAD-At5g55530 full-length proteins (data not shown). Similar results were observed with the Arabidopsis TGA2, TGA5 and TGA6 proteins (Figure 5.9, sectors 3, 4 and 5). The Y2HS interactions between At1g50570 and TGA class-II factors verified that the YRWH2 histidine prototrophy phenotype is resulted from the At1g50570-TGA2.2 interaction. However, how is the inverted cDNA insert expressed in YRWH2 cells? The answer remains unresolved.





Sector	Bait (*TRP1* marker)	Prey (*LEU2* marker)	Growth on SD lacking histidine
1	GAL4BD-TGA2.2	GAL4AD-NPR1	+++
2	GAL4BD-TGA2.2	GAL4AD-At1g50570	+++
3	GAL4BD-TGA2	GAL4AD-At1g50570	+++
4	GAL4BD-TGA5	GAL4AD-At1g50570	+++
5	GAL4BD-TGA6	GAL4AD-At1g50570	+++
6	GAL4BD	GAL4AD-At1g50570	-

Figure 6.9. Y2HS assays of interactions between At1g50570 and TGA proteins.

HF7c cells containing pGBT9/TGA2.2 + pGAD424/NPR1 (1), pGBT9/TGA2.2 + pGAD424/At1g50570 F (2), pGBT9/TGA2 + pGAD424/At1g50570 F (3), pGBT9/TGA5 + pGAD424/At1g50570 F (4), pGBT9/TGA6 + pGAD424/At1g50570 F (5), pGBT9 + pGAD424/At1g50570 F (6) were grown for 3 days at 30 °C on selective SD medium lacking leucine, tryptophan and histidine, supplemented with 5 mM 3-AT.

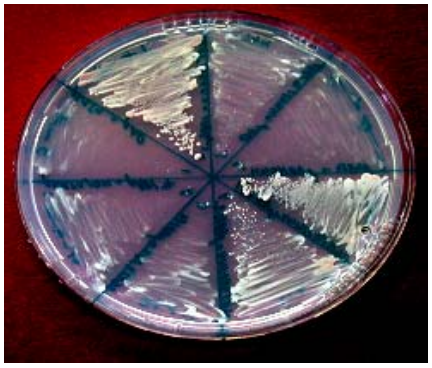
To define the region in TGA2.2 that interacts directly with At1g50570 and At5g55530 proteins, a *TGA2.2* gene fragment encoding the carboxyl part, which does not include the bZIP domain, was cloned into the pGBT9 vector. This truncated GAL4BD-TGA2.2 C-terminal protein was coexpressed with GAL4AD-At1g50570 in the HF7c strain and the transformants were assayed for the histidine prototrophy. Similarly to the full-length TGA2.2 results, yeast coexpressing the TGA2.2 C-terminus part (downstream of the bZIP region) and At1g50570 had a histidine prototrophy phenotype (data not shown). This suggests that the C-terminus region of TGA2.2 contributes to the TGA2.2-At1g50570 interaction. It is most likely that the C2 domain is not involved in TGA2.2-At1g50570 interaction. The *At1g50570* cDNA isolated (abbreviated as P) in the

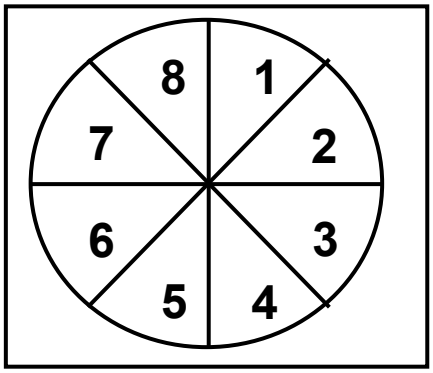
MY1HS, which encodes a truncated protein that lacks 32 amino acids of the C2 domain still interacts with TGA2.2 in the Y2HS assays.

6.4.3 At1g50570 Interacts with Class II of Tobacco TGA Factors

To further determine the specificity of the TGA2.2-At1g50570 interactions, a bait construct containing the *GAL4BD-At1g50570 P* coding sequence was generated in order to test the interactions with other TGA factors from tobacco using of the Y2HS. First, the *GAL4BD-At1g50570 P* construct was tested for *HIS3* reporter gene auto-activation in the HF7c strain. The bait protein seems to have a low level of transactivation activity (Figure 6.10, sector 1). The *GAL4AD-TGA2.2*, *GAL4AD-TGA2.1*, *GAL4AD-TGA1a* or *GAL4AD-TGA10* were cotransformed with *GAL4BD-At1g50570 P* into HF7c yeast. The histidine prototrophy was monitored in the resulting transformants on media supplemented with 5 mM 3-AT. As observed for TGA2.2, At1g50570 P interacts strongly with TGA2.1 (Figure 6.10, sectors 3 and 4). Surprisingly, the *TGA1a* and *TGA10* transformants showed only background levels of HF7c growth on media lacking histidine, indicating that the At1g50570 did not interact with TGA1a or TGA10 (Figure 6.10, sectors 5 and 6).

The lack of reporter gene expression is not related to poor expression of the TGA1a and TGA10 proteins as previous analysis showed that both proteins interact with GAL4BD-TGA2.2 and this interaction conferred transcriptional activation of the reporter gene in yeast (Niggeweg *et al.*, 2000b; Schiermeyer *et al.*, 2003). In conclusion, the At1g50570 protein interacts specifically with the C-terminus part of tobacco TGA class-II factors.





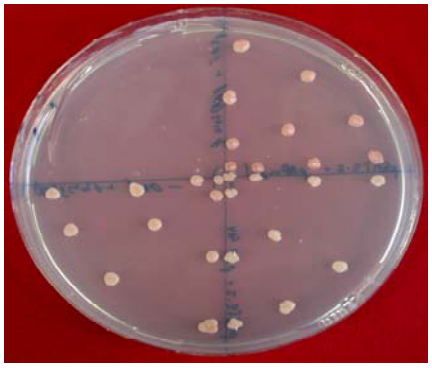
Sector	Bait (*TRP1* marker)	Prey (*LEU2* marker)	Growth on SD lacking histidine
1	GAL4BD-At1g50570 P	GAL4AD	-
2	GAL4BD	GAL4AD-At1g50570 P	-
3	GAL4BD-At1g50570 P	GAL4AD-TGA2.1	++
4	GAL4BD-At1g50570 P	GAL4AD-TGA2.2	++
5	GAL4BD-At1g50570 P	GAL4AD-TGA1a	-
6	GAL4BD-At1g50570 P	GAL4AD-TGA10	-
7	GAL4BD	GAL4AD	-
8	GAL4BD-TGA2.2	GAL4AD-NPR1	++

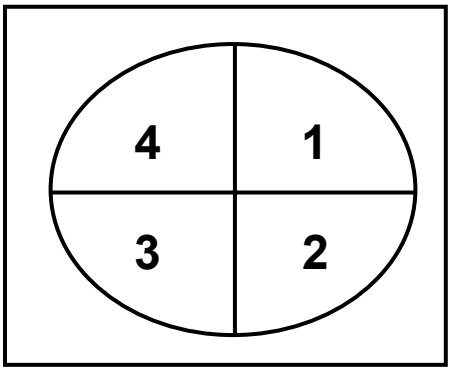
Figure 6.10. Y2HS assays of interactions between At1g50570 P and TGA proteins.

HF7c cells containing pGBT9/At1g50570 P + pGAD424 (1), pGBT9 + pGAD424/At1g50570 P (2), pGBT9/At1g50570 P + pGAD424/TGA2.1 (3), pGBT9/At1g50570 P + pGAD424/TGA2.2 (4), pGBT9/At1g50570 P + pGAD424/TGA1a (5), pGBT9/At1g50570 P + pGAD424/TGA1a (6), pGBT9 + pGAD424 (7) and pGBT9/TGA2.2 + pGAD424/NPR1 (8) plasmids were grown for 3 days at 30 °C on selective SD medium lacking leucine, tryptophan and histidine, supplemented with 5 mM 3-AT.

6.4.4 Function of At1g50570 and At5g55530 as Transcription Activators in Yeast

To test whether At1g50570 and At5g55530 are able to confer autoactivation function in yeast, the *At1g50570* and *At5g55530* full-length coding sequences were inserted downstream of the GAL4BD in the pGBT9 plasmid. These constructs were introduced into the yeast strain HF7c and assayed for histidine prototrophy. Yeast cells transformed with the *GAL4BD-At1g50570* and *GAL4BD-At5g55530* constructs were able to grow on media lacking histidine, indicating the activation of the *HIS3* reporter gene by the fusion proteins (Figure 6.11, sectors 3).





Sector	Bait (*TRP1* marker)	Prey (*LEU2* marker)	Growth on SD lacking histidine Media
1	GAL4BD-TGA2.2	GAL4AD-NPR1	+++
2	GAL4BD-TGA2.2	GAL4AD-At1g50570	+++
3	GAL4BD-At1g50570	GAL4AD	+++
4	GAL4BD	GAL4AD-At1g50570	-

Figure 6.11. Function of At1g50570 as transcription activator in yeast.

HF7c cells containing pGBT9/TGA2.2 + pGAD424/NPR1 (1), pGBT9/TGA2.2 + pGAD424/At1g50570 (2), pGBT9/At1g50570 + pGAD424 (3) and pGBT9 + pGAD424/At1g50570 (4) plasmids were grown for 3 days at 30 °C on selective SD medium lacking leucine, tryptophan and histidine, supplemented with 5 mM 3-AT.

Additional experiments indicated that conditional expression of the At1g50570 P protein from the *Met25* promoter (encoded in pBD-/At1g50570 P) activates expression of the *HIS3* reporter gene by the interaction with TGA2.2 expressed from the same promoter (encoded in pLEU/Met25::TGA2.2) in the YRWH2 strain (data not shown). In conclusion, the At1g50570 and At5g55530 proteins can act as transcription activators in yeast.

6.4.5 At1g50570 Interacts with TGA2.2 *In Vitro*

To corroborate the direct physical interactions between TGA2.2 and At1g50570 protein, GST-pull-down (Section 4.5.8), Far Western (Section 4.5.7) and EMSA (Section 4.5.12) *in vitro* experiments were conducted. For this purpose, the *At1g50570* full-length coding sequence was subcloned into the pGEX4T plasmid downstream of the *GST* cDNA frame. The *TGA2.2* coding sequence was subcloned into the pET28a plasmid downstream of a *6x His tag* coding sequence. The 6x His-TGA2.2 and GST-

At1g50570 proteins were expressed in *E. coli* and purified as described in methods (Section 4.5.3). Purified proteins were analyzed by SDS-PAGE (Figure 6.12)

Figure 6.12. SDS-PAGE analysis of purified 6x His-TGA2.2 and GST-At1g50570 proteins.

10 µl of purified GST, GST-At1g50570 and 6x His-TGA2.2 proteins were separated on the 12% gel by SDS-PAGE and subjected to Coomassie Blue staining. Arrows indicates purified proteins positions.

6.4.5.1 GST Pull-down Analysis of At1g50570 and TGA2.2 Interaction

For the GST pull-down assay, 1 ml of *E. coli* lysate expressing the 6x His-TGA2.2 was mixed with glutathione-sepharose beads loaded with GST or GST-At1g50570 and the mixtures were then collected by centrifugation. The resulting complexes were analyzed by immunoblotting using antibodies against the N-terminus of TGA2.2. Immunoblot analysis showed that the TGA2.2 protein copurified with At1g50570 (Figure 6.13, lane 2), demonstrating that At1g50570 physically associates with TGA2.2 *in vitro*.

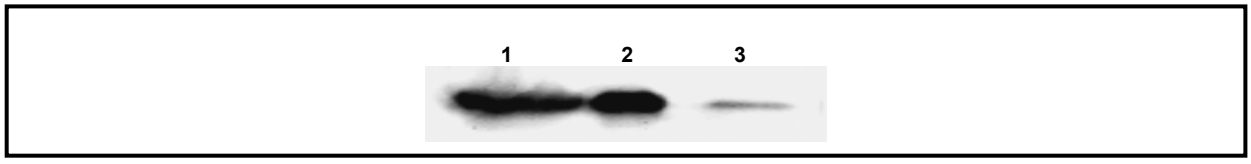


Figure 6.13. Pull-down assay of GST-At1g50570 binding to 6x His-TGA2.2 protein.

E. coli lysate expressing 6x His-TGA2.2 was incubated with glutathione-sepharose beads loaded with GST-At1g50570 or GST for 1 h at room temperature. The beads were collected by centrifugation and washed 3 times in 1x PBS before final collection. 10 µl of TGA2.2 cleared lysate (1), GST-pull down At1g50570 (2) and GST (3) Sepharose bound proteins were run on a 12% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and then immunoblotted with α TGA2.2 antibody.

6.4.5.2 Far Western Analysis of At1g50570 and TGA2.2 Interaction

At1g50570 and TGA2.2 interaction was further analyzed by means of the Far Western technique. 1 µg recombinant GST-At1g50570 protein blotted on a nitrocellulose membrane was hybridized with an excess of purified 6x His-TGA2.2 (~10 µg) and the binding of 6x His-TGA2.2 was revealed with a specific TGA2.2 antibody in an immunoblot analysis. The 6x His-TGA2.2 protein interacts physically with the GST-At1g5070, whereas no band was detected with the GST protein (Figure 6.14).

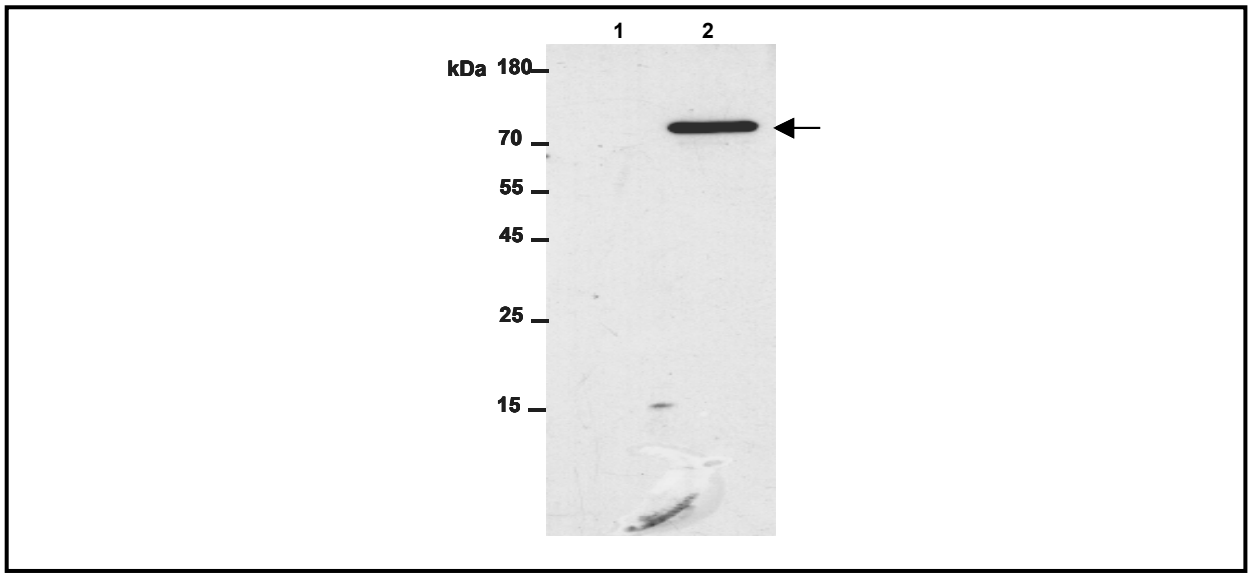


Figure 6.14. Far western analysis of 6X His-TGA2.2 protein binding to GST-At1g50570.

1 µg of GST (1) or GST-At1g50570 (2) were run on a 12% SDS-polyacrylamide gel and then transferred to a nitrocellulose membrane. The membrane was incubated with an excess of 6x His-TGA2.2 (~10 µg) and then immunoblotted with α TGA2.2 antibody.

6.4.5.3 At1g50570 Interacts with *As-1* Bound TGA2.2

To determine whether At1g50570 interacts with TGA2.2 at the *as-1* element, an EMSA was performed using an *in vitro* translated TGA2.2 (donated by G. Lyss), purified GST-At1g50570 and a mixture of *in vitro* translated TGA2.2 and GST-At1g50570 proteins. A super-shift in TGA2.2 mobility was detected in the presence of GST-At1g50570 protein (Figure 6.15, lane 3). This demonstrates that a complex between GST-At1g50570 and TGA2.2 can form at the *as-1* element. By contrast, GST-

At1g50570 alone did not bind the *as-1* element, indicating that the super-shifted TGA2.2 band is a result of TGA2.2-At1g50570 interaction (Figure 6.15, lane 4). To demonstrate that this mobility shift was due to the interaction of At1g50570 with TGA2.2 and not other nonspecific proteins in the preparation, a control reaction was carried out using GST protein. The GST protein alone did not alter the TGA2.2 mobility (data not shown).

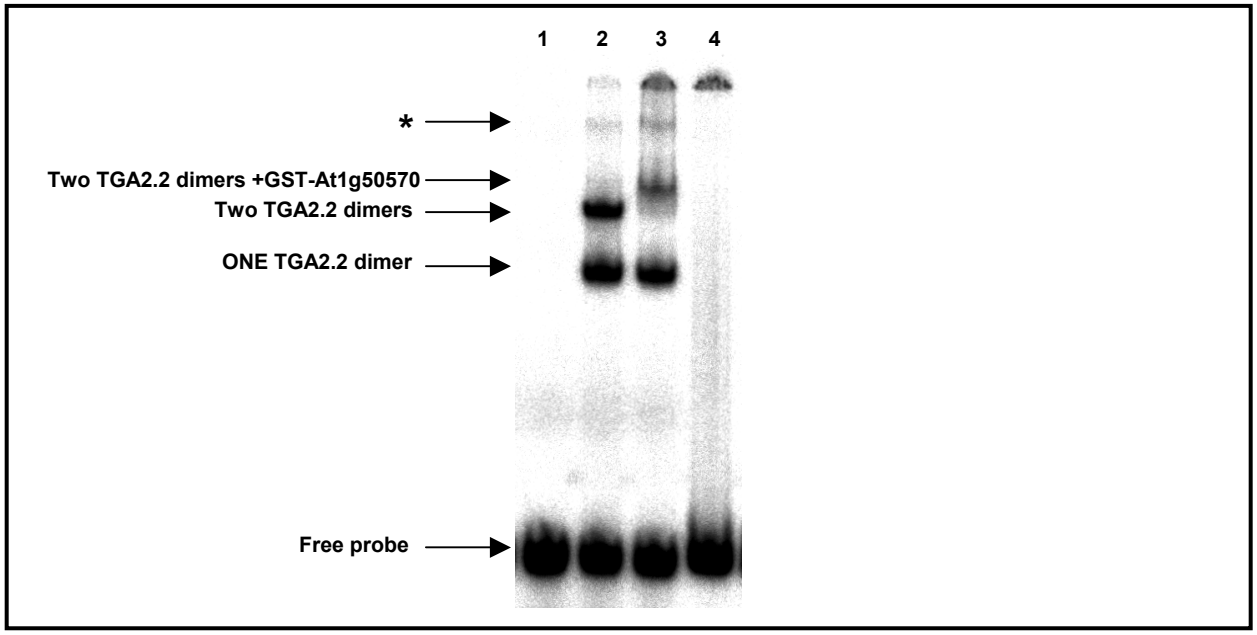


Figure 6.15. EMSA analysis of At1g50570-TGA2.2 interaction.

EMSA was done using *as-1* radioactive probes (lane 1), 2 µl of *in vitro* translated TGA2.2 protein alone (lane 2), a mixture of 2 µl of *in vitro* translated TGA2.2 with 2 µg of GST-At1g50570 protein (lane 3) or GST-At1g50570 alone (lane 4). The mixtures were run on a 5% native PAGE gel. The protein-DNA complexes were detected by autoradiography. Asterisk indicates non-specific DNA binding activity while arrows indicate specific band shifting.

6.4.6 Expression Analysis of *At1g50570* Gene

To examine the expression patterns of the *At1g50570* transcripts in Arabidopsis, RNA gel blot hybridization and RT-PCR experiments were performed. Total RNA samples isolated from roots, leaves, stems, flowers and green siliques were subjected to RT-PCR analysis to investigate the expression pattern of *At1g50570*. After RT-PCR, a specific 1170-bp fragment corresponding to *At1g50570* was detected in all tissues analyzed (Figure 6.16). The observed transcripts were consistent with the observation

that *Arabidopsis* has 20 *At1g50570* ESTs that are isolated from different organs. The expression level of *At1g50570* was similar in roots, leaves, stems, flowers and green siliques. The control *actin* gene was expressed at a similar level in all tissues analyzed (Figure 6.16). To ensure that the observed transcripts are not related to any DNA contaminations, a control *actin* gene PCR reaction was performed using Arabidopsis genomic DNA (an intron region of about 180 bp exists in the *actin* gene) and one of the RT-PCR products.

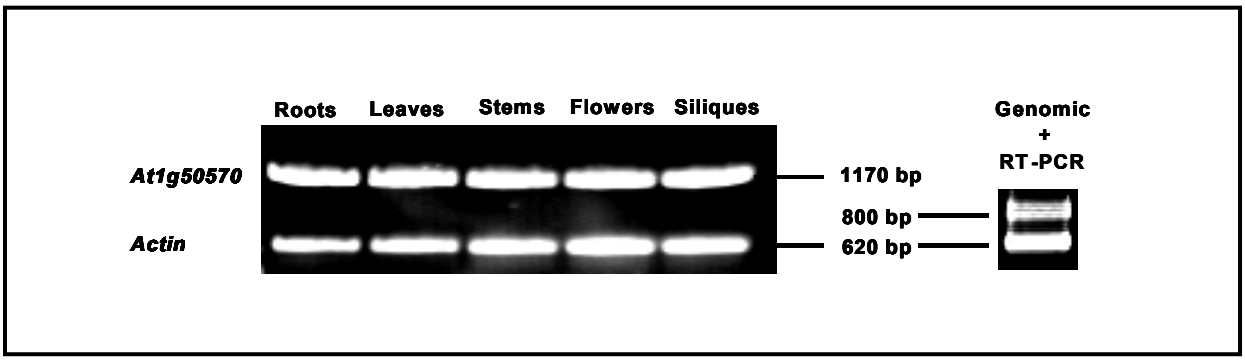


Figure 6.16. Detection of *At1g50570* mRNA in different Arabidopsis tissues by RT-PCR.

Total RNA isolated from six weeks old plants parts (roots, leaves, stems, flowers and siliques) were detected for *At1g50570* mRNA transcripts using RT-PCR. The *actin* control is shown at the bottom of the gel. A PCR control reaction for *actin* using genomic DNA and one of the RT-PCR products is shown on the left.

To characterize the level of accumulation of the *At1g50570* transcript in Arabidopsis leaves in response to SAR inducers, total RNA from whole plants sprayed with 1 mM SA or challenged with *P. syringae* pv. maculicola ES4326 with or without the *avrRpt2 R* gene at various time points were subjected to RNA gel blot analysis. There was no significant *At1g50570* mRNA accumulation within 2 or 24 hours after SA treatment, whereas a moderate accumulation of the *At1g50570* mRNA after 24 h of pathogen challenge was observed (Figure 6.17). In conclusion, the transcription of the *At1g50570* gene was not strongly activated by SAR inducers.

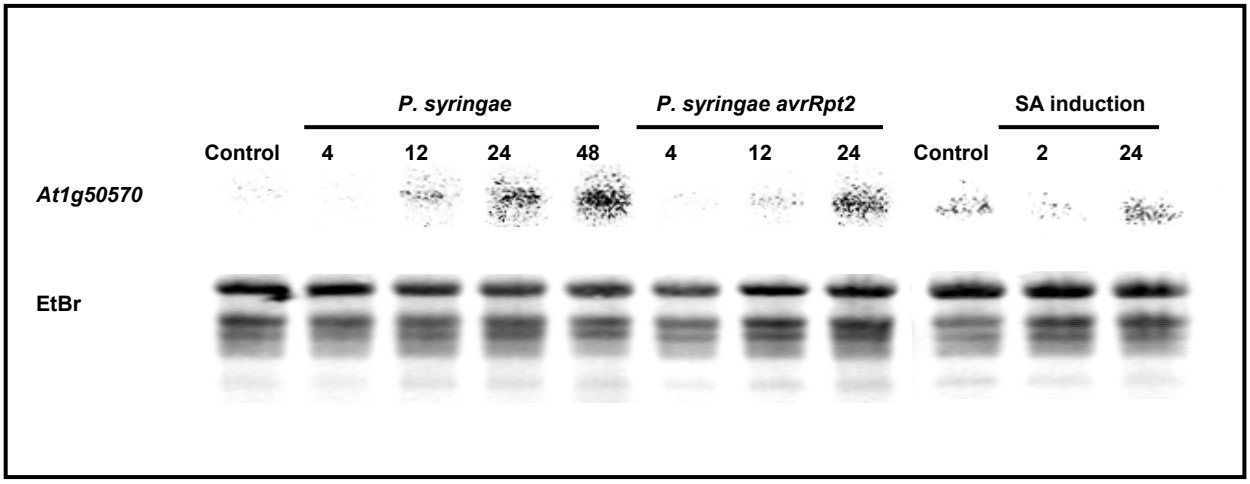


Figure 6.17. RNA gel blots analysis of *At1g50570* gene expression after SA or pathogen treatments.

Arabidopsis plants were grown for 4 weeks on soil before spraying with 1 mM SA or challenged with *P. syringae* pv. maculicola ES4326 with or without the *avrRpt2 R* gene. Total RNA was isolated from plants at different time points (in hours) and 10 µg were separated on denaturing gels in the presence of ethidium bromide (EtBr), photographed to assess equal loading, and examined by Northern blot analysis. A radioactive *At1g50570* full-length cDNA probe was hybridized to the membrane, which was then examined by autoradiography.

6.4.7 Subcellular Localization of At1g50570 Protein

To examine the subcellular localization of At1g50570, the *At1g50570* full-length coding sequence was inserted in-frame upstream of the *GFP* coding sequence in the HBTL/GFP plasmid (Figure 6.18A). The construct was expressed under the control of the *HBT* chimeric promoter in BY-2 protoplast. A control plasmid with only the GFP protein was included as a control. Protoplasts transfected with the *GFP* control construct showed green fluorescence throughout the entire cytoplasm and nucleus (Figure 6.18B). In contrast, the At1g50570-GFP fusion protein was localized mainly to the nuclear envelope and the endoplasmic reticulum (Figure 6.18B). Some residual At1g50570 protein signal in the nucleus appears to be due to the saturation of the nuclear envelope by the overexpressed protein.

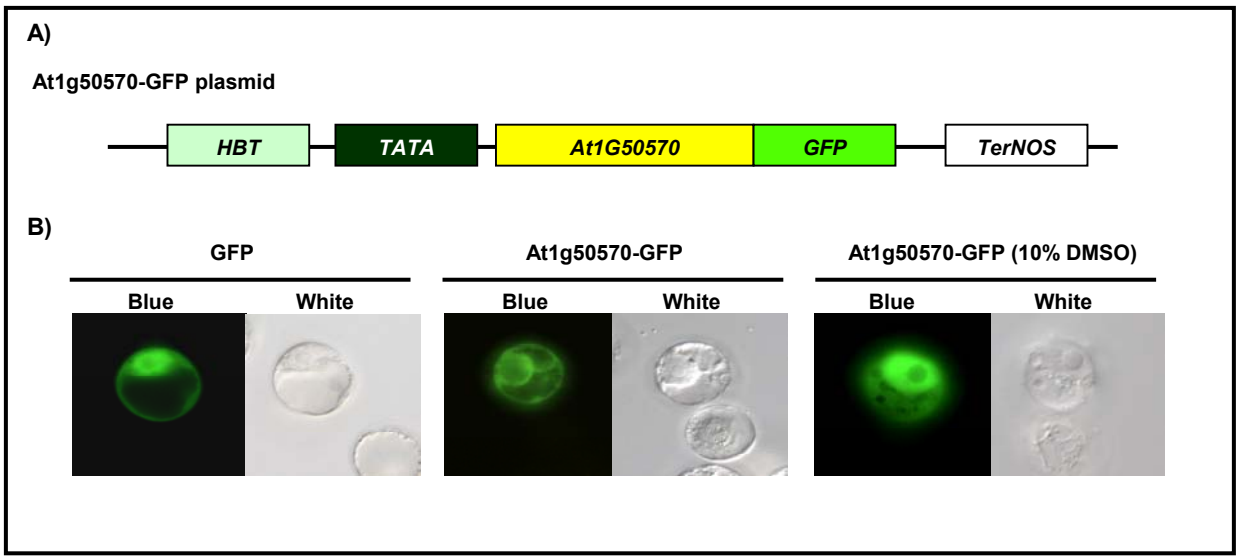


Figure 6.18. Subcellular localization of At1g50570-GFP protein.

A) Schematic diagram of HBTL/At1g50570-GFP plasimd used in subcellular localization experiments.

B) BY-2 protoplasts expressing GFP and At1g50570-GFP.

BY-2 protoplasts were transfected with the *GFP and At1g50570-GFP* constructs, and was visualized using a BX 51 fluorescent microscope using the blue and white light fields.

As described above, At1g50570 protein sequence contains a typical C2 domain. The C2 domain is responsible for translocation of proteins to the cell membrane compartments in a Ca^{2+}-dependent manner (Nalefski and Falke, 1996). The fact that At1g50570 is localized to the nuclear envelope and the endoplasmic reticulum hints that the C2 domain might mediate the At1g50570 targeting to those compartments. To examine if the C2 domain mediates the observed localization of At1g50570, the subcellular localization of At1g50570-GFP transfected BY-2 protoplasts was examined after treatments with 10 mM $CaCl_2$ (as a Ca^{2+} source), 5 mM EGTA (as a Ca^{2+} chelator). No alteration in the subcellular localization pattern of At1g50570 was observed after 10 mM $CaCl_2$ and 5 mM EGTA treatments (data not shown). In contrast, treatment of At1g50570-GFP transfected protoplast with 10% DMSO (known for altering membrane fluidity (Trubiani *et al.*, 2003)) prompted protein accumulation in the nucleus, however the protoplast membrane integrity was severely affected (Figure 6.18B).

6.4.8 Function of At1g50570 as Transcription Activators in Protoplasts

To determine whether the At1g50570 protein is capable of transactivating *as-1* element-dependent transcription in plant cells, transactivation experiments using protoplasts prepared from tobacco BY-2 cells were performed. The reporter plasmid consisted of a copy of the *as-1* element put upstream of a plant minimal *TATA*-containing promoter to control the *uidA* gene encoding the GUS enzyme (Figure 6.19A). To construct the effector plasmid, the full-length *At1g50570* coding sequence was put under the control of the *HBT* chimeric promoter (a *CaMV 35S* promoter derivative) (Figure 6.19A).

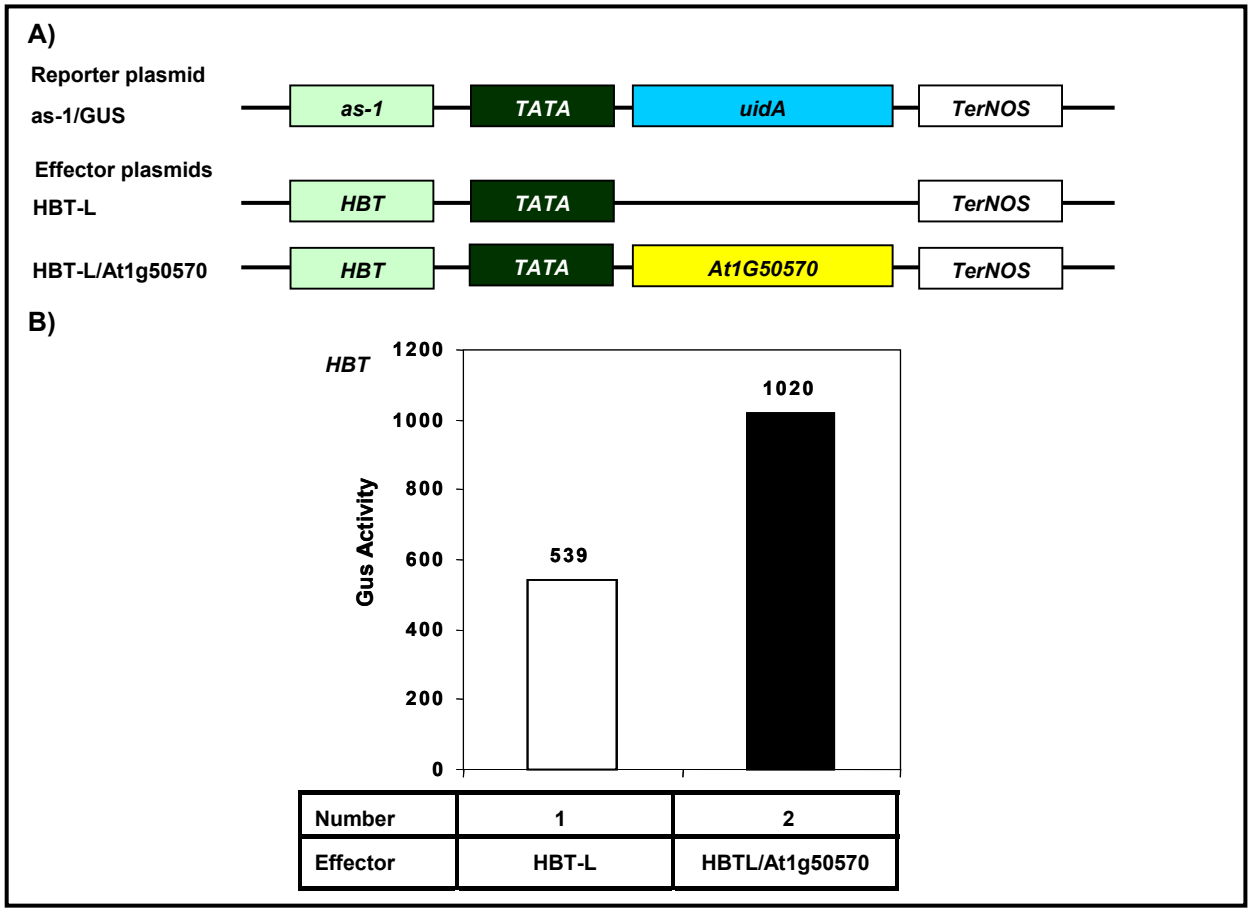


Figure 6.19. Transactivation assay of At1g50570 in BY-2 protoplasts.

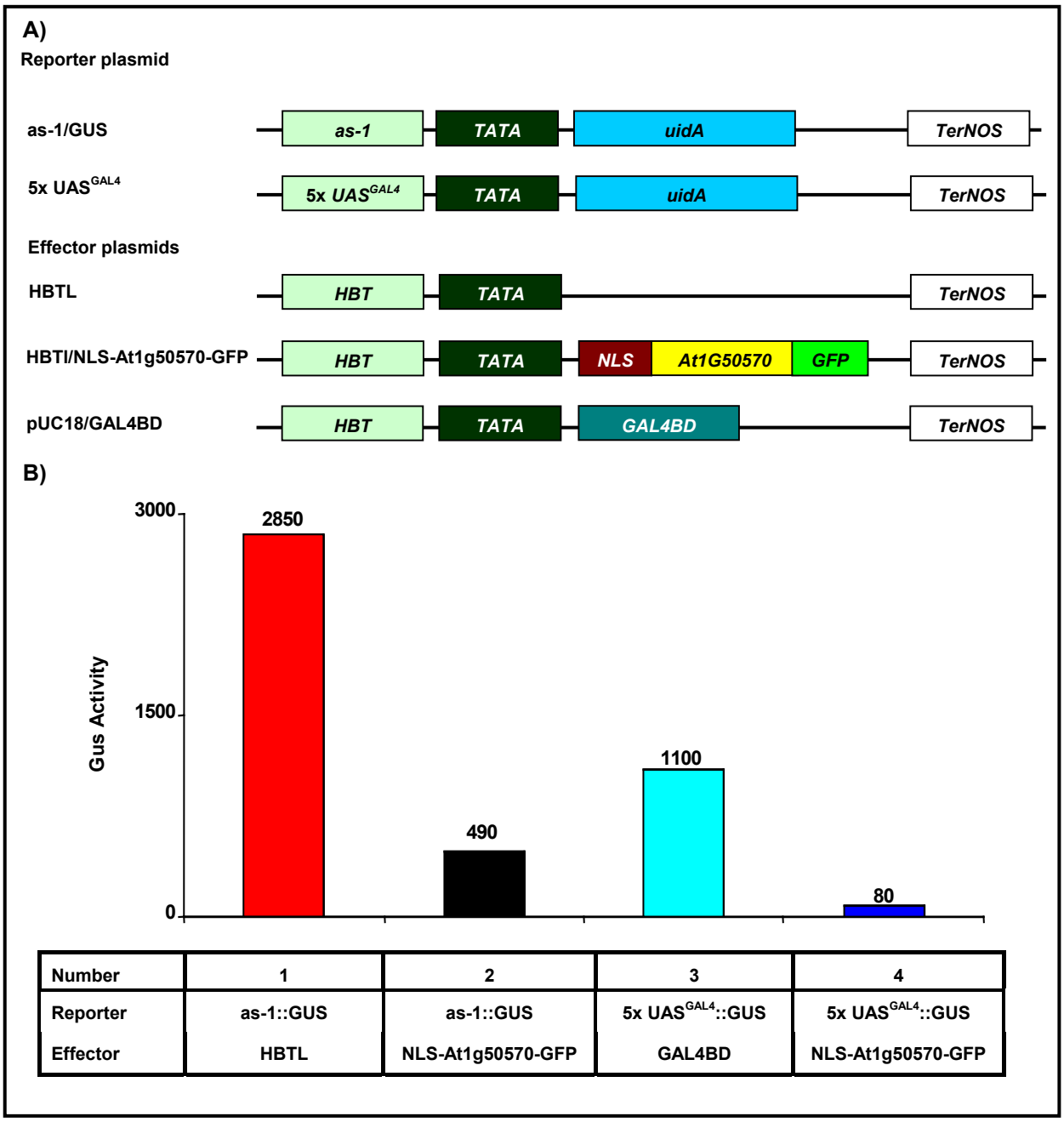
A) Schematic diagram of the effector and reporter plasmids used in cotransfection experiments.

B) Transactivation of the *as-1::GUS* fusion reporter gene by At1g50570 protein.

The reporter gene was cotransfected with 10 µg of *as-1::GUS* reporter and 25 µg of *At1g50570* effector plasmid or the empty vector as control treatment. GUS Activity was estimated as described in methods.

The effector plasmid (termed HBTL/At1g50570) and a control effector empty plasmid (HBTL) were cotransfected separately with the reporter plasmid (termed as-1::GUS) into tobacco BY-2 protoplasts by electroporation. GUS activities from three independent transfections were measured. At1g50570 protein expressed in protoplasts could activate the *uidA* reporter gene to about 2-fold (Figure 6.19B, no. 1 vs. 2). These results would suggest that At1g50570 protein might function as a transcriptional activator that is involved in the *as-1* element-dependent responsive gene expression. It is possible that the low nuclear localized At1g50570 levels were responsible for the moderate transactivation activity observed (Figure 6.18B).

To further demonstrate if the At1g50570 protein nuclear localization will lead to transactivate *as-1* element-dependent transcription in protoplasts, transactivation experiment was conducted using an At1g50570 protein fused to a NLS (found to be exclusively targeted to nucleus; data not shown). The effector plasmid HBTL/At1g28480 was constructed by inserting the *At1g508570* full-length coding sequence in-frame with a *NLS* sequence of the SV40 large antigen downstream of the *HBT* chimeric promoter and upstream of *GFP* coding sequence (Figure 6.20A). The NLS-At1g50570 protein repressed the *as-1::GUS* reporter transactivity when compared to the HBTL plasmid (Figure 6.20B). These results indicate that the NLS-At1g28480 expression might have a toxic effect on the BY-2 protoplasts. The general toxic effect on BY-2 protoplasts was verified using a *5x UAS^{GAL4}::uidA* reporter gene as a control.



Number	1	2	3	4
Reporter	as-1::GUS	as-1::GUS	5x UAS^{GAL4}::GUS	5x UAS^{GAL4}::GUS
Effector	HBTL	NLS-At1g50570-GFP	GAL4BD	NLS-At1g50570-GFP

Figure 6.20. Transactivation assay of NLS-At1g50570-GFP in BY-2 protoplasts.

A) Schematic diagram of the effector and reporter plasmids used in cotransfection experiments.

B) Transactivation of the reporter genes by NLS-At1g50570-GFP protein.

The reporter gene was cotransfected with 10 µg of *as-1::GUS* or *5x UAS^{GAL4}::GUS* reporter genes and 25 µg of *NLS-At1g50570-GFP* effector plasmid or the empty vector as control treatment. GUS Activity was estimated as described in methods.

6.4.9 Analysis of the *In Vivo* Roles of At1g50570 by Generation of At1g50570 Antisense Lines

To investigate the role of At1g50570 in plants, the full-length coding sequence of *At1g50570* was cloned into pFGC5941 vector in the sense and antisense orientations (Figure 6.21A). The pFGC5941/At1g50570 vector contains a *CaMV 35S* promoter that drives the transcription of a sense and an antisense *At1g50570* sequences that are separated by an intron designed so that hairpin RNA structures are formed (Wesley *et al.*, 2001). These double-stranded RNAs are degraded and lead to the production of 21- to 23-bp short interfering RNA (siRNA) sequences that will lead to the degradation of the endogenous *At1g50570* transcript. The pFGC5941/At1g50570 plasmid was transformed into the Arabidopsis -90-GUS transgenic plants (Redman *et al.*, 2002), which contain a copy of the *as-1* element upstream of the *uidA* gene encoding the GUS enzyme using flower dip method. The transformed plants were selected by BASTA herbicide spraying.

The *At1g50570* RNA levels in T2 segregating plants of Basta resistant lines were analyzed using RNA gel blot analysis. In wild-type plants, *At1g50570* was expressed constitutively but at a low level, and its transcript level increased slightly after bacterial inoculation (Figure 6.17). Among ten antisense lines tested, the *At1g50570* transcripts levels varied compared to control plants. For instance, lines #1, #7, #8, #9 and #10 showed normal accumulation of the *At1g50570* transcripts, while elevated RNA levels were observed in lines #2, #5 and #6 (Figure 6.21B). Lines #3 and #4 had diminished *At1g50570* gene expression as compared with the other plants (Figure 6.21B). Based on those observations, the At1g50570 antisense line #4 was selected for further analysis.

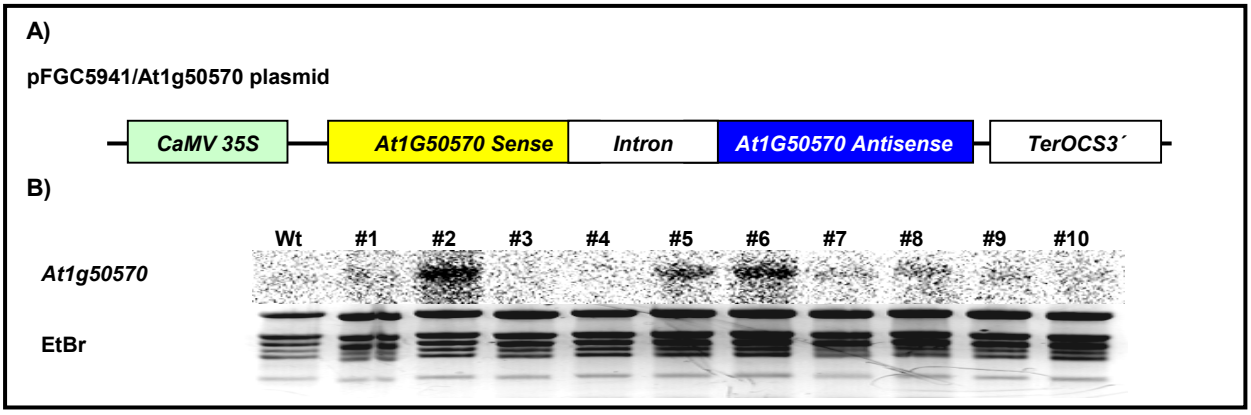


Figure 6.21. Analysis of *At1g50570* gene expression in different At1g50570 antisense lines.

A) Schematic diagram of pFGC5941/At1g50570 antisense plasmid used in the generation of antisense plants.

B) RNA gel blots analysis of *At1g50570* gene expression in different At1g50570 antisense lines.

Plants were grown for 4 weeks on soil and total RNA was isolated from Arabidopsis -90-GUS plants (control) and Arabidopsis At1g50570 antisense lines. 7.5 µg were separated on denaturing gels in the presence of ethidium bromide (EtBr), photographed to assess equal loading, and examined by Northern blot analysis. A radioactive *At1g50570* full-length cDNA probe was hybridized to the membrane, which was then examined by autoradiography.

The effect of the *At1g50570* repression on the expression of *PR-1* gene in response to SA induction was examined. The SA-inducible expression of *PR-1* gene was not affected in At1g50570 antisense line #4 as compared to the wild type plant (Figure 6.22). These results indicated that the *At1g50570* expression repression did not cause any significant effect on *PR-1* gene expression. Similar results were obtained when antisense line #4 was challenged with *P. syringae* pathogen (data not shown).

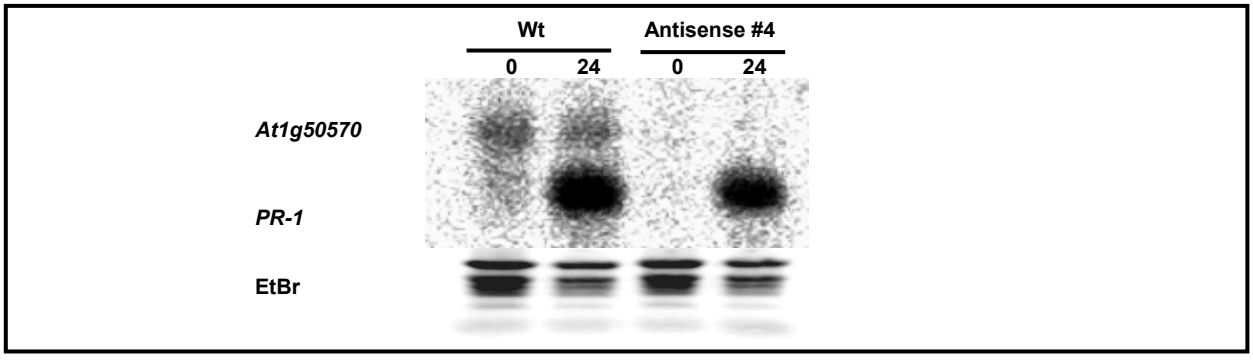


Figure 6.22. RNA gel blots analysis of *PR-1* gene expression in At1g50570 antisense line #4.

Plants were grown for 4 weeks on soil before spraying with 1 mM SA. Total RNA was isolated from plants at different time points (in hours) and 10 µg were separated on denaturing gels in the presence of ethidium bromide (EtBr), photographed to assess equal loading, and examined by Northern blot analysis. A radioactive *At1g50570* full-length cDNA and *PR-1* cDNA probes were hybridized to the membrane, which was then examined by autoradiography.

6.4.10 Analysis of the *In Vivo* Roles of At1g50570 by Generation of At1g50570 Overexpression Lines

To elucidate the putative function of At1g50570, Arabidopsis overexpression lines were generated. Arabidopsis -90-GUS transgenic plants were transformed with a *3x HA tag* fused in-frame to *At1g50570* transgene, which is under the control of the strong *2x CaMV 35*S promoter (Figure 6.23A). The Alligator2/At1g50570 binary vector, harboring the *At1g50570* overexpression cassette, contains a *GFP* selection marker gene driven by the seed storage protein *At2S3* promoter. The *GFP* selection marker gene allows the selection of At1g50570 overexpression transgenic lines based on their seeds fluorescence level under blue light illumination (Figure 6.23B). The presence of 3x HA tag allows protein detection in protein blot analysis using a HA antibody.

T2 plants from seven At1g50570 overexpression lines, showing GFP fluorescence, were examined for the expression of *At1g50570* transcripts by using RNA and protein gel blot analysis. RNA gel blot analysis showed that four lines (#1, #2, #3 and #7) exhibited substantially elevated levels of *At1g50570* transcripts (Figure 6.23C). Similarly, protein gel blot and immunoprecipitation analysis confirmed that lines #1, #2 and #3 had significantly increased 3x HA-At1g50570 protein levels compared with the wild type plants (Figure 6.23D and E). Based on the previous analysis, the At1g50570 overexpressor line #3 transgenic plant was selected for further analysis.

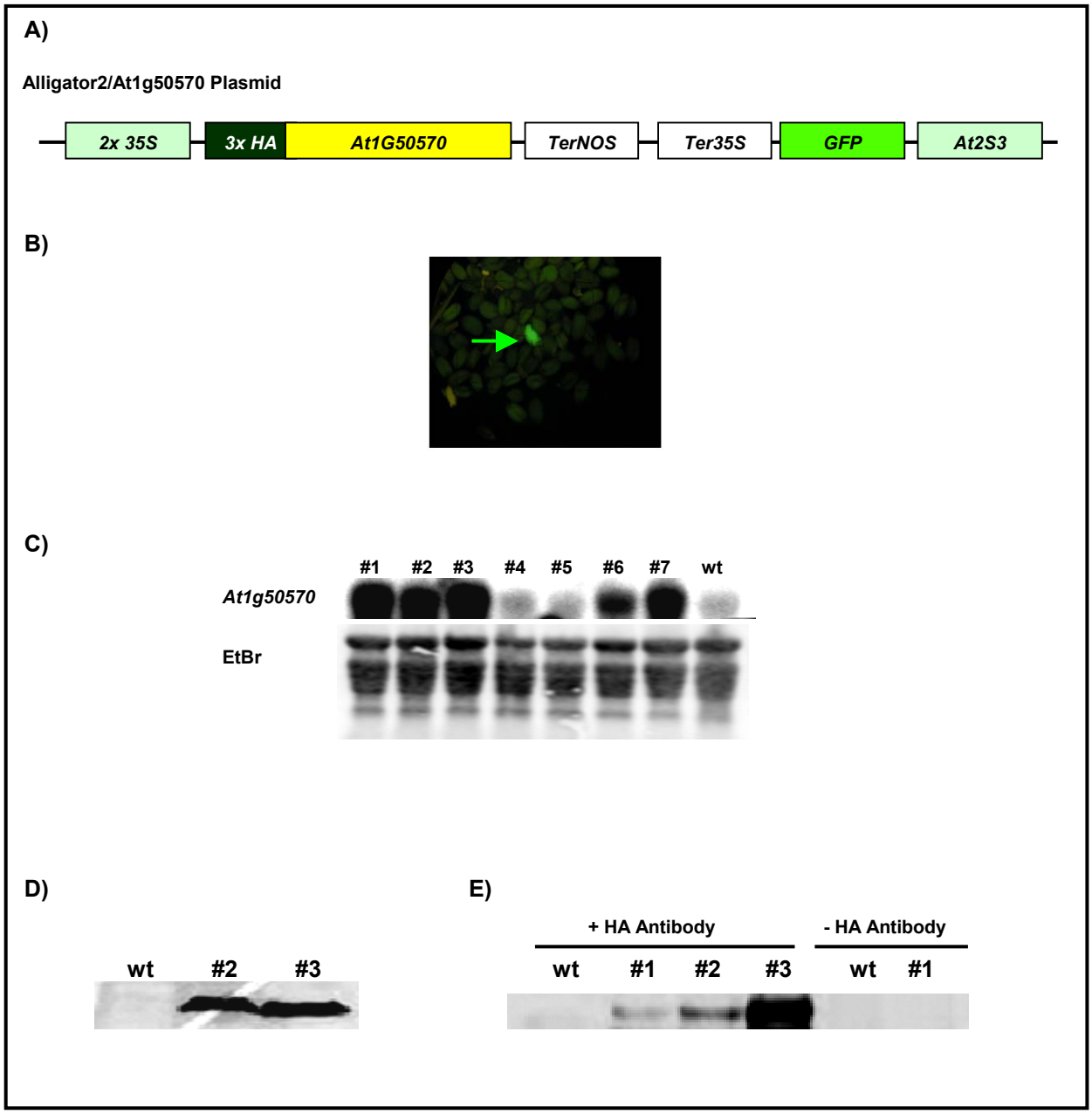


Figure 6.23. Analysis of At1g50570 overexpression lines.

A) Schematic diagram of Alligator2/At1g50570 plasmid used in the generation of At1g50570 overexpression plants.

B) Selection of -90-GUS Alligator2/At1g50570 transgenic seeds based on GFP florescence.

C) RNA gel blots analysis of *At1g50570* gene expression in different At1g50570 overexpression lines

Total RNA was isolated from -90-GUS plants (control) and -90-GUS Alligator2/At1g50570 transgenic lines. 10 µg were separated on denaturing gels in the presence of ethidium bromide (EtBr), photographed to assess equal loading, and examined by Northern blot analysis. A radioactive *At1g50570* full-length cDNA probe was hybridized to the membrane, which was examined by autoradiography.

D) Protein gel blot analysis of 3x HA-At1g50570 protein expression levels in At1g50570 overexpression lines #2 and #3.

E) Immunoprecipitation analysis for 3x HA-At1g50570 protein detection in At1g50570 overexpression lines #1, #2 and #3.

To determine the contribution of *Atg50570* overexpression to SAR, the expression of *PR-1* gene in response to SA induction was examined. *At1g50570* overexpression plant did not exhibit any altered *PR-1* expression in comparison to wild type plant (Figure 6.24). Similar results were observed after *P. syringae* virulent strain infection (data not shown). These results indicated that the *At1g50570* overexpression did not have any significant effect on *PR-1* gene expression.

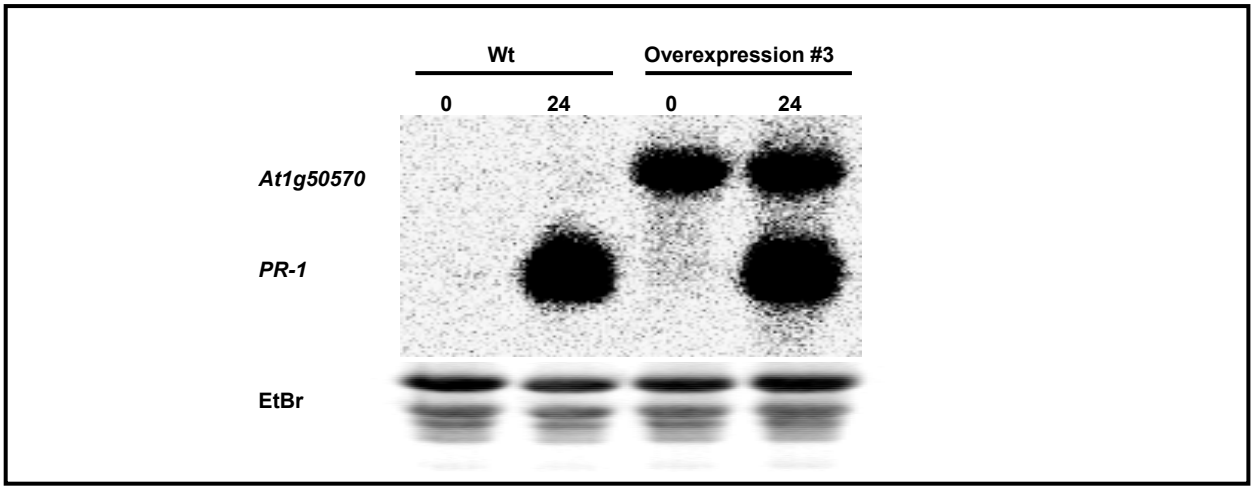


Figure 6.24. RNA gel blots analysis of *PR-1* gene expression in At1g50570 overexpression line #3.
Plants were grown for 4 weeks on soil before spraying with 1 mM SA. Total RNA was isolated from plants at different time points (in hours) and 7.5 µg were separated on denaturing gels in the presence of ethidium bromide (EtBr), photographed to assess equal loading, and examined by Northern blot analysis. A radioactive *At1g50570* full-length cDNA and *PR-1* cDNA probes were hybridized to the membrane, which was examined by autoradiography.

To examine the influence of the *At1g50570* overexpression on the *GUS* reporter gene activity, a histochemical staining assay was performed. Two weeks soil grown -90-GUS and At1g50570 overexpression line #3 plants were treated with 1 mM SA or 0.1 mM 2,4-D. The *as-1*-dependent reporter gene expression was activated after SA and 2,4-D treatments in both transgenic lines (Figure 6.25). However, no constitutive GUS activity was detected in At1g50570 overexpression line #3. In conclusion, it is clearly demonstrated that the *At1g28480* overexpression or repression did not lead to any altered gene expression related to SAR or TGA transcription factors.

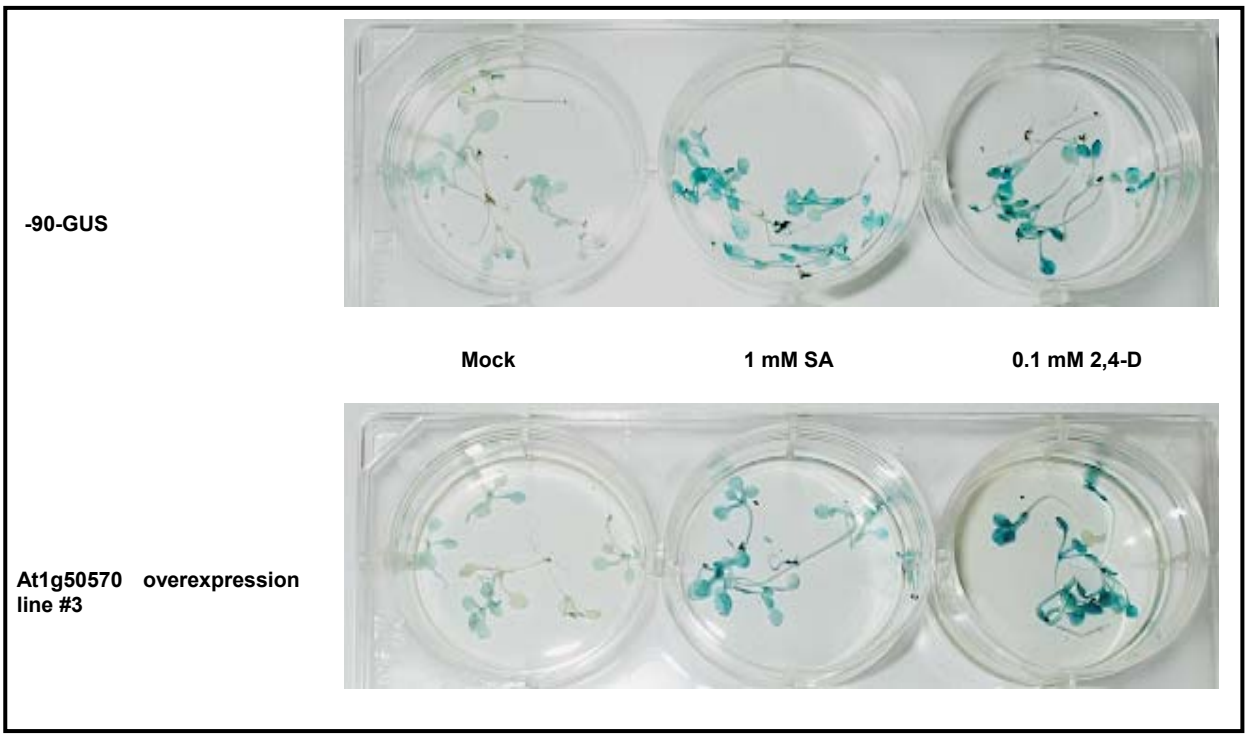


Figure 6.25. Histochemical staining of GUS activities in At1g50570 overexpression line #3.

Two weeks soil grown -90-GUS transgenic and At1g50570 overexpression line #3 plants were treated with mock, 1 mM SA or 0.1 mM 2,4-D for 24 hours and then stained for GUS.

6.5 Functional Analysis of *At1g28480* Isolated cDNA Clone

6.5.1 Structural Analysis of *At1g28480* Gene

Among the four groups isolated in the MY1HS screens, clone 5 was the most abundant cDNA insert (Table 6.1, group four). It was isolated twenty-two times using the Arabidopsis cDNA library as a prey. DNA sequence analysis revealed that clone 5 contains a full cDNA coding sequence was fused in-frame to the GAL4AD, a translation stop codon, a 3´-untranslated region and a poly (A) tail of adenosine residues. A BLAST search of the Arabidopsis databases showed sequence identity with the *At1g28480* gene that contains an open reading frame of 137 amino acids encodes a putative glutaredoxin protein with a predicted molecular mass of 14.75 kDa (Figure 6.26). The complete sequence of *At1g28480* gene was found on the F3M18 BAC clone (GenBank Accession no.: AC010155).

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                attaccaaaattgttgtatatagtctggtgattccgttttgatggtcgagatcgaaagaagcaagcatccactatgtggtcgtcacatgcgaatatttctaagtatacaaccatct
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gtgacgcacatccttacgtaaccatcgttgacgctagactttcctctctgatctctctttcttcatgtatatataacaaaaccttcctttcctaattggtatctatctttaaaaacatacttgaaaggt

M  Q  G  T  I  S  C  A  R  N  Y  N  M  T  T  T  V  G  E  S  L  R  P  L  S  L  K  T  Q  G  N  G
ATGCAAGGAACGATTTCTTGTGCAAGAAATTATAACATGACGACAACCGTCGGGGAATCTCTGCGGCCGCTATCGCTTAAAACGCAGGGAAACGGC

E  R  V  R  M  V  V  E  E  N  A  V  I  V  I  G  R  R  G  C  C  M  C  H  V  V  R  R  L  L  L  G
GAGAGAGTTCGGATGGTGGTGGAGGAGAACGCGGTGATTGTGATTGGACGGAGAGGATGTTGCATGTGTCATGTGGTGAGGAGGCTGCTTCTTGGA

L  G  V  N  P  A  V  L  E  I  D  E  E  R  E  D  E  V  L  S  E  L  E  N  I  G  V  Q  G  G  G  G
CTTGGAGTGAATCCGGCGGTCCTTGAGATTGATGAGGAGAGGGAAGATGAAGTTTTGAGTGAGTTGGAGAATATTGGAGTTCAAGGCGGCGGAGGT

T  V  K  L  P  A  V  Y  V  G  G  R  L  F  G  G  L  D  R  V  M  A  T  H  I  S  G  E  L  V  P  I
ACGGTGAAGTTACCGGCGGTTTATGTAGGAGGGAGGTTGTTTGGAGGGTTAGATAGGGTTATGGCTACTCATATCTCCGGTGAGTTAGTTCCAATT

L  K  E  V  G  A  L  W  L  *
CTTAAGGAAGTTGGGGCTCTGTGGTTGTGAttgtaaattaataatttaaaattatttttttttctttttaattaagaatcttgattggtaattgttgtttacggtttataattgaatcgt
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ttatcaacctttgtgaaacggaaagaatttattactgtgttagactgttaggtgatagtgatttaaatgtgaccttaatgctcttgtttggggtacgtggtagtttatcagatttgaatattcacaaa
tgccaaaatatgagagatcctttа
```

Figure 6.26. Full-length sequence of *At1g28480* cDNA.

The red background represents the redox active center, the yellow background represents a conserved hydrophobic surface, and the blue background points for a putative glutathione binding site.

Glutaredoxins, also known as thioltransferases, are ubiquitous proteins that catalyze reductions of disulfides (protein-S-S) or mixed disulfides formed between proteins and glutathione (protein-S-SG) in a coupled system with glutathione, NADPH, and glutathione reductase. The major function of glutaredoxins is glutathione-dependent hydrogen donor for ribonucleotide reductase enzyme (Figure 6.27; Holmgren and Åslund, 1995). Glutaredoxin protein sequence contains three characteristic regions: the dithiol/disulfide active site or redox active center with the sequence YCPYC, a glutathione binding site, and a hydrophobic surface area (Figure 6.26; Lundberg *et al.*, 2001).

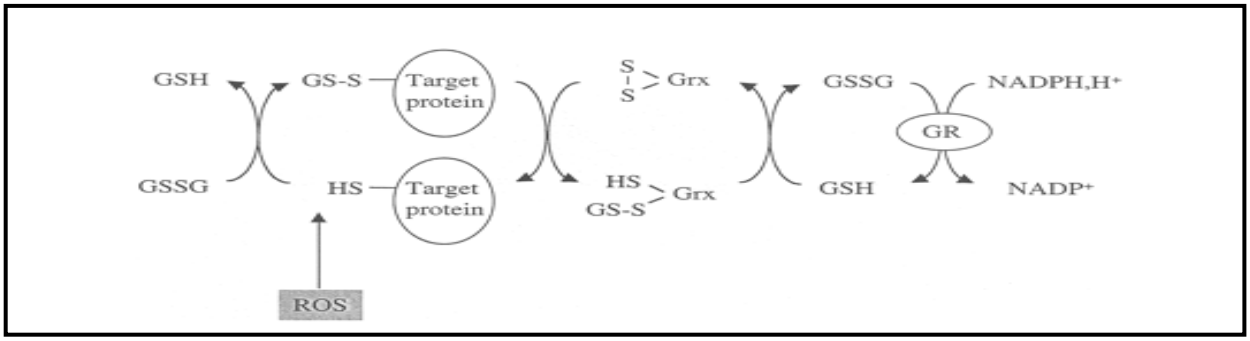


Figure 6.27. Glutaredoxins redox reactions (Lundberg *et al.*, 2001).

Glutathione (GSH or GSSG)-glutaredoxin (Grx) redox reactions of a target protein; ROS: reactive oxygen species.

The amino-acid sequences of the 24 Arabidopsis glutaredoxins proteins were aligned and a neighbor-joining phylogenetic tree was generated from the protein alignment using the Vector NTi software (Figure 6.28). The At1g28480 protein clustered with the monocysteinic At1g03850 glutaredoxin. Arabidopsis plant has at least 24 genes encoding proteins with homology to glutaredoxin, however, little is known about their function in plant (Meyer *et al.*, 1999). Only two Arabidopsis glutaredoxins have the YCPYC redox active site conserved motif, while the majority of Arabidopsis glutaredoxins are highly variable in their redox active center sequence (Rouhier *et al.*, 2002a).

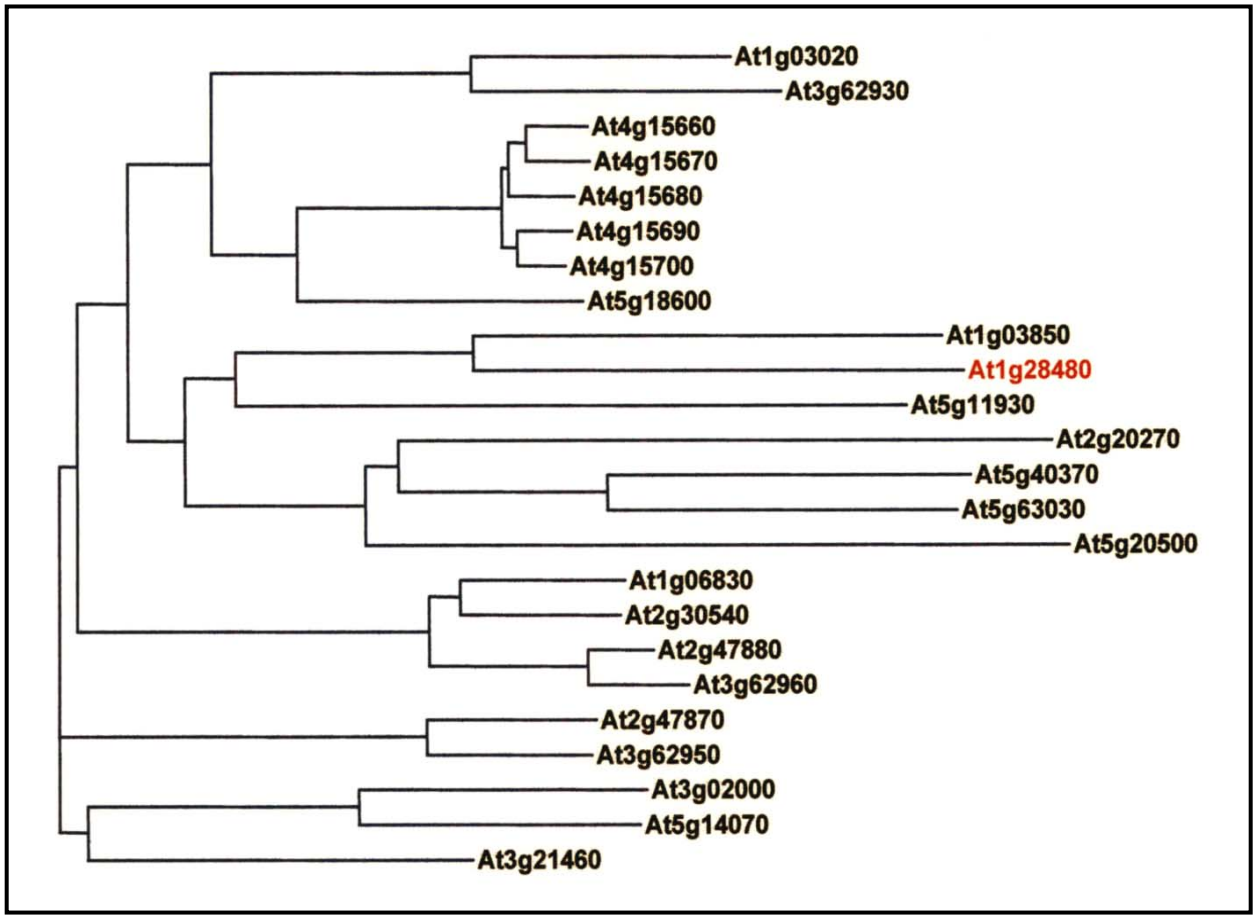
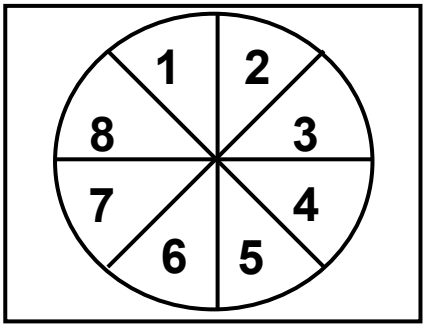


Figure 6.28. Phylogeny of the Arabidopsis glutaredoxin family. At1g28480 is shown in red.

6.5.2 TGA2.2 Interacts with At1g28480 in Y2HS

To verify that Arabidopsis At1g28480 protein interacts with TGA2.2 and to test whether it can interact with Arabidopsis homologs, a classical Y2HS assay was conducted. The *GAL4DB-TGA2.2*, *GAL4BD-TGA2* and *GAL4AD-TGA6* constructs were cotransformed with the pGAD10/At1g28480 into the HF7c strain and the transformants were assayed for histidine prototrophy in the presence of 5 mM 3-AT. In consistence with the MY1HS results, the TGA2.2, TGA2 and TGA6 proteins interacted with At1g28480 in the Y2HS assay (Figure 6.29, sector 3, 5 and 7). These results were further confirmed using a domain swap experiment, in which GAL4BD-At1g28480 the GAL4AD-TGA2.2 transformants were used in similar assays (data not shown).



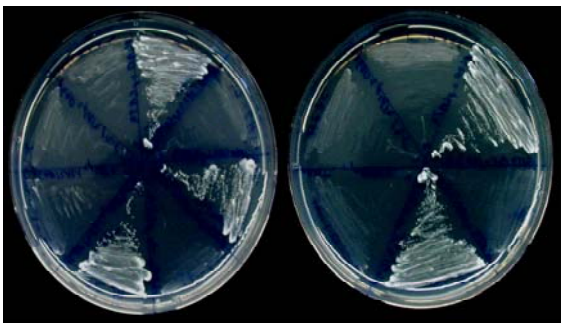
Sector	Bait (*TRP1* marker)	Prey (*LEU2* marker)	Growth on SD lacking histidine
1	GAL4BD-TGA2.2	GAL4AD-NPR1	++
2	GAL4BD	GAL4AD-At1g28480	-
3	GAL4BD-TGA2.2	GAL4AD-At1g28480	++
4	GAL4BD-TGA2.2	GAL4AD	-
5	GAL4BD-TGA2	GAL4AD-At1g28480	++
6	GAL4BD-TGA2	GAL4AD	-
7	GAL4BD-TGA6	GAL4AD-At1g28480	++
8	GAL4BD-TGA6	GAL4AD	-

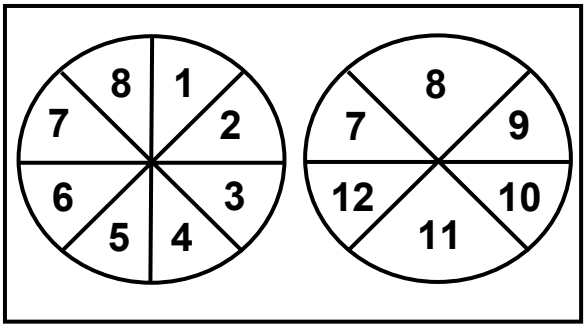
Figure 6.29. Y2HS assays of interactions between At1g28480 and TGA proteins.

HF7c cells containing pGBT9/TGA2.2 + pGAD424/NPR1 (1), pGBT9 + pGAD424/At1g28480 (2), pGBT9/TGA2.2 + pGAD424/At1g28480 (3), pGBT9/TGA2.2 + pGAD424 (4), pGBT9/TGA2 + pGAD424/At1g28480 (5), pGBT9/TGA2 + pGAD424 (6), pGBT9/TGA6 + pGAD424/At1g28480 (7) and pGBT9/TGA6 + pGAD424 (8) plasmids were grown for 3 days at 30 °C on selective SD medium lacking leucine, tryptophan and histidine, supplemented with 5 mM 3-AT.

6.5.3 At1g28480 Interacts with All Identified Members of Tobacco TGA Factors

The interaction of At1g50570 with other TGA factors from tobacco was analyzed using the Y2HS in the HF7c strain. The bait construct containing the *GAL4BD-At1g28480* coding sequence was tested for interactions with other TGA factors from tobacco. The GAL4BD-At1g28480 protein was not able to transactivate the *HIS3* reporter gene (Figure 6.30, sector 7). At1g28480 interacts strongly with other identified tobacco TGA factors (Figure 6.30, sectors 3, 5, 9 and 11).





Sector	Bait (*TRP1* marker)	Prey (*LEU2* marker)	Growth on SD lacking histidine
1	GAL4BD-TGA2.2	GAL4AD-NPR1	++
2	GAL4BD	GAL4AD-NPR1	-
3	GAL4BD-At1g28480	GAL4AD-TGA2.2	++
4	GAL4BD	GAL4AD-TGA2.2	-
5	GAL4BD-At1g28480	GAL4AD-TGA2.1	++
6	GAL4BD	GAL4AD-TGA2.1	-
7	GAL4BD-At1g28480	GAL4AD	-
8	GAL4BD	GAL4AD	-
9	GAL4BD-At1g28480	GAL4AD-TGA1a	++
10	GAL4BD	GAL4AD-TGA1a	-
11	GAL4BD-At1g28480	GAL4AD-TGA10	++
12	GAL4BD	GAL4AD-TGA10	-

Figure 6.30. Y2HS assays of interactions between At1g28480 and TGA proteins.

HF7c cells containing pGBT9/TGA2.2 + pGAD424/NPR1 (1), pGBT9 + pGAD424/NPR1 (2), pGBT9/At1g28480 + pGAD424/TGA2.2 (3), pGBT9 + pGAD424/TGA2.2 (4), pGBT9/At1g28480 + pGAD424/TGA2.1 (5), pGBT9 + pGAD424/TGA2.1 (6), pGBT9/At1g28480 + pGAD424 (7), pGBT9 + pGAD424 (8), pGBT9/At1g28480 + pGAD424/TGA1a (9), pGBT9 + pGAD424/TGA1a (10), pGBT9/At1g28480 + pGAD424/TGA10 (11) and pGBT9 + pGAD424/TGA10 (12) plasmids were grown for 3 days at 30 °C on selective SD medium lacking leucine, tryptophan and histidine, supplemented with 5 mM 3-AT.

To define the region in TGA2.2 that interacts directly with At1g28480, the C-terminal domain of TGA2.2 was coexpressed as a GAL4BD fusion with GAL4AD-At1g28480 in the HF7c strain and the transformants were assayed for the histidine prototrophy. The TGA2.2 encoded C-terminus is sufficient to confer interaction with At1g28480 (data not shown). To determine the specificity of the TGA2.2-At1g50570 interaction, Y2HS interaction analysis between TGA2.2 and two other glutaredoxins, At5g20500 and At5g40370, was conducted. Constructs containing the *GAL4BD-At5g*20500 or *GAL4BD-At5g40370* and *GAL4AD-TGA2.2* coding sequences were cotransformed into HF7c. The histidine prototrophy assay showed no interaction between TGA2.2 and the At5g20500 and At5g40370 glutaredoxins (data not shown). These results indicate that the interaction between At1g28480 glutaredoxin and TGA2.2 is indeed specific.

6.5.4 Generation of *At1g28480* and *TGA2.2* Mutants by Site-directed Mutagenesis

As described above, glutaredoxin catalyzes reductions of disulfides via its redox active site. To explore if the two-cysteine residues at active site (Cys^{52} and Cys^{55}) of At1g28480 plays an important role in the interaction with TGA2.2 site-directed mutagenesis techniques (Section 4.3.12.2) were used to create point mutations in one or both cysteine residues converting them to serine residues (C52S (GSM); C52S/C55S (GDM) (Figure 6.31A). Using this strategy, two glutaredoxin mutants, designated as GSM and GDM, were generated.

If TGA transcription factors undergo redox changes that are modulated by At1g28480 protein, the presence of cysteine residues in their sequences is crucial. Protein sequence analysis of TGA2.2 reveals the presence of a single conserved cysteine residue at the C-terminal part of the protein (Cys^{181}). This single conserved cysteine is also found in TGA2.1, TGA2, TGA5, TGA6 and TGA10 that contains other cysteines beside it. The TGA2.2 conserved cysteine residue was point mutated into serine using the site-directed mutagenesis strategy (Section 4.3.12.2). The new TGA2.2 mutant was designated as $TGA2.2^{Cys181Ser}$ (Figure 6.31B).

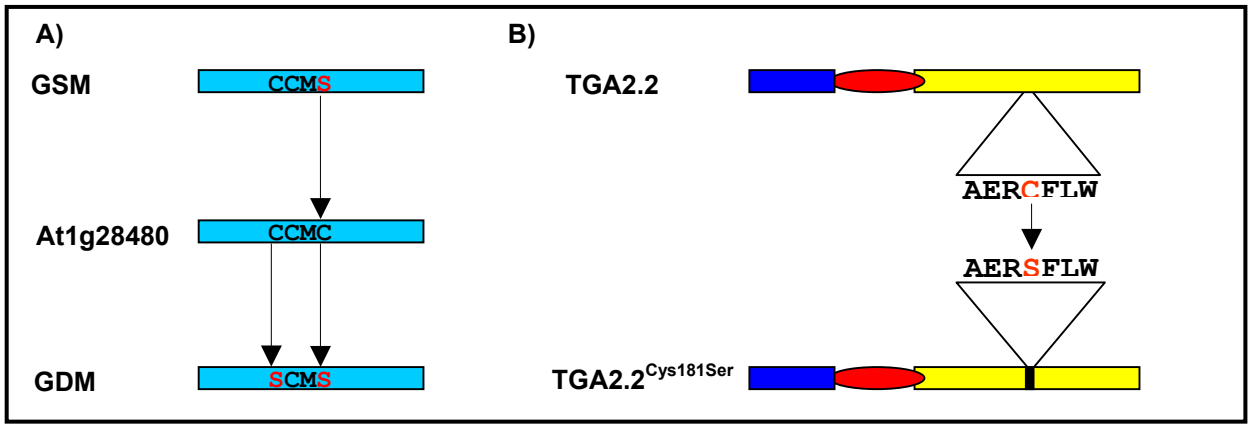


Figure 6.31. *TGA2.2* and *At1g28480* mutants used in this study.

6.5.5 Analysis of Disulfide Bridge Formation in TGA2.2

To determine whether the conserved cysteine in TGA2.2 is involved in the formation of intramolecular or intermolecular disulfide bridges, an immunoblot analysis was performed using the 6x His-TGA2.2 and 6x His-TGA2.2^{Cys181Ser} (encoded in pET28a/ TGA2.2^{Cys181Ser} plasmid) proteins under oxidizing or reducing conditions. The 6x His-TGA2.2^{Cys181Ser} protein was produced in *E. coli* and purified as described above. Before subjecting proteins to SDS-PAGE, both proteins were incubated with 1 mM of the oxidizing agent diamide for 15 minutes in order to oxidize them. Putatively oxidized proteins were reduced by incubation with an excess of the reducing agent ß-ME or left in its oxidized form. Both oxidized and reduced proteins forms were subjected to SDS-PAGE analysis followed by an immunoblot analysis using a TGA2.2 specific antibody.

SDS-PAGE analysis under reducing conditions of the purified 6x His-TGA2.2 and 6x His-TGA2.2^{Cys181Ser} indicates that both proteins migrate as a 55-kDa band (Figure 5.30). Under non-reducing conditions, oxidized TGA2.2 produces two bands, a 55-kDa band comprising the majority of the monomeric 6x His-TGA2.2, and an ~130 kDa band shifted to position of approximately triple the size of the 55-kDa band observed under reducing conditions, suggesting oligomer formation (Figure 6.32, arrow). Under the same oxidizing conditions, no oligomerization band was observed with TGA2.2^{Cys181Ser}, indicating that the TGA2.2 conserved cysteine is responsible for TGA2.2

oligomerization (Figure 6.32). Although the oligomerization band contains considerably lower amounts of TGA2.2, the results indicate that the TGA2.2 conserved cysteine is involved in the formation of intermolecular disulfide bonds.

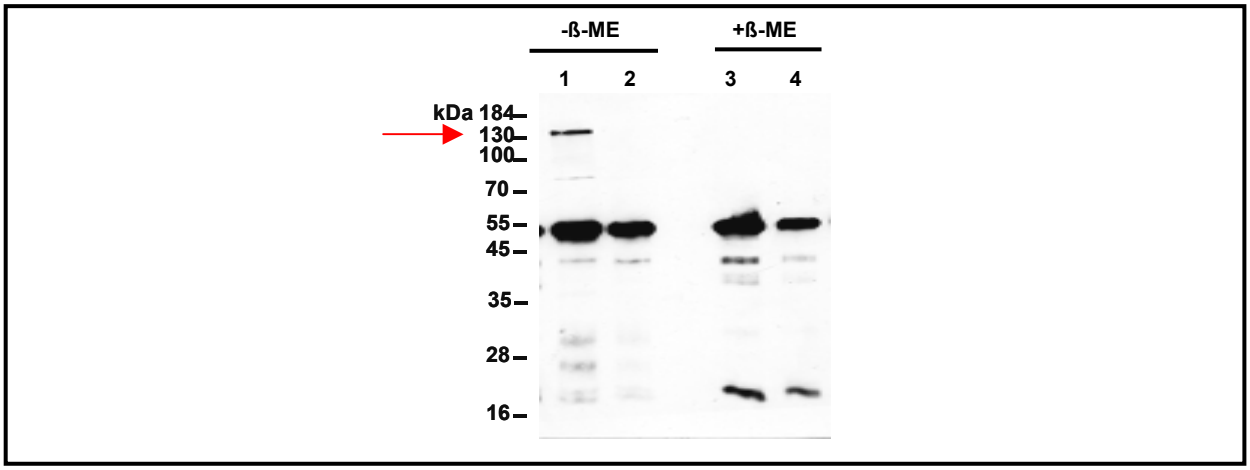


Figure 6.32. Analysis of disulfide bond formation in TGA2.2.

6x His-TGA2.2 (1 and 3) and 6x His-TGA2.2^{Cys181Ser} (2 and 4) purified proteins were incubated with 1 mM diamide for 30 min then subjected to non-reducing SDS-PAGE and immunoblot analysis was conducted thereafter. The oxidized 6x His-TGA2.2 and 6x His-TGA2.2^{Cys181Ser} were either reduced with ß-ME (3 and 4, respectively) or left in its oxidized form (1 and 2, respectively) before loading for SDS-PAGE. Immunoblot analysis using α TGA2.2 antibody was performed. Arrow indicates the oligomer band.

6.5.6 The Interactions of *TGA2.2* and *At1g28480* Mutants in Yeast

To assess whether the At1g28480 and its generated mutants are still able to interact with TGA2.2 and TGA2.2^{Cys181Ser}, an interaction assay was conducted in yeast. The full-length coding sequences of GSM and GDM mutants were inserted downstream of the *GAL4AD* in the pGAD424 plasmid, while the *TGA2.2^{Cys181Ser}* coding sequence was inserted downstream of *GAL4BD* in the pGBT9 plasmid. The GAL4BD-TGA2.2, GAL4BD-TGA2.2^{Cys181Ser} bait proteins were assayed for interactions with GAL4AD-At1g28480, GAL4AD-GSM and GAL4AD-GDM in the HF7c yeast.

Histidine prototrophy assay showed interaction between GAL4BD-TGA2.2 and the GAL4AD-GSM mutant (Table 6.3, no. 5). However, no interaction was observed between GAL4BD-TGA2.2 and GAL4AD-GDM (Table 6.3, no. 7). Therefore, it was assumed that the At1g28480 redox active site is crucial for interaction with TGA2.2,

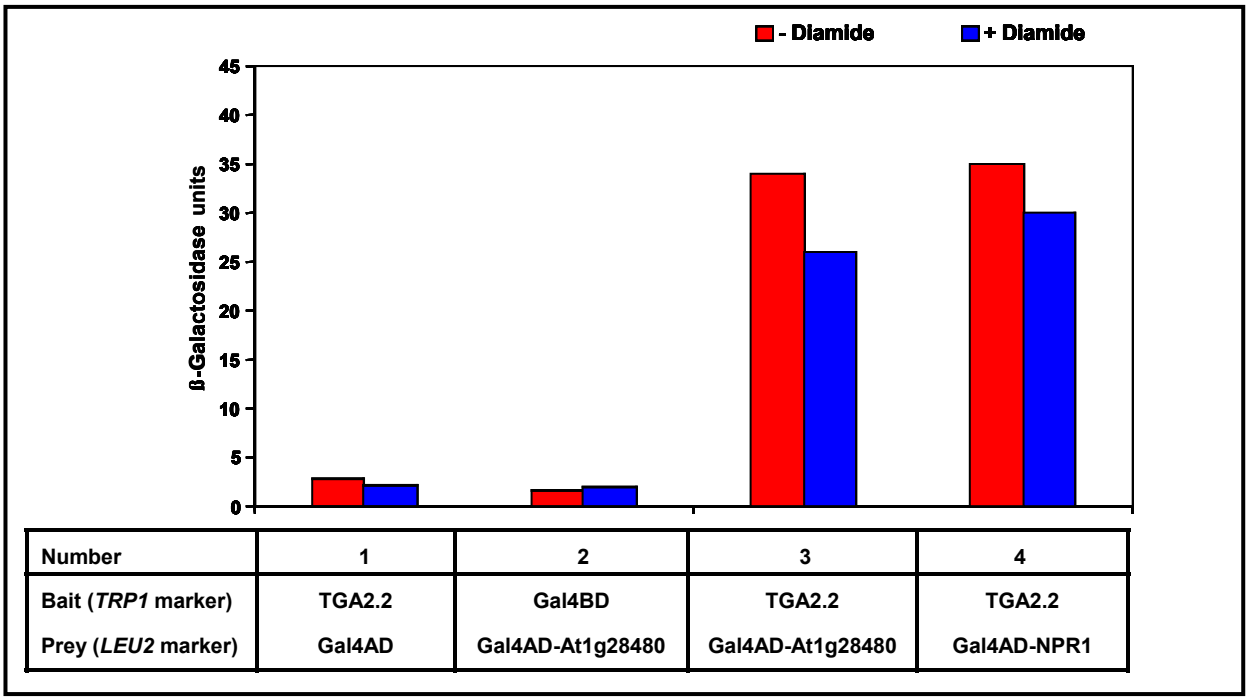
suggesting that TGA2.2 might undergo a redox change modulated by Domain swap experiments supported the observation that the interaction was severely reduced, as only a weak interaction was observed as compared with At1g28480-TGA2.2 control interactions (Table 6.3, no. 13). Unfortunately, no protein expression analysis was conducted due to antibody limitations. The GAL4BD-TGA2.2^{Cys181Ser} protein interacts with GAL4AD-At1g28480 in yeast (Table 6.3, no. 17).

Table 6.3. Interaction of TGA2.2 and At1g28480 mutants in HF7c yeast strain.

HF7c cells transformed with different bait and prey plasmids combinations (see table) were grown for 3 days at 30 °C on selective SD medium lacking leucine, tryptophan and histidine, and supplemented with 5 mM 3-AT.

Number	Bait (*TRP1* marker)	Prey (*LEU2* marker)	Growth on SD lacking Histidine Media
1	GAL4BD-TGA2.2	GAL4AD-NPR1	+++
2	GAL4BD-TGA2.2	GAL4AD	-
3	GAL4BD-TGA2.2	GAL4AD-At1g28480	+++
4	GAL4BD	GAL4AD-At1g28480	-
5	GAL4BD-TGA2.2	GAL4AD-GSM	+++
6	GAL4BD	GAL4AD-GSM	-
7	GAL4BD-TGA2.2	GAL4AD-GDM	-
8	GAL4BD	GAL4AD-GDM	-
9	GAL4BD-At1g28480	GAL4AD-TGA2.2	+++
10	GAL4BD-At1g28480	GAL4AD	-
11	GAL4BD-GSM	GAL4AD-TGA2.2	+++
12	GAL4BD-GSM	GAL4AD	-
13	GAL4BD-GDM	GAL4AD-TGA2.2	+
14	GAL4BD-GDM	GAL4AD	-
15	GAL4BD-TGA2.2^{Cys181Ser}	GAL4AD	-
16	GAL4BD-TGA2.2^{Cys181Ser}	GAL4AD-NPR1	+++
17	GAL4BD-TGA2.2^{Cys181Ser}	GAL4AD-At1g28480	+++
18	GAL4BD-TGA2.2^{Cys181Ser}	GAL4AD-GSM	+++
19	GAL4BD-TGA2.2^{Cys181Ser}	GAL4AD-GDM	-

To determine whether At1g28480 affects TGA2.2 transcriptional activity under oxidative stress, the effect of diamide treatment on the interaction between the two proteins was assayed using the YRWZ2 yeast strain harboring the *lac-Z* reporter gene under the control of the *as-1* elements. The *Met25::TGA2.2* and *GAL4AD-At1g28480* constructs were cotransformed into YRWZ2 strain and the transformants were assayed for *lac-Z* reporter gene activity using an ONPG assay. YRWZ2 transformants were grown on SD medium to an OD_{600} of 0.6 and then split into two equal aliquots, which were subjected to diamide stress or left untreated for 4 h. In consistence with the histidine prototrophy assay, At1g28480 interacted with TGA2.2 and a transactivation of the TGA2.2-dependent *lacZ* reporter gene was observed (Figure 6.33, no. 3). YRWZ2 cells subjected to oxidative stress exhibited the same reporter gene activity as the control (Figure 6.33, no. 3 vs. 4). These data establish that the At1g28480-TGA2.2 interaction is not affected by oxidative stress conditions.



Number	1	2	3	4
Bait (*TRP1* marker)	TGA2.2	Gal4BD	TGA2.2	TGA2.2
Prey (*LEU2* marker)	Gal4AD	Gal4AD-At1g28480	Gal4AD-At1g28480	Gal4AD-NPR1

Figure 6.33. Interaction of TGA2.2 and At1g28480 in YRWZ2 yeast strain under oxidative stress.

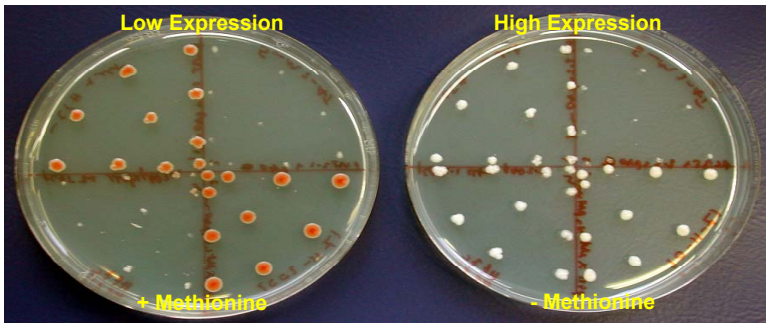
YRWZ2 cells containing pBL/TGA2.2 + pGAD424 (1), pBL+ pGAD424/At1g28480 (2), pBL/TGA2.2 + pGAD424/At1g28480 (3) and pBL/TGA2.2 + pGAD424/NPR1 (4) plasmids were grown on SD medium lacking tryptophan and leucine to an OD_{600} of 0.6 and then split into two equal aliquots, which were subjected to diamide stress or left untreated for 4 h. Cells were then processed and *lac-Z* activity was measured as described in methods.

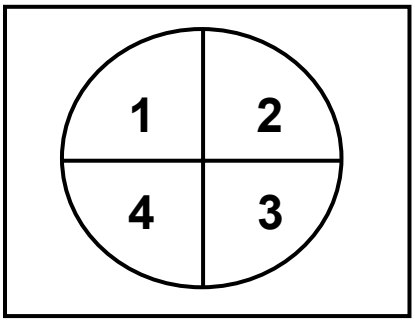
6.5.7 At1g28480 Enhances TGA2.2 Expression in Yeast

As described above, the presence of the leucine zipper motif in TGA factors facilitates their dimerization. Previous analysis showed that homodimerization between two TGA2.2 factors is possible and was detectable in EMSAs (Niggeweg *et al.*, 2000b). However, in contrary to the EMSA data, no interaction was observed between two GAL4BD-TGA2.2 and GAL4AD-TGA2.2 hybrid proteins in the Y2HS (C. Thurow, unpublished observations). This TGA2.2 behavior in yeast hints to the possibility that the TGA2.2 factors could undergo a posttranscriptional modification enabling them to dimerize.

To test whether the At1g28480 protein can enhance TGA2.2 dimerization in yeast, the At1g28480 protein was expressed conditionally as a third protein using the *Met25::HA-NLS-At1g28480::PGK* cassette harbored in the pBD vector. The *TGA2.2* coding sequence was inserted in-frame downstream of the *GAL4BD* cassette in the pBD vector harboring the *Met25::HA-NLS-At1g28480::PGK* cassette. The newly cloned plasmid, designated pBDTGA2.2/Met25::At1g28480, will allows the expression of two proteins simultaneously in the same yeast cell.

The GAL4AD-TGA2.2 and GAL4BD-TGA2.2 proteins were assayed for their interactions in the presence or absence of HA-NLS-At1g28480 protein in the HF7c Y2HS strain. As anticipated, GAL4BD-TGA2.2 does not interact with GAL4AD-TGA2.2 in yeast (Figure 6.34, sector 2). Also, the solo coexpression of At1g28480 with GAL4BD-TGA2.2 didn't lead to any reporter gene activation (data not shown). Strikingly, the inducible expression of At1g28480 in the presence of GAL4BD-TGA2.2 and GAL4AD-TGA2.2 proteins led to *HIS3* reporter gene activation (Figure 6.34, sector 4: **-**methionine plate). In the contrary, At1g28480 repression abolished the observed activation (Figure 6.34, sector 4; +methionine plate). Similar results were obtained when a HA-NLS-GDM protein was used (data not shown). These results indicate that the At1g28480 protein can facilitate dimer formation between two TGA2.2 factors in yeast. However, it was not clear whether the observed effect is related to ternary complex formation between the three proteins or is due to other unknown mechanisms.





Sector	Bait (*TRP1* marker)	Bridge	Prey (*LEU2* marker)	Growth on SD lacking histidine	
				- Methionine	+ Methionine
1	GAL4BD-TGA2.2	-	GAL4AD-NPR1	+++	+++
2	GAL4BD-TGA2.2	-	GAL4AD-TGA2.2	-	-
3	GAL4BD-TGA2.2	-	GAL4AD-At1g28480	+++	+++
4	GAL4BD-TGA2.2	HA-NLS-At1g28480	GAL4AD-NPR1	+++	-

Figure 6.34. Y2HS assays of interactions between At1g28480 and TGA2.2 proteins.

HF7c cells containing pGBT9/TGA2.2 + pGAD424/NPR1 (1), pGBT9/TGA2.2 + pGAD424/TGA2.2 (2), pGBT9/TGA2.2 + pGAD424/At1g28480 (3) and pBDTGA2.2/Met25::At1g28480 + pGAD424/TGA2.2 (4) plasmids, were grown for 3 days at 30 °C on selective SD medium lacking leucine, tryptophan and histidine supplemented with 5 mM 3-AT.

To understand how At1g28480 affects TGA2.2 interaction, the DNA binding activity of the TGA2.2 protein expressed in yeast was examined. EMSA assay was conducted using native protein extracts made from HF7c yeast cells expressing TGA2.2 alone (encoded in pLEU/Met25::TGA2.2) or coexpressing TGA2.2 and At1g28480 (encoded in pBD/Met25::At1g28480). As expected, a retarded band corresponding to TGA2.2 was present in the binding mixture from both protein extracts (Figure 6.35A). The retarded band is most likely formed by the specific binding of TGA2.2 to the *as-1* element as incubation of the *as-1* element with a yeast extract expressing At1g28480 alone did not yield any retarded band (data not shown). The coexpression of TGA2.2 and At1g28480 cause a substantial increase in the amount of TGA2.2 complex formed on the *as-1* element when compared with yeast cell extract expressing TGA2.2 alone (Figure 6.35A).

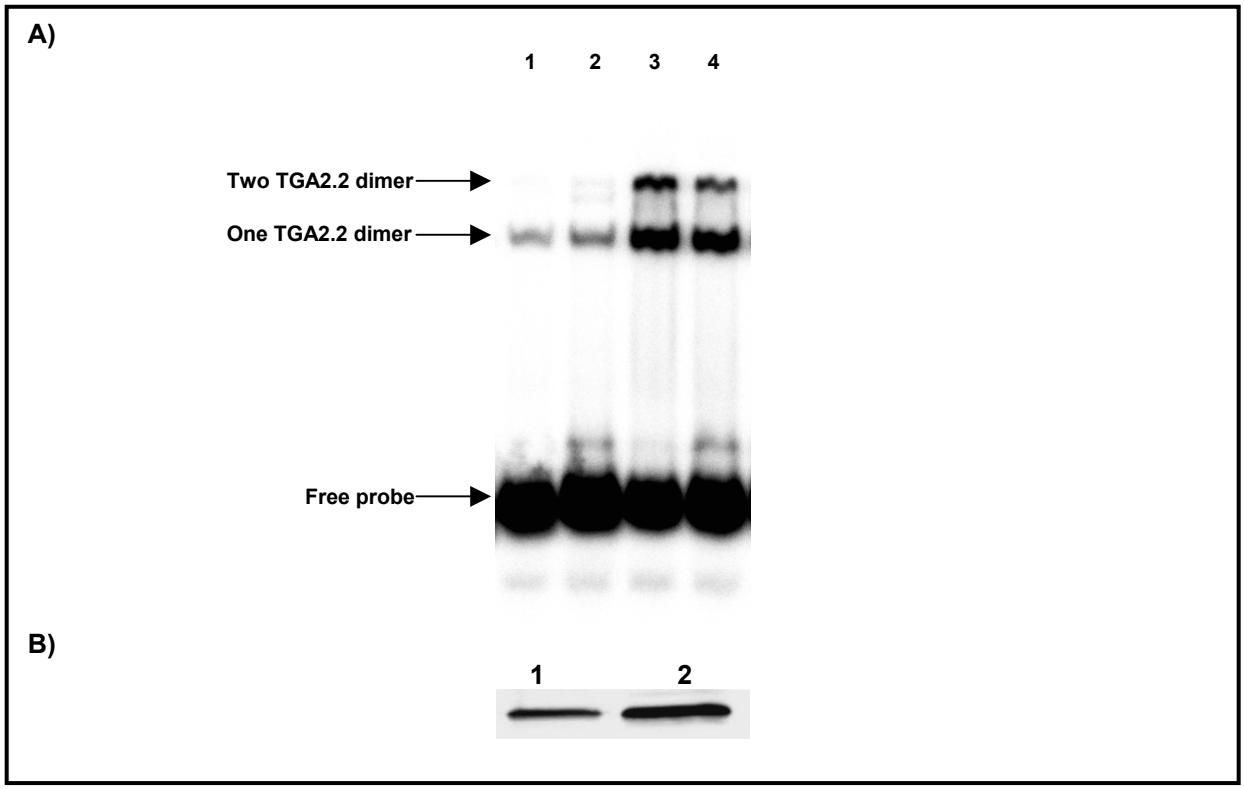


Figure 6.35. At1g28480 enhances indirectly the *in vitro* binding of TGA2.2 to *as-1* element by increasing TGA2.2 expression levels in yeast.

A) EMSA analysis of TGA2.2 binding to *as-1* element.

20 µg of yeast native protein extracts expressing TGA2.2 (lanes 1 and 2) or coexpressing TGA2.2 and At1g28480 (lane 3 and 4) were incubated with *as-1* radioactive probes, and the mixtures were loaded on native PAGE gel. The protein-DNA complexes were detected by autoradiography.

B) Protein gel analysis of TGA2.2 protein expression levels in HF7c yeast cells.

20 µg of yeast native protein extracts expressing TGA2.2 (lanes 1) or coexpressing TGA2.2 and At1g28480 (lane 2) were analyzed on a 12% SDS-polyacrylamide transferred to a nitrocellulose membrane, and then immunoblotted with TGA2.2 antibody.

Surprisingly, the protein extracts from yeast cells coexpressing TGA2.2 and At1g28480 showed higher expression levels (about ~3 fold higher) of the TGA2.2 protein as compared with extracts expressing TGA2.2 alone (Figure 6.35B). These findings indicate that At1g28480, through unknown mechanism, increases TGA2.2 expression levels and thereby leads indirectly to a higher DNA binding activity of the TGA2.2.

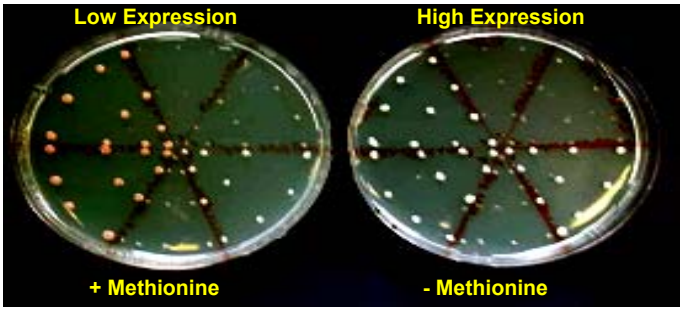
6.5.8 At1g28480 Interacts via TGA2.2 with NPR1 in Yeast

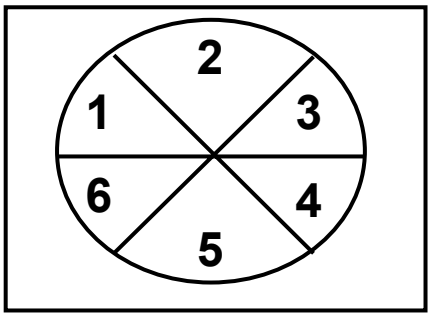
In order to investigate possible interactions between At1g28480, TGA2.2 and NPR1, *i.e.,* ternary complex formation, the interaction between the three proteins was examined in Y3HS experiments. The yeast strain HF7c was then transformed with constructs expressing TGA2.2, GAL4BD-At1g28480 and GAL4AD-NPR1. The TGA2.2 protein was expressed conditionally as a third protein using the *Met25::TGA2.2::PGK* cassette. The *Met25::TGA2.2::PGK* cassette was cloned into the pGBT9/*At1g28480* and pGBT9/GDM plasmid. The newly cloned plasmids were designated pBDAt1g28480/Met25::TGA2.2 and pBDAt1g28480/Met25::TGA2.2, respectively.

As expected, no interaction between GAL4BD-At1g28480 and GAL4AD-NPR1 was observed in yeast (Figure 6.36, sector 2). Also, the coexpression of GAL4BD-At1g28480 with TGA2.2 didn't lead to any reporter gene activation (Figure 6.36, sector 5). When the yeast cells expressed the TGA2.2, GAL4BD-At1g28480 and GAL4AD-NPR1, a robust growth in medium lacking histidine was observed (Figure 6.36, sector 6). Noticeably, low TGA2.2 expression levels have also reinforced the formation of a ternary complex (Figure 6.36, sector 6, + methionine). Also the GAL4BD-GDM protein was able to form ternary complex with NPR1 and TGA2.2 (?, sector 4). These findings would suggest that the At1g28480 is able to interact with NPR1 in the presence of TGA2.2, however it is not clear whether the interaction nature is direct or indirect one.

In order to determine whether other TGA transcription factors involved in ternary complex formation, TGA1 was used instead of TGA2.2 in the described Y3HS. TGA1 was selected because previous studies indicates that it does not interact with NPR1 in the Y2HS (Section 1.9). The coexpression of GAL4BD-At1g28480 with TGA1 alone leads to *HIS3* reporter gene activation. Based on this observation it was not feasible to distinguish if there is any ternary complex formed between the three proteins on a histidine prototrophy assay. To overcome this obstacle, An ONPG assay was conducted to quantify the *lac-Z* reporter gene activity. Cells expressing the TGA1, GAL4BD-At1g28480 and GAL4AD-NPR1 proteins had higher *lac-Z* reporter gene activity as compared to cells expressing the GAL4BD-At1g28480 and TGA1 proteins

(Table 6.4, no. 3). These results would suggest that the TGA1 is able to interact with NPR1 via At1g28480. However, in domain swap experiments the interaction between GAL4BD-NPR1, TGA1 and GAL4AD-At1g28480 was lost, adding many question marks on the nature of the TGA1-NPR1 interaction in yeast systems.





Sector	Bait (*TRP1* marker)	Bridge	Prey (*LEU2* marker)	Growth on SD lacking histidine	
				- Methionine	+ Methionine
1	GAL4BD-TGA2.2	-	GAL4AD-NPR1	+++	+++
2	GAL4BD-At1g28480	-	GAL4AD-NPR1	-	-
3	GAL4BD-GDM	TGA2.2	GAL4AD	-	-
4	GAL4BD-GDM	TGA2.2	GAL4AD-NPR1	+++	++
5	GAL4BD-At1g28480	TGA2.2	GAL4AD	-	-
6	GAL4BD-At1g28480	TGA2.2	GAL4AD-NPR1	+++	+++

Figure 6.36. At1g28480 interacts with NPR1 via TGA2.2 in HF7c yeast strain.

HF7c cells containing pGBT9/TGA2.2 + pGAD424/NPR1 (1), pGBT9/At1g28480 + pGAD424/NPR1 (2), pBDGDM/Met25::TGA2.2 + pGAD424 (3), pBDGDM/Met25::TGA2.2 + pGAD424/NPR1 (4), pBDAt1g28480/Met25::TGA2.2 + pGAD424 (5) and pBDAt1g28480/Met25::TGA2.2 + pGAD424/NPR1 (6) plasmids, were grown for 3 days at 30 °C on selective SD medium lacking leucine, tryptophan and histidine, supplemented with 5 mM 3-AT.

Table 6.4. Interaction of TGA1, At1g28480 and NPR1 in HF7c yeast strain.

HF7c cells containing pGBT9/At1g28480 + pGAD424/NPR1 (1), pBDAt1g28480/Met25::TGA1+ pGAD424 (2) and pBDAt1g28480/Met25::TGA1+ pGAD424/NPR1 (3) plasmids were grown on SD medium (lacking methionine) to an OD_{600} of 0.6 and were processed and *lac-Z* activity was measured as described in methods.

Number	Bait (*TRP1* marker)	Bridge	Prey (*LEU2* marker)	ß -Galactosidase units
1	GAL4BD-At1g28480	-	GAL4AD-NPR1	0.30
2	GAL4BD-At1g28480	TGA1	GAL4AD	2.5
3	GAL4BD-At1g28480	TGA1	GAL4AD-NPR1	5.5

6.5.9 At1g28480 Transactivation Assays in Protoplasts

In order to assess the effect of At1g28480 protein on *as-1* element-dependent reporter gene transcription activation, transient assays using BY-2 protoplasts were conducted. The effector plasmid HBTL/At1g28480 was constructed by inserting the *At1g28480* full-length coding sequence downstream of the *HBT* chimeric promoter (Figure 6.37A). The HBTL/At1g28480 and HBTL (control) plasmids were cotransfected separately with the *as-1-GUS* reporter plasmid into tobacco BY-2 protoplasts. In comparison with the control, the At1g28480 protein had repressed the *as-1-GUS* reporter gene activity (Figure 6.37B).

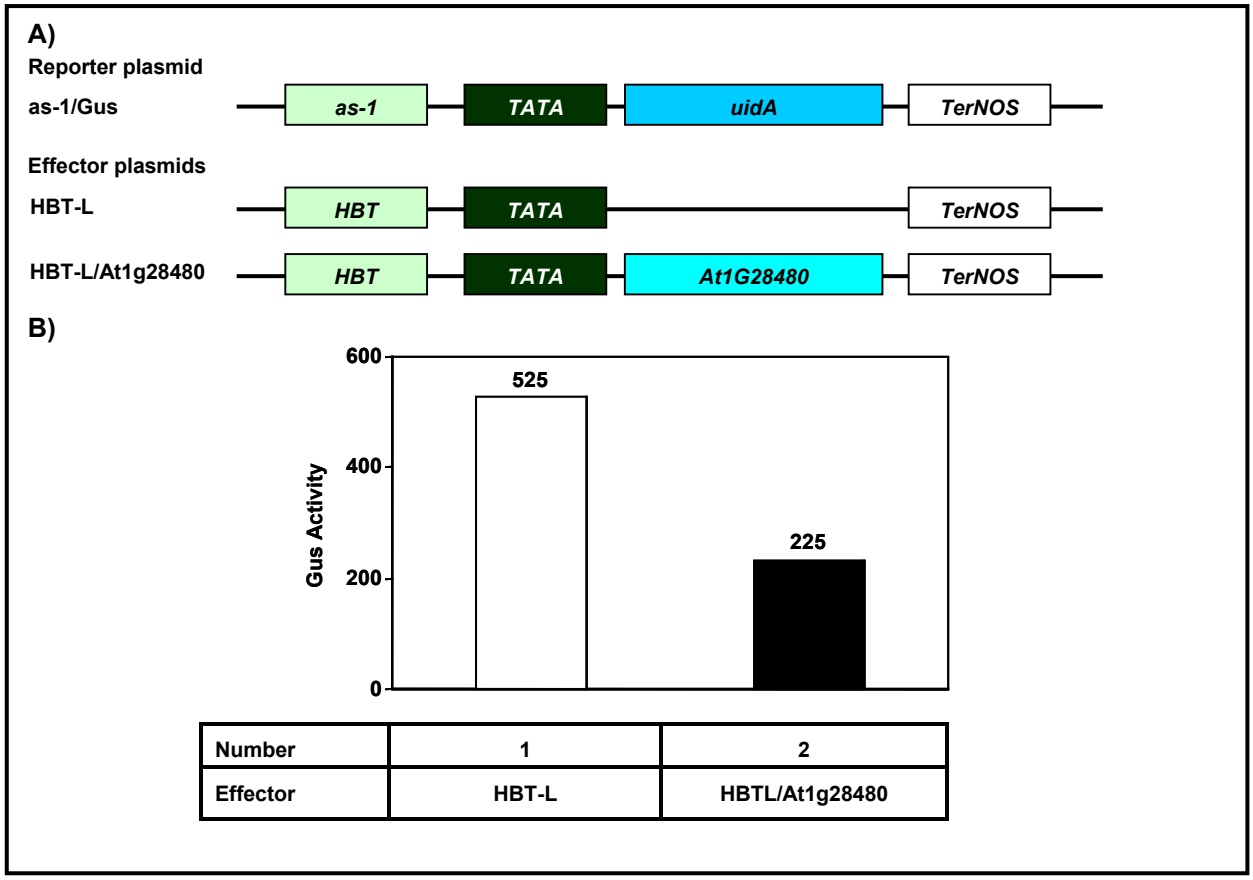


Figure 6.37. Transactivation assay of At1g28480 in BY-2 protoplasts.

A) Schematic diagram of the effector and reporter plasmids used in cotransfection experiments.

B) Transactivation of the *as-1::GUS* fusion reporter gene by At1g28480 protein.

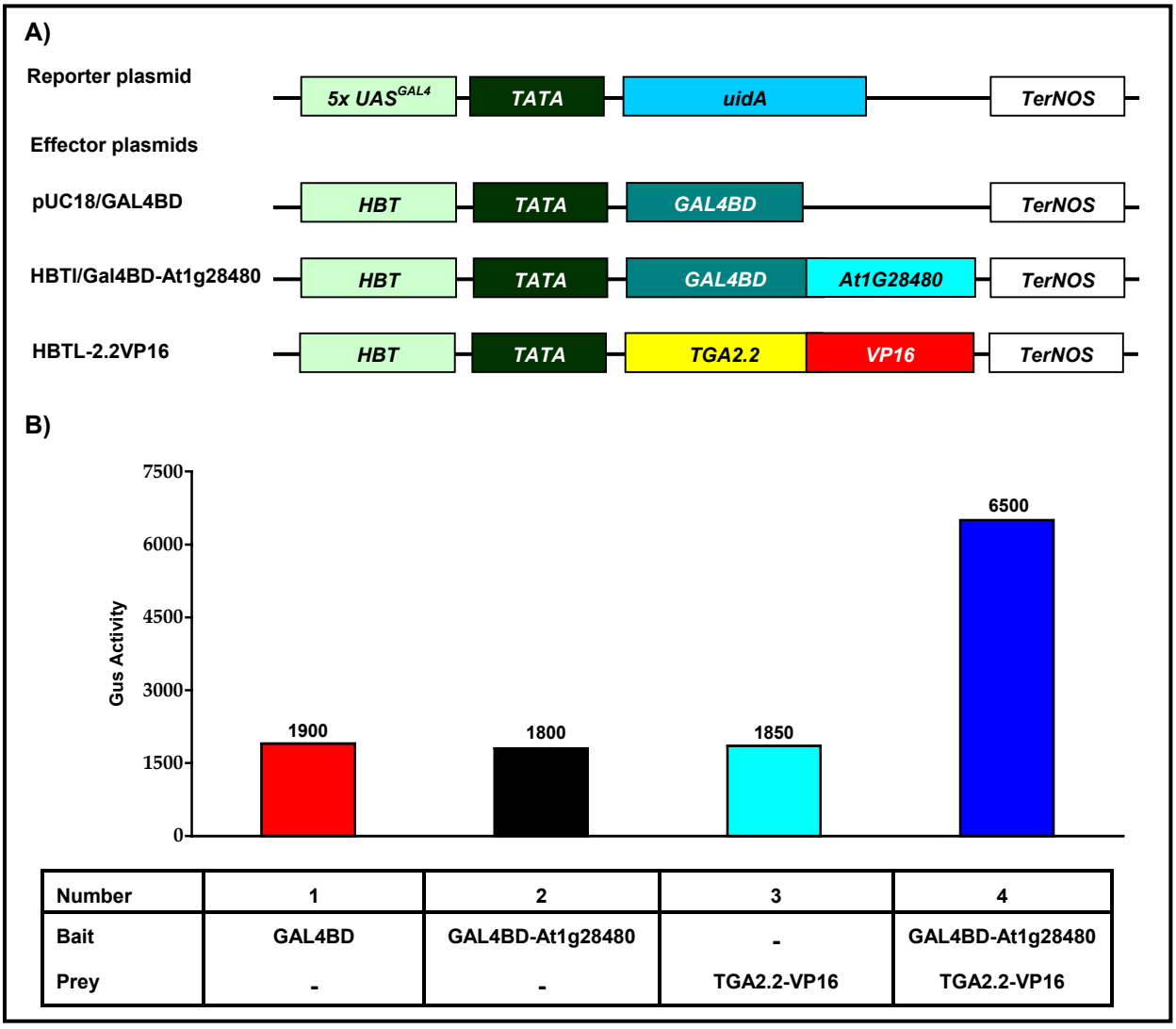
The reporter gene was cotransfected with 10 µg of *as-1-GUS* reporter and 25 µg of *At1g28480* effector plasmid or the empty vector as control treatment. GUS Activity was estimated as described in methods.

The At1g28480 repression effect is most likely due to a toxic effect on the BY-2 protoplast. In consistent with this assumption, transient assay using the *5x UAS^{GAL4}::uidA* reporter gene and its positive control GAL4BD-VP16 effector (strongly activate the *5x UAS^{GAL4}::uidA* reporter gene), a similar repression effect was observed with this control system, indicating a general toxic effect on the BY-2 protoplasts (data not shown).

6.5.10 Interaction between At1g28480 and TGA2.2 *In planta*

To determine whether the direct physical interactions between TGA2.2 and At1g28480 protein could be accomplished by other means rather than yeast hybrid systems, the interaction between both proteins was assessed using a protoplast transient assay similar to the Y2HS. This method was deployed after the failure to detect any TGA2.2 and At1g28480 protein complex using the conventional *in vitro* assays described above. For this purpose, the *At1g50570* full-length coding sequence was ligated in-frame with the GAL4BD coding sequence downstream of the *HBT* chimeric promoter into the HBT-L plant expression vector. This bait effector plasmid was designated as HBTL/GAL4BD-At1g28480 (Figure 6.38A). The *TGA2.2-VP16* coding sequence was cloned downstream of the *HBT* chimeric promoter into the HBT-L vector, designated as HBT-2.2VPs, was used as an effector prey plasmid (Figure 6.38A; Tharow, 2002).

The described effector plasmids were cotransfected into BY-2 protoplasts, and the At1g28480-TGA2.2 interaction was assayed for the expression of a *5x UAS^{GAL4}::uidA* reporter gene. BY-2 protoplast were also transfected with the reporter gene along with the effector constructs pUC18/GAL4BD, HBTL/GAL4BD-At1g28480 and HBT-2.2VPs separately to serve as internal controls. Protoplasts cotransfected with the reporter plasmids along with GAL4DB-At1g28480 and *TGA2.2-VP16* constructs showed a substantial and distinguishable increase of *GUS* reporter gene activity as compared with other controls (Figure 6.38B, no. 4). These results indicate that TGA2.2 can interact with At1g28480 *in planta*.



Number	1	2	3	4
Bait	GAL4BD	GAL4BD-At1g28480	-	GAL4BD-At1g28480
Prey	-	-	TGA2.2-VP16	TGA2.2-VP16

Figure 6.38. At1g28480 interacts with TGA2.2 in BY-2 protoplasts.

10 µg of *5x UAS^{GAL4}-uidA* reporter gene plasmid were transfected with 25 µg pUC18/GAL4BD, 25 µg HBTL/GAL4AD-At1g28480 and 25 µg HBTL/TGA2.2-VP16 or cotransfected with 12.5 µg HBTL/GAL4AD-At1g28480 + 12.5 µg HBT-2.2VPs effector plasmids. β-Glucuronidase Activity estimated as described in methods.

6.5.11 At1g28480 Subcellular Localization

The subcellular localization of At1g28480 was assayed in BY-2 protoplasts as described above. The *At1g28480* coding region was cloned into HBTL/GFP plasmid, in-frame to the 5' end of a *GFP* gene (Figure 6.39A). As a positive control, protoplasts were transfected with HBTL/GFP plasmid (Figure 5.39A). The At1g28480-GFP fusion protein was evenly distributed in the cytoplasm and the nucleus (Figure 6.39B).

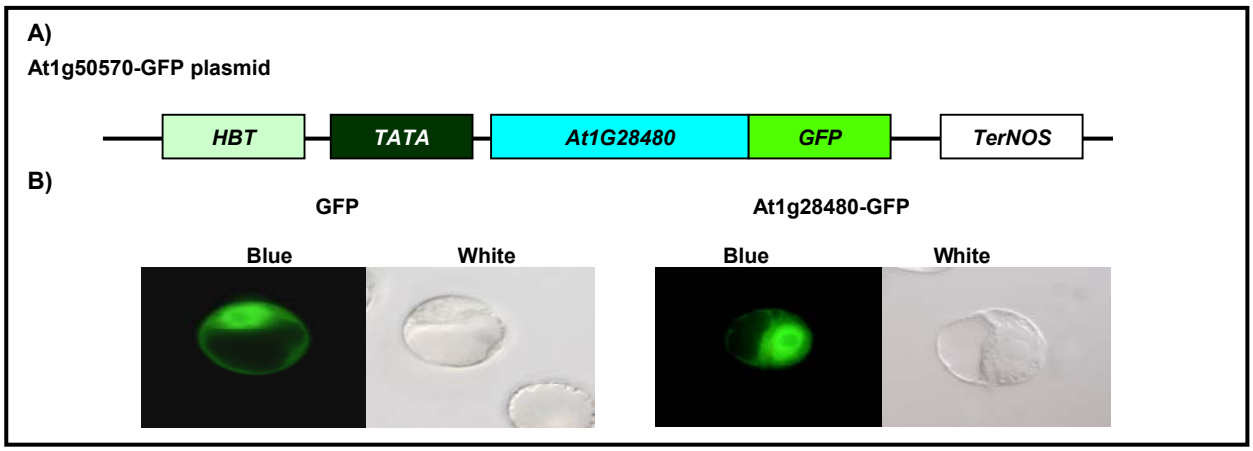


Figure 6.39. Subcellular localization of At1g50570-GFP protein.

A) Schematic diagram of HBTL/At1g28480-GFP plasimd used in subcellular localization experiments.

B) BY-2 protoplasts expressing GFP and At1g28480-GFP.

BY-2 protoplasts were transfected with the *GFP and At1g28480-GFP* constructs, and was visualized using a BX 51 fluorescent microscope using the blue and white light fields.

6.5.12 Expression Analysis of *At1g28480* Gene

In order to analysis the *At1g28480* tissue-specific expression patterns, gene transcripts from different organs were analyzed using the RT-PCR method. *At1g28480* mRNA was detected in flowers, siliques, inflorescence stems, and rosette leaves, and roots, indicating the presence of *At1g28480* transcripts in all Arabidopsis plant tissues (Figure 6.40). The a*ctin* RT-PCR reactions were used as described above (section 5.4.6).

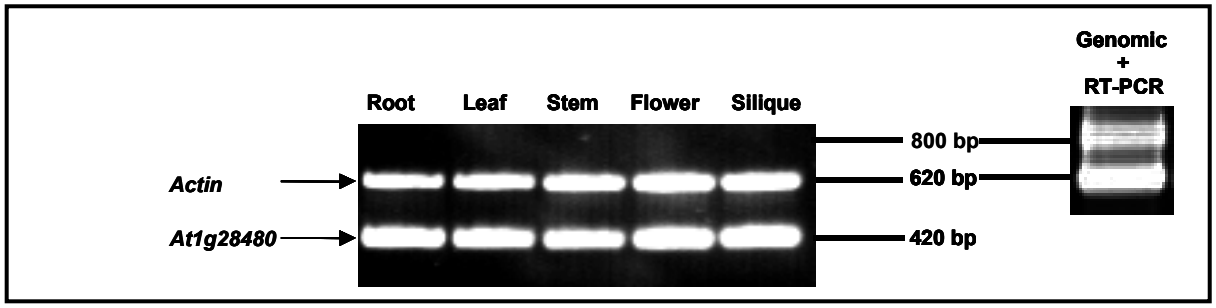


Figure 6.40. Detection of *At1g28480* mRNA in different Arabidopsis tissues by RT-PCR.

Total RNA isolated from 4 weeks old plants parts (roots, leaves, stems, flowers and siliques) were detected for *At1g28480* mRNA transcripts using RT-PCR. The *actin* control is shown at the bottom of the gel. A PCR control reaction for *actin* using genomic DNA and one of the RT-PCR products is shown on the left.

The expression of *At1g28480* in response to SA treatment and *P. syringae* pv. maculicola ES4326, with or without the *avrRpt2 R* gene, pathogen challenging was analyzed using RNA blot analysis. Plants sprayed with 1 mM SA for 2 hours showed significant higher *At1g28480* mRNA accumulation compared to untreated plants, whereas after 24 hours of SA treatment At1g50570 mRNA accumulation was simliar as untreated plants (Figure 6.41). Inoculation with the *P. syringae*, virulent or avirulent strain, led to an increase in the expression of *At1g28480* starting 12 hr after pathogen inoculation up to 24 hr and then decreasing during the next 48 hr (Figure 6.41). After *P. syringae*, virulent infection, *At1g28480* was induced earlier than *PR-1*, whose transcription was maximal after 24 hr (Figure 6.41). These results indicate that the transcription of the *At1g28480* gene is strongly activated by SAR inducers.

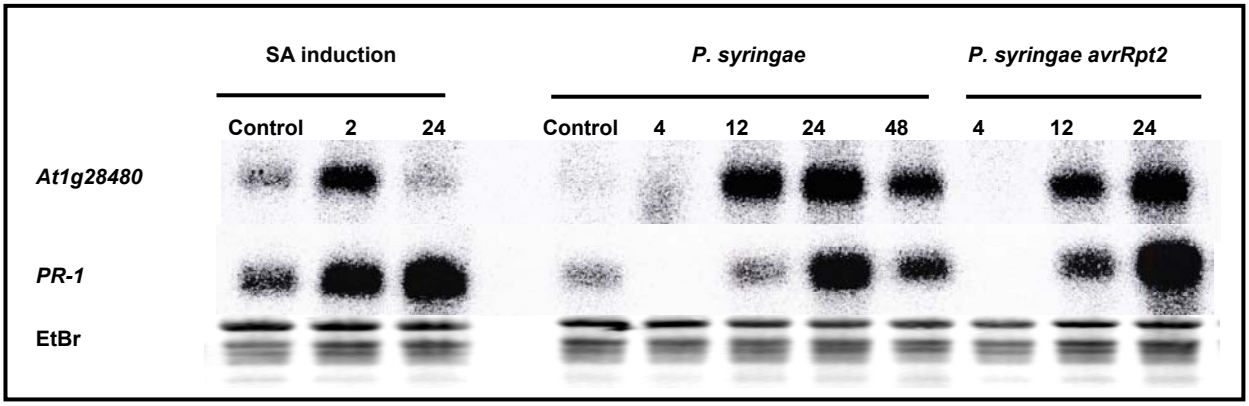


Figure 6.41. RNA gel blots analysis of *At1g28480* gene expression after SA or pathogen treatments.

Arabidopsis plants were grown for 4 weeks on soil before spraying with 1 mM SA or challenged with *P. syringae* pv. maculicola ES4326 with or without the *avrRpt2 R* gene. Total RNA was isolated from plants at different time points (in hours) and 10 µg were separated on denaturing gels in the presence of ethidium bromide (EtBr), photographed to assess equal loading, and examined by Northern blot analysis. A radioactive *At1g28480* and *PR-1* full-length cDNA probes were hybridized to the membrane, which was then examined by autoradiography.

6.5.13 Analysis of the *In Vivo* Roles of At1g28480 by Generation of At1g28480 Antisense Lines

To determine if At1g28480 has a role in the SA-mediated gene expression signaling, suppression of *At1g28480* gene expression in plants via antisense (RNAi)

approach was conducted. For this purpose, the full-length cDNA fragment of *At1g28480* was cloned into the pFGC5941 vector in the sense and antisense orientations (Figure 6.42A). The pFGC5941/At1g28480 vector was transformed into the Arabidopsis -90-GUS transgenic plants using the flower dip method. The transformed plants were selected by spraying them with Basta herbicide.

RNA blot analysis was used to analysis the *At1g28480* RNA levels in T2 segregating lines after SA treatment for four hours in comparison to the wild type control. In contrary to the *At1g28480* RNA transcripts from the control plant that showed a clear band for *At1g28480*, the antisense lines didn't showed any distinguishable band corresponding to the *At1g28480* gene transcripts (Figure 6.42B). The RNA blot analysis was repeated twice with similar *At1g28480* transcripts pattern in the antisense lines.

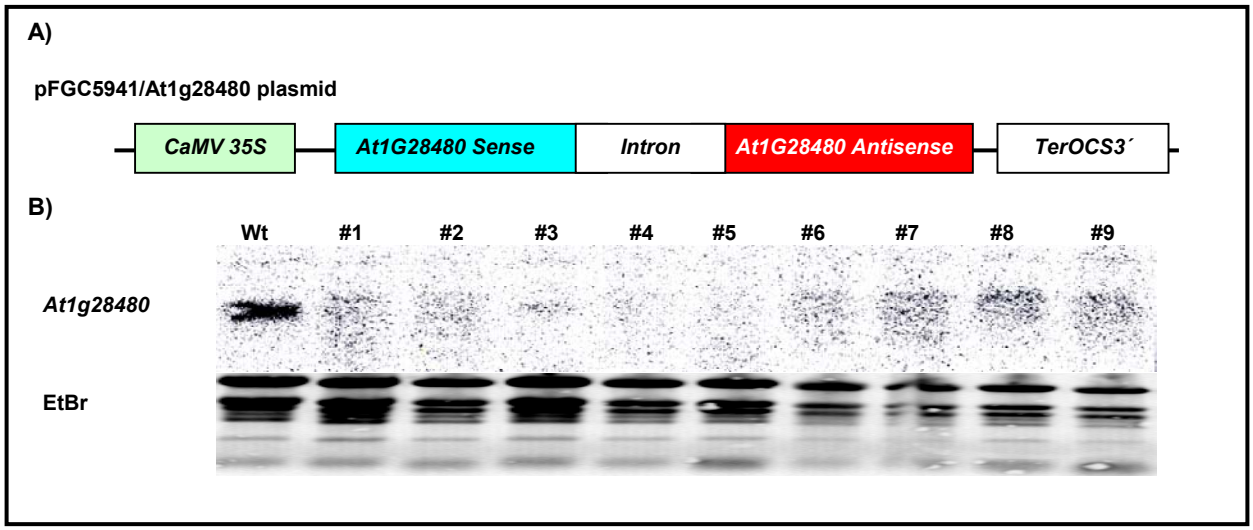


Figure 6.42. Analysis of *At1g28480* gene expression in different At1g28480 antisense lines.

A) Schematic diagram of pFGC5941/At1g28480 antisense plasmid used in the generation of antisense plants.

B) RNA gel blots analysis of *At1g28480* gene expression in different At1g28480 antisense lines.

Plants were grown for 4 weeks on soil and total RNA was isolated from Arabidopsis -90-GUS plants (control) and Arabidopsis At1g28480 antisense lines. 7.5 µg were separated on denaturing gels in the presence of ethidium bromide (EtBr), photographed to assess equal loading, and examined by Northern blot analysis. A radioactive *At1g28480* full-length cDNA probe was hybridized to the membrane, which was then examined by autoradiography.

The expression of *PR-1* gene in response to SA induction in the At1g28480 antisense lines was examined. The SA-induced *PR-1* expression after 24 hours was not affected in *At1g28480* antisense lines as compared to the wild type plants (Figure 6.43). These results indicated that the *At1g28480* transcript suppression didn't have any effect on the *PR-1* gene expression.

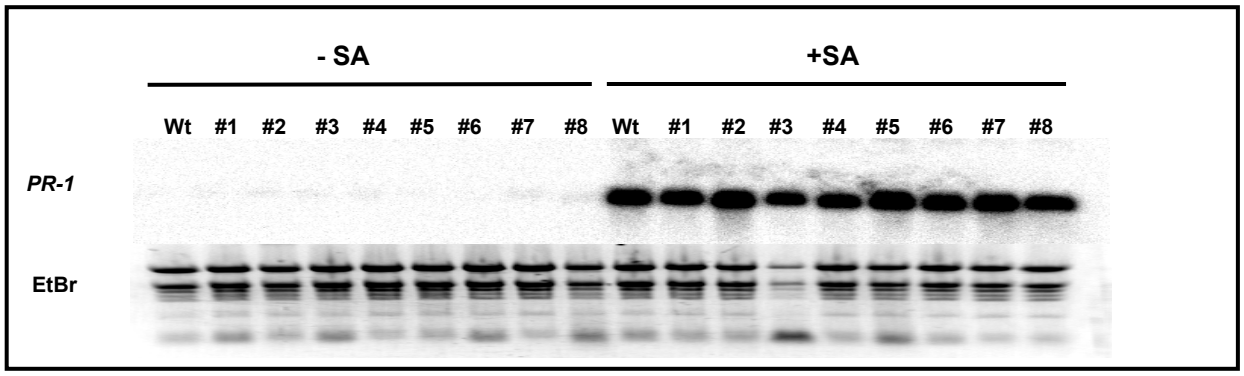


Figure 6.43. RNA gel blots analysis of *PR-1* gene expression in At1g28480 antisense lines.

Plants were grown for 4 weeks on soil before inducing them with 1 mM SA for 24 hours. Total RNA was isolated from untreated and SA treated plants and 10 µg were separated on denaturing gels in the presence of ethidium bromide (EtBr), photographed to assess equal loading, and examined by Northern blot analysis. A radioactive *PR-1* cDNA probe was hybridized to the membrane, which was then examined by autoradiography.

6.5.14 Analysis of the *In Vivo* Roles of At1g28480 by Generation of At1g28480 Overexpressor lines

To examine the effect of *At1g28480* overexpression on the SA-dependent gene expression in Arabidopsis plant, -90-GUS transgenic plants were transformed with the Alligator2/At1g28480 binary vector harboring a *3x HA tag* sequence fused in-frame to the *At1g28480* coding sequence (Figure 6.44A). Protein gel blot analysis of T1 At1g28480 overexpressors lines, showing GFP fluorescent seeds, using a HA specific antibody revealed that lines #3, #4 and #6 accumulates higher protein levels compared with the wild type (Figure 6.44B).

Interestingly, the T1 At1g28480 overexpression line #7 plant showed a distinguishable phenotype as compared with the -90-GUS transgenic plants (Figure 6.44C). The At1g28480 overexpression line #7 plant has phenotype of an uneven or

irregular leaf shape, cotyledon epinasty, late flowering time and crinkled fruits. Examining T2 seeds for the segregation of the T-DNA insertion revealed that the T-DNA insertion and the corresponding phenotype segregated together (3:1 ratio). It is possible that this phenotype is related to a T-DNA insertion mutation rather than to At1g28480 overexpression effect. Based on the previous analysis, the At1g28480 overexpressor line #6 was selected for further analysis.

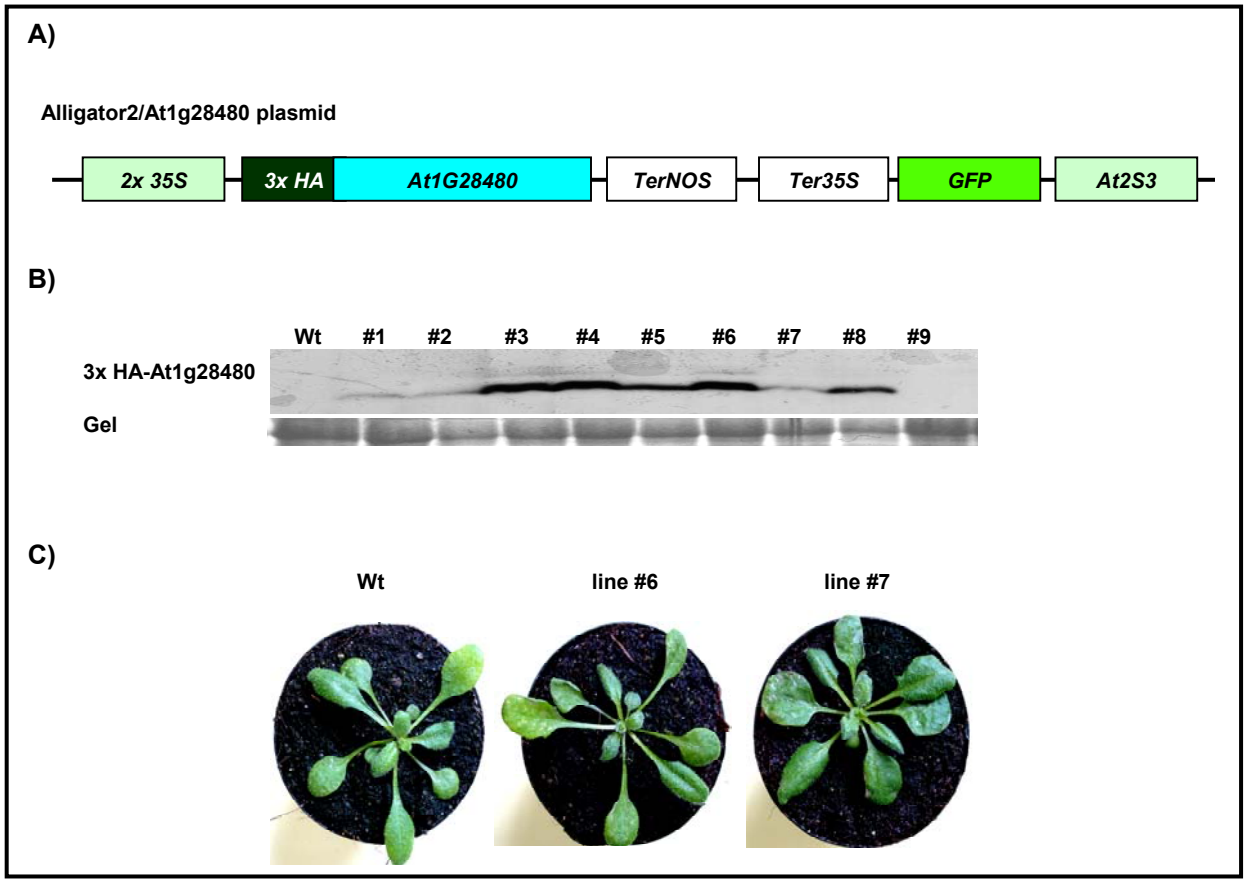


Figure 6.44. Analysis of At1g50570 overexpression lines.

A) Schematic diagram of Alligator2/At1g28480 plasmid used in the generation ofAt1g28480 overexpressor lines.

B) Protein gel blot analysis using αHA antibody of 3x HA-At1g28480 levels in Alligator2/At1g28480 transgenic lines. Coomassie stained gel serve as loading control

C) Alligator2/At1g28480 transgenic lines phenotypes.

The effect of the *At1g28480* overexpression on the expression of genes induced by SA was examined. The expression patterns of *GST6* and *PR-1* after SA treatment were examined in the At1g28480 overexpressor line #6 plants and were then compared

to those of wild type plants. *At1g28480* overexpression didn't have any significant effect on the SA-inducible gene expression (Figure 6.45A and B).

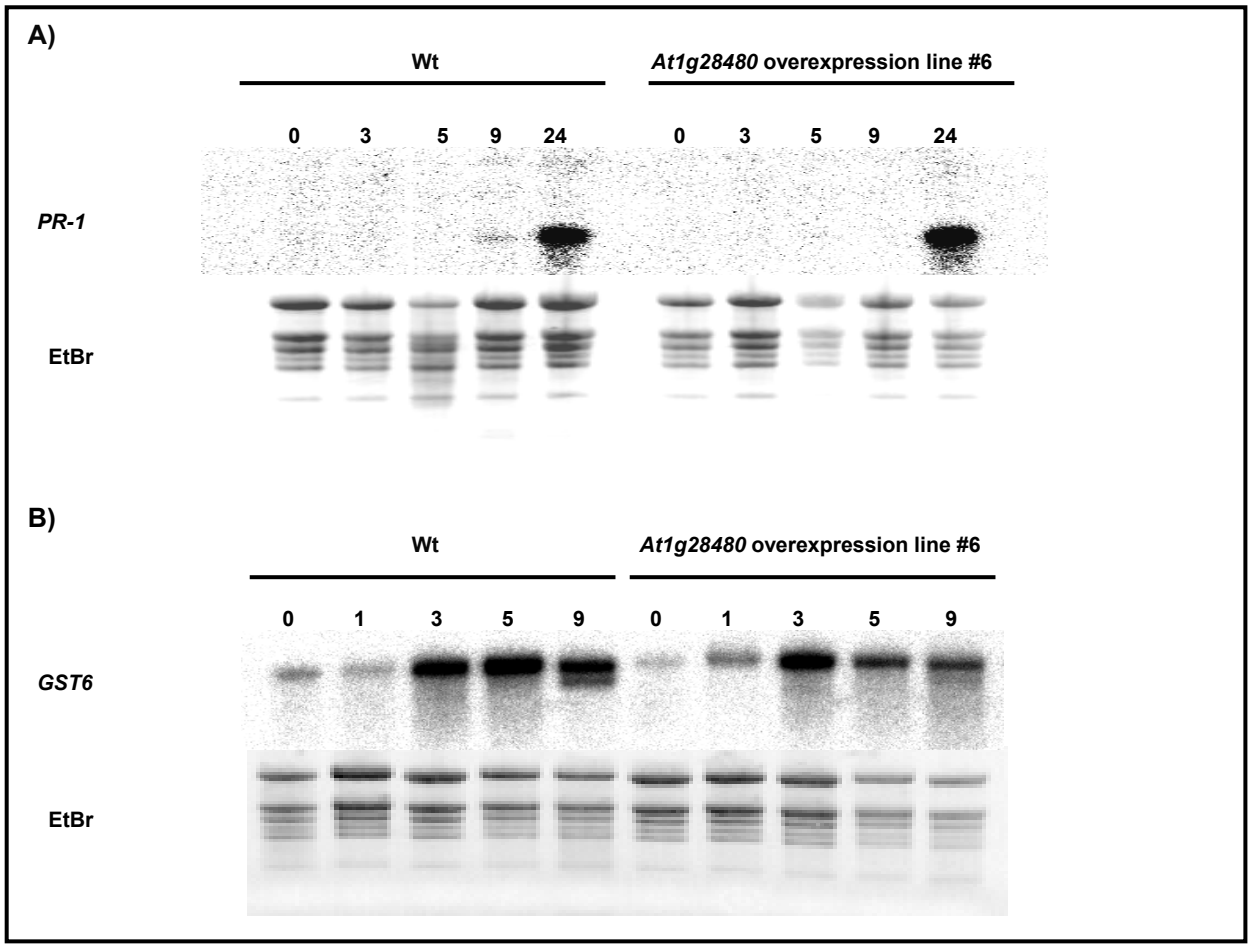


Figure 6.45. RNA gel blots analysis of *PR-1* and *GST6* expression in At1g28480 overexpressor line #6.

Plants were grown for 4 weeks on soil before spraying with 1 mM SA. Total RNA was isolated from plants at different time points (in hours) and 10 µg were separated on denaturing gels in the presence of ethidium bromide (EtBr), photographed to assess equal loading, and examined by Northern blot analysis. A radioactive *PR-1* **(A)** and *GST6* **(B)** cDNA probes were hybridized to the membrane, which was examined by autoradiography.

The expression pattern of the *GUS* reporter gene was analyzed using histochemical GUS staining in At1g28480 overexpressor lines and in the wild-type background after 2,4-D treatment. As expected, the *as-1*-dependent reporter gene expression was activated after 2,4-D treatments in the wild type background (Figure

6.46). However, the 2,4-D inducible expression pattern of *as-1*::*GUS* in At1g28480 overexpressor line #3 was severely repressed when compared with that of -90-GUS plants. In conclusion, it is seems that the *At1g28480* overexpression had led to repress auxin-dependent gene expression.

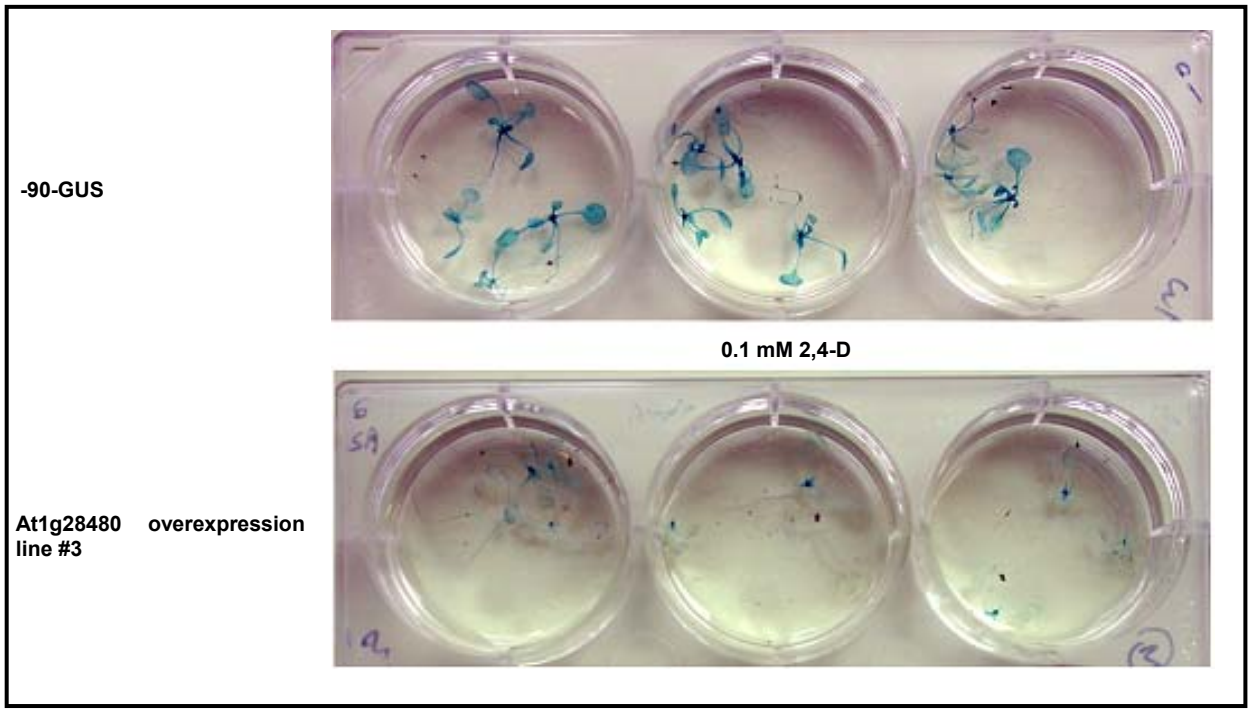


Figure 6.46. Histochemical staining of GUS activities in At1g28480 overexpression line #3.

Two weeks soil grown -90-GUS transgenic and At1g28480 overexpression line #3 plants were treated with 0.1 mM 2,4-D for 24 hours and then stained for GUS.

7 Discussion

7.1 The MY1HS as a Method to Isolate TGA2.2 Partners

TGA2.2 is a transcription factor that mediates SA- and auxin- regulated gene expression. Being expressed constitutively, the activity of TGA2.2 must be regulated by either post-transcriptional modification or protein-protein interactions. Based on evidence for the later mechanism (Jupin and Chua, 1996; Stange *et al.*, 1997), we tried to identify regulatory proteins that can interact with TGA2.2. A MY1HS strategy was established and employed using a TGA2.2 bait protein bound to an *as-1* element found upstream of a *HIS3* reporter gene, to screen a cDNA expression library from Arabidopsis and tobacco vegetative tissues for interacting proteins.

Recently, several different yeast hybrid screens were conducted in our group to isolate TGA interacting partners. For instance, Thurow (2002) was successful in isolating a tobacco NPR1 homolog using TGA2.1-GAL4BD as a bait in a classical Y2HS approach. In another approach, utilizing TGA2.1 in a split-protein sensor system that is based on the reconstitution of ubiquitin-mediated cleavage of a reporter protein, a DEAD box RNA-helicase and an E2 ligase were identified as TGA2.1-interacting partners (Krawczyk, 2003). Siemsen (2002) identified a scarecrow-like transcription factor that acts as a putative TGA2 coactivator protein using a MY1HS that utlized TGA2 from Arabidopsis as a bait protein and an Arabidopsis cDNA library, which was not fused to any activation domain.

In order to identify TGA2.2-interacting proteins, a MY1HS that utilizes TGA2.2 bound to the *as-1* element was deployed (Figure 6.1). This novel screening method should identify prey proteins that are able to interact with TGA2.2 bait protein to assemble an *as-1* bound activation complex, which will lead to the activation of a reporter gene. Similar approaches, termed as the one-and-a-half hybrid system (Serebriiskii *et al.*, 2001), were described previously in a number of instances, such as

the identification of regulatory proteins that are involved in activation of *ftz*-dependent promoters (Yu *et al.*, 1999).

The MY1HS screening conditions were optimized to work with established positive controls. Bait protein expression is considered to be a limiting criterion before performing any yeast hybrid screen (Toby and Golemis, 2001). The TGA2.2 bait protein was under the control of the *Met25* promoter cassette that can regulate the transcription of the *TGA2.2* coding sequence upon adding or omitting methionine in the culture medium, *i.e.,* an "on" or "off" switch system for the expression of the TGA2.2 bait protein. TGA2.2 overexpression was toxic to the YRWH2 screening strain. The problem was resolved by lowering TGA2.2 expression levels in YRWH2 through methionine application to the screening media. The toxic effect of TGA2.2 on the YRWH2 cells might be due to TGA2.2 binding to other *cis*-elments found in yeast promoter regions. The quality of a cDNA library is critical to the success of any yeast hybrid screen. In the current study, the cDNA inserts (from the Arabidopsis and tobacco cDNA library plasmids) were driven by a truncated *ADH1* promoter. The weak expression of cDNA inserts is considered as a serious pitfall for any yeast hybrid screen (Agatep *et al.*, 1998). The detection of protein expressed from the truncated *ADH1* promoter using an immunoblot analysis was not applicable.

The MY1HS screens were successful in identifying several TGA2.2-interacting proteins (Table 6.1 and Table 6.1). In this regard, it might be significant to mention that two types of cDNA clones were expected to activate reporter gene expression: those that bind directly to the *as-1* element and those that interact directly with TGA2.2 but do not bind to the *as-1* elements. In the MY1HS screens using the tobacco cDNA library, two TGA transcription factor-related cDNAs, TGA2.1 and TGA10 were isolated (Table 6.2). The observation that a *TGA10* cDNA clone was isolated in the MY1HS using a cDNA library prepared from tobacco leaves suggests that the *TGA10* mRNA might be expressed in vegetative tissues. However, RT-PCR and protein immunoblot expression analysis demonstrated that TGA10 is a root specific TGA transcription factor

(Schiermeyer *et al.*, 2003). Even though, it is necessary to conduct an independent expression analysis to confirm these results.

The MY1HS screens had identified other candidates that can interact with TGA2.2 without binding to the *as-1* element. A screen with an Arabidopsis cDNA library yielded four putative TGA2.2-interacting candidates (Table 6.1). However, there were distinct and overlapping interaction patterns among the four putative candidates with the TGA transcription factors. For instance, the Y2HS experiments revealed that the isolated *At4g00270* cDNA clone did not interacted with TGA2.2 prey protein when it was fused to the GAL4BD domain (data not shown). Among the isolated TGA2.2 interacting proteins, the focus was concentrated on the characterizing of two cDNA groups, groups 3 and 4, respectively (Table 6.1 and Table 6.1).

Group 3 cDNA inserts, encoding the At1g50570 and At5g55530 proteins, respectively, were selected for multiple reasons. First, both cDNA inserts encode for two homologous proteins. Second, the presence of the conserved C2 domain, which is involved in a variety of signal transduction pathways. Finally, the group interacts exclusively with the class-II of TGA factors. The group 4 cDNA insert, encoding the At1g28480 protein, was selected because of its ability to interact with all identified TGA factors, sequence similarity to glutaredoxins and its potential ability to mediate changes of the redox state of TGA factors.

7.2 Functional Analysis of At1g50570 and At5g55530 Proteins

Both the At1g50570 and At5g55530 proteins contain a typical C2 domain (Figure 6.8), which was originally identified in protein kinase C and has been described in more than 60 proteins (Nalefski and Falke, 1996; Rizo and Südhof, 1998). Numerous genes encoding proteins with C2 domains are expressed in plants and are speculated to be involved in a variety of signal transduction pathways (Kopka *et al.*, 1998).

Recently, Arabidopsis C2 domain containing proteins have been identified and their role as signalling proteins was confirmed (Jambunathan *et al.*, 2001; Qin and

Wang, 2002). For instance, a humidity-sensitive Arabidopsis mutant, which encodes a copin protein that contains two C2 domains, had an increased resistance to virulent bacterial and oomyceteous pathogens (Jambunathan *et al.*, 2001). The amino acid sequences comparison of At1g50570 and At5g55530 with other sequences of different plant species such as tomato, potato, soybean, rice, maize and corn (http://tigr.org) revealed that the At1g50570 and At5g55530 amino acid sequences are conserved within the plant kingdom. In Arabidopsis, the amino acid sequences of At1g50570 and At1g55530 showed consensus amino acids similarity (~38%) to a third protein, known as At5g12300. The At5g12300 interaction with TGA2.2 was not studied, however, it might be possible that the three proteins have redundant functions.

Although the role of C2 domain in mediating protein-protein interactions is well documented (Rickman and Davletov, 2003; Zhang, 2003A), it is most likely that it is not involved in the At1g50570 and At5g55530 interactions with TGA2.2 because the *At1g50570* cDNA isolated in the MY1HS, encodes a truncated protein that lacks 32 amino acids of the C2 domain. Probably, the interaction region is defined to the conserved regions that are found downstream of the C2 domain (Figure 6.8).

Transient expression of At1g50570-GFP protein in BY-2 protoplast revealed that the At1g50570-GFP was targeted to the nuclear envelope and to the endoplasmic reticulum (Figure 6.18). As TGA-factors are staidly localized to the nucleus, it has to be postulated that an interaction can only occur upon a stimulus that changes the localization of At1g50570. Indeed, the membrane binding activity of C2-domain containing phospholipid binding proteins can be changed in response to calcium. Usually, the C2 domain binds Ca^{2+} and mediates Ca^{2+}-dependent membrane targeting of proteins, *i.e.,* the C2 domain containing proteins under resting state (low Ca^{2+} levels) are dispersed in the cytoplasm and the nucleus (Cho, 2001). The activation of 5-lipoxygenase protein involves its calcium-dependent translocation from the cytoplasm and the nucleus to the nuclear envelope via a C2-like domain (Kulkarni *et al.*, 2002). In addition, there are C2 domains whose binding to membranes does not require calcium. Das *et al.*, 2003, showed that C2 domain of PTEN, a tumor suppressor protein, was

necessary and sufficient for the Ca^{2+}-independent targeting of PTEN from cytoplasm and the nucleus to the plasma membrane. Intuitively, one can assume the presence of C2 domain-inducing conditions (high Ca^{2+} levels) for the BY-2 protoplasts that mediate the constitutive targeting of At1g50570-GFP to membranes. Experiments designed to observe a difference in At1g50570 localization upon addition of SA or auxin were unsuccessful. However, as protoplast represent de-differentiated cell with putatively not-active signal transduction pathways, experiments have to be repeated with onion epidermal cells, using particle bombardment technique.

The mRNA accumulation analysis suggests that *At1g50570* transcripts are expressed constitutively in Arabidopsis plants (Figure 6.16 and Figure 6.17). The observed *At1g50570* expression profile is in congruence with the presence of over 20 ESTs that show complete sequence identity with the *At1g50570* gene and were isolated from different organs and challenged plants (http://arabidopsis.org). Time course experiments showed that the transcription of *At1g50570* was not influenced by SA treatment (Figure 6.17). However, *At1g50570* expression did increase moderately, when Arabidopsis plants were challenged with *P. syringae* (Figure 6.17). The *At1g50570* response was late, appeared after 24 and 48 hours post pathogen infection, which resembles the induction kinetics of other SAR marker genes such as *PR-1* gene (Figure 6.17 *vs.* Figure 6.41). Interestingly, in plants challenged with *P. syringae* virulent strain, the *PR-1* transcripts accumulated transiently (start increasing after 12 hours, reached maximum after 24 and then decreased after 48 hours), whereas the *At1g50570* mRNA continued to increase steadily after 12 hours from pathogen infection (Figure 6.17 *vs.* Figure 6.41). These observations might indicate that the At1g50570 is not involved in mediating the *PR-1* gene accumulation in response to treatments that induce SAR signalling. However, it is possible that the observed *At1g50570* transcript accumulation after pathogen challenge is mediating other resistance responses against pathogen rather than SAR.

The At1g50570 and At5g55530 proteins were demonstrated to function as transcriptional activators in yeast cells (Figure 5.11). These results strongly suggest

that both proteins can mediate *as-1*-dependent gene activation by interaction with TGA2.2, *i.e.,* once bound to the TGA2.2, At1g50570 and At5g55530 can act as coactivator that might be recruited to the general transcription machinery. The sequence analysis of the *At1g50570* isolated cDNA insert revealed the absence of in-frame fusion with the GAL4AD, indicating that the truncated At1g50570 protein was able to interact with TGA2.2 and to transactivate the reporter gene expression. This behavior of the At1g50570 truncated protein mimics the strategy used by Siemsen (2002), where a cDNA library that is not fused to any AD was used to identify TGA2 coactivator proteins. Surprisingly, GAL4BD-At1g50570 truncated protein (encoded in the At1g50570 isolated cDNA) showed low transactivation activity levels in yeast when compared with the GAL4BD-At1g50570 full-length protein (Figure 6.10 vs. Figure 6.11). It is most likely that length of At1g50570 protein is crucial to achieve its full activation potential, however, this low transactivation activity of the GAL4BD-At1g50570 truncated protein could be attributed to the yeast hybrid system itself. For instance, Zhang *et al.*, 1999, reported that the N-terminal region of NPR1 has low levels of reporter gene transcriptional activity when compared with the full-length protein, which didn't activate the expression of reporter gene at all. The nature of the transactivation domain is not clear, however, it is most likely located downstream of the C2 domain, as the comparison of deduced amino acids sequence of the At1g50570 and At5g55530 upstream of the C2 domain showed no significant sequence identity (Figure 6.8).

The results showed that At1g50570 protein could function as transcription activator of an *as-1*-reporter gene when transfected into protoplasts as effector plasmids (Figure 6.19). The At1g50570 transactivation levels were considerably moderate (~2 fold-induction) when compared with the basal transactivation levels of the *as-1*-reporter gene alone. These moderate levels of *as-1*-dependent reporter gene transactivation could be attributed to the presence of trace levels of At1g50570 protein in the nucleus (Figure 6.18B). In order to test whether At1g50570 nuclear targeting can enhance *as-1*-dependent reporter gene transactivation, *as-1-GUS* reporter

transactivation assays were conducted using 10% DMSO, which was shown to induced higher At1g50570-GFP protein accumulation in the nucleus. Unfortunately, the high DMSO treatment was extremely toxic for BY-2 protoplasts, thus it was unfeasible to test the effect of DMSO-dependent At1g50570 nuclear localization on the *as-1*-dependent transcriptional activity. Additionally, the *as-1*-reporter gene transcriptional activity was tested using an At1g50570 protein fused to an NLS (exclusively targeted to nucleus (data not shown). The NLS-At1g50570-GFP protein had repressed the *as-1-GUS* and the *5x UAS^{GAL4}::uidA* (used as a control) reporter genes activities, indicating the presence of a general toxic effect on the BY-2 protoplasts (Figure 6.20). In conclusion, the yeast and protoplasts transactivation experiments indicate that At1g50570 can act as a coactivator protein *in vivo*. The open question still is: what are the mechanisms that regulate the At1g50570 targeting to nucleus where it can function as a transcriptional activator. The At1g50570 lethal effect might suggest a potential function in HR establishment.

In Y2HS, both the At1g50570 and At5g55530 interacted strongly with class-II of tobacco and Arabidopsis TGA transcription factors, whereas, no interactions with tobacco TGA1a and TGA10 factors were detected (Figure 6.10). This result suggests that the interactions between At1g50570 and the class-II of tobacco TGA factors may be highly specific. Interestingly, the At1g50570 interaction with TGA factors behaves similar to the NPR1 protein. The Arabidopsis NPR1 protein didn't interact with the tobacco TGA1a and TGA10 factors in the Y2HS (Thurow, 2002). NPR1 was found to interact with Arabidopsis TGA2, TGA3, TGA5, TGA6, and TGA7 factors in yeast, whereas no interaction with TGA1 and TGA4 was observed (Després *et al.*, 2000). Similarly to the NPR1 protein, the specificity of the interaction is defined to the C-terminal part of TGA2.2 downstream of the bZIP region (data not shown). In conclusion, the At1g50570 interaction with TGA2.2 mimics that of NPR1. One can assume that the interaction between At1g50570 and the TGA1 factor requires the redox control of TGA1 (Després *et al.*, 2003). To interact with NPR1, two critical cysteine residues within the TGA1 should be reduced. The formation of a TGA1 intramolecular disulfide bridge

inhibits the interaction with NPR1. It might be possible that At1g50570 needs to interact with a reduced form of TGA1 or TGA1a.

The interaction between TGA2.2 and At1g50570 was further confirmed in GST pull-down, Far Western and EMSA experiments (Figure 6.13, Figure 6.14 and Figure 6.15). GST-At1g50570 protein interacts with two TGA2.2 dimers bound to the *as-1* element palindromes in an EMSA. These results suggest that the interaction between At1g50570 and the class-II of TGA factors has a high value of specificity (Figure 5.15). Krawczyk *et al.* (2002) have shown that the conserved spacing between the two centers of the palindromes of the *as-1* element is essential for conferring full transcriptional activity *in vivo*. In this regard, the presence of a coactivator that associates only with two correctly spaced TGA dimers was postulated. To further confirm these observations, the interaction between TGA2.2 and At1g50570 was tested in a MY1HS using YTSH2 strain, which carries a *HIS3* reporter gene downstream of three-times tandemly repeated *as-1-2* elements (the *as-1* lacked two bp between the two palindromes) (Siemsen, 2002). However, At1g50570 does interact with TGA2.2 in the YTSH2 strain, indicating that the conserved spacing in the *as-1* element was not crucial for the interaction to take place (data not shown).

As observed above, several lines of evidence suggest that At1g50570 and At5g55530 proteins might activate expression of *as-1* responsive genes by interacting with TGA class-II transcription factors. However, does the At1g50570 protein act as a positive regulator of TGA-responsive genes in plants? To answer this question, At1g50570 antisense and overexpression transgenic lines were generated and analyzed. Although At1g50570 antisense approach was successful, no increase or decreases in the basal levels of *PR-1* gene expression were observed (Figure 6.21 and Figure 6.22). At1g50570 and At5g55530 are highly homologous and exhibit similar TGA2.2 interaction patterns, thus it might be possible that both proteins are functionally redundant.

The overexpression of *At1g50570* failed to produce any phenotype related to gene expression under non-induced conditions (Figure 6.24 and Figure 6.25). It might

be possible that the At1g50570 protein is not able to interact with TGA factors under these conditions. At1g50570 might undergo some kind of relocalization, which might be mediated by SA or auxin treatments, that will enable it to interact with TGA factors. Overexpression of *NPR1*, which interacts with TGA factors, did not lead to any constitutive *PR* gene expression under non-inducing conditions but the *PR* gene induction after pathogen infection was stronger, but not quicker, when compared to wild type plants (Cao *et al.*, 1998). These observations suggested that SAR inducers modulate an NPR1 activation step. Mou *et al.* (2003) showed that INA, a SAR inducer, activates NPR1 by converting it from an oligomeric form (oxidized), which is arrested in cytoplasm, to a monomeric form (reduced) that is localized to the nuclear. For At1g50570, it is tempting to speculate that the mechanism of At1g50570 activation requires its nuclear localization. A logical assumption is that under non-inducing conditions, At1g50570 is localized to cellular membranes rather than the nucleus. The presence of C2 domain in At1g50570 strengthens this assumption (Figure 6.18). As described above, At1g50570 might respond to a certain stimulus that will lead eventually to its release from the cellular membranes and make it available to interact with TGA factors in the nucleus.

In summary, two novel proteins, At1g50570 and At5g55530, were found to interact strongly with the TGA2.2 transcription factor in yeast hybrid systems and *in vitro*. In future experiments, the At1g50570 and TGA2.2 interactions should be verified *in vivo* using the tandem affinity purification tag system that allows rapid purification of native protein complexes (Rigaut *et al.*, 1999). The redundant or overlapping function that might exist between the two homologous proteins might be resolved by the generation of a double knockout mutant. Also, it might be interesting to demonstrate the *in vivo* function of At1g50570 by generating NLS-At1g50570 overexpression transgenic lines or by regulating the subcellular localization of At1g50570 by generating a fusion with the rat glucocorticoid receptor HBD in a double knockout mutant background (Picard *et al.*, 1988). The rat glucocorticoid receptor HBD system was used to control the nuclear transport of various transcriptional regulators in Arabidopsis (Kinkema *et*

al., 2000; Wagner *et al.*, 1999). The outputs of these experiments might give a conclusive evidence for the roles of these proteins in regulating TGA factors activity.

7.3 Functional Analysis of At1g28480 Protein

The identification of At1g28480, a glutaredoxin protein, as a TGA2.2 interacting protein emerged when several recent reports demonstrated the importance of redox regulation in SAR establishment. There is a growing body of evidence that indicates that an altered redox state of the cell can activate different signaling pathways in plants. For instance, the generation of ROS, *e.g.,* H_2O_2, during the oxidative burst is one of the earliest cellular responses to pathogens (Apel and Hirt, 2004; Lamb and Dixon, 1997). Increasing evidences suggest that H_2O_2, which is generated during the oxidative burst, functions as a signaling molecule in the plant immune system. For instance, H_2O_2 induces the expression of defense-related genes such as the Arabidopsis *GSTs* and *PR-1* (Chen and Singh, 1999; Klessig *et al.*, 2000). Microarray analysis of H_2O_2-induced gene expression in Arabidopsis revealed that the *as-1* promoter element is a potential H_2O_2-responsive *cis*-element (Desikan *et al.*, 2001). In the contrary, Garreton *et al.* (2002) reported that binding of the TGA transcription factors to *as-1* element is responsive to oxidative species other than H_2O_2. In consistence with these observations, the TGA1 and NPR1 transcriptional regulators were found to undergo some kind of redox modifications (Després *et al.*, 2003, and Mou *et al.*, 2003). What is the molecular basis of the redox signal that mediates the modification of both proteins? Both authors speculate the involvement of thioredoxins, glutaredoxins and Ref-1, an apurinic/apyrimidinic endonuclease that is known to mediate the redox regulation of many eukaryotic transcription factors. The identification of the At1g28480 glutaredoxin appeared as the missing link that could regulate TGA factors by redox processes.

Glutaredoxins, also known as thioltransferases, are small disulfide reducing enzymes that have been shown to catalyze the reactivation of many oxidatively modified proteins (Holmgren, 1989). Glutaredoxins are part of the thioredoxin superfamily of proteins that includes thioredoxin, GST and glutathione peroxidases (Åslund and Beckwith, 1999). Usually, gluatredoxin has a conserved active site

consensus sequence of “YCPYC” that is involved in the reversible oxidation by the formation of a disulfide bond between the two active site cysteine residues (Figure 6.26; Berardi and Bushweller, 1999). The glutaredoxin reactivity with glutathione is mediated by a glutathione-binding site (Bushweller *et al.*, 1994). The glutathione-binding site mediates the formation of glutathione-containing mixed disulfides bridge, implying that glutaredoxin has a role in glutathionylation and deglutathionylation of target proteins. Beside its essential function for the glutathione-dependent reduction of ribonucleotides to deoxyribonucleotides by the ribonucleotide reductase enzyme (Holmgren, 1979), glutaredoxin has been shown to be a molecule that operates in the redox-regulation of gene expression via modulation of transcription factors. For instance, the activation OxyR transcriptional factor of *E. coli* is reversed by cellular disulfide-reducing machinery that involves a glutaredoxin (Zheng *et al.*, 1998). Several studies have demonstrated that the glutaredoxin can modulate the DNA binding and transcriptional activities of several transcription factors, *e.g.,* NF*κ*B, AP-1 and NFI (Bandyopadhyay, *et al.*, 1998, Hirota, *et al.*, 2000).

Glutaredoxins have been isolated and characterized from different organisms such as *E. coli* (Åslund *et al.*, 1996), yeast (Grant, 2001) and humans (Lundberg *et al.*, 2001). However, studies about plant glutaredoxin enzymatic properties, structure and characteristics are scarce (Meyer *et al.*, 1999). Minakuchi *et al.* (1994) had characterized the first plant glutaredoxin cDNA from rice, while Morell *et al*. (1994) had identified and localized the first glutaredoxin in spinach leaves. The first hint about glutaredoxin function in plants came after the detection of glutaredoxin in phloem sap of different plant species (Szederkenyi *et al.*, 1997). Since this discovery, only little information on glutaredoxin function in plants has emerged. Furthermore, the target proteins of glutaredoxins in plants are not known. For instance, a poplar phloem peroxiredoxin, which was isolated from a xylem/phloem cDNA library and known for its role in regulating intracellular levels of H_2O_2, was found to accept protons from a poplar glutaredoxin (Rouhier *et al.*, 2002b). In the genome of Arabidopsis, there are at least 24 glutaredoxins or glutaredoxin-like proteins (Meyer *et al.*, 1999). Only two Arabidopsis

glutaredoxins, At5g20500 and At5g40370, showed the conserved active site consensus sequence of “YCPYC”. The information about Arabidopsis glutaredoxins is scarce.

In the present study, At1g28480 clone accounted for 24 of 45 positives clones identified in the YM1HS, although its low expression does not imply an overrepresentation of the clone in the cDNA library (Table 6.1). The interaction between the At1g28480 glutaredoxin and all identified members of the tobacco TGA transcription factors was demonstrated using the Y2HS (Figure 6.30). Interestingly, the At1g28480 glutaredoxin was able to interact strongly with class-I of tobacco TGA transcription factors, which does not interact with NPR1 and At1g50570 proteins in yeast. This result might suggest that At1g28480 could modulate the redox state of class-I TGA transcription factors by reducing the previously described conserved cysteine residues in their amino acid sequences (Després *et al.*, 2003). The specificity of the interaction between TGA2.2 and At1g28480 glutaredoxin was demonstrated by testing the interaction with two other glutaredoxins, At5g20500 and At5g40370. Both classical glutaredoxins failed to interact with TGA2.2 in Y2HS, indicating that the interaction between TGA2.2 and At1g28480 is indeed specific. The At5g20500 and At5g40370 glutaredoxins showed amino acids similarity to At1g28480 of 48% and 52%, respectively, indicating that their sequence similarities with At1g28480 are considerably low. The highest sequence similarity with At1g28480 (45% identity, 60% similarity) was found with monocysteinic At1g03850 glutaredoxin (Figure 5.26). At1g03850 is predicted to be a chloroplastic-targeted protein. Therefore the interaction between At1g03850 with TGA2.2 was not studied. The amino acid sequences comparison of At1g28480 with other sequences of different plant species revealed that the its amino acid sequence is conserved within plant kingdom (http://tigr.org).

In TGA2.2, the C-terminal part downstream of the bZIP region was defined as the interaction region with At1g28480 (data not shown). Interestingly, sequence analysis of the tobacco and Arabidopsis class-II TGA transcription factors revealed that they contain a single conserved cysteine in their putative interaction region. Therefore, the nature of the interaction between TGA2.2 and At1g28480 was analyzed further by

using different *At1g28480* and *TGA2.2* cysteine point mutations (Figure 6.31). A small percentage of TGA2.2 protein was able to form an oligomer under oxidizing conditions when compared with the TGA2.2^{Cys181Ser} mutant protein (Figure 6.32). These results indicate that the TGA2.2 single conserved cysteine might be involved in disulfide formation and it could be sensitive to redox changes. Interestingly, Lenk (2001) had identified a similar band in his immunoblot analysis using protein extracts prepared from SNN tobacco plants overexpressing a TGA2.1 deleted form (lacking 29 amino acids from the TGA2.1 N-terminal region). Lenk (2001) speculated that a heterodimer between TGA2.2 and TGA2.1, which might be mediated by the conserved cysteine residue, is responsible for the corresponding band.

The TGA2.2^{Cys181Ser} mutant protein was found to interact with the At1g28480 glutaredoxin, indicating that the single conserved cysteine of TGA2.2 is not involved in the interaction with the At1g28480 protein. Several previous reports had shown that mutations in cysteine residues of many proteins, which had shown *in vitro* redox sensitivity, did not show redox-regulated functions *in vivo*. For instance, Ordway *et al.* (2003) found that a mutation in cysteine 64 of mouse Ref-1, which was previously implicated in reduction of oxidized cysteine residues within the DNA binding domains of several transcription factors *in vitro*, didn't have any influence on the *in vivo* AP-1 transcription factor DNA binding activity.

An At1g28480 cysteine single mutant (GSM; CCMS; C55S), which retains only cysteine 52 in its redox active site, displays the same binding specificity for TGA2.2 as At1g28480 (Table 6.3). These observations indicate that active site cysteine 55 is not required for At1g28480-TGA2.2 interaction. When two point mutations in both At1g28480 glutaredoxin active site cysteine residues (GDM; SCMS; C52S and C55S) were introduced, an alteration in the TGA2.2-At1g28480 interaction affinities was observed (Table 6.3). This suggests that the At1g28480 glutaredoxin redox active center might be involved in modulating the interaction with TGA2.2. Intriguingly, the GAL4AD-GDM was not able to interact with TGA2.2, while GAL4BD interacted weakly when compared with GAL4AD- and GAL4BD-At1g28480, respectively (Table 6.3). It

could be possible that the TGA2.2-GDM loss of interaction is related to weak expression levels of GDM in yeast. Unlike classical glutaredoxins that have a conserved active site sequence of “YCPYC”, At1g28480 glutaredoxin contains an additional conserved cysteine residue at position 53 (Figure 6.26). The presence of CCMC sequence as the redox active site might suggest that cysteine 53 might substitute the cysteine 52 if mutated. Data supporting such assumptions are still lacking.

The confirmation of the interaction between TGA2.2 and At1g50570 by *in vitro* means and the analysis of At1g28480 glutaredoxin activity in a redox assay were not feasible. At1g28480 production in *E. coli* expression systems yielded very low amounts of At1g28480 protein (data not shown). Rouhier *et al.* (2002c) had reported low expression of a poplar glutaredoxin in *E. coli* and they proposed a strategy used to optimize it production in *E. coli* through the manipulation of its cDNA sequence. However, the interaction between At1g28480 and TGA2.2 was confirmed by a protoplast two-hybrid system (Figure 6.38). At1g28480 was detected in cytoplasm and nucleus (Figure 6.39). These findings demonstrated that both proteins could interact in plant cells.

To better understand the function of At1g28480 in regulating TGA2.2, several experiments were conducted in yeast to analyze the putative function of At1g28480. The effect of At1g28480 or GDM overexpression on the interaction between TGA2.2 monomers was studied using Y3HS. The results clearly showed that At1g28480 enhanced indirectly *in vivo* TGA2.2 interaction and *in vitro* binding to the *as-1* element by increasing TGA2.2 protein levels in yeast cells (Figure 6.34 and Figure 6.35). Similar results were obtained using the GDM protein (data not shown). It is most likely that At1g28480 acts like a chaperone, *i.e.,* provides a suitable microenvironment where TGA2.2 is more stable or free to interact. Molecular chaperones are a class of polypeptide-binding proteins that are implicated in protein folding, protein targeting to membranes, protein renaturation or degradation after stress and the control of protein-protein interactions (reviewed in Hartl and Hayer-Hartl, 2002). Several glutaredoxin or

glutaredoxin like proteins have been shown to display a chaperone-like activity (Kern *el al.*, 2003; Lundström-Ljung and Holmgren, 1995). However, the mode of action of the At1g28480 glutaredoxin protein in increasing the TGA2.2 protein levels in yeast is unknown.

A property of signalling proteins in general is that treatments that activate them often enhance expression of their genes. *At1g28480* expression levels respond markedly to pathogen infection and SA treatment (Figure 6.41). The onset of expression of *At1g28480* after virulent pathogen infection was earlier than the onset of the *PR-1* gene. Similarly, *At1g28480* transcript appeared after two hours after SA treatment. Somehow, the increase in *At1g28480* transcription is correlated to subsequent *PR-1* transcript accumulation (Figure 6.41). These induction patterns suggest that the accumulation of the *At1g28480* transcript might be involved functionally in the regulation of plant defense or SA-mediated responses through At1g28480 interaction with TGA factors. The overall picture of such speculated events are still unclear and should be investigated in the future. Interestingly, the observation that At1g28480 can form a ternary complex with TGA factors and NPR1 in yeast might link the function of At1g28480 to the NPR1, which is the key regulator of *PR* genes expression. The ternary complex formation might be responsible for the modulation of DNA-binding specificity and/or transcriptional activity of TGA transcription factors in plants. As described above, At1g28480 glutaredoxin might function as a chaperone, thus it might be possible that At1g28480 stabilizes the interaction between NPR1 and TGA2.2 or it might increase the interaction affinity between both proteins. However, the significance of the At1g28480-TGA2.2-NPR1 ternary complex still unclear and further characterization should be carried out.

To test the above hypothesis, transgenic plants that overexpress or repress expression of *At1g28480* were analyzed. The *At1g28480* encoding mRNA was severely reduced in the RNAi lines, while At1g28480 protein levels significantly accumulated in the overexpressor lines (Figure 6.42 and Figure 6.44). One At1g28480 overexpressor line revealed a distinguishable phenotype under normal conditions (Figure 6.44). The

At1g28480 overexpression line #7 plant phenotypes of irregular leaf shape, cotyledon epinasty, late flowering time and crinkled fruits mimic those of *JAW* locus, which produces a microRNA that can guide messenger RNA cleavage of several *TCP* genes, which encode transcription factors, that control leaf development (Palatnik *et al.*, 2003). It might be interesting to characterize this transgenic line and to verify if it is corresponding to *At1g28480* overexpression or not. Similarly to *At1g50570*, the overexpression or repression of *At1g28480* failed to produce any phenotypes related to SA-inducible gene expression (Figure 6.43 and Figure 6.45). These observations should be interrupted carefully as the selected gene promoters contain, in addition to *as-1* element, both positive and negative regulatory elements (Lebel *et al.*, 1998). Therefore, regulation of these promoters is certainly more complicated than what is thought. It might be possible that other promoter elements besides *as-1*, or other transcription factors in addition to TGA transcription factors, are important for enhancing or repressing the expression of the selected marker genes and as a subsequence they will mask the function of At1g28480 or At1g50570. As discussed above, the TGA factors can function as both positive and negative regulators of SA-dependent gene expression, which make it difficult to define the function of any specific factor. In contrast, At1g28480 overexpressor transgenic lines revealed weak GUS expression after 2,4-D induction. TGA transcription factors regulate several auxin-induced genes such as *GNT35* and *Nt103*, which are involved in plant defense against xenobiotic stresses (Johnson *et al.*, 2001b; Niggeweg *et al.*, 2000; Pascuzzi *et al.*, 1998). One likely mechanism suggested here, which involves At1g28480, is the preventing of DNA binding of TGA transcription factor to the *as-1* element. It might be possible that the At1g28480 glutaredoxin activity quenches an oxidative stress signal that leads to the activation of TGA transcription factors (Garreton *et al.*, 2002). Further analysis should be conducted to determine the role and the mechanism by which At1g28480 represses the auxin inducible gene expression.

In summary, the At1g28480 protein seems to interact with TGA2.2 as a chaperone in yeast hybrid systems. In future experiments, the At1g28480 and TGA2.2 interactions should be verified by *in vitro* and *in vivo* (TAP-tag) methods. The redox

properties of At1g28480 should be demonstrated using purified proteins or through complementation assay in *E. coli* as described previously (Bick *et al.*, 1998; Rouhier *et al.*, 2003). The influence of At1g28480 on auxin-dependent gene expression should be further analyzed using transgenic plants that overexpress the GDM mutant, which might help into better understand the mechanism by which the At1g28480 influence the auxin inducible gene expression. The DNA microarray analysis could be powerful approach for the elucidation of At1g28480 function and its regulatory networks (Schenk *et al.*, 2000).

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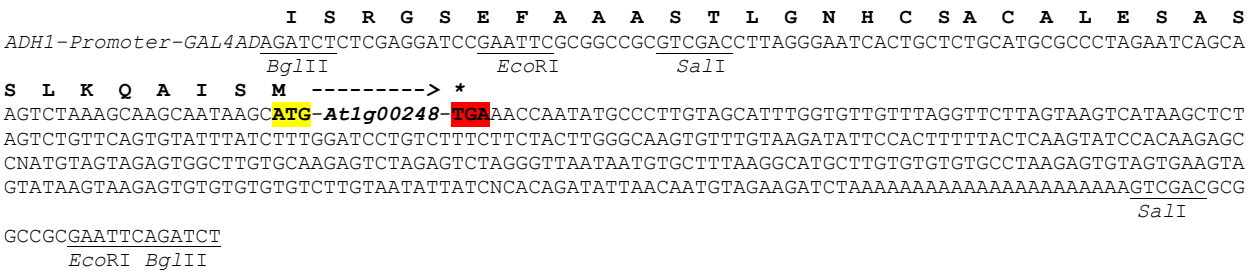
9 Appendix

8.1 Sequences

8.1.1 Arabidopsis Isolated cDNA inserts

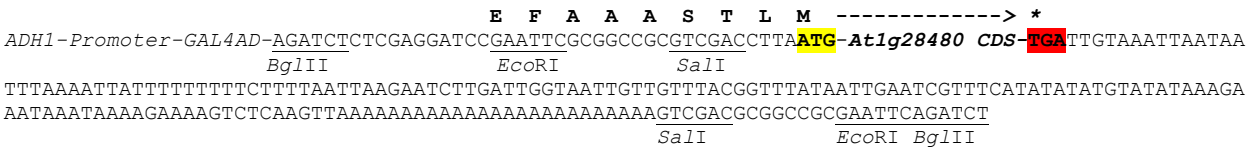
8.1.1.1 pGAD10/At4g00270

Rest sequence: pGAD10



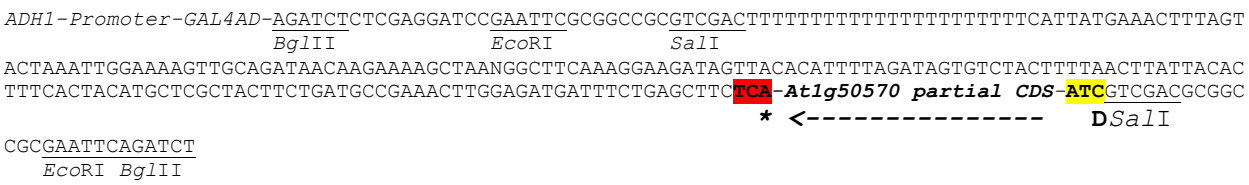
8.1.1.2 pGAD10/At1g28480

Rest sequence: pGAD10



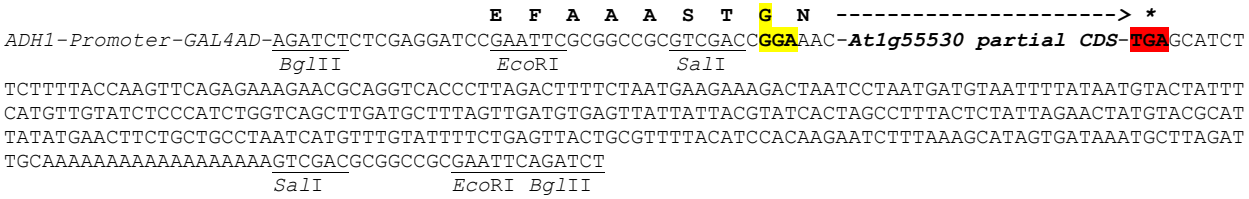
8.1.1.3 pGAD10/At1g50570

Rest sequence: pGAD10



8.1.1.4 pGAD10/At5g55530

Rest sequence: pGAD10



8.1.2 *At1g28480* and *TGA2.2* mutants

8.1.2.1 GDM

```
M  Q  G  T  I  S  C  A  R  N  Y  N  M  T  T  T  V  G  E  S  L  R  P  L  S  L  K  T  Q  G  N  G
ATGCAAGGAACGATTTCTTGTGCAAGAAATTATAACATGACGACAACCGTCGGGGAATCTCTGCGGCCGCTATCGCTTAAAACGCAGGGAAACGGC

E  R  V  R  M  V  V  E  E  N  A  V  I  V  I  G  R  R  G  S  C  M  S  H  V  V  R  R  L  L  L  G
GAGAGAGTTCGGATGGTGGTGGAGGAGAACGCGGTGATTGTGATTGGACGGAGAGGATCTTGCATGTCTCATGTGGTGAGGAGGCTGCTTCTTGGA

L  G  V  N  P  A  V  L  E  I  D  E  E  R  E  D  E  V  L  S  E  L  E  N  I  G  V  Q  G  G  G  G
CTTGGAGTGAATCCGGCGGTCCTTGAGATTGATGAGGAGAGGGAAGATGAAGTTTTGAGTGAGTTGGAGAATATTGGAGTTCAAGGCGGCGGAGGT

T  V  K  L  P  A  V  Y  V  G  G  R  L  F  G  G  L  D  R  V  M  A  T  H  I  S  G  E  L  V  P  I
ACGGTGAAGTTACCGGCGGTTTATGTAGGAGGGAGGTTGTTTGGAGGGTTAGATAGGGTTATGGCTACTCATATCTCCGGTGAGTTAGTTCCAATT

L  K  E  V  G  A  L  W  L  *
CTTAAGGAAGTTGGGGCTCTGTGGTTGTGA
```

8.1.2.2 GSM

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M  Q  G  T  I  S  C  A  R  N  Y  N  M  T  T  T  V  G  E  S  L  R  P  L  S  L  K  T  Q  G  N  G
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E  R  V  R  M  V  V  E  E  N  A  V  I  V  I  G  R  R  G  S  C  M  C  H  V  V  R  R  L  L  L  G
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L  G  V  N  P  A  V  L  E  I  D  E  E  R  E  D  E  V  L  S  E  L  E  N  I  G  V  Q  G  G  G  G
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T  V  K  L  P  A  V  Y  V  G  G  R  L  F  G  G  L  D  R  V  M  A  T  H  I  S  G  E  L  V  P  I
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L  K  E  V  G  A  L  W  L  *
CTTAAGGAAGTTGGGGCTCTGTGGTTGTGA
```

8.1.2.3 TGA2.2^{Cys181Ser}

```
M  A  D  I  S  P  S  T  S  T  D  A  D  T  E  D  K  N  R  F  L  N  S  Q  Q  L  G  A  V  A  S  D
ATGGCTGATATCAGTCCTAGTACATCAACAGATGCCGATACGGAAGATAAGAACAGGTTCCTAAATTCTCAACAACTGGGTGCGGTAGCTTCTGAT

G  S  D  R  T  R  D  Q  K  T  L  R  R  L  A  Q  N  R  E  A  A  R  K  S  R  L  R  K  K  A  Y  V
GGAAGTGACAGGACAAGAGATCAGAAGACACTTCGTAGACTTGCCCAAAATCGTGAAGCAGCTCGAAAAAGTCGTCTAAGGAAAAAGGCATATGTT

Q  Q  L  E  S  S  R  M  K  L  T  Q  L  E  Q  E  L  Q  R  A  R  Q  Q  G  I  F  I  S  G  S  G  D
CAACAGTTAGAGAGCAGCCGGATGAAGCTGACACAACTAGAGCAGGAACTTCAACGAGCTCGACAACAGGGCATATTTATTTCAGGTTCAGGAGAT

Q  S  Q  S  M  S  G  N  G  A  L  A  F  D  V  E  Y  A  R  W  L  E  E  Q  N  R  R  I  N  E  L  R
CAATCACAGTCGATGAGCGGAAATGGAGCTTTGGCATTTGATGTAGAATATGCCCGGTGGTTGGAGGAGCAGAACCGACGAATTAATGAGCTAAGG

G  A  V  N  S  H  A  G  D  G  E  L  R  I  I  V  D  G  I  L  A  H  Y  D  D  I  F  R  I  K  G  D
GGAGCTGTAAATTCTCATGCTGGTGATGGTGAACTTCGCATAATTGTCGACGGTATCTTAGCACACTATGATGACATATTCAGGATAAAAGGGGAT

A  A  K  S  D  V  F  H  I  L  S  G  M  W  K  T  P  A  E  R  S  F  L  W  L  G  G  F  R  S  S  E
GCTGCAAAGTCCGACGTTTTTCACATATTGTCGGGCATGTGGAAAACTCCAGCAGAGAGATCCTTCTTGTGGCTTGGTGGATTCCGTTCGTCTGAA

L  L  K  L  L  I  N  Q  L  E  P  L  T  E  Q  Q  L  L  A  I  N  N  L  Q  Q  S  S  Q  Q  A  E  D
CTCCTCAAGCTCCTCATTAACCAGTTGGAGCCTTTAACCGAACAACAATTATTGGCAATCAACAACTTGCAACAGTCATCCCAACAGGCTGAAGAT

A  L  S  Q  G  M  E  A  L  Q  Q  S  L  A  E  T  L  A  G  S  L  G  P  S  S  S  S  G  N  V  A  N
GCTTTATCCCAAGGAATGGAGGCACTGCAGCAGTCTTTGGCTGAGACTCTGGCGGGGTCCCTTGGACCTTCAAGTTCCTCAGGGAATGTTGCCAAT

Y  M  G  Q  M  A  M  A  M  G  K  L  G  T  L  E  G  F  I  R  Q  A  D  N  L  R  Q  Q  T  L  Q  Q
TATATGGGTCAAATGGCCATGGCAATGGGGAAGCTCGGAACTCTCGAGGGCTTCATACGACAGGCTGATAACCTTCGGCAACAAACATTGCAGCAA

M  H  R  I  L  T  T  R  Q  S  A  R  A  L  L  A  I  S  D  Y  F  S  R  L  R  A  L  S  S  L  W  L
ATGCATCGTATATTGACAACTCGCCAATCAGCTCGTGCTCTTCTTGCGATCAGTGATTATTTCTCTCGGCTTCGAGCACTGAGCTCTCTCTGGCTT

A  R  P  R  E  *
GCTCGCCCCCGGGAATAA
```

8.2 List of Figures

8.3 List of Tables

8.4 Acknowledgments

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8.5 Curriculum vitae

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