Characterization Of An *Arabidopsis* Glutaredoxin That Interacts With Core Components Of The Salicylic Acid Signal Transduction Pathway – Its Role In Regulating The Jasmonic Acid Pathway

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To my grandparents who taught me that “the fear of the Lord is the beginning of wisdom, and the knowledge of the Holy One is understanding”.
To my father and my mother, my heroes, whose courage inspires me to keep pressing on.

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1 Abstract

Salicylic acid (SA) is a plant signalling molecule that mediates the induction of defense responses upon attack by a variety of pathogens. *Activation sequence-1* (*as-1*) type *cis* elements and their cognate basic/leucine zipper (bZIP)-type transcription factors of the TGA family regulate transcription in response to SA and in response to xenobiotic chemicals. TGA factors interact with NPR1 (NON EXPRESSOR OF PR GENES 1), a central regulator of many SA-induced defense responses. Changes in the redox state of both TGA1 and NPR1 have been observed under inducing conditions. In addition to induction of SA-inducible genes, NPR1 is also involved in suppression of jasmonic acid (JA)-inducible genes under conditions of the simultaneous presence of increased level of SA and JA in the cell.

A yeast protein interaction screen with tobacco NtTGA2.2 as a bait and an *Arabidopsis thaliana* cDNA prey library had previously identified a member of the glutaredoxin family (GRX480, encoded by *At1g28480*) as a TGA interacting protein. Glutaredoxins are candidates for mediating redox regulation of proteins because of their capacity to catalyze disulfide transitions. GRX480 is localized in the cytosol and the nucleus of plant protoplasts. A ternary GRX480/NtTGA2.2/NPR1 complex could be detected in the yeast three hybrid assay.

In this study, we used the yeast and plant protoplast two-hybrid assays to assess the influence of the two catalytic cysteines of GRX480 on the interaction with AtTGA2. Individual yeast clones showed a considerable variability with respect to the interaction that did not correlate with the expression of the interacting proteins. Nevertheless it can be concluded that the redox-deficient GRX480 still interacts with AtTGA2 in yeast. This interaction seems to be compromised in plant protoplasts. Furthermore, the interaction does not depend on the GRX480-specific N terminus. AtTGA2 does not interact with the related glutaredoxin GRX370 (*At5g40370*).

GRX480 is expressed in response to SA and pathogen challenge. SA-induced expression depends on NPR1 and TGA factors. Though the gene is not inducible by JA, JA can enhance SA-induced expression by a factor of 2.

Arabidopsis lines ectopically expressing GRX480 show reduced transcriptional activation from the truncated *CaMV 35S* promoter that contains the *as-1* element as the only cis regulatory element. This was not observed upon ectopic expression of GRX370 indicating that the interaction with TGA factors might be important for this effect.

The JA-responsive defensin gene *PDF1.2*, that is subject to the SA/JA antagonism, is also negatively regulated by GRX480, suggesting that GRX480 is a regulatory component of

this cross-talk. GRX480 with cysteine to serine exchanges in the active centre did not mediate this effect. Epistasis analysis showed that GRX480 functions independently or downstream of NPR1. The functionality of the SA/JA antagonism in *grx480* knock out plants suggests that redundant mechanisms exist in *planta* that lead to the strong suppression of PDF1.2 expression in Arabidopsis.

2 Introduction

2.1 Plant struggle for survival – studying defense mechanisms from a molecular perspective

The interaction between plants and their environment is critical for their survival and productivity. Plants in different environments are exposed to abiotic stresses such cold, heat, salinity, water logging, uv-irradiation, as well as to biotic stresses such as invertebrate feeding and pathogen infestation. Pathogens ranging from viral and fungal to bacterial types are able to invade plants. Because they are immobile, plants are unable to escape. In order to compensate for this, they make use of their preformed and inducible defense responses. Unlike vertebrate immune responses where specialized cells are mobilized to the site of infection to eliminate invading organisms, the plant non-circulatory defense mechanism minimizes infections. Their innate immunity contributes to basic resistance. Though pathogen invasion patterns evolve with time, the plants are also able to actively recognize pathogens and trigger general and specific responses leading to defense. Plants are also able to trigger an acquired resistance, which is induced after a first exposure to infection. This is able to spread to tissues distant from the original site of infection.

Various model systems have been used for research in order to gain a better understanding of the molecular mechanisms involved. Among them, the *Arabidopsis thaliana* of the Brassicaceae family has been used, which has the advantages of a short life cycle (6 weeks), a small genome size (125Mb in 5 chromosomes), availability of an extensive genetic and physical map (completed for the Colombia ecotype by 2000) as well as an efficient method of transformation for the purposes of genetic modification. In addition to all these, a growing number of mutant lines (T-DNA, transposon insertion and EMS mutagenesis lines) are continuously being made available which makes reverse genetic approaches possible. Other genetic resources such as DNA Chips and microarrays are increasingly being made available over the past five years. These are a few among diverse possibilities which are hands on tools, that make the *Arabidopsis* a plant model system exciting to work with. The knowledge acquired here makes a significant contribution to understanding not only the differences in cell physiology and molecular biology between kingdoms, but broadens the scope of opportunities of enhancing crop productivity, which is at the base of the food chain of life.

2.2 Plant pathogen interactions

Passive defense in plants is constituted by preformed structural barriers (cuticle and rigid cell walls), and the production of toxic anti-microbial substances that hinder the penetration of invading pathogens. Once inside the plant, the pathogen uses one of three strategies to use the host plant as a substrate in its cycle of pathogenesis.

First of all, biotrophic pathogens attack and come into intimate intracellular contact with plant cells, but leaving minimal damage to the cells. The plant cells remain alive throughout the time of infection. The host rage for biotrophic pathogens is narrow and consists of fungal mildews, rusts, viruses, endoparasitic nematodes and *Pseudomonas* spp. bacteria.

A second but extreme set of pathogens that colonize plants consist of the necrotrophic type, which secret cell wall degrading enzymes and host toxins, which end up in the death of the plant tissue and complete colonization by the pathogen. The range of hosts broadly includes rotting bacteria like *Erwinia* and rotting fungi like *Botrytis cinerea*.

A third set of pathogens known as hemibiotrophic pathogens have an initial biotrophic phase and a terminal necrotic phase, during which extensive tissue damage occurs. *Phytophthora infestans*, which causes potato late blight disease, is a good example in this case.

Pathogens could either quickly proliferate in the plant or could be tolerated to certain extent. In other cases they could be completely prevented from developing in the plant. This depends on one hand on the recognition of the products of the pathogen avirulence genes (Avr) by the plant host receptors, which are encoded by plant disease resistance genes (R-genes).

A ***"gene for gene model***" was proposed by Flor (1971) that both plant resistance and pathogen virulence are inherited. It predicts that plant resistance occurs when a plant possesses a dominant resistance gene (R) and the pathogen expresses a complimentary dominant avirulence gene (Avr). The interaction between the plant and pathogen in this case is incompatible. The recognition of the pathogen virulence factors by resistance host elicits plant defense, which betrays the pathogen to the plant's surveillance system.

A compatible interaction occurs when there is an alteration or loss of plant resistant gene or of the pathogen avirulence gene leading to disease. The gene products of the pathogen in this case act as virulent factors, leading to virulence) The phytopathogenic bacteria for

example, evolve specialized strategies like the use type III effector proteins, toxins, and other factors to inhibit host defenses (Abramovitch and Martin, 2004).

This ***'gene for gene'*** otherwise known as ***'race specific' resistance*** explains this phenomenon of resistance in which the recognition and interaction of avirulent gene products with resistance genes leads to a hypersensitive response (HR, see Figure 2.8). The HR involves local cell death of plant cells at the site of infection, thereby destroying and/or containing the pathogens and prevents further spread of the pathogenic organisms.

A compatible interaction between pathogen and plant takes place when environmental conditions are favorable and preformed defenses are insufficient, leading to infection and disease. Here the plant fails to detect the pathogen or the activated defense responses are not effective enough.

Recognition of pathogen Avr protein occurs either in the apoplast or it may be injected into the host cell as in the case of *Pseudomonas syringae* and other bacteria pathogens that use the evolutionarily conserved type III secretion system to deliver their effectors into the eukaryotic host. (Kjemtrup *et al*., 2000).

The *Arabidopsis* genome encodes about 125 R-genes, while the rice genome encodes about 600 R-genes, indicating that a considerable portion of the plant genomes are invested into cultivating R- gene families (Nimchuk *et al.,* 2003)

Based on structural motive types, R proteins can be classified into at least 5 types.

Class 1, is made up of the **Pto** from tomato has a serine/threonine kinase catalytic region and a myristylation motif at its N-terminus.

Class 2 comprises proteins with leucine rich repeats (LRR), putative nucleotide binding site (NB), and an N-terminal leucine zipper (LZ) or coiled coil sequence (CC).

Class 3 is similar to class 2 except that the CC sequence is replaced by a region similar to the N-terminus cytoplasmic domain of *Drosophila* TOLL and human interleukin-1 (IL-1) receptor (**TIR**).

The R-proteins belonging to the first three classes lack a trans-membrane domain (TM) and are thought to be localized intracellularly.

Class 4 consists of the **Cf -** proteins of tomato, which lack an NBS, have a TM domain, an extracellular LRR and a small putative cytoplasmic tail.

Class 5 consists of the **Xa21** protein from rice, which has an extracellular LRR domain, a TM domain and a cytoplasmic serine/threonine kinase domain.

Other R-proteins which do not fall into the first 5 structural classes are placed into a 6th class. (For review of all the classes, see Gregory *et al.,* 2003)

A majority of R loci in *Arabidopsis* encode the **NBS-LRR** proteins, with a distinct N-terminal domain: either a putative **CC**-domain or a **TIR domain**. The NBS-LRR class has been genetically linked only to disease resistance function while other structural classes of LRR containing R proteins are associated with plant development, hormone perception as well as R function. (Wang *et al*., 2001). The NBS domain is similar the pro-apoptotic protein APAF-1 which has some ATP binding and hydrolytic properties in signaling like some NBS-LRR proteins Mi and Is (Tameling *et al*., 2002). The LRR domain is important in protein-protein interaction, and confers recognition specificity to the R-proteins in their interaction with Avr proteins of pathogens.

The NBS-LRR protein RPM1 of *Arabidopsis,* for example, recognizes the *P. syringae* expressing either AvrRpm1 or AvrB type III effector proteins. The same is true for many of its alleles (Tornero *et al.,* 2002). Some R genes like the tobacco N gene, are able to generate multiple splice variants, whose encoded proteins may be important in conferring complete resistance to the TMV (Dinesh-Kumar and Baker, 2000).

R protein – protein intra or inter-molecular interactions may lead to negative regulation of R proteins, as some mutations in the LRR or NBS domains leads to constitutive activation of the R genes in the absence of pathogen (Shirano *et al*., 2002). The mutation of the R gene SSI4, confers constitutive expression of several PR (pathogenesis-related) genes, induces SA accumulation, triggers programmed cell death, and enhances resistance to bacterial and oomycete pathogens, in a manner requiring EDS1 (Enhanced disease susceptibility protein 1; *eds1* mutation results in enhanced disease susceptibility to necrotic pathogens, see Feys *et al*., 2005). Direct evidence for intramolecular interaction has been further shown for the Rx protein of potato (Moffett *et al.,* 2002) and the SLH1 (sensitive to low humidity 1) protein in *Arabidopsis* which has a dual function as an R protein and regulates transcription of target genes by its WRKY domain, which also negatively regulates disease resistance signaling (Noutoshi *et al*, 2005).

It was further envisioned that other proteins that interact with NBS-LRR proteins may stabilize R-protein complexes. They may act as co-factors for Avr binding, partner in early signaling, regulate R-protein activity or a combination of these.

A ***"guard hypothesis"*** (Van der Biezen and Jones, 1998) stipulated that the R-proteins may not directly recognize pathogen virulence factors, but the cellular consequence of their action in the host cells, on in other terms, that the targets of virulence factors are associated with R proteins.

In other cases, the resistance of an entire plant species to all isolates of a microbial invading species constitutes a phenomenon known as "***non-host"*** or "***species resistance"*** (Jones and Takemoto, 2004). The plant species attacked is unable to support the life-strategy requirements of the particular heterologous pathogen and thus is considered a ***non-host*****.** Whole classes of ***pathogen associated molecular patterns*** (PAMP) that are characteristic of whole classes of microbial organisms are recognized by plants. Their PAMP perception systems and PAMP induced signal cascades resemble those conserved in animals for the recognition of non-self. The establishment of an infection in susceptible plants is associated to the suppression of plant species resistance and development of virulence factors by the so called homologous pathogens (Gabriel and Rolfe, 1990). PAMP induced defenses are required for basal resistance since they are insufficient to stop infection in susceptible host plants.

PAMPs generally constitute highly conserved determinants, typical of whole classes of pathogens, which have an elicitor and defense inducing capacity. Examples include among several others, the surface exposed peptide motive of *Phytophthora* cell-wall transglutamase (Pep13), which is recognized by several plant species in response to infection by various species of *Phytophthora*; and a cold shock inducible RNA binding protein (RNP-1) from various gram-positive bacteria (Nürnberger and Lipka, 2005; Brunner *et al.,* 2002, Felix and Boller, 2003).

Race specific resistance and ***non-host resistance*** are two complimentary elements of plant innate immunity that use similar mechanisms for recognition and response.

RPS2 and RPM1 are CC-NBS-LRR plasma membrane localized R proteins which are important in regulating both gene for gene and PAMP downstream signaling responses. Recent work has shown that in *Nicotiana benthamiana*, a rapid (HR) develops upon transient expression of RPS2. This is blocked by expressing RPS2 in the presence of RIN4 (an **R**PM1 **In**teracting protein) a small plasma membrane localized protein, which also regulates PAMP signaling. This recapitulates the ability of RIN4 to negatively regulate RPS2-mediated resistance in *Arabidopsis*. The RPS2-mediated HR in the

presence of RIN4 can nevertheless be restored by the type III mediated delivery of AvrRpt2 into the cell membrane, in the presence of RPS2 and RIN4.

It has furthermore been shown that the *Pseudomonas syringae* type III effectors, AvrRpt2 and AvrRpm1, are able to inhibit PAMP-induced signaling and thus compromise the host's basal defense system. In doing so, AvrRpt2 normally targets RIN4 to degradation while AvrRpm1 normally targets RIN4 to phosphorylation, which correlates with the activation of RPM1. AvRpt2 dependent cleavage and release of RIN4 from the membrane consequently prevents AvRpm1 or AvrB from activating RPM1. This is nevertheless able to hyper activate RPS2 to a lethal extent.

The R proteins, RPS2 and RPM1 are able to sense type III effector-induced perturbations of RIN4, to get activated. Thus, R proteins guard the "guardee" - RIN4 against type III effector manipulation, which aim to manipulate host defense mechanisms. The R protein probably detects the modified RIN4 and the virulent effector as a complex. The protein RIN4 acts as a sensor for the target R-proteins. It functions as a molecular switch regulating at lest two independent R- proteins and modulates their downstream defense signaling events, further elucidating the "guard hypothesis". (McDowell *et al.,* 2003 ; Han-suk *et al.,* 2005; Kim *et al.,* 2005; Day *et al.,* 2005). A similar pattern is observed in several other R-protein mediated responses.

2.3 Signaling pathways in plant defense responses

Several signaling pathways are important in fine tuning defense responses during plant pathogenic attack. Salicylic acid dependent signaling is required for local and systemic resistance mechanisms in plants (Malamy *et al.,* 1990). Signaling pathways involving SA can be grouped into those occurring upstream of SA synthesis (i.e. downstream of R-gene activation), and those occurring downstream of SA synthesis, which lead to SA dependent responses.

Upstream of SA synthesis, there are at least 3 partially independent pathways that result in transcriptional reprogramming and gene activation. Two of these pathways are defined by mutations either in the EDS1 (Enhanced disease Susceptibility 1), PAD4 (Phytoalexin –Deficient 4), SAG101 (Senescence Associated Gene 101) or NDR1 (Non-race specific Disease Resistance 1) genes. PAD4, EDS1 and SAG101 physically interact with each other *in vivo* (Feys *et al.*, 2005) and affect the same spectrum of R genes. The combined

activities of SAG101 and PAD4 are necessary for programmed cell death triggered by the TIR-NBS-LRR immune receptor in response to avirulent pathogen isolates and in restricting the growth of normally virulent pathogens.

NDR1 encodes a probable glycosylphosphatidylinositol (GPI) anchored protein and might be associated in lipid rafts with receptor complexes, important in signaling (Nimchuk *et al.*, 2003). Though most CC type R-proteins depend on NDR1 for signaling while most TIR type R-proteins depend on EDS1/PAD4 for signaling, there are exceptions of CC-type R-proteins that signal independent of NDR and EDS/PAD4, and seem to be independent of SA signaling (Bittner-Eddy *et al.,* 2001). In one example described in 2001 by Bittner-Eddy, RPP13-Nd-mediated resistance remained unchanged in a background of salicylic acid depletion (nahG). RPP13 mediated resistance is not altered in eds1/ndr1 double mutant plants.

Other genes have been reported, whose mutants are deficient in SA accumulation, and in R-gene mediated defense pathways. EDS5 and EDS16 (also ICS1 or SID2) fall into this category (Nawrath *et al.,* 2002). Exogenous applications of SA or its analogues restores resistance in many mutants compromised in signaling steps upstream of SA production (Parker *et al*., 1996; Century *et al,* 1995).

Isochorismate synthase (encoded by ICS1) is a chloroplast localized enzyme that catalyses a critical step in the synthesis of SA (Wildermuth *et al*., 2002, Figure 2.9). EDS5 encodes a chloroplast localized transmembrane protein, which might be important in transporting SA to the cytoplasm (Métraux, 2002)

Downstream targets of SA include a carbonic anhydrase (CA/SABP3), which has been shown to be important for avrPto -mediated hypersensitive response in disease resistance in tobacco (Slaymaker *et al.,* 2002). CA as an antioxidant may control the level of reactive oxygen species (ROI) produced in the oxidative burst during an HR. Another SA binding protein previously identified is a catalase (SABP).

Another SA binding protein, a lipase (SABP2) was identified as a high affinity SA receptor that is required for the plant immune response (Kumar and Klessig, 2003). It is activated by binding to SA, and its loss of function critically compromises local and systemic disease resistance.

So SA may therefore act through multiple effector-proteins in plants. SA independent signaling pathways also occur in plants after pathogen challenge.

Mitogen activated protein (MAP) kinase signaling in plant defense is also activated by several Avr/R interactions, and appears to be independent of upstream ROI production (Romeis *et al.,* 1999; Ligterink *et al.*, 1997). Phosphorylated modified downstream targets are translocated to the nucleus where it might interact with transcription factors that induce expression of defense genes. In *Arabidopsis*, the flg22 signal of bacterial flagellin is the effector which triggers the MAP kinase pathway. The FLS2 receptor sensor is upstream of the MAPKKK, MAPKK and MAPK, the MEKK1, MKK4/5 and MPK3/6 respectively. This response appears to regulate the immediate early flg22 responsive expression of WRKY-type transcription factors. Constitutive or transient over-expression of MKK4/5 or MEKK1 enhances resistance to *P. syringae* and *Botrytis cinerea.* Similar pathways seem to occur in tobacco, tomato, rice and parsley (Nakagami *et al.,* 2005, See Figure 2.3A).

Nitric Oxide (NO), which controls a number of physiological processes in animal cells has also been reported to be important for full R-gene-triggered HR responses in soybean cells and *Arabidopsis*. NO as well as Cyclic GMP and cyclic ADP ribose (which signal downstream of NO in animal cells) also activate phenylalanine ammonium lyase (PAL, important in SA synthesis, see Figure 2.9) and PR1 (Delledonne *et al.,* 1998; Durner *et al.,* 1998).

Both NO and ROI are synergistically important in generating an HR. Positive feed back loops involving SA, NO and ROI are also important in potentiating the responses both in upstream synthesis and downstream responses (Shirasu *et al.,* 1997; Wendehenne *et al.*, 2001).

Research has also revealed other SA independent disease resistance mechanisms involving jasmonic acid and ethylene. Jasmonic acid (JA) and ethylene (ET) signaling control the expression of genes encoding antimicrobial peptides such as thionin and defensins.

Methyl JA, first discovered as the major component of the jasmine scent, is a major hormone that regulates developmental processes such as fruit ripening, root growth, pollen development, leaf abscission, senescence, tuberization, as well as defense processes such as responses to ozone exposure, environmental stress, water deficit, wounding attack by necrotic pathogens as well as responses to insect feeding (Penninckx *et al*, 1996; McConn *et al*., 1997; Pieterse *et al*., 1998; Reymond and Farmer, 1998;

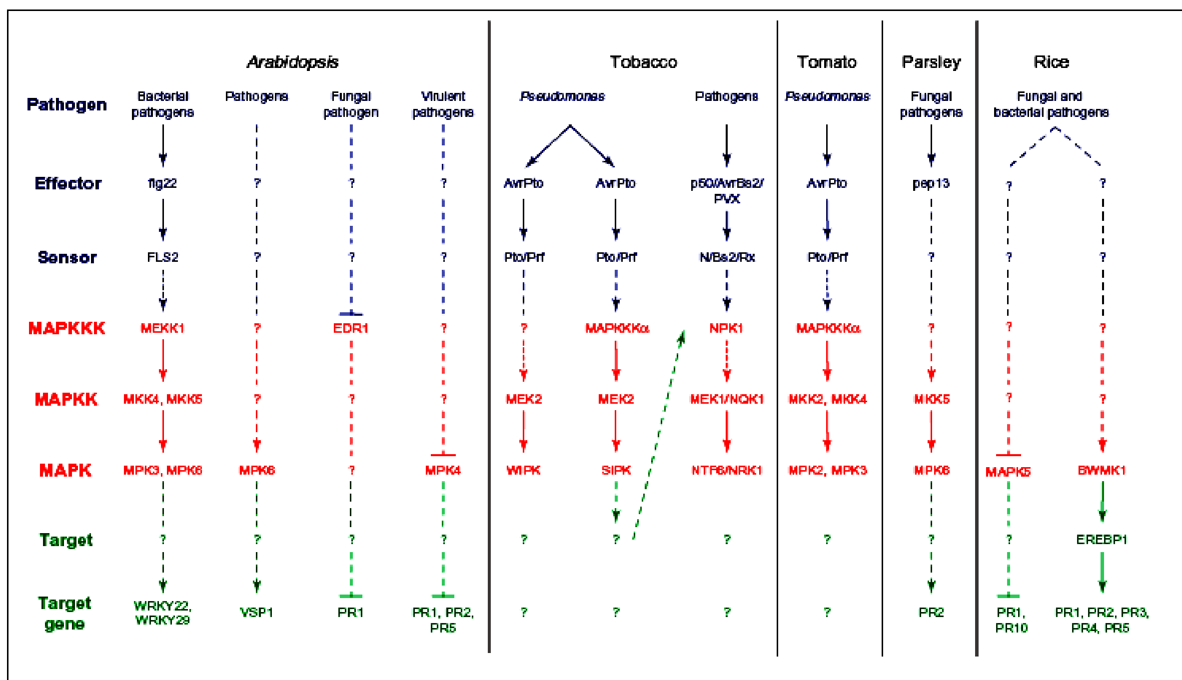

Figure 2.3A: Model describing the role of Arabidopsis, tobacco, tomato, parsley and rice MAPK pathways in pathogen defense.
Broken arrows indicate hypothetical pathways; question marks indicate unknown factors (Nakagami et al., 2005)

Staswick *et al.*, 1998; Farmer *et al.,* 2003). Various genes regulate the different steps involved in JA biosynthesis, and are outlined in Figure 2.3B (ARA cyc tool, http://www.arabidopsis.org; Farmer and Ryan, 1990; Creelman *et al.,* 1992; Creelman and Mullet, 1995; Reymond and Farmer, 1998; Seo *et al.*, 2001).

JA inducible gene expression is also regulated by several genes, which have been identified by mutant screens. COI1 (for Coronatine Insensitive 1), which is an F-box, leucine rich repeat (LRR) protein, forms part of a functional E3-type ubiquitin ligase complex, otherwise known as SCF^{COI} (skip-cullin-F-box) complex. It functions in targeting repressors or JA response genes for ubiquitin mediated degradation. It is also required for defense to *A. brassicicola.* (Feys *et al.*, 1994; Xie *et al.,* 1994). One of the putative targets of COI1 is a lumazine synthase (COS1, for Coronatine insensitive1 Suppressor), identified in a screen for suppressors of the *coi1* mutation. When the *cos* mutation is present in the *coi* mutant background, it restores sensitivity to meJA. It restores the defects in JA inducible gene expression of VSP, Lox2, as well as the expression of senescence associated genes SEN4 and SAG12. It is thought to exert its function downstream of COI (Xio *et al.,* 2004).

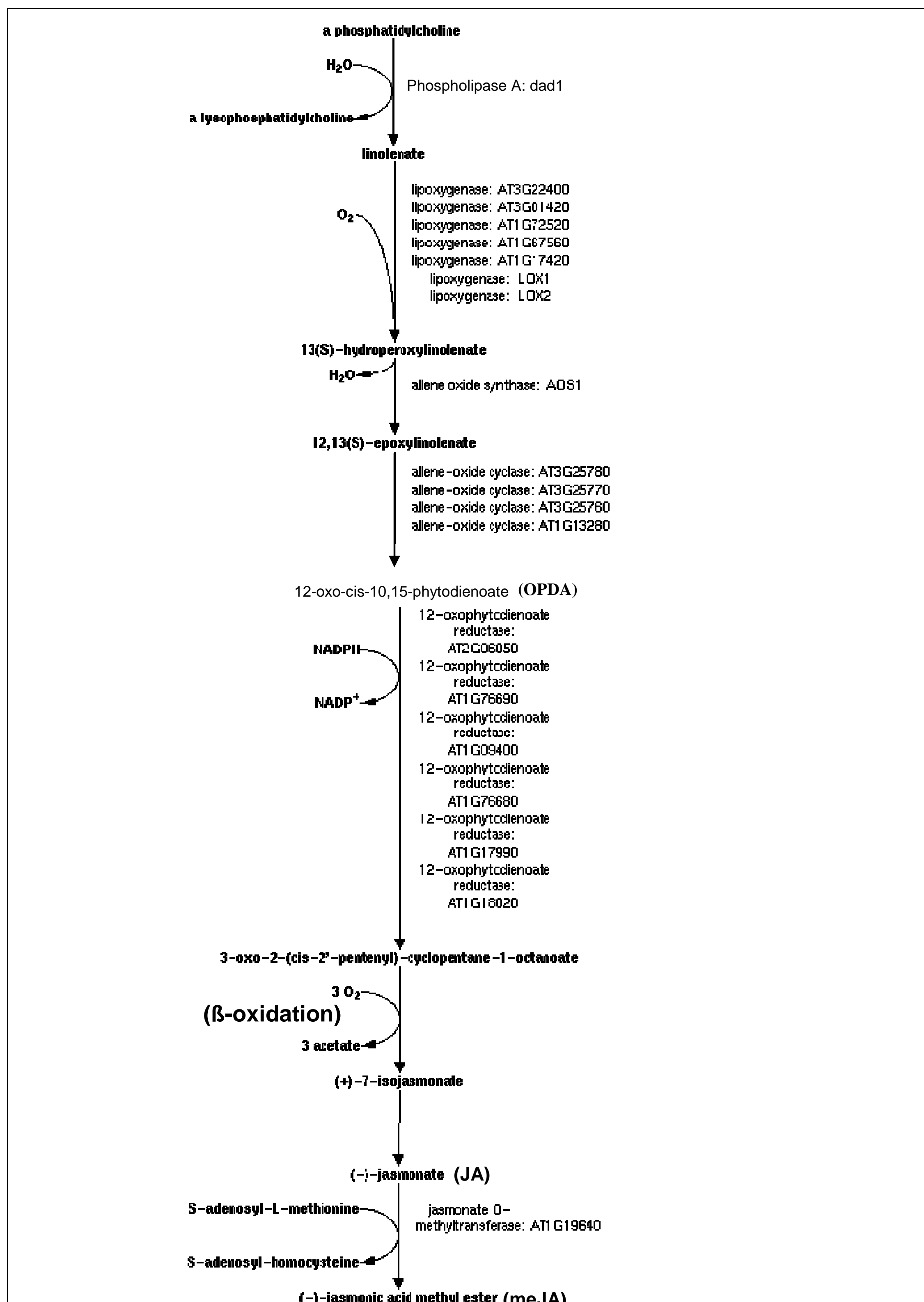

Figure 2.3B: Jasmonic acid biosynthesis pathway.
Scheme shows genes coding for enzymes regulating differing steps in the biosynthesis of jasmonic acid, including their AGI codes. (ARA cyc tool, http://www.arabidopsis.org)

Other JA signaling genes form part of the ubiquitin-proteosome pathway. These include an auxin resistant gene AXR1, which is a RUB-activating enzyme E1 (Xu *et al*., 2002; Tiryaki *et al.,* 2002) and JAR1.

JAR1/JIN4/JAI2, is JA-aminosynthase, whose mutation shows enhanced sensitivity to *Pythim irregulare.*

AtMYC2 also known as JIN1/JAI1 is a bHLHzip transcription factor, localized in the nucleus. It is an important protein in the differential regulation and expression different JA responses in *Arabidopsis*. On one hand, genes that are involved in the defense response against necrotic pathogens are repressed by AtMYC2. On the other hand, genes involved in JA-mediated systemic responses to wounding are activated by AtMYC2. These two branches of regulation are shown to be antagonistically regulated by the ERF1 transcription factor, which mediates repression of wounding response genes (VSP, Lox, Thi2.1) and mediates expression of pathogen response genes (PDF1.2, bCHI, HEL. AtMYC2 is rapidly upregulated by JA and abscissic acid in a COI1 dependent manner.

The mutants: *coi, jar1, jin1* and *jai3* (in ein3 background) show reduced root growth inhibition on medium containing 50µM JA compared to the Col-O and *ein3-3* background plants (Staswick *et al*., 1992; Berger *et al*., 1996; Lorenzo *et al*., 2004).

Ethylene signaling also occurs in plants, leading to the regulation of gene expression associated with defense. Its function has been attributed to developmental processes such as germination, flower and leaf senescence, fruit ripening, leaf abscission, root nodulation, programmed cell death, and in fitness responses such as responsiveness to stress and pathogen attack. A combination of genetic and molecular analyses of ethylene response mutants has defined a largely linear ethylene response pathway leading from hormone perception at the membrane to transcriptional regulation in the nucleus (Reviewed by Guo and Ecker, 2004). Ethylene is perceived by a family of membrane associated receptors, including ETR1/ETR2, ETHYLENE RESPONSE SENSOR1 (ERS1)/ERS2 and EIN4 in *Arabidopsis*. Ethylene binds to its receptors mediated by a copper co-factor. Genetic studies predict that hormone binding results in the inactivation of receptor function. In the absence of ethylene, therefore, the receptors are thought to be in a functionally active form that constitutively activates a Raf-like serine/threonine (Ser/Thr) kinase, CTR1, which is also a negative regulator of the pathway (*Kieber et al.,*

1993). EIN2, EIN3, EIN5, and EIN6 are positive regulators of ethylene responses, acting downstream of CTR1. EIN3 is a nuclear localized transcription factor that regulates the expression of its immediate target genes such as ETHYLENE RESPONSE FACTOR1 (ERF1). ERF1 belongs to a large family of APETALA2- domain-containing transcription factors that were initially referred to as ETHYLENE RESPONSE ELEMENT BINDING PROTEINS (EREBPs) but later found to function in a diverse range of process. They bind to a GCC-box present in the promoters of many ethylene inducible and defense-related genes. ERF1 also regulates other hormone responses, particularly the jasmonate (JA) - mediated defense response. Ethylene and JA mediate defense responses against pathogen attack partly by inducing the expression of defense genes, such as PLANT DEFENSIN1.2 (PDF1.2). Ethylene and JA have been shown to induce several plant defense genes synergistically. The GCC box required for ERF1 binding in the PDF1.2 promoter has also been identified as a JA-responsive element (Lorenzo *et al*., 2003).

AtMPK4, a MAP kinase, is required for repressing SAR by repressing a step upstream of SA synthesis. It is also required for the JA-responsive expression of PDF1.2 and Thi2.1 (Petersen *et al.,* 2000). Other genes, which regulate by repressing JA response genes, were isolated, by identifying their mutants which show a constitutive JA response. They include *cet1-9, cex1, cev1, joe1, joe2* and *cas1* mutants, which show constitutive expression of different JA inducible genes and genes involved in JA synthesis (Lorenzo and Solano, 2005).

Another SA independent resistance pathway requiring SA and JA response pathways is the induced systemic resistance (ISR) pathway (Pieterse *et al.,* 1996). It is also independent of pathogenesis related gene expression, but leads to broad spectrum disease resistance (Discussed in section 2.12). It is mediated by JA and ET signaling.

Abscisic acid (ABA) signaling mediates the inhibition of seed germination. Other physiological processes such as plant defenses against pathogens and wounding are also thought to be mediated by a cross talk between JA and ABA. The induction of the protease inhibitor gene (PIN) in response to wounding for example needs ABA perception (Carrera *et al.,* 1998). They are antagonized by gibberellins (GA), which breaks seed dormancy in some plants which require stratification to induce germination. The ABA/GA interaction is an important factor regulating the developmental transition from embryogenesis to seed germination. (Gomez-Cadenas *et al*., 2001)

2.4 Cross-talk occurring among signaling pathways

SA and JA/ET pathways are an example of how cross talk occurs between signaling pathways, in order to fine-tune the cells defenses depending on the need. SA and JA act in a mutually antagonistic manner. MPK4 is required for the induction of JA mediated defense genes, PDF1.2 and THI2.1. It represses SA-regulated PR genes as high levels of SA accumulate in the *mpk4* mutant (Petersen *et al.,* 2000). The MAP kinase cascade is therefore involved in simultaneously repressing SA biosynthesis and promoting JA responses.

A cross talk pattern is also seen to be mediated by the ERF1, which promotes expression of ET and JA response genes but reduces tolerance of *Pseudomonas syringae* (Berrocal-Lobo *et al.,* 2004). In tobacco, meJA induced expression of basic PR genes was inhibited by SA treatment, whereas, meJA treatment inhibited SA-induced expression of acidic PR genes (Niki *et al*., 1998). It has also been reported that SA accumulation after *Pseudomonas* infection or exogenous application of SA prevents JA accumulation and represses JA response genes. This has been shown to be critically mediated by NPR1, which interacts with TGA family of bZIP transcription factors and activates the expression of PR genes in an SA dependent manner (Spoel *et al.,* 2003). Upon simultaneous treatment of wild type plants with SA and JA, SA suppresses JA inducible gene expression. This is compromised in *npr1-1* mutant plants, showing that it is required for SA mediated suppression of JA responsive gene expression. Spoel and colleagues in 2003 also showed that the nuclear localization of NPR1 is not required for the SA mediated suppression of the JA inducible gene, PDF1.2, to take place. SA might be required to activate NPR1 for suppression since without SA induction; mutations in NPR1 which lead to its constitutive monomerization and nuclear localization do not affect JA inducible VSP expression. PR expression is nevertheless observed. (Mou *et al*., 2003; Beckers and Spoel, 2005). The SA induced monomer of NPR1 may therefore be involved in the cross talk.

Another regulator of cross talk between the SA and JA pathways is the WRKY transcription factor. It is up-regulated by SA, and down-regulated by JA. Overexpression of WRKY70 results in increased SA-responsive gene expression and an enhanced resistance to virulent pathogens. On the other hand, antisense suppression leads to the

activation of JA-inducible gene expression, suggesting that WRKY70 may serve as a molecular switch between both pathways (Li *et al.,* 2004).

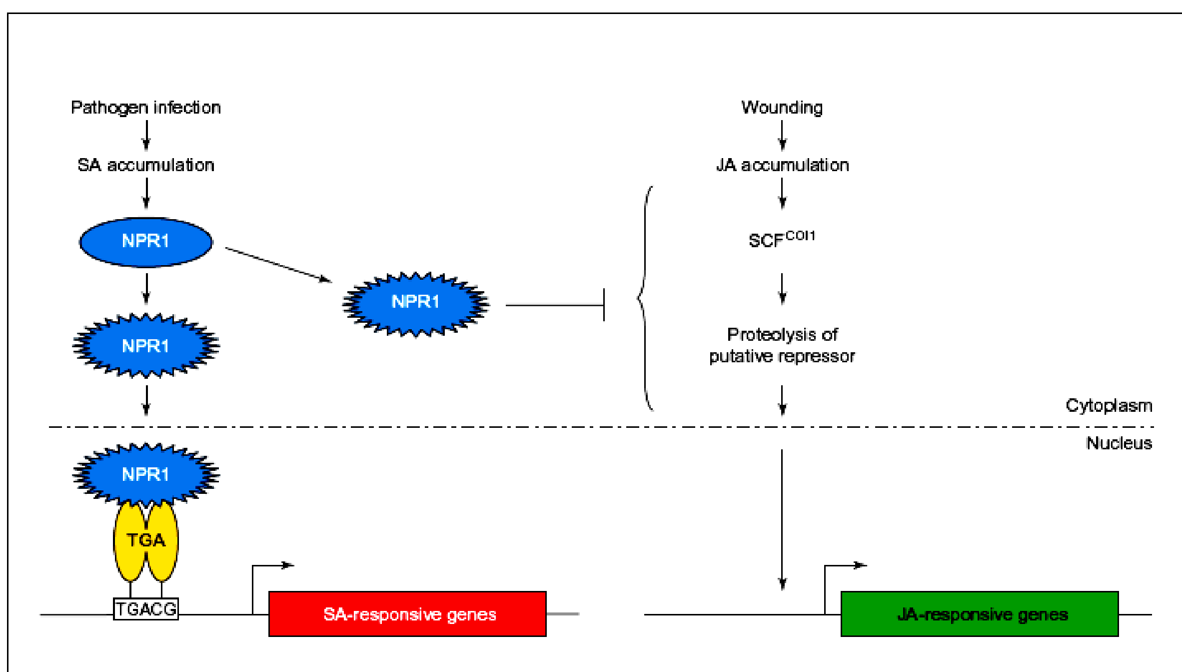

Figure 2.4: Illustration of cross talk between SA and JA pathways.
Infection by a necrotizing pathogen results in the accumulation of SA, redox changes in the cell and the activation of NPR1. Activated NPR1 is then translocated into the nucleus where it interacts with TGA transcription factors, resulting in the activation of SA-responsive genes. Wounding, such as that caused by feeding insects, results in the accumulation of JA. A putative repressor of JA-responsive gene expression is then ubiquitinated by a SCF^{COI1} *ubiquitin-ligase complex that target proteins for degradation by the proteasome. Removal of the putative repressor protein results in the activation of JA-responsive genes. Inhibition of JA signaling by SA is regulated by a cytosolic function of SA-activated NPR1, but its site of action is unclear. (Pieterse and Van Loon, 2004)*

2.5 Transcription factors in plant defense response

Transcription factors are critical in regulating gene expression changes in the cell. They occur at the end of signals occurring before gene expression. Plants devote a large portion of their genome capacity to transcription, with the *Arabidopsis* genome dedicating over 5% of it's genome to code for over 1500 transcription factors (Riechmann *et al*., 2000). ERF transcription factors belong to a subfamily of APETALA2 (AP2)/ethylene-responsive-element-binding protein (EREBP) transcription factor family, and this is unique to plants. In *Arabidopsis*, about 124 ERF proteins are known, and share a conserved homology domain in a stretch of about 59 amino acids, called the ERF domain (Fujimoto *et al*., 2000). They bind to two similar *cis*-elements: the GCC box found in the

promoters of most pathogenesis-related genes where they confer responsiveness to ethylene, and to the C-repeat (CRT)/dehydration responsive element (DRE) motif, where they control expression of dehydration and low temperature responsive genes. The accumulation of RNA transcripts of specific *ERF* genes is regulated by cold, drought, pathogen infection, wounding or treatment with salicylic or jasmonic acid, as reviewed by Singh *et al* in 2002. Post translational control of an ERF protein has also been reported for the PTO-INTERAXTING4 ERF protein, which is phosphorylated by the *Pseudomonas* tomato resistance (PTO) kinase, an R-gene product, resulting in an enhanced binding to the GCC box. (Gu YQ *et al*) A subset of ERF proteins have also been reported to repress transcription.

Loss of function mutants of ERF genes has not shown any phenotype, possibly because of a functional overlap between the family members. Over-expression of ERF genes generally shows an up-regulation of GCC or CRT/DRE-motif-containing genes, an enhanced activation of downstream responses and resistance to specific stresses. For example, over-expression of *Arabidopsis ERF1* caused an enhanced resistance to the necrotrophic fungi *Botrytis cinerea* and *Plectosphaerella cucumerina*, but reduced resistance to *Pseudomonas syringae*. (Berrocal-Lobo *et al*., 2002), thus showing a negative cross-talk between the ethylene and SA signaling pathways.

The bZIP transcription factors form another large family of transcription factors with about 75 members represented in *Arabidopsis*. A class of bZIP proteins associated with stress responses is the TGA/octopine synthase (*ocs*)-element-binding factor (OBF) proteins, generally called "TGA" factors. They selectively bind to an *activation sequence-1* (*as-1*) element which regulates the expression of some stress responsive genes such as PR1, Glutathione-S-transferase (GST6) and genes associated with defense and detoxification (Lebel *et al*., 1998, Chen *et al*., 1999). These 'TGA factors' encode a variable N-terminal domain followed by a highly conserved basic/leucine zipper (bZIP) domain. The approximately 250 amino acid C-terminal domain is moderately conserved (at least 52% identical amino acids).

Some of those characterized so far in *A. thaliana* contain homologues in *N. tabacum.* Based on sequence homology, they can be classified into at least 6 subclasses, as shown

on Table 2.5 below. PERIANTHA is exemplified by its extensively long N-terminus (167aa vs 48aa in TGA2). TGA7, also has a long N-terminus but shows no similarity to the other TGA proteins (Schiermeyer, 2001). These transcription factors were first called activating sequence factor-1(ASF-1) because they are present in nuclear extracts which bind to the *as-1* element. *Arabidopsis* TGA2 comprises approximately 33 and 50% of the ASF-1 activity detected in root and leaf nuclear extracts respectively (Lam and Lam, 1995)

Table 2.5: Classification of TGA factors from A. thaliana and N. tabacum.

TGA Class	*Arabidopsis thaliana*	*Nicotiana tabacum*
Subclass I	TGA1 [Schindler *et al*., 1992] TGA4 (OBF4) [Zhang *et al*., 1993]	TGA1a [Katagiri *et al.,* 1989] PG13 [Fromm *et al*., 1991]
Subclass II	TGA2 (AHBP1b) [Kawata *et al*., 1992] TGA5 (OBF5) [Zhang *et al.,* 1993] TGA6 [Xiang *et al.,* 1995] [Xiang *et al.,* 1997]	TGA2.1 [Niggeweg *et al*., 1997] TGA2.2 [Niggeweg *et al*., 2000a] [Niggeweg *et al*., 2000b]
Subclass III	TGA3 [Miao *et al*., 1994] AtbZIP50 [Jakoby *et al*., 2002]	
Subclass IV	PERIANTHIA [Chuang *et al*., 1999]	TGA7 [Schiermeyer, 2001]
Subclass V	AtbZIP21 [Jakoby *et al.,* 2002]	
Subclass VI	AtbZIP65 [Jakoby *et al.,* 2002]	TGA10 [Schiermeyer, 2003]

2.6 The *as-1* type regulatory elements in promoters of defense genes

TGA-bZIP binding motives exist on the promoters of SA mediated target genes. Two of them contain the core motive (TGACG) and are important for INA induced activation of PR1. One of these - the LS7 is an *as-1 (activating sequence-1)* – type promoter *cis* element and acts as an activator element, while the other - LS5 functions as a weak silencer element (Lebel *et al.,* 1998). In vitro binding of TGA2 to both LS7 and LS5 is enhanced in the presence of NPR1, which appears to alter the activity of transcription factors (Despres *et al.,* 2000). SA-induced expression in *Arabidopsis* is mediated by the *as-1*–type promoter *cis* element (*LS7*) which is recognized in vitro by TGA factors. Johnson *et al.,* in 2003 reported that two NPR1-interacting TGA factors, TGA2 and TGA3, are the principal contributors to the *LS7* binding activity of leaves and is enhanced by SA through NPR1. These *as-1* type elements are common to the promoters of PR and glutathione *S*-transferase genes and that confer transcription in response to defense hormones and xenobiotic stress signals (Niggeweg *et al*., 2000a).

The *as-1* element was first identified in the cauliflower mosaic virus (CaMV) 35S promoter. In leaves and protoplasts, expression is inducible by salicylic acid or auxin (Qin *et al*., 1994; Lui *et al.,* 1994, Ulmasov *et al.,* 1994). Since their original discovery, *as-1*-like elements have been identified as functional elements of other viral promoters and promoters of the *Agrobacterium tumefaciens* encoded T-DNA. They were also found in the promoters of other genes that were identified either as auxin-inducible genes such as NtParA (Sakai *et al.,* 1998); AtGST6 otherwise known as 'immediate early' SA-inducible genes. Even though the sequence can deviate quite substantially from the consensus sequence, spacing of 12 bp between the two centers of the palindromes is conserved in all *as-1*-like elements that respond to auxin and SA (Figure. 2.6).

On the other hand, the spacing found in *as-1*-like elements of the so-called 'late' SA-inducible promoters is less well conserved.
In 2002, using electrophoretic mobility shift assays (EMSA), Krawczyk *et al* showed that insertions of bases between the palindromes of the *as-1* element decrease factor binding *in vitro* while deletions and insertions reduce its transcriptional activation capacity.

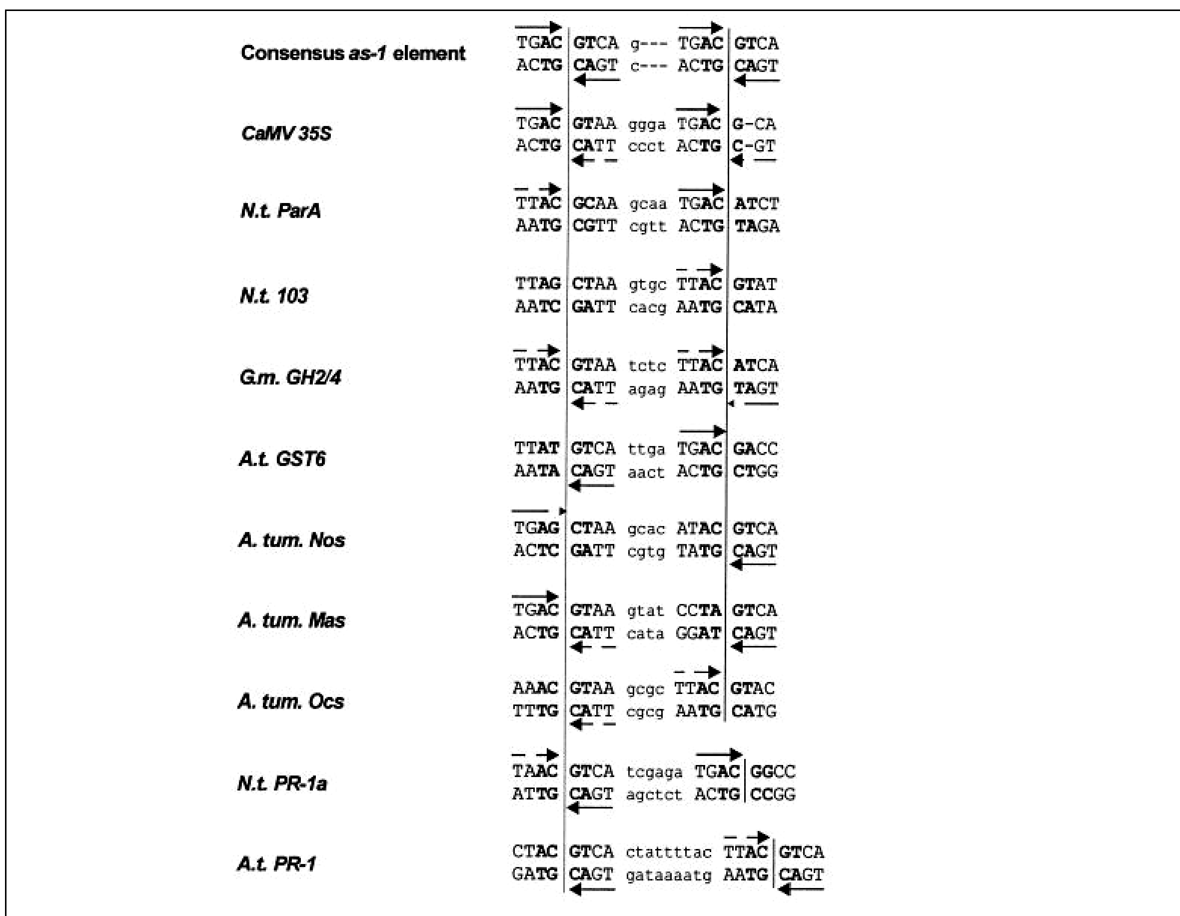

Figure 2.6. Alignment of different* as-1*-like elements from viral promoters [CaMV 35S], T-DNA promoters [*ocs, nos, mas*] and plant genes [*ParA *;* Nt103*;* GH2/4*;* GST6 *;* PR-1a*);* PR-1 *].
Palindromes are shown in capital letters, the sequence of the spacer is shown in small letters. Positions that are not defined in the consensus sequence are indicated with n. The TGAC half sites of the 8 bp palindromes are marked by arrows. TGAC sequences carrying one mutation are marked by interrupted arrows; half sites with more than one mutation are not marked. The central 4 bp (ACGT in the consensus sequence) are indicated by bold letters. The centers of the palindromes are marked by vertical lines. (From Krawczyk et al., 2001)

2.7 Early and late defense gene responses

SA inducible genes can therefore either be classified as "***immediate early***" or as "***late defense***" genes. Immediate early induced genes are those whose activation does not require *de novo* protein synthesis at the time of SA induction. They are activated independent of NPR1. Their expression is usually transiently observed as early as 1-2 hours after SA induction. Examples include the genes coding for glutathione-S-transferases (AtGST6, Nt103) and glucosyltransferases (EIGT). Evidence for this has been shown by Chen *et al.* (1996) and Uquillas *et al.* (2004).

On the other hand, lately expressed defense genes require *denovo* protein biosynthesis. Their expression is inhibited by cycloheximide. They are induced after 10-12 hours and are long lasting. Their induction requires the function of NPR1 as discussed in section 2.10. Examples include the pathogenesis related genes [AtPR-1 (Zhang *et al*., 1999); NtPR-1a (Strompen *et al.,* 1998)].

2.8 Systemic Acquired Resistance (SAR) in plants

Plants use distinct signal transduction pathways to defend themselves against invading pathogens. A primary local infection in resistant plants results in a hypersensitive reaction (HR, Figure 2.8), and an oxidative burst, a process in which the plant quickly recognizes the pathogen (Lam *et al.,* 2001). The formation of necrotic lesions in this process limits the growth and spread of the pathogen. Distant or systemic tissues become resistant to infection by a broad range of fugal, bacterial, oomycetes and viral pathogens. (Hunt and Ryals, 1996, Durant and Dong, 2004) This resistance state attained could be long lasting and could sometimes even persist throughout the lifetime of the plant (Ryals *et al*., 1996; Sticher *et al*., 1997).

This biologically induced resistance in systemic tissues is called "***Systemic Acquired Resistance"*** (SAR) and has been reported in several plant species. It was reported as early as 1960 by Ross who showed that tobacco plants infected by the TMV virus subsequently developed an increased resistance in distant tissues (Ross, 1961)

Associated with SAR is the activation and coordinated expression of a broad range of pathogenic related (PR) genes in both local and systemic tissues (Ryals *et al*., 1996; Maleck *et al.,* 2000, Figure 2.8), whose concerted action is thought to bring about the SAR. They are useful molecular markers for the onset of SAR. They encode small secreted vacuolar targeted proteins. Even though most of them have anti-microbial properties *in vitro*, the function of each in the defense response is not clearly defined (Van Loon *et al.,* 1999).

Their concerted action is nevertheless thought to be important in bringing about the SAR response.

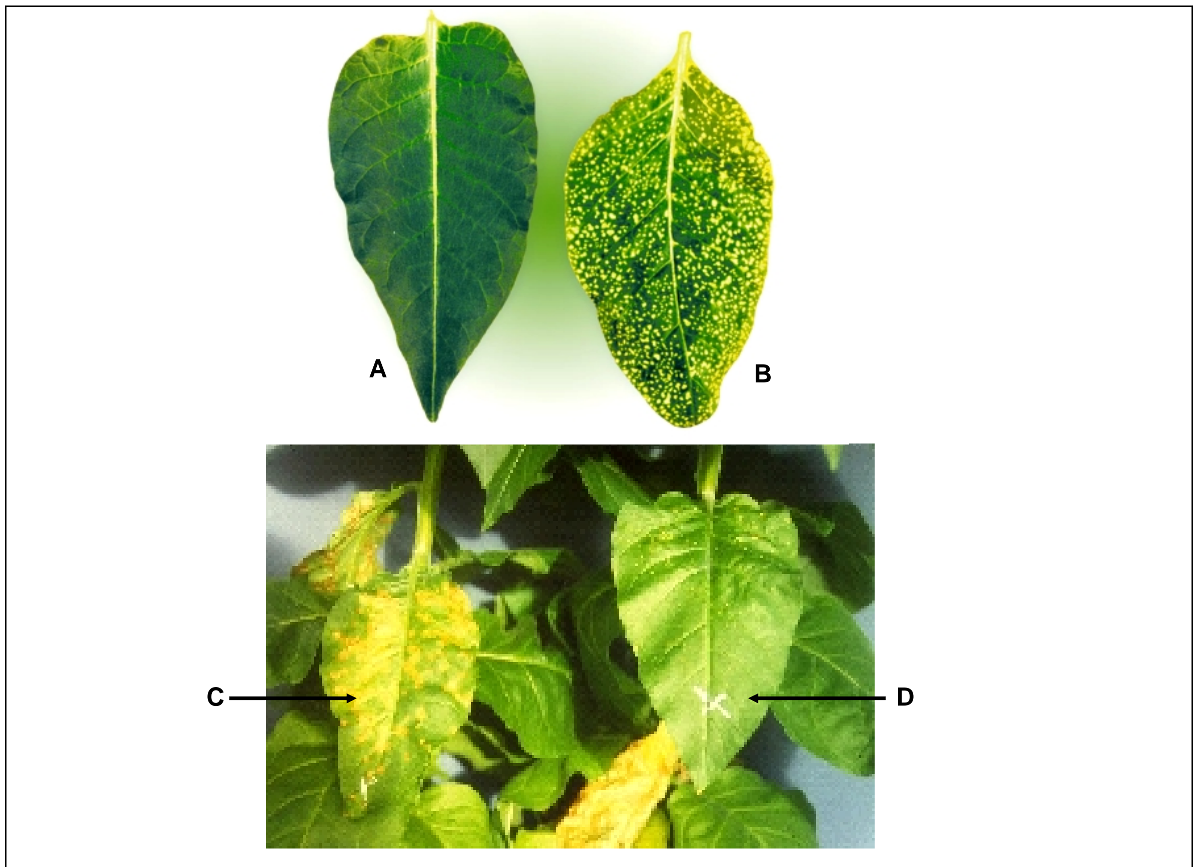

Figure 2.8: Tobacco mosaic virus/N gene interaction: a classic hypersensitive response (HR) and Systemic Acquired Resistance (SAR) model system. *Leaves of a tobacco (Nicotiana tabacum) cultivar containing the N resistance gene were either mock treated (A) or inoculated with TMV (B and C). After-inoculation, TMV replication leads to formation of HR lesions that efficiently restrict the virus to the inoculated regions, as in B and C. (Lam et al., 2001). When a systemic leaf is later on inoculated (D), it does not produce the same HR lesion types as in C, because of the development of a systemic acquired resistance.*

A lipid-based molecule may function as a mobile signal for SAR. Maldonado *et al.* (2003) showed that the *dir1* (defective in induced resistance 1) mutant has normal local resistance to pathogens but is unable to develop SAR or express PR genes in systemic leaves. Therefore, wild-type DIR1, which has sequence similarity to lipid transfer proteins (LTPs), might function in the generation or transmission of the mobile signal.

2.9 Salicylic acid mediates SAR signaling

In 1979, White reported that when tobacco leaves are treated with SA, aspirin (acetyl SA) or benzoic acid, PR genes are induced and the tobacco plants become resistant to TMV invasion. It was also observed later that infection of tobacco with TMV correlates with the rise in the levels of SA in the plant, as well as the expression of PR genes, giving evidence to the fact that SA is a signal for SAR (Malamy *et al.,* 1990, Métraux *et al*., 1990).

Other SA analogues which induce the same set of PR genes include 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole S-methyl esther (BTH) (Friedrich *et al.,* 1996, Görlach *et a*l., 1996, , Lawton *et al.,* 1996, Ward *et al.,* 1991). Gaffney *et al* further showed that when a bacteria gene encoding a salicylate hydroxylase (NahG) is expressed in plants, SA is hydrolyzed to chatechol. Transgenic *Arabidopsis* and tobacco plants containing the nahG transgene accumulate very low levels of SA after pathogen induction, and therefore fail to express PR genes and are thus compromised in mounting an SAR response (Delaney *et al.,* 1994; Gaffney *et al.,* 1993).

2.10 NPR1 and the regulation of SAR

NPR1 is a key element involved in regulating changes in gene expression mediated by SA. The NPR1 gene encodes a protein containing a bipartite nuclear localization sequence (NLS) and two potential protein-protein interaction domains: one ankyrin repeat domain and one BTB/POZ (broad-complex, tramtrack, and brica` -brac/poxvirus, zinc finger) domain (Cao *et al*., 1997; Kinkema *et al.,* 2000).

The *Arabidopsis non expressor of PR-1 genes (npr1)* mutant was first isolated in a screen for plants that failed to express PR genes after SAR induction (Cao *et al.,* 1994). Other alleles like the *non-inducible immunity-1* (*nim1)* and the *salicylic acid insensitive mutant -1 (sai1)* were also isolated in screens for mutants in the SAR pathway (Glazebrook *et al.,* 1996; Shah *et al.,* 1997). These mutants are compromised in SAR, as well as in basic resistance, and show enhanced symptoms of disease when infected with virulent pathogens. Expression of essentially all genes demonstrated to be upregulated by SAR is affected in the *npr 1-1* mutant (Maleck *et al*., 2000).

NPR1 is constitutively expressed, with the transcript level increasing only to two fold levels upon SA induction, giving a hint that post transcriptional modification at the protein level is needed for its activation (Cao *et al*., 1998).

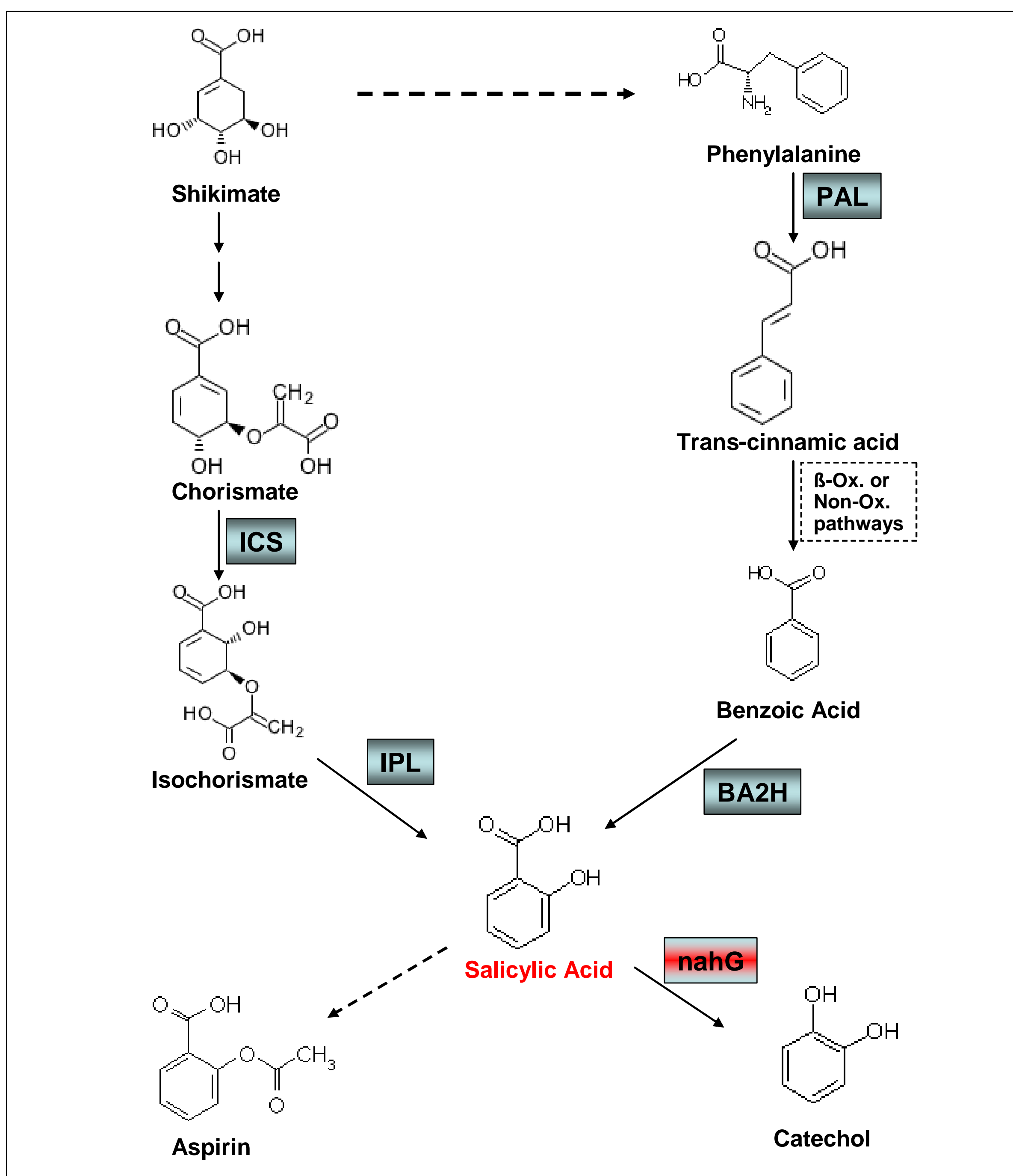

Figure 2.9: Biosynthetic pathway of Salicylic Acid.
Adapted from Ryals et al., (1996); Shah (2003) and Wildermuth et al., 2002.
ICS: Isochorismate synthase, IPL: Isochorismate lyase, PAL: Phenylalanine ammonium lyase; BA2H: Benzoic Acid 2- Hydroxylase.

In 2003, Mou *et al* in a series of ground breaking experiments clearly showed that NPR1 exists in the cytoplasm as an oligomer, formed through intermolecular disulfide bonds. Upon SAR induction, there is a rapid oxidative burst. A biphasic change in cellular reduction potential occurs, resulting in a reduced environment and the reduction of NPR1 to a monomeric form. Two cysteine residues Cys82 and Cys216 are critical for this step, whose mutation results in constitutive monomerization and nuclear accumulation.

An unmasking of the NPR1- NLS also occurs in the process of reduction and monomerization. Monomeric NPR1 accumulates in the nucleus and activates gene expression by its interaction with the TGA sub-family of bZIP transcription factors (Zhang *et al.,* 1999). It regulates their binding to a regulatory element on the promoter of PR1 gene, thereby regulating its expression (Despres *et al.,* 2000; Figure 2.13).

Redox mediated modification of TGA factors has also been demonstrated in the case of TGA1 and TGA4 although TGA1 does not interact with NPR1 in yeast two-hybrid assays (Despres *et al.,* 2003). In an uninduced state, two conserved cysteine residues in these proteins are oxidized, forming intramolecular disulfide bonds. Upon SA induction, the disulfide bond is broken and NPR-TGA1 interaction occurs to mediate gene expression. Site directed mutagenesis of the conserved cysteines leads to their interaction in yeast, indicating that TGA1 relies on the oxidation state of the Cys residues to mediate the interaction with NPR1. An intramolecular disulfide bridge in TGA1 prevents interaction with NPR1, which can only stimulate the DNA binding activity of the reduced form of TGA1 (See Figure 2.13).

The *Arabidopsis* genome contains six NPR1-related genes. Liu *et al*. (2005) reported that an NPR1-like gene, NPR4 codes for a protein which shares 36% identity with NPR1 and interacts with the same spectrum of TGA transcription factors in yeast two-hybrid assays. It also plays a role in resistance to virulent bacterial pathogen *Pseudomonas syringe* pv. tomato DC3000 and to the fungal pathogen *Erysiphe cichoracearum.* NPR4 mRNA levels increase following pathogen challenge or SA treatment. NPR4 is nevertheless speculated to be required for basal defense against pathogens as the mutant plant *npr4* but does not differ markedly from wild type in its interaction with virulent and avirulent strains of the oomycete *Peronospora parasitica.*

2.11 Salicylic acid mediated regulation of transcription and gene expression patterns

TGA factors are able to form homo and hetero dimmers, which bind to the *as-1* element to mediate transcription. Modifications of the protein-protein interacting basic domain of TGA factors interfere with their ability to bind DNA and mediate transcription from the *as-1* element (Miao et *al.,*1995, Rieping *et al.,* 1994). A dominant negative version of the TGA2, which lacks the N-terminal and bZIP domains important in DNA binding and dimerization was expressed in *Arabidopsis* by Fan and Dong in 2002. This produced a phenotype which resembled that of the *npr1-1* mutant plant. They could show by this that though the dominant negative version still interacted with NPR1, both the properties of protein-protein interaction and the ability to bind to DNA are necessary for the function of TGA factors. In a similar manner, Thurow *et al* in 2005 have shown that the expression of a TGA2.2 mutant which is unable to form heterodimers with the endogenous pool of TGA factors led to a reduced inducibility of Nt103, an immediate early gene, indicating the importance of the native leucine zipper in positively enhancing transcription.

Yeast two hybrid screens and other *invitro* and *invivo* screens have been used to isolate other proteins which interact with TGA factors. NPR1 is the key protein which interacts with TGA subclass of basic leucine zipper transcription factors, NtTGA2.2, AtTGA2, AtTGA5 and AtTGA6 (Zhang *et al.,* 1999; Niggeweg *et al.,* 2000b). NtTGA2.2 is highly homologous to AtTGA2, AtTGA5 and AtTGA6, which have also been shown to have redundant but essential roles in SAR and SA inducible expression of PR-1 genes (Zhang *et al.,* 2003). Zhang and co also found that SA induced PR gene expression was significantly blocked only in the *tga2 tga5 tga6* triple knockout mutant, and not as much in the single and double mutants. This triple mutant also showed reduced tolerance to high levels of exogenous SA, a phenotype that is also observed in the *npr1* mutant. However, unlike *npr1*, the triple mutant does not show significantly enhanced susceptibility to virulent pathogens, indicating that other NPR1- interacting TGA factors (including the redox-sensitive TGA1 and TGA4) may be responsible for the expression of genes that are involved in basal resistance.

Because of the complex nature of gene expression, it was necessary to characterize the endogenous function of other TGA interacting proteins, in order to identify putative activators and suppressors of *as-1* mediated gene expression.

One negative regulator of SAR is SNI1, which was identified in a genetic screen for suppressors of *npr1* (Li *et al*., 1999). SA-induced PR gene expression is restored in the *npr1sni1* double mutant, giving a hint for an SA dependent but NPR1 independent transcription factor that might be involved in controlling PR gene expression. A candidate gene requiring validation is that coding for the *A. thaliana* Whirly 1 transcription factor (AtWhy1) with single stranded DNA binding activity. It was shown by Desveaux *et al*., in 2002 to bind to DNA in an SA-dependent but NPR1-independent fashion. The recognized motives are present on some PR promoters. Mutations in the AtWhy1 gene compromised SA-mediated PR gene expression and resistance.

The NIMIN proteins, NIMIN-1, NIMIN-2, and NIMIN-3 are also SA inducible proteins which able to interact via NPR1/NIM1 with basic leucine zipper transcription factors of the TGA family(Weigel *et al.,* 2001). They modulate the PR gene expression in *Arabidopsis* by negatively regulating SA induced PR gene induction. The compromised induction of SAR by NIMIN-1 overexpessing plants resembles the phenotype of *npr1* mutants. They exert their activity possibly through their EAR repressor motives. In *nimin* mutant plants, PR1 gene induction is super induced (Weigel *et al.,* 2005).

WRKY transcription factors may also play a role in regulating PR gene expression as the W-boxes which they recognize are represented in the regulon of the PR1 promoter. They have also been shown to be important in the PR1 promoter activity (Lebel *et al.,* 1998). Of this family, the WRKY70 transcription factor has been shown to positively regulate PR1 gene expression (Li *et al.,* 2004).

2.12 Induced Systemic Resistance (ISR)

Apart from SAR, another induced defense pathway described is the Induced Systemic Resistance (ISR). ISR is triggered by non-pathogenic *Pseudomonas fluorescens* rhizobacteria (Ton *et al.,* 2002). It was shown to be predominantly effective against pathogens that are sensitive to JA/ET dependent defenses (Pieterse *et al*., 1998). It is postulated that the activation of pathogen - induced SAR and rhizobacteria-mediated ISR constitute a reinforcement of existing SA- or JA/ET-dependent basal defense responses

respectively. It follows what Ton and colleagues reported in 2002, that rhizobacteria-mediated ISR provided significant protection against the necrotrophic fungus *Alternaria brassicicola,* whereas pathogen-induced SAR was shown to be ineffective. On the other hand, activation of SAR resulted in a high level of protection against *Peronospora. parasitica* and Turnip crinkle virus (TCV), whereas ISR conferred only weak and no protection against *P. parasitica* and TCV, respectively.

ISR and SAR pathways are independent but have an overlapping requirement for NPR1. Recently, the role of NPR1 in ISR was reaffirmed using the *Pseudomonas fluorescens* CHA0 strain as the inducer and *P. parasitica Noco* as the challenging pathogen. ISR is initiated in roots, whereas SAR is initiated in leaves, suggesting that these two responses may not be in competition for NPR1 (Iavicoli *et al.,* 2003).

In the *npr1* mutant, the induction of induced systemic resistance (ISR) by non-pathogenic rhizobacteria is blocked. This resistance response is independent of SA as nahG plants are still capable of mounting an ISR. It nevertheless requires regulators of ethylene and jasmonic acid (JA) signaling, ETR1 and JAR1, respectively [Pieterse *et al.,* 1998].

2.13 Redox regulation in defense responses

As discussed above (Section 2.10), TGA1 and NPR1 are subject to redox regulation in connection with the regulation of the expression of PR genes.

Glucose-6-phosphate 1-dehydrogenase is main source of the reducing power for the cell. It is important in providing the reduced state that leads to NPR1 monomerization in the cytoplasm, under SA inducible conditions. Though it is pathogen inducible, it is not clear whether it is induced in an NPR1 independent manner to carry out its function (Dong, 2004).

The question remains to be answered about what proteins mediate these redox changes. Thioredoxins and glutaredoxins have also been postulated to be important in plant defense response signaling, and might function by regulating the redox status of some target proteins in the cell.

Rivas *et al.* (2004) identified CITRX (Cf-9 Interacting Thioredoxin) as a novel TRX that interacts specifically with the tomato Cf-9 resistance protein. Virus induced gene silencing of CITRX resulted in accelerated activation of Cf-9/Avr9 defense responses and disease resistance.

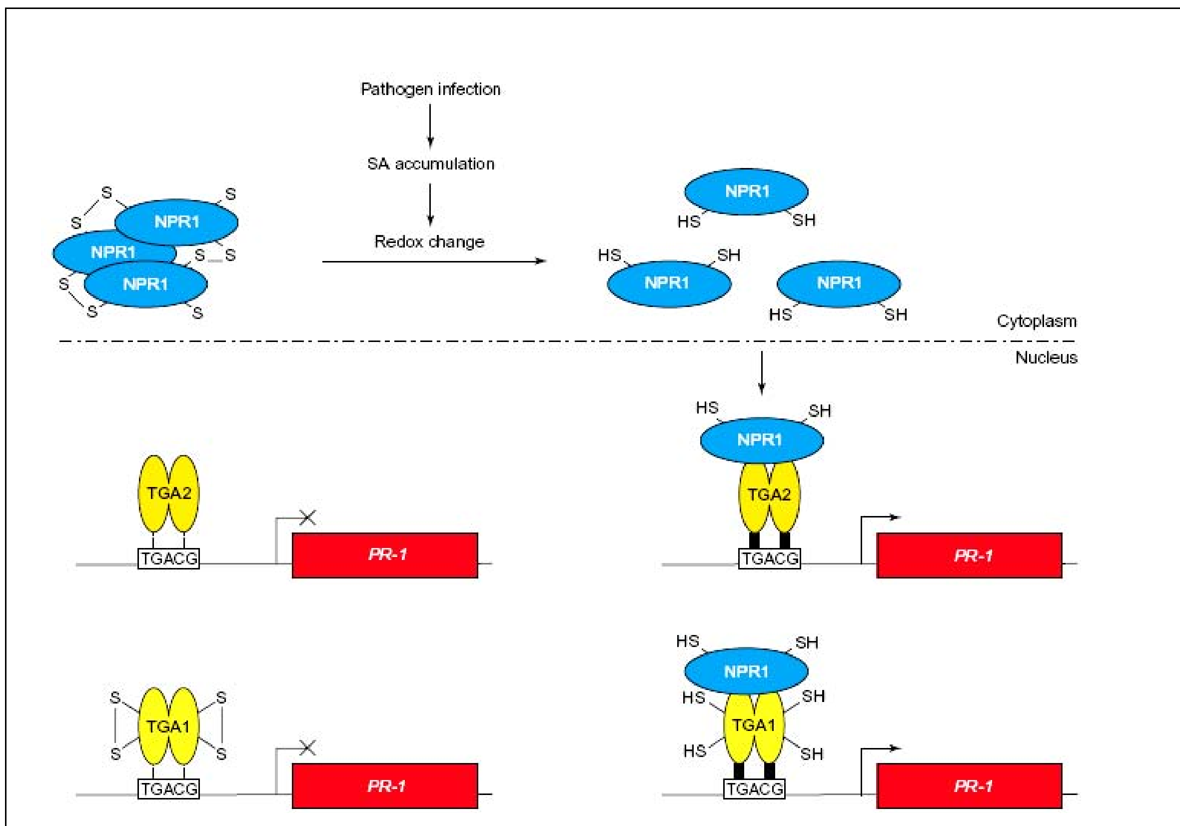

***Figure 2.13 : Illustration of SA-mediated redox regulation, associated with PR gene expression.** NPR1 exists in an oxidized state as inactive oligomers that remain in the cytosol under regular uninduced conditions. Binding of TGAs to the as-1 like (SA-responsive) promoter elements (TGACG) (indicated by dotted lines) is not sufficient to activate the expression of PR-1 genes. Upon pathogen infection, SA levels rise and a reducing environment is attained in the cells, possibly because of the accumulation of antioxidants. Under these conditions, NPR1 is reduced from an inactive oligomeric complex to an active monomeric state through the reduction of intermolecular disulfide bonds.*
Monomeric NPR1 is then translocated into the nucleus where it interacts with TGAs, such as TGA2. The binding of NPR1 to TGAs stimulates the DNA-binding activity of these transcription factors to the as-1 like cis element (represented by black boxes), resulting in the activation of PR-1 gene expression. In non-induced cells, TGAs that do not interact with NPR1 in yeast two-hybrid assays, such as TGA1, are oxidized and form intramolecular disulfide bridges that prevent interaction with NPR1. Upon accumulation of SA in planta, the change in redox status reduces the disulfide bonds in these TGAs, resulting in a conformational change that allows interaction with NPR1. (Pieterse and Van Loon, 2004).

Furthermore, the transient overexpression of CITRX compromised Cf-9/Avr9-dependent defense responses. Rivas and colleagues propose that CITRX could negatively regulate the Cf-9 resistance gene function.

Furthermore, Laloi and colleagues (2004) also identified an *Arabidopsis* cytosolic thioredoxin h5, which is inducible by oxidative stress and the *P. syringae*-derived elicitor peptide flg22. Its expression is also regulated by the WRKY6 transcription factor, which binds to WRKY sites in its promoter. Knock out mutants nevertheless show no obvious

phenotype with respect to resistance responses, indicating that there might be a functional redundancy, possibly contributed by other redox proteins.

2.14 Glutaredoxins in stress responses

Glutaredoxins are small redox proteins which belong to the superfamily of thioredoxins (TRX). Thioredoxins are involved in thiol-disulfide exchange reactions. They are characterized by a primary conserved active site sequence :[W-C-G(P)-P-C] and share a typical TRX structural fold: β1, α1, β2, α2, β3, α3, β4, β5, α4 (Martin, 1995). The five major classes in this super family include thioredoxin, glutaredoxin (GRX), protein disulphide isomerase (PDI), glutathione-S-transferase (GST) and glutathione peroxidase.

Over 40 years ago, thioredoxin function was first attributed to being an electron donor to ribonucleotide reductase in *E.coli* (Laurent *et al*. 1964). Based on sequence and structural comparisons as well as sub-cellular localization, the 19 types of TRXs in *Arabidopsis* can be classified into 5 groups (Lemaire *et al,* 2003). They are distributed in the cytoplasm, chloroplast and mitochondria.

Glutaredoxins are able to catalyze the reduction of disulfides or glutathione (GSH) mixed disulfides. They are reduced by GSH, generated from NADPH, in a reaction catalyzed be glutathione reductase (GR). They are alternative hydrogen donors of ribonucleotide reductase.

About 31 different GRXs are present in *Arabidopsis*. Three groups of GRXs exist based of structural classification: the classical CPYC group, the CGFS group and the CC-type group, which is specific for higher plants.

GRXs can reduce disulfides either through a monothiol or a dithiol mechanism (Vlamis-Gardikas and Holmgren, 2002). In the ***dithiol mechanism***, following a nucleophilic attack of the disulphide by the most N-terminal cysteine of the active site, a transient mixed disulfide is formed between the GRX and the target substrate. In a second step, the second cysteine serves as an electron donor, resulting in the reduced substrate and an oxidized GRX. *Dithiol GRXs can therefore reduce both protein disulfides and GSH-thiol disulfides.*

In a ***monothiol mechanism***, where only one cysteine is present in the active site of GRX, GRX reduces a mixed disulfide between glutathione and a protein thiol. The mixed

disulfide formed between GRX and GSH is reduced by GSH, to form oxidized GSH (GSSG) and reduced GRX. GSSG is regenerated to GSH through the action of GR. *Monothiol GRXs can thus reduce only GSH-thiol disulfides.*

GRX genes are induced in oxidative stress conditions (Prieto-Alama *et al*. 2000). Their diverse functions reported so far include function as a dehydroascorbate reductase in human neutrophils (Park and Levine 1996), GR (Collinson *et al*. 2002) and glutathione-S-transferase (Collionson and Grant 2003). They also reduce certain peroxiredoxins and participate in the regulation of several transcription factors related to oxidative stress signaling in mammals. (Rouhier *et al*. 2002). A human GRX-homologue called PICOT (protein Kinase C- interacting cousin of thioredoxin), has been shown to be a negative regulator of protein kinase C-θ which is involved in the activation of AP-1 and NF-kB transcription factors. (Witte *et al*. 2000)

A CC- type *Arabidopsis* glutaredoxin called ROXY1 has also been reported to regulate the development of flower primordia, by inhibiting the AGAMOUS gene. (Xing *et al*. 2005)

Glutaredoxins thus have diverse functions. Even though their functions sometimes overlap with that of TRX, GRXs have a unique role in the reactivation of proteins modified post-translationally after oxidative stress conditions, by deglutathionylation of cysteine residues, a function which cannot be carried out by TRX (Lemaire, 2004).

The abundance of GRX in phloem sap (Ishiwatari *et al*. 1997) suggests its possible role in long distance signaling and oxidative stress responses in plants (Juang and Thomas 1996; Nulton-Persson *et al*. 2003).

2.15 Purpose of the study

In a search for interacting partners for the TGA2.2 transcription factor, an *in vitro* Y1HS was previously used. A glutaredoxin was isolated, which interacts with the spectrum of all bZIP TGA transcription factors. The identity AGI code was At1g28480(Abdallat, 2004).

The purpose of this study was to characterize the function of the isolated glutaredoxin using reverse genetic and biochemical approaches.
Knock out lines, lines containing an RNA-interference construct, as well as constitutively expressing lines were to be characterized, and compared to wild type lines.
The expression pattern of At1g28480 was to be characterized.
The function of At1g28480 in modulating or mediating *as-1* related gene expression and defense was to be investigated *in vitro* and *in vivo.*
The effect of At1g28480 on transgenes, as well as endogenous target genes was to be studied.
The specificity of the effects was also to be analyzed by studying a classical glutaredoxin, At5g40370, using similar approaches.
An epistasis analysis was to be used to determine the position of At1g28480 in the signal transduction pathway leading to a modulation of *as-1* mediated gene expression patterns in plants.

3 Manuscript for publication

SA-induced Arabidopsis glutaredoxin interacts with TGA factors and suppresses JA-induced PDF1.2 transcription

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Running title: Glutaredoxin interacting with TGA factors

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Abstract

Salicylic acid (SA) is a plant signalling molecule that mediates the induction of defense responses upon attack by a variety of pathogens. *Activation sequence-1* (*as-1*) type *cis* elements and their cognate basic/leucine zipper (bZIP)-type transcription factors of the TGA family regulate transcription in response to SA and in response to xenobiotic chemicals. TGA factors interact with NPR1 (NON EXPRESSOR OF PR GENES 1), a central regulator of many SA-induced defense responses. Changes in the redox state of both TGA1 and NPR1 have been observed under inducing conditions. In order to identify further proteins interacting with TGA factors, a yeast protein interaction screen with tobacco TGA2.2 as a bait and an *Arabidopsis thaliana* cDNA prey library was performed and led to the identification of a member of the glutaredoxin family (GRX480, encoded by *At1g28480*). Glutaredoxins are candidates for mediating redox regulation of proteins because of their capacity to catalyze disulfide transitions. Transgenic Arabidopsis plants ectopically expressing *GRX480* show wild-type expression of standard marker genes for SA- and xenobiotic-inducible expression. However, jasmonic acid (JA)-inducible transcription of defensin gene *PDF1.2* is suppressed in these plants. As SA is known to interfere with JA-dependent transcription, GRX480, which is induced after SA-treatment, may constitute one of the regulatory compounds controlling *PDF1.2* promoter activity. In contrast to the NPR1-mediated antagonism between SA- and JA-dependent signal transduction networks described earlier, the GRX480-mediated repression does not affect expression of other JA-inducible genes like *ERF1*, *LOX2* and *VSP* indicating that NPR1 and GRX480 act through separate pathways.

Introduction

TGA factors constitute a conserved plant subfamily of basic domain/leucin zipper (bZIP) transcriptional regulators whose genomic targets include *glutathione S-transferase* and *pathogenesis-related* (*PR*) genes that are associated with detoxification and defense (Zhang *et al.*, 2003; Thurow *et al.*, 2005). TGA factors bind to *as-1*–type elements (Lam *et al.*, 1989) that autonomously confer transcription in response to defense hormones like salicylic acid (SA) and xenobiotic stress cues (Liu and Lam, 1994; Qin *et al.*, 1994; Ulmasov *et al.*, 1994).

Based on sequence similarities, TGA factors are grouped into different classes (Miao *et al.*, 1994). Loss-of-function studies have shown that especially class II TGA factors play important roles in SA-mediated gene expression: Arabidopsis plants lacking TGA factors AtTGA2, AtTGA5 and AtTGA6 are deficient in *PR-1* expression and SAR establishment (Zhang *et al.*, 2003). Likewise, reduction of tobacco class II factors NtTGA2.2 and

NtTGA2.1 in 2.2/2.1RNAi tobacco plants correlates with reduced expression of *glutathione S-transferase Nt103* and *PR-1a* (Thurow *et al.*, 2005).

As TGA factors are primary transcription factors that pre-exist under non-inducing conditions (Qin *et al.*, 1994), their activity must be tightly regulated. SA-inducible phosphorylation of TGA2 has been reported, but the biological significance remained elusive (Kang and Klessig, 2005). A sensible approach to understand the regulation of gene expression by TGA factors is the search for interacting proteins. Most importantly, NPR1 (NON EXPRESSOR OF *PR* GENES1), a central regulatory protein of SA-dependent processes, has been identified as a TGA-interacting protein (Zhang *et al.*, 1999). NPR1 functions in a signal pathway leading from salicylic acid (SA) or its analogs such as 2,6-dichloroisonicotinic acid (INA) to the induction of *PR* genes and the onset of a global defense program known as systemic acquired resistance (SAR)(Cao *et al.*, 1997; Ryals *et al.*, 1997). TGA factors AtTGA2 and AtTGA3 are recruited to the *PR-1* promoter only in the presence of SA and NPR1 (Johnson *et al.*, 2003). In contrast, *as-1*-containing promoters of *glutathione S-transferase* genes are activated independently of NPR1 implicating different regulatory mechanisms controlling *as-1*-mediated gene expression (Uquillas *et al.*, 2004). In addition to NPR1, members of the Dof (Zhang *et al.*, 1995) and ERF family (Buttner and Singh, 1997) of transcription factors interact with TGA factors and are likely to contribute to the promoter specificity of TGA factor binding.

SA treatment and xenobiotic stress lead to changes in the cullular redox homeostasis and the redox state of NPR1 (Mou *et al.*, 2003) and TGA1 (Despres *et al.*, 2003). NPR1 protein activation is correlated with the reduction of two of its cysteines resulting in a transition from an oligomeric to a monomeric form (Mou *et al.*, 2003). The monomeric and reduced form of NPR1 is then translocated from the cytosol to the nucleus where it activates *PR-1* gene expression (Kinkema *et al.*, 2000). In yeast, NPR1 interacts constitutively with AtTGA2, AtTGA5 and AtTGA6, whereas interaction with TGA1 occurs only when the intramolecular disulfide bridge of TGA1 is reduced, a modification that is induced in SA-treated plant cells (Despres *et al.*, 2003).

In addition to SA, the signalling molecule jasmonic acid (JA) plays a crucial role in plant defense responses (Beckers and Spoel, 2006). Whereas SAR is efficient against biotrophic pathogens that feed on a living host cell, JA protects the plant from insect infestation and necrotrophic pathogens. NPR1 has emerged as a critical modulator of the cross-talk between the SA and JA signals. NPR1 does not only repress genes involved in JA synthesis, but also interferes with the signal transduction chain leading from JA to the expression of target genes like *PDF1.2* and *VSP* (Spoel *et al.*, 2003). Whereas NPR1 needs to be transported into the nucleus for activation of SA-induced genes, nuclear localization is not required for suppression of JA-inducible responses.

Here we report the isolation of a glutaredoxin as a TGA-interacting protein. Glutaredoxins catalyze thiol disulfide reducing reactions rendering these proteins candidates for controlling the redox state of regulatory proteins (Lemaire, 2004). We show evidence that the TGA-interacting glutaredoxin, which is synthesized under conditions of elevated SA levels, is a negative regulator of jasmonic acid (JA)-inducible expression of *PDF1.2* (Spoel et al., 2003) and thus represents a potential regulatory component of the SA/JA antagonism.

Results

Identification of TGA interacting protein GRX480 by a yeast "one-and-a-half hybrid" screen

To identify proteins that interact with TGA factors, a yeast "one-and-a-half hybrid" screen was performed (Serebriiskii *et al.*, 2001). This screen constitutes a combination of the yeast "one hybrid system" with the yeast "two hybrid system". The "one hybrid" part consists of three copies of the *as-1* element upstream of the *HIS3* selectable marker gene as well as a library encoding Arabidopsis cDNAs fused to the GAL4 activation domain. The "half hybrid" part is represented by the actual bait, which is the TGA factor expressed *in trans* under the control of the *Met-25* promoter. As AtTGA2 and its ortholog NtTGA2.2 do not encode an activation domain that is active in yeast cells, binding of these factors to the *as-1* elements upstream of the *HIS3* gene does not lead to growth on selective medium. However, if interacting proteins fused to an activation domain are recruited to this promoter, growth is restored. This screening strategy reduces the risk of loosing potential TGA interaction partners whose affinities might be lowered because of the N-terminal fusion of the bait with the GAL4 DNA binding domain commonly used in "two hybrid" screens. Moreover, the TGA factor is bound to its target sequence thus mimicking a situation present in plant cells. Expression of NPR1 together with NPR1-interacting protein NIMIN1 fused to the GAL4 activation domain restored histidine prototrophy in these yeast cells demonstrating the functionality of the system (Weigel *et al.*, 2005).

After transformation of a library of Arabidopsis cDNAs fused to the GAL4 activation domain (Weigel *et al.*, 2001) into the yeast strain harboring NtTGA2.2 bound to three *as-1* elements upstream of the *HIS3* reporter gene, 28 clones out of 1 x 10^6 yeast transformants were able to grow on selective media. Restriction analysis and sequencing of the inserts led to the classification of four groups of recombinant plasmids. The largest group (22 members) encoded full length cDNAs being identical to *At1g28480* that were fused in frame with the GAL4 activation domain. The protein deduced from this sequence

belongs to the family of glutaredoxins and was subsequently called GRX480. Recovered prey plasmids were retransformed into the appropriate yeast cells expressing or lacking TGA2.2, respectively. Histidine prototrophy was restored only in the presence of TGA2.2 (data not shown) indicating that the gene product of *At1g28480* is recruited to the *as-1* element via TGA2.2.

GRX480 is a CC type glutaredoxin with a unique N-terminal domain

Figure 1A depicts the genomic DNA and deduced protein sequence of *At1g28480*. Glutaredoxins (GRXs) are small redox proteins of approximately 12 kDa that are able to catalyze the reduction of disulfides or glutathione (GSH) mixed disulfides (Lemaire, 2004). The Arabidopsis genome encodes 30 *GRX* genes that have been classified into three major subgroups according to the sequence of the active center: (1) the CPYC group present also in yeast, *Escherichia coli* and mammals; (2) the CGFS group; and (3) the plant specific CC group. GRX480 encodes a CCMC sequence and thus belongs to the CC group with two potentially active cysteines (C). 24 of the Arabidopsis *glutaredoxin* genes only encode a 100 amino acid long core protein, whereas 6 *glutaredoxin* genes, including GRX480, possess protein specific N-terminal extensions of ca. 30 to 70 amino acids.

Consistent with the lack of SA-inducible expression of GRX480 in the *npr1-1* and *tga2tga5tga6* triple mutants (Fig. 1B,C), a number of TGA binding motifs were found in promoter. Perfect binding site for TGA dimers is the palindromic sequence TGAC/GTCA, but the first five basepairs (TGAC/G) are already sufficient for recognition (Spoel *et al.*, 2003). Within 1036 bp of the *At1g28480* sequence upstream of the putative transcriptional start site, six of these TGAC/G motifs are found. Typical *as-1*-like elements are characterized by two binding sites with 12 bps between the palindromic centres. In this arrangement, the sequence requirement is less stringent (Krawczyk *et al.*, 2002). A putative *as-1* element (TGAC/GCACnnnnTTAC/GTAA) is located between positions –80 and -101 relative to the putative transcriptional start site, which corresponds to its location within the *CaMV 35S* promoter. In addition to TGA binding sites, *cis* elements recognized by WRKY transcription factors are generally overrepresented in pathogen-inducible promoters (Maleck *et al.*, 2000). Indeed, four binding sites for WRKY transcription factors are located in the *GRX480* promoter.

GRX480 transcription is induced by salicylic acid and depends on TGA factors and NPR1

The potential relevance of GRX480 in the biological context of SA-dependent defense responses was supported by Northern blot analysis that revealed increased steady state *GRX480* transcript levels after SA treatment (Fig. 1B). As described for *PR-1*, expression of *GRX480* is severely reduced in the *npr1-1* mutant (Cao *et al.*, 1994), though some background transcript levels remain. The *tga2tga5tga6* triple mutant (Zhang *et al.*, 2003) revealed compromised inducibility with respect to *GRX480* transcript levels (Fig. 1C) but not with respect to *PR-1* transcript levels, clearly identifying a difference between the regulation of these promoters. Previous analysis of the *tga2tga5tga6* triple mutant had revealed compromised *PR-1* expression after INA treatment (Zhang *et al.*, 2003), indicating that SA and INA are not equivalent inducers with respect to the requirement of *PR-1* expression for class II TGA factors. Consistent with earlier results, basic *PR-1* levels were enhanced in the *tga2tga5tga6* triple mutant. As expected, *GRX480* expression was also induced after challenge of plants with *Pseudomonas syringae*, irrespective of whether a virulent or an avirulent strain was used (data not shown). Jasmonic acid (JA) and 2.4D, that induce NPR1-independent *glutathione S-transferase* promoters encoding an *as-1* element (Wagner *et al.*, 2002; Blanco *et al.*, 2005), did not efficiently induce *GRX480* (supplementary data fig 6.2).

GRX480 is both localized in the nucleus and the cytosol

The subcellular localization of GRX480 was determined by transient expression of a GRX480:GFP (green fluorescent protein) fusion in BY-2 protoplasts. As documented in Figure 1D, GRX480:GFP is localized both in the nucleus and the cytosol. As the majority of TGA2 accumulates in the nucleus (Kang and Klessig, 2005), it seems feasible that GRX480 can interact with TGA factors *in planta*.

GRX480 interacts with different TGA factors in the yeast two hybrid system

In order to test whether GRX480 would also interact with TGA factors in the classical yeast two hybrid system, the prey plasmid isolated in the original screen (pGAD10-GRX480) was transformed into yeast HF7c cells containing either NtTGA2.2, AtTGA2 or AtTGA6 fused to the GAL4 DNA binding domain (in pGBT9) and assayed for growth on histidine drop-out medium (Table 1). Prototrophic growth was detected whenever GRX480 was co-expressed with one of the TGA factors tested. This indicates that GRX480 does not only interact with NtTGA2.2 but also with the Arabidopsis homologues AtTGA2 and AtTGA6. Interaction with NtTGA2.2 was confirmed by a

domain swap experiment that used yeast cells expressing GRX480 fused to the GAL4 DNA binding domain and NtTGA2.2 fused to the GAL4 activation domain. Using this experimental setup, interaction with other TGA factors encoding an activation domain could be tested. Clearly, TGA factors of distinct classes like NtTGA1a and NtTGA10 interact with GRX480 in this assay.

As NPR1 is a redox regulated protein (Mou *et al.*, 2003), we asked the question whether the interaction with TGA factors might be able to recruit GRX480 into the vicinity of NPR1. Therefore, a yeast “bridge assay” was carried out with BD-GRX480 and AD-NPR1 fusion proteins expressed together along with TGA2.2. Growth under selective conditions occurred only when TGA2.2 was provided as a bridging component (Table 1). Thus, it is possible that these three proteins form a ternary complex *in planta*.

The N terminus end of GRX480 is not essential for the interaction with TGA factors

As outlined above, Arabidopsis encodes 30 related *GRX* genes raising the probability of functional redundancy. A unique feature of GRX480 is that it encodes a specific 31 amino acid N-terminal domain. To test, whether this domain is essential for the interaction with TGA factors, an N-terminal deletion of GRX480 (GRX480ΔN) was tested in a two hybrid system. In addition, GRX370 (*At5g40370*), a classical CPYC type glutaredoxin, which is highly represented in the EST data bases, was challenged for its interaction with AtTGA2 in the same assay. Individual yeast clones expressing either GRX480 or GRX480ΔN varied dramatically with regard to their interaction to TGA factors, independently from the amounts of proteins synthesized as analyzed by Western blot analysis. Still, it can be concluded that AtTGA2 interacts with GRX480 and with GRX480ΔN but not with GRX370 (Fig. 2).

Ectopic expression of GRX480 negatively regulates as-1-mediated gene expression

The effect of GRX480 on *as-1*-mediated gene expression was analyzed in transgenic Arabidopsis lines expressing the *GRX480* coding sequence under the strong constitutive *Cauliflower Mosaic Virus (CaMV) 35S* promoter. The construct was transformed into Arabidopsis lines that had been previously transformed with a construct encoding the *ß-glucuronidase* gene (*GUS*) under the control of the “truncated” *CaMV 35S* promoter (*as-1::GUS*). This promoter fragment comprises the sequences between +1 to -90 of the CaMV 35S promoter and contains the *as-1* element as a single upstream regulatory element (Redman *et al.*, 2002). *GRX480* expressing transgenic Arabidopsis lines were selected by Western blot analyses using an αHA antibody (data not shown) that detects the HA_3-tagged transgenic protein. The effect of constitutive expression of GRX480 on

the “truncated” CaMV 35S promoter was analyzed after auxin induction of the *as-1::GUS* transgene. Using auxin rather than SA had the advantage that the inducibility of *as-1::GUS* is higher than its inducibility by SA. Second, the relative degree of GRX480 overexpression is higher in auxin- than in SA-treated tissue because the endogenous *GRX480* transcript levels do not increase after auxin treatment. As shown in Figure 3A, *GUS* transcript levels decreased in the three lines with high HA_3:GRX480 expression, whereas transgenic lines with only slightly enhanced HA_3:GRX480 levels showed a response almost identical to the control plant. Thus, GRX480 negatively affects *as-1*-mediated gene expression. However, this negative effect was not observed for *GST6*, which contains a functional TGA binding site in its promoter (Chen and Singh, 1999). SA-induced expression of *PR-1* was twofold reduced (Fig. 3B) in line #3. Ectopic expression of HA_3:GRX370, which does not interact with TGA factors, did not have any influence on the expression of the reporter gene, though the protein was clearly detectable by Western blot analysis (Supplementary data, Fig.6.5C).

Ectopic expression of GRX480 interferes with induction of PDF1.2

The repressive effect of GRX480 on expression from the “truncated” *CaMV 35S* promoter prompted us to test the hypothesis whether GRX480 might be involved in the down-regulation of genes after SA treatment. One well-known example of such a gene is the JA-responsive gene *PDF1.2* (Spoel *et al.*, 2003), the induction of which is antagonized by SA. In order to analyze whether GRX480 affects *PDF1.2* induction, *CaMV35S::HA₃:GRX* plants (line #3) and control plants were treated with JA and analyzed for *PDF1.2* expression in a time course experiment. As a matter of fact, *PDF1.2* expression was strongly impaired (Fig. 4A), indicating that GRX480 might be involved in the down-regulation of JA responses. *LOX2* and *VSP*, two other JA-inducible genes that were shown previously to be negatively regulated by SA, were repressed to a much lesser extent (twofold) than *PDF1.2*.

To make sure that the negative effect of GRX480 on *PDF1.2* expression is not due to an artefact created by the HA_3 tag, a second construct leading to the expression of an untagged GRX480 was generated and transformed into Arabidopsis plants. As shown in Figure 4B, expression of an untagged derivative also suppressed *PDF1.2* expression. The GRX480 specific N-terminal domain, which was shown in Fig. 2 to be dispensable for the interaction with TGA factors, is also dispensable for the repressive activity.

The negative effect of GRX480 on PDF1.2 expression is mediated either independently from or downstream of NPR1

It has been described previously that SA-mediated suppression of JA-responsive genes requires the presence of NPR1 (Spoel *et al.*, 2003). This could be due to the absence of GRX480 in the *npr1-1* mutant (Fig. 1B). Alternatively, GRX might regulate the redox status of NPR1. Our results on the potential formation of a TGA/GRX480/NPR1 ternary complex (Table 1) prompted us to analyze whether NPR1 is required for the GRX480-mediated repression of *PDF1.2* transcription. HA_3:GRX480 was ectopically expressed in the *npr1-1* mutant. Transformants and the appropriate control plants (wild-type, *npr1-1*) were subjected to a "cross-talk" experiment that included treatment with SA, JA, SA/JA and the solvent ethanol. The SA-mediated suppression of JA-induced *PDF1.2* expression is clearly observed in Col-0 wild-type plants. As described previously (Spoel *et al.*, 2003), this negative effect of SA was partially compromised in the *npr1-1* mutant (Fig. 5). However, GRX480 is functional in the absence of NPR1 as documented by the transgenic line expressing HA_3:GRX480 in the *npr1-1* mutant background, indicating that GRX480 interacts independently from or downstream of NPR1.

Rehybridization of the blot with a probe encoding for ethylene response factor 1 (ERF1) showed that this global regulator of JA-related defense responses is also under the control of the NPR1-mediated SA/JA antagonism. As constitutive expression of *ERF1* leads to constitutive expression of *PDF1.2* (Solano *et al.*, 1998), it can be hypothesized that the SA/JA-responsive expression pattern of *ERF1* determines the expression pattern of *PDF1.2* However, *ERF1* expression was not subject to the negative regulation by GRX480, suggesting that *PDF1.2* is controlled by an additional mechanism acting downstream of the more global NPR1-mediated level. When *GRX480* transcription is driven by the endogenous promoter, it will not be induced in the *npr1-1*. Thus, in the absence of NPR1, SA fails to suppress induction of *PDF1.2*.

Another gene, which can be activated by ectopic expression of *ERF1,* is the JA/ethylene-induced *PR* gene *HEL*. As shown in Figure 5, *HEL* is also subject to the negative effect exerted by SA in wild-type plants. However, the *npr1-1* mutant, which shows induced *ERF1* expression when treated with SA and JA simultaneously, still shows very low *HEL* transcription under these conditions. Thus, the *HEL* promoter might be controlled at the global level by the NPR1-regulated levels of ERF1, but in addition, it is subject to an NPR1-independent negative control mechanism. Thus, both, the *PDF1.2* and the *HEL* promoter might be regulated by NPR1-controlled ERF1 levels under conditions that favour SA and JA biosynthesis. However, different NPR1-independent mechanisms also exist that are specific for the two individual promoters analyzed.

The TGA/GRX480 interaction might directly act at the PDF1.2 promoter.

The lack of influence of GRX480 on *ERF1* transcription might implicate that GRX480-mediated suppression affects directly the *PDF1.2* target promoter. It has been shown previously that the *PDF1.2* promoter contains a binding site for TGA factors at positions -445 to -441 relative to the predicted translational start of the PDF1.2 gene product (Spoel *et al.*, 2003). Using chromatin immunoprecipitation experiments with an antiserum directed against the C-terminal regions of AtTGA2 and AtTGA5 we were able to show that TGA factors indeed bind to the *PDF1.2* promoter. The PCR signal was lacking in the *tga2tga5tga6* triple mutant supporting the specificity of the assay (Fig. 6). Thus, it is possible that – within certain promoter contexts - TGA factors recruit GRX480 to the promoter which in turn might recruit further regulatory proteins that interfere with gene expression.

Discussion

The family of TGA transcription factors is involved in the regulation of *PR* genes and the establishment of SAR (Zhang *et al.*, 2003). Moreover, TGA factors activate detoxification genes in response to xenobiotic stress (Johnson *et al.*, 2001). As TGA factors are primary transcription factors that pre-exist in the cell in the absence of the inducing stimulus, identification of TGA-interacting proteins constitutes an important step to understand the mechanism of their activation. Using a yeast screen set up to identify proteins that interact with TGA factors, a clone encoding a glutaredoxin was isolated.

The isolation of a glutaredoxin as a TGA-interacting factor seemed probable given that conditions activating TGA-dependent transcription lead to changes in the redox status of the cell. Furthermore, critical cysteines of TGA1 and NPR1 are reduced *in planta* after SA treatment (Despres *et al.*, 2003; Mou *et al.*, 2003). The promiscuous interaction of GRX480 with TGA factors from several subgroups (Table 1) opens the possibility that it might be involved in different TGA-mediated functions. However, though ectopic expression GRX480 negatively affected *as-1::GUS* and *PDF1.2* expression (Figs. 5 and 6), the SAR gene *PR-1* and the xenobiotic stress-responsive gene *GST6* were not significantly affected. In the course of this study we tried several *in vitro* approaches to analyze whether GRX480 catalyzes the reduction of TGA1. So far, we were unable to obtain any evidence for this. Also, analysis of the *in vitro* interaction between NtTGA2.2 and GRX480 by pull-down assays, Southwestern analysis and electrophoretic mobility assays was not successful, raising the question whether the TGA/GRX480 interaction needs additional factors that are not present in the respective *in vitro* experiments. NtTGA2.2 and AtTGA2 contain a single cysteine which can be mutated to a serine without altering the DNA binding or GRX480 interaction properties (data not shown). Thus, it

does not seem evident that GRX480 regulates the redox status of these and other class II TGA factors.

Though we have shown that AtTGA2 does not interact with GRX370, it still seems possible that functional redundancy with several of the other 30 glutaredoxins exists. This seems likely, given that the GRX480-specific N terminus does not contribute to the interaction with TGA factors (Fig. 2) nor to its function as a repressor of *PDF1.2* (Fig. 4). The potential redundancy within the glutaredoxin family has to be addressed before initiating the generation and analysis of loss of function mutants that might unravel function that cannot be observed in gain of function mutants.

Glutaredoxin might be involved in fine-tuning the cross talk between the two stress signalling molecules SA and JA

Ectopic expression of *GRX480* negatively regulates two promoters that contain TGA binding sites, namely the truncated *CaMV 35S* promoter, the expression of which is solely dependent on the *as-1* element, and the *PDF1.2* promoter that is bound by TGA factors as demonstrated by chromatin immunoprecipitation experiments (Fig. 6). The mechanism of this repression is presently unclear, but it can be envisioned that TGA factors recruit GRX480 to the promoter, which in turn recruits other factors leading to impaired promoter activity. As the repression of the *PDF1.2* promoter is more stringent than the repression of the truncated *CaMV 35S* promoter, other *cis* elements on the *PDF1.2* promoter seem to support the repressive effect.

Expression of *GRX480* is induced upon SA treatment (Fig. 1B,C) and enhanced by a factor of two by additional JA (Fig. 5). This pattern supports our hypothesis that the endogenous GRX480 is involved in the SA-triggered suppression of *PDF1.2*. However, GRX480 exerts its negative effect in the absence of NPR1 (Fig. 6) which was previously shown to have crucial role in the cross-talk. Moreover, ectopically expressed GRX480 does not significantly repress transcription of *ERF1*, *VSP* and *LOX2*, which are subject to the NPR1-mediated SA/JA antagonism. Thus, GRX480 is not involved in the general negative SA/JA crosstalk mediated by NPR1, but rather acts specifically on selected promoters, at least on the *PDF1.2* promoter.

In Figure 7, a model is put forward that shows the position of GRX480 in this regulatory network. We have shown that NPR1 mediates its antagonistic effect at the level of *ERF1* transcription, which depends on the COI1-mediated degradation of a repressor protein. It has been hypothesized that SA-activated NPR1 interferes with this degradation machinery (Beckers and Spoel, 2006), leading to reduced levels of ERF1. This NPR1 function is

supposed to be localized in the cytosol and is therefore unlikely to involve the primarily nuclear localized TGA factors. NPR1-dependent suppression of ERF1 and related factors might already lead to reduced expression of target genes *PDF1.2* and *HEL*. Under conditions of increased levels of SA and JA, *GRX480* expression is induced and might subsequently repress *PDF1.2* transcript levels that might arise due to ERF1-independent residual *PDF1.2* transcription. *HEL* transcription also seems to be under the control of an additional repressive effect, because under conditions of elevated ERF1 expression, which should be sufficient for activation, SA still exerts a negative effect, which is independent of NPR1 (Fig. 5). However, this repressive effect is not due to GRX480 function. The expression pattern of *HEL* supports the idea that - in addition to a global NPR1-dependent mechanism of the SA/JA antagonism – NPR1-independent promoter-specific mechanisms of the SA/JA cross-talk exist. The GRX480/TGA complex is a good candidate for representing a regulatory component acting directly at the *PDF1.2* promoter.

Experimental Procedures

Yeast screen, assays, strains and plasmids

The yeast strain carrying the *HIS3* reporter gene driven by a minimal promoter with three tandemly repeated *as-1* DNA binding sites is described in Weigel et al., 2004. To express *NtTGA2.2* under the *Met-25* promoter, the *NtTGA2.2* coding region was cloned as a *Nco*I/*Bam*HI fragment (isolated from pGBT9-TGA2.2 (Niggeweg *et al.*, 2000)) into a modified pBridge plasmid (Weigel *et al.*, 2001) opened with *Nco*I and *Bgl*II, resulting g in pBL/TGA2.2. The cDNA library was constructed using pGAD10 (Weigel *et al.*, 2001). The *At1g28480* cDNA insert was excised as a 550 bp *Bgl*II fragment and ligated into pGAD424 or pGBT9 opened with *Bam*HI to obtain the fusion proteins with the GAL4 DNA binding or activation domain, respectively. Bait plasmids pGBT9/TGA2 and pGBT9/TGA5 were obtained by cloning their coding regions from the respective pGAD424 plasmids into pGBT9 (Weigel *et al.*, 2001). Prey plasmids containing the coding regions of *NtTGA2.1*, *NtTGA2.2*, *NtTGA1a* ((Niggeweg *et al.*, 2000), *NtTGA10* (Schiermeyer *et al.*, 2003) and *AtNPR1* (Weigel *et al.*, 2001) are described in the indicated references. For the bridge assay, the *Met-25::TGA2.2::PKG* expression cassette was out from pBL/TGA2.2 and cloned into the *Pvu*II site of pGBT9/GRX480.

To analyze the interaction between GRX480, GRX480ΔN and GRX370 with AtTGA2, the vectors pDEST/GBKT7/myc and pDEST/GAD/HA were used. Yeast vectors pDEST/GBKT7/myc and pDEST/GAD/HA are derivatives of pGBKT7 and pGADT7/Rec (http://orders.clontech.com/clontech/techinfo/vectors/catabc.shtml) containing the GATEWAY^{TM} cassette inserted into the *Sma*I site (B. Causier and B.

Davies, Leeds, UK, unpublished data). PCR fragments were generated from cDNA templates using the primer combinations listed below to facilitate recombination events using the GATEWAY^{TM} technology.

GRX480: *At1g28480gatewayFWD* (5'- GGGG-*attB$_1$*-AT-GCAAGGAACGATTTCTTG-3') and *At1g28480gatewayBCK* (5'- GGGG-*attB$_2$*-ATTAATTTACAATCACAACC-3').

GRX370: At5g40370gateway FWD (5'-GGGG-attB$_1$-AT-GGCGATGC-3') and At5g40370gatewayREV (5'-GGGG-*attB$_2$*-ATATGGATGTG-3').

GRX480ΔN: *GRX480ΔN30gatewayFWD* (5'- GGGG-attB$_1$-ATGGAGAGAGTTCGGATG GTGG-3') and *At1g28480gatewayBCK.*

The attB$_1$ sequence is: ACAAGTTTGTACAAAAAAGCAGGCTCC, the attB$_2$ sequence is: ACAAGTTTGTACAAAAAAGCAGGCTCC

By using GATEWAY^{TM} technology, coding sequences of *GRX480* and *GRX370* were recombined into pDONR^{TM}207 (Invitrogen GmbH, Karlsruhe, Germany) by an attB × attP (BP) recombination reaction according to the manufacturer's instructions. The *GRX480ΔN* PCR fragment was recombined into pDONR^{TM}223. The *GRX* fragments in the pDONR^{TM} vectors were subsequently recombined into the yeast vector pDEST-GBKT7-myc by an attL × attR (LR) recombination reaction at the GATEWAY^{TM} recombination sites located 5` to the GAL4 DNA binding domain. The resulting vectors contain an N-terminal myc tag and Gal4 DNA binding domain in frame with the *GRX* genes. The pENTRY201 vector containing the full length coding sequence of the TGA2 transcription factor (http://jicgenomelab.co.uk/libraries/arabidopsis/tf.html?no_cache=1) was subjected to a LR recombination reaction with pDEST-GAD-HA vector, to generate an expression vector with an N-terminal HA_3 tag and Gal4 activation domain in frame with the TGA2 coding sequence.

The interaction assays using the pGBT and pGAD derived plasmids were performed in yeast HF7c cells grown on selective SD medium lacking leucine, tryptophane and histidine, supplemented with 5 mM 3-AT. The interaction assays using the pDEST plasmids were performed in yeast MAV203 cells (Invitrogen GmbH, Karlsruhe, Germany). LacZ assays were performed as described (Niggeweg *et al.*, 2000). The yeast screen was pursued in the presence of 30 mM 3-AT.

Plant Growth Conditions and Chemical Treatments

Arabidopsis plants (Col-0 background) containing an *as-1::GUS* reporter construct (Redman *et al.*, 2002) were provided by Jonathan Arias, University of Maryland, College Park, MD 20742, USA. Arabidopsis *npr1-1* and *tga2tga5tga6* mutants were obtained from the Nottingham Arabidopsis Stock Centre and by Y. Zhang (University of British Columbia, Vancouver, British Columbia, Canada), respectively. Arabidopsis plants were grown in soil under controlled environmental conditions (21/19°C, 150 $\mu E/m^2/s$, 16-hrs-

light/8-hrs-dark cycle, and 60% relative humidity). Soil was autoclaved for 10 minutes at 90°C before usage. Before sowing the seeds, pots were irrigated with water containing a starter fertilizer (0.5ml/l Wuxal® Liquid, AgNOVA Technologies, Eltham, Australia) and an insecticide (CONFIDOR® WG70 Bayer, Leverkusen, Germany). All seeds on moist soil were vernalized at 4°C for 2 days before placing them in a growth environment. To maintain moderately high humidity, plant trays were covered with a lid with air inlet, during the first three weeks of growth. Plants were watered within two day intervals.

For phytohormone treatments, three weeks old plants were carefully uprooted using aqua plant forceps. The roots washed twice in beakers containing tap water, till the soil was completely removed. Much care was taken not to wound the plants. 15 plants were subsequently transferred in 30 ml in 0.5 mM sodium phosphate buffer containing the respective chemicals (0.01% ethanol) in petri-dishes and allowed to float for the indicated time points. Chemical treatment included 20 µM MeJA, 1 mM SA, 20 µM MeJA/1 mM SA, and 0.1 mM 2.4D. Chemicals were purchased from Sigma-Aldrich, St. Louis, Mo. For each timepoint, a pool of 15 plants was collected. Samples were immediately frozen and crushed with pre-cooled mortar and pistils in liquid nitrogen. 200 mg of pooled sample was used for each RNA preparation.

RNA Gel Blot Analysis

Total RNA was extracted from 150-300 mg plants using the TRIZOL method (Invitrogen GmbH, Karlsruhe, Germany) (Chomczynski and Mackey, 1995)). 10-20 µg RNA was loaded on a 1.2% agarose/2.5% formaldehyde agarose gel, photographed under ultraviolet illumination to allow for comparison of RNA loading, and transferred on a Hybond N+ nylon membrane (Amersham Biosciences, Freiburg, Germany). RNA blots were pre-hybridized for 1 hour at 65°C in Church buffer (250 mM sodium phosphate, pH 7.2 , 1 mM EDTA, pH 8.0, 7% (w/v) sodium dodecyl sulphate (SDS)). Probes were amplified from either cDNA or plasmid templates using appropriate primers: *PR-1/At2g14610* (Uknes *et al.*, 1992), *PDF1.2/ At5g44420* (Penninckx *et al.*, 1998), *VSP*/At5g24770 (Berger *et al.*, 1995), *GST6*/*At2g47730*, (Chen *et al.*, 1996), *LOX2*/*At3g45140* (Bell *et al.*, 1995), *ERF1*/*At3g23240* (Solano *et al.*, 1998), *GUS* (Thurow *et al.*, 2005), and *GRX480/At1g28480* (this manuscript). Hybridization was performed in Church buffer for 14–16 h at 65°C with 100 mg/ml salmon sperm carrier DNA and [α-^{32}P]- dATP labelled probe (Mega-prime DNA labelling system (Amersham Biosciences, Freiburg, Germany)). Washing was done with 2 x SSC, 0.1% SDS at 65 °C for 20-30 min. Quantification of RNA levels was accomplished by Bioimager analysis (Fuji Bas 1000; Fuji, Tokyo) after exposure for 24 h on a PhosphorImagerscreen (Molecular Dynamics, Sunnyvale, CA).

Plant vectors and transformation

For transient expression of the GRX480:GFP fusion protein, the *At1g28480* cDNA was subcloned as a *Acc*651/*Pst*I restriction fragment into previously modified vector containing the *35SC4PPDK-sGFP (S65T)* construct (Chiu *et al.*, 1996; Butterbrodt *et al.*, 2005; Thurow *et al.*, 2005). Transformation of tobacco BY-2 cells was performed as described (Krawczyk *et al.*, 2002). After an overnight incubation at 25°C in the dark, the transformed cells were subjected to microscopic analysis using BX51 fluorescent microscope (Olympus, Hamburg, Germany) with filter sets for UV light (excitation filter EF 395-449; 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).

In order to generate binary vectors for the expression of HA_3-tagged glutaredoxin proteins GRX480, GRX370, and GRX480ΔN (in which the first 31 amino acids are deleted), the respective pDONR^{TM} vectors (see above) were incubated with binary destination vector pAlligator2 (Bensmihen et al., 2004)(http://www.psb.ugent.be/gateway/index.php) that contain the GATEWAY^{TM} recombination sites located downstream of a sequence encoding a HA_3 tag. The final construct expresses GRX derivatives N-terminally fused to the HA_3 tag under the control of the *CaMV 35S* promoter. Using a cloning strategy involving overlapping PCR, the CaMV35S::HA:GRX480 construct was modified so that the resulting GRX480 open reading frame starts directly at the methionine of the HA_3 tag.

For generation of transgenic plants, binary plasmids were electroporated (GenePulser II, BioRad, Munich, Germany) into *Agrobacterium tumefaciens* strain GV3101 (pMP90). The resulting Agrobacteria were used to transform Col-0 (containing *as-1::GUS*) or *npr1-1* plants using the floral dipping method (Clough and Bent, 1998). Transgenic seeds containing pAlligator2 construct were scored for the seed-specific green fluorescent protein (GFP) marker under a fluorescent microscope (BX51, Olympus, Hamburg, Germany) using light of wavelength 460 nm. If all of the seeds of an F2 silique were GFP-positive, the plant was scored as homozygous.

Yeast Protein Extracts and Western blots

Yeast cells were grown in a 20 ml YPED medium at 30°C until an OD_{600} between 0.8 and 1.3 was reached. 4 ml of culture was removed for the LacZ assay (Niggeweg *et al.*, 2000) and the rest of the cells was collected by centrifugation (1000 rpm, 5 mins, at 4°C) and washed in ice cold water. The cells were frozen in liquid nitrogen and re-suspended in a normalized volume (75 µl/7.5 OD_{600} units of cells) pre-warmed 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, 10µl/ml ß-mercaptoethanol, 4.42 mM PMSF and 70 µl/ml protease inhibitor cocktail for plants (P-9599, Sigma-Aldrich, St. Louis, Mo). The samples were transferred to safe lock Eppendorf tubes containing 80 µl of glass beads (Sigma-Aldrich, St. Louis, Mo), heated at 70°C for 10 minutes and further vortexed for 1 minute. The cell debris, unbroken cells and glassbeads were removed by centrifugation leading to the first supernatant. The pellet was boiled in a water bath for 5 minutes and vortexed vigorously for 1 minute. After centrifugation the supernatant was combined with the first supernatant. 30µl of each extract separated on a 10% SDS polyacrylamide gel, which was stained with coomassie brilliant blue (0.01% Coomassie brilliant blue F-250 in 10% glacial acetic acid). The band signal intensities of the gel were quantified (PCBAS2.09 and TINA2.0 software) and used to calculate the amounts for equal loading of proteins on the gel. Fractionated proteins on 10% polyacrylamide gels were transferred to nitrocellulose membranes in transfer buffer (3.03 g/l tris base, 14.4 g/l glycine and 20% methanol) by tank blotting. Quantitative transfer of proteins was verified by the simultaneous transfer of pre-stained protein markers (Fermentas GmbH, St. Leon-Rot, Germany), and by staining the gel with coomassie brilliant blue after the transfer.

Non-specific binding of proteins was blocked with 5% non-fat dry milk in PBS-T (6 mM NaCl, 58 mM Na_2HPO_4, 17 mM NaH_2HPO_4, pH7.4 with 0.05% v/v Tween 20) for 2 hours. Filters were then incubated overnight with antibody diluted in PBS-T/5% non-fat dry milk (1:5000 dilution of αmyc monoclonal antibody (New England Biolabs, Frankfurt, Germany) for detection of GRX expression, 1:5000 dilution of αHA monoclonal antibody (Santa Cruz Biotechnology, Heidelberg, Germany). Incubation with the secondary antibody was done in PBS-T for one hour (horseradish peroxidase-coupled anti mouse IgG). The Enhanced Chemiluminescence Plus^{TM}-(ECL+) kit (Amersham Biosciences, Freiburg, Germany) was used for detection of specific signals.

Generation of an antiserum directed against the C termini of TGA2 and TGA5

To express the C-terminal domains of AtTGA2 and AtTGA5 as fusion proteins with glutathione *S*-transferase (GST) for antigen production, the cDNA sequences encoding aa 64 to aa 329 were amplified by PCR and cloned into the pGEX4T-1 vector (Amersham Biosciences, Freiburg, Germany). Expression and purification of GST-AtTGA-Cterm for generation of a polyclonal antiserum and the generation of the antiserum was done as described (Thurow *et al.*, 2005).

Chromatin immunoprecipitations

5 g of leaf material from plants grown for 6 weeks under a 16-h-light/8-h-dark cycle light regime was placed into a plastic basket (15 cm diameter) that was put into 500 ml of 1% formaldehyde in 50 mM KH_2PO_4/K_2HPO_4, pH 5.8. Vacuum was applied twice for 5 min and samples were afterwards left for another 20 min in this buffer. Subsequently, the leaf material was into a buffer containing 0.3 M glycine in 50 mM KH_2PO_4/K_2HPO_4, pH 5.8. Vacuum was applied twice for 5 min with subsequent incubation for 5 min. Leaves were washed twice in water, dried with paper towels, frozen and stored in liquid nitrogen till further processing.

Nuclei were isolated according to a modified protocol (Folta and Kaufman, 2000). Briefly, the frozen tissue was ground with a pistil under liquid nitrogen, resuspended in 20 ml extraction buffer (1 M hexylene glycol, 0.5 M PIPES KOH, pH 7.0, 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol) and homogenized for 5 min using a Miccra-D8 homogenizer (ART Labortechnik, Mühlheim, Germany). The homogenate was passed through a double layer of Miracloth (Calbiochem, San Diego, CA). Triton X-100 (25%) was added dropwise to the resulting liquid fraction with constant stirring to a final concentration of 1% to lyse organelle membranes. The lysate was gently layered on top of a 6 ml 35% percoll cushion (Sigma-Aldrich, St. Louis, Mo) in gradient buffer (0.5 M hexylene glycol, 0.5 M PIPES KOH, pH 7.0, 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 1% Triton X-100). After centrifugation at 2000 x g for 30 min in a swinging bucket rotor, the nuclei were found as a pellet at the bottom of the tube. Nuclei were resuspended in 21 ml of gradient buffer and again gently layered on top of a 6 ml 35% percoll cushion. After centrifugation as above, the pellet was resuspended in 1 ml of gradient buffer and centrifuged at 2000 x g for 10 min. The pellet containing the nuclei was stored for not more than a week at -80°C.

Chromatin extraction and chromatin immunoprecipitation (ChIP) was performed as described previously (Turck *et al.*, 2004) with slight modifications. Formaldehyde cross-linked nuclei from 5 g of leaf material were first resuspended in 1 ml sonication buffer (10 mM HEPES/NaOH, pH 7.4, 1 mM EDTA, 0.5% SDS and diluted with 1 ml sonication buffer without SDS. Chromatin was sheared to an average size of 2000 base pairs by repetitive sonication ((MSE Soniprep150, Sanyo-Gallen-Kamp, Loughboro, Leicestershire, UK) 4 times 20 s in an ethanol/ice bath, interrupted by 1 min cooling steps) at 12 amplitude microns. The final centrifugation was performed at 11200 x g for 20 min at 4°C. The supernatant was shock frozen in liquid nitrogen and stored at -80 °C for up to 6 weeks. To normalize different samples for equal DNA content, the OD_{260} was measured after the following purification steps: 50 µl of the chromatin were brought to a volume of 450 µl with sonication buffer containing 0.25% SDS, incubated first in the presence of 10 µg of Proteinase K for 1 h at 37°C and subsequently at 65°C for 16 h for

de-crosslinking. Free DNA was purified from the solution by phenol/chloroform/isoamylalcohol (25:24:1) extraction followed by chloroform/isoamylalcohol (24:1) extraction. DNA was precipitated by the addition of 10 µg glycogen (G-8751, Sigma-Aldrich), one-tenth volume of 3 M Na acetate and 2.5 volumes of ethanol at -80 °C for at least 3 h. After centrifugation, DNA was resuspended and used for OD_{260} measurements. Equal amounts of chromatin as measured by DNA content (15 µg) were brought to a total volume of 200 µl with sonication buffer (10 mM HEPES/NaOH, pH 7.4, 1 mM EDTA, 0.25% SDS). After adding 300 µl RIPA buffer (50 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% deoxycholate), samples were incubated in the presence of preimmune serum (5 µl) for 30 min at 4°C on a rotary shaker. Next, 50 µl Protein A agarose beads (Sigma-Aldrich, St. Louis, MO) 50% slurry in RIPA) were added and incubated for additional 30 min. Beads were pelleted and 50 µl of the supernatant was removed for the input control (see below). The residual supernatant was incubated with 4 µl of the immune serum for 4 h at 4 °C on a rotary shaker. Subsequently, 50 µl Protein A agarose beads (50% slurry in RIPA) were added. After incubation for 2 h on a rotary shaker at 4°C, immunoprecipitates were washed three times in 1 ml of RIPA buffer supplemented with SDS (0.1%), followed by an additional wash with 800 µl and a transfer into a fresh tube. Immunocomplexes were then eluted from the beads by two sequential incubations in 150 µl of elution buffer (0.1 M glycine, 0.5 M NaCl, and 0.05% Tween 20, pH 2.5) followed by centrifugation and addition of 150 µl 1 M Tris/HCl, pH 8.0 to the combined eluates. As input control, 50 µl of the supernatant from pre-immune incubations (10% of the sample) was brought to a volume of 450 µl with sonication buffer (10 mM HEPES/NaOH, pH 7.4, 1 mM EDTA, 0.25% SDS). Eluted DNA and DNA of the input control were treated with Proteinase K, heat treated and purified as described above. Precipitation of the DNA was done at –80°C for at least 3 h. DNA was resuspended in 35 µl (ChIP DNA) or 175 µl (input control) of water for PCR analysis. Comparison of the amounts of PCR products yielded with immunoprecipitated and input DNA, respectively, allowed us to estimate that roughly 0.8% of the input promoters are precipitated.

Quantitative Real Time PCR analysis

Real-time PCR quantification was performed using the SYBR™ Green (Invitrogen GmbH, Karlsruhe, Germany) technology in a Mini Opticon Real PCR device from Biorad, Munich, Germany. PCR amplifications of 2.5µl of the template DNA were performed in the presence of 0.25 µM of each primer, 200 µM deoxynucleotide triphosphate, 1.5 µM $MgCl_2$, a 10^{-5} dilution of the SYBR™ Green stock with 5 U Immolase Taq polymerase-mix (Bioline, Luckenwalde, Germany) in the buffer provided by the company. The PCR regime was: 95°C/7 min; 35 cycles of 95°C/20 s, 60°C/20 s, 72°C/28 s; 72°C/4 min; 95°C/1 min; 55°C/1 min. The oligonucleotide sequences (*PDFsense: TTCAGTAATAGGTGTGTCCCAGG*; *PDFantisense:*

ACGGCTGGTTAATCTGAATGG) lead to the amplification of a 323 bp promoter fragment from positions -262 to -585.

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Figure Legends

Figure 1

Sequence (A), SA-dependent expression (B, C) and localization of *At1g28480*

A, Sequence of *At1g28480* from position −1180 to the 3'-end of the cDNA. Black arrows indicate single TGACG sequences representing TGA binding sites; white arrows indicate binding sites for WRKY transcription factors. The boxed sequence indicates the position of an *as-1* element consisting of two TGA binding sites. The putative TATA box is underlined. The bent arrow designates the transcription start site (+1). The open reading frame is given in the one letter code, the shaded box highlights the GRX480 specific 31 aa N-terminal domain and the presumed active centre (CCMC).

B, C, Three weeks old wild-type, *npr1-1* (B) and *tga2tga5tga6* (C) plants were treated with 1 mM SA for the time periods indicated above the lanes. 20 µg of RNA was loaded and the blot was hybridized with probes for *GRX480* and *PR-1*. Ethidium bromide stained RNA is shown to document equal loading.

D, Localization of the GRX480:GFP fusion protein in protoplasts of tobacco BY-2 cells. Protoplasts were transfected with plasmids encoding GFP or GRX480:GFP as indicated and analyzed by fluorescence microscopy. Corresponding bright field images are shown on the right.

Figure 2

Analysis of the interaction of different glutaredoxin variants with AtTGA2 in a quantitative yeast two hybrid assay.

A, Coding regions of GRX480, GRX480ΔN and GRX370 were fused to the GAL4 DNA binding domain (BD:480, BD:ΔN, BD:370), AtTGA2 was fused to the GAL4 activation domain (AD:AtTGA2) After transformation of the respective plasmids in yeast MAV203 cells, ß-galactosidase activity was measured. Three to five independent clones were taken for each construct in a single experiment. Results represent the average of 5 independent experiments. Values were normalized to the LacZ value of yeast cells transformed with the empty vectors, which was set to 1 relative ß galactosidase unit.

B, Western blot analysis of extracts from three independent yeast clones transformed with vectors encoding BD:480, BD:ΔN and BD:370, respectively. Expression of the proteins was analyzed by immunodetection with the αMyc antibody. Though expression of the proteins and of AD:AtTGA2 (data not shown) was comparable in different yeast clones, expression of the reporter gene was quite variable (see error bars in A).

Figure 3

Expression of *as-1::GUS*, *GST6* and *PR-1* in independent transgenic Arabidopsis lines ectopically expressing HA_3-tagged GRX480. Three weeks old plants were treated with 100 µM 2.4D (A) or 1 mM SA (B) for the time spans indicated above the lanes. 20 µg of RNA was loaded and the blot was hybridized with the indicated probes. Ethidium bromide stained RNA is shown to document equal loading.

Figure 4

Expression of JA-responsive genes in transgenic Arabidopsis lines transformed with different *GRX* constructs. Three weeks old plants were treated with 20 µM MeJA for the time spans indicated above the lanes (A) or for six hours (B). 20 µg of RNA was loaded and the blot was hybridized with the indicated probes. Ethidium bromide stained RNA is shown to document equal loading. HA: H_3-tagged GRX480; w/o tag: GRX480; ΔN: GRX480 lacking the 31 N-terminal amino acids.

Figure 5

SA/JA antagonism of JA-responsive genes in *npr1-1* mutants ectopically expressing HA_3-tagged GRX480. Three weeks old plants old plants were treated with SA, JA, SA/JA and solvent 0.01% ethanol for four hours as indicated above the lanes. 20 µg of RNA was loaded and the blot was hybridized with the indicated probes. Ethidium bromide stained RNA is shown to document equal loading.

Figure 6

In vivo TGA factor binding to the *PDF1.2* promoter as revealed by chromatin immunoprecipitation analysis. Leaves from Col-0 and *tga2tga5tga6* plants were incubated in 1% formaldehyde before chromatin preparation. Chromatin samples were subjected to immunoprecipitation using 5 µl of the αAtTGA2-C antiserum. The DNA was recovered after reversal of the cross-links and analyzed for the enrichment of *PDF1.2* promoter sequences by quantitative Real time PCR. C_T-values are given as a parameter to quantify the amount of PCR products. C_T-values obtained from the respective input controls are shown. C_T-values above 30 cycles were not taken into account, as polymerase chain reactions carried out without specific templates yielded C_T-values in this range.

Figure 7

Proposed schematic model illustrating the role of GRX480 in the regulation of SA-induced suppression of JA-dependent defense signalling. JA activates COI1 at the protein level (white arrows) leading to transcription of *ERF1*, which in turn activates transcription of *HEL* and *PDF1.2.* SA activates NPR1, which leads to suppression of COI1 function in the cytosol. NPR1, which is mobilized to the nucleus, activates together with TGA factors

transcription of *GRX480*. GRX480 interacts with TGA factors and interferes with expression from *PDF1.2*. The question mark indicates yet unknown factors that suppress transcription of *HEL* in the presence of SA. White arrows mark activation events targeted to the protein, black arrows indicate transcription or translation.

Figure 1

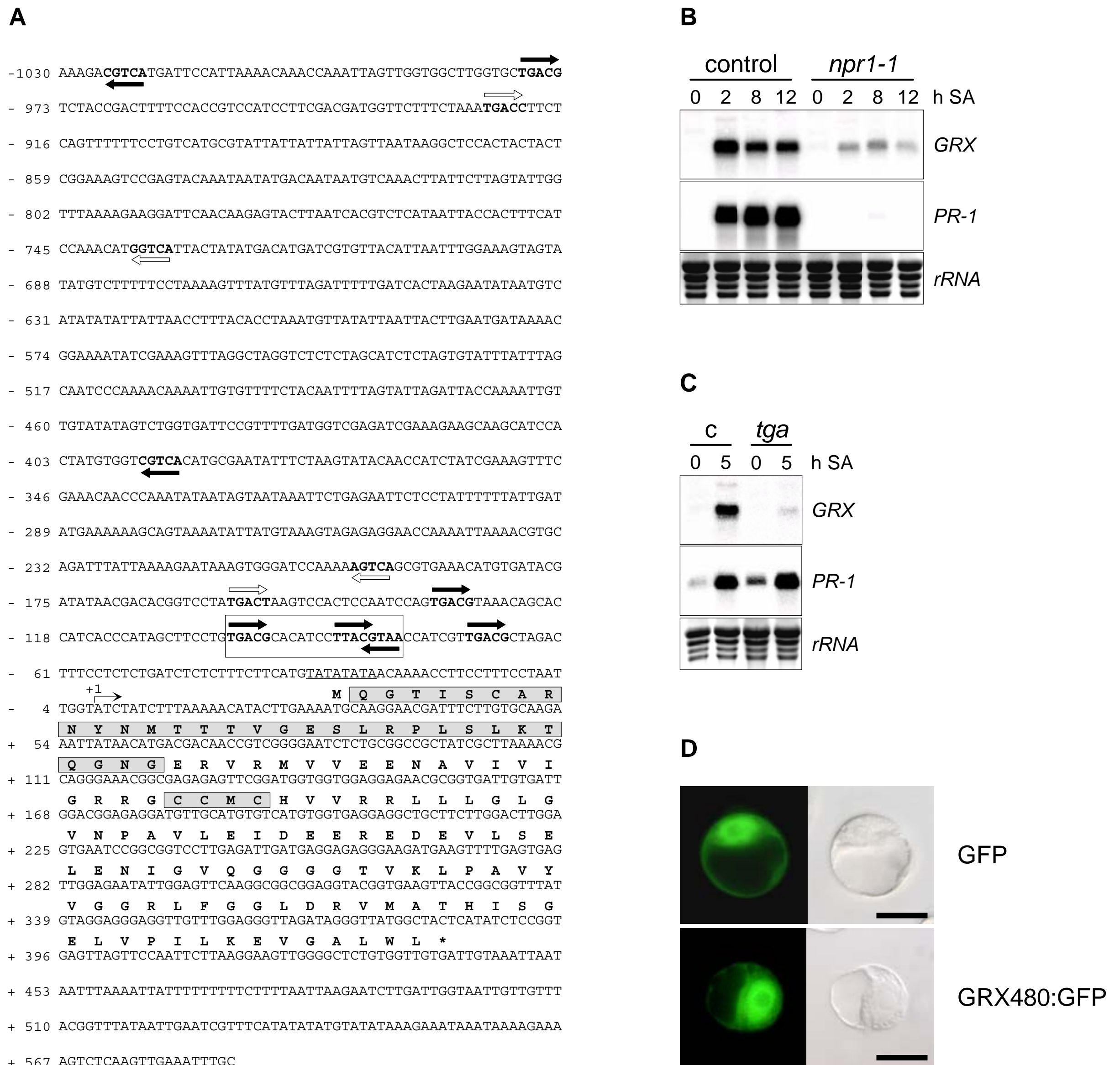


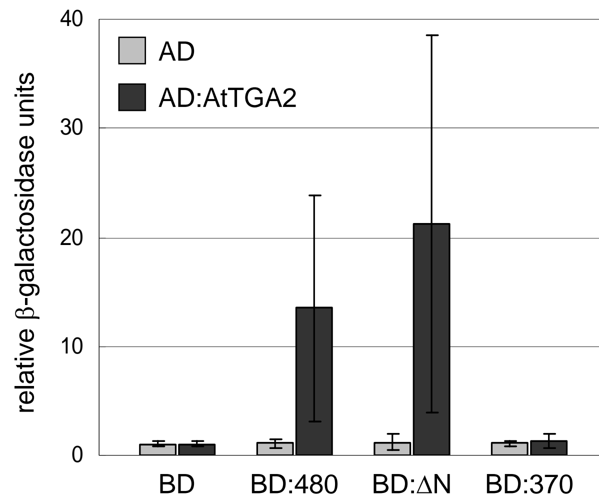
Table 1

Bait	Prey	Growth
BD	AD:GRX480	-
BD:NtTGA2.2	AD	-
BD:NtTGA2.2	AD:GRX480	+
BD:AtTGA2	AD	-
BD:AtTGA2	AD:GRX480	+
BD:AtTGA6	AD	-
BD:AtTGA6	AD:GRX480	+
BD:GRX480	AD	-
BD	AD:NtTGA2.2	-
BD:GRX480	AD:NtTGA2.2	+
BD	AD:NtTGA2.1	-
BD:GRX480	AD:NtTGA2.1	+
BD	AD:NtTGA1a	-
BD:GRX480	AD:NtTGA1a	+
BD	AD:NtTGA10	-
BD:GRX480	AD:NtTGA10	+

Bait	Bridge	Prey	Growth
BD:GRX480	-	AD:NPR1	-
BD:GRX480	NtTGA2.2	AD	-
BD:GRX480	NtTGA2.2	AD:NPR1	+

Figure 2

A



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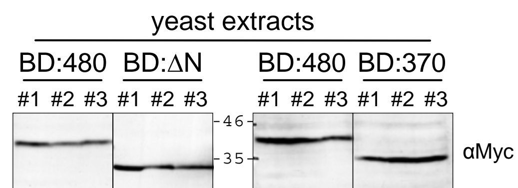
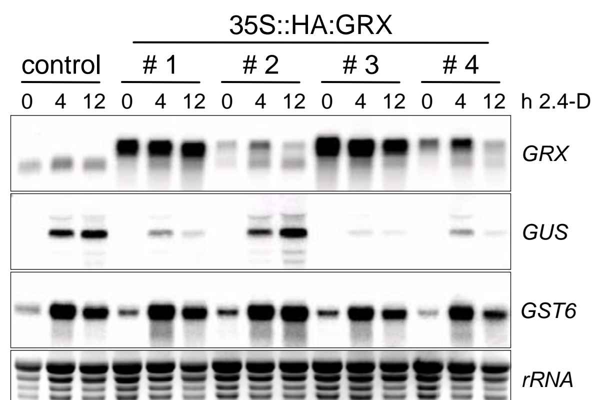


Figure 3



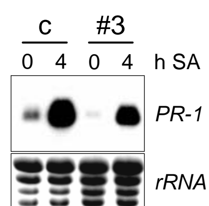
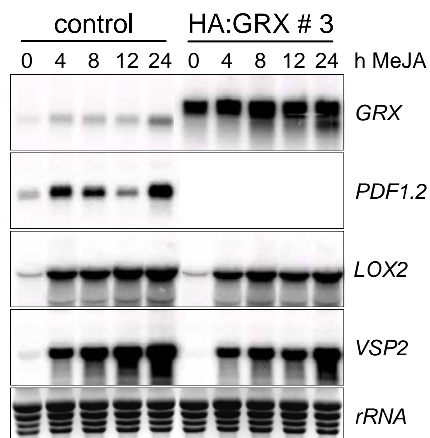


Figure 4



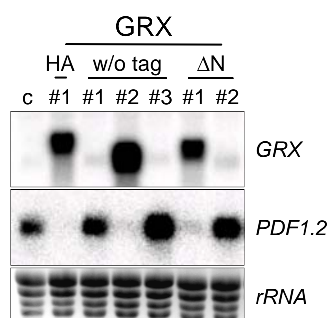


Figure 5

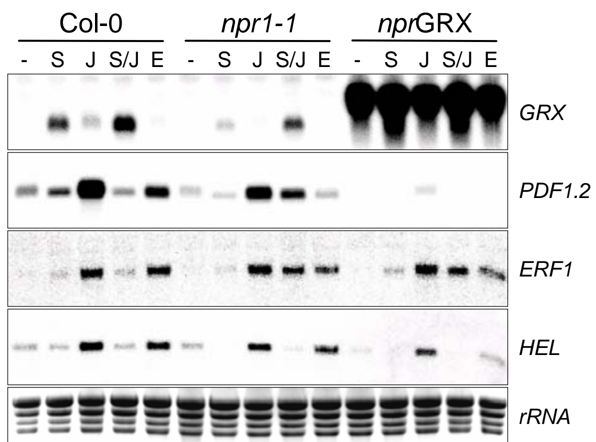


Figure 6

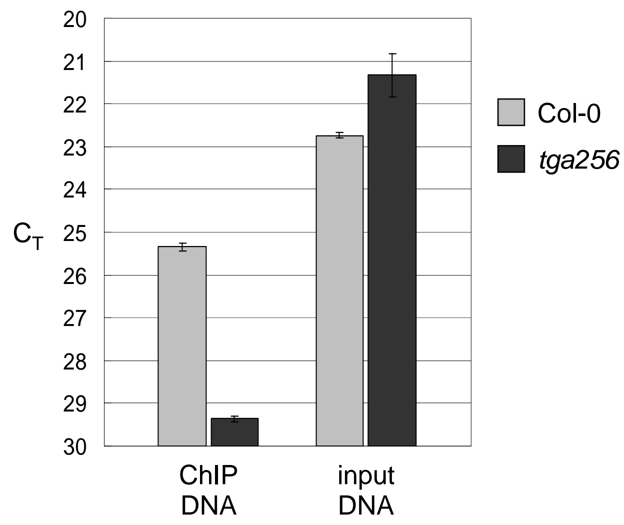
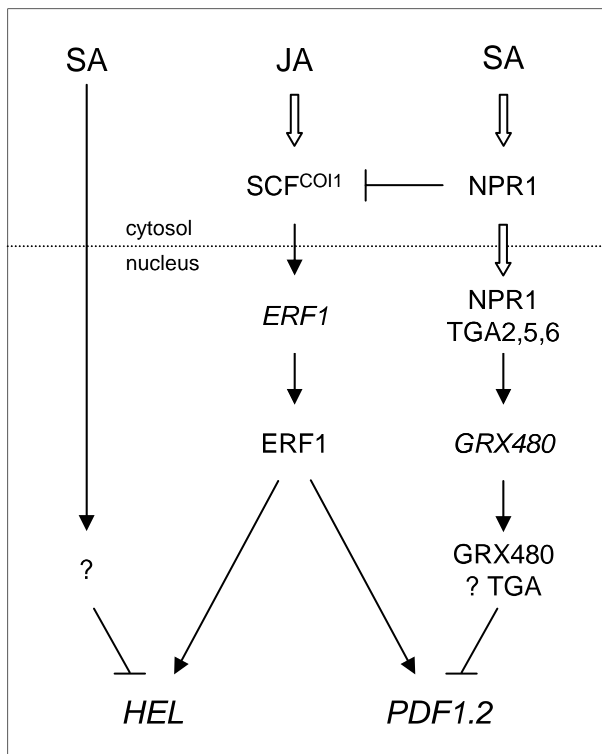


Figure 7



4 Supplementary 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 DB3.1	*F-; gyrA462, endA1, glnV44, Δ(sr1-recA) mcrB mrr hsdS20(r_B^-, m_B^-) ara14 galK2 lacY1 proA2 rpsL20(Sm^r) xyl5 Δleu mtl1*	Invitrogen
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 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

4.1.2 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
MAV203	*MAT*α, *leu*2-3,112, *trp*1-901, *his*3Δ200, *ade*2-101, *gal*4Δ, *gal*80Δ, *SPAL*10::*URA*3, *GAL*1::*lac*Z, *HIS*3$_{UAS\ GAL1}$::*HIS*3@*LYS*2, *can*1R, *cyh*2R	Vidal, 1997, Invitrogen
PJ69-4A	*MATα, trp1-901, leu2-3,112, ura3-52, his3-200, gal4Δ, gal80Δ, GAL2-ADE2, LYS2 ::GAL1-HIS3, met2::GAL7-lacZ*	James *et al.*, 1996

4.1.3 Plants

Plant	Description	Reference
as1-90-GUS	*Arabidopsis thaliana* Columbia-0 transgenic plant containing an *as-1* element upstream of the uidA (GUS) reporter gene, km^R.	Redman *et al.,* 2002
GRX480-DM overexpressors	*as1*-90-GUS plant containing the Alligator2/GRX480-DM (double mutant in active site cysteines) plasmid with a 3x HA-At1g28480 construct under the control of 2x CaMV 35S promoter, $strp^R$.	This work
GRX480 Ohne HA overexpressors	*as1*-90-GUS plant containing the Alligator2/GRX480 plasmid without a 3x HA-GRX480 construct under the control of 2x CaMV 35S promoter, $strp^R$.	This work
GRX480 overexpressors	*as1*-90-GUS plant containing the Alligator2/GRX480 plasmid with a 3x HA-GRX480 construct under the control of 2x CaMV 35S promoter, $strp^R$.	Ayed Abdallat, 2004
GRX480 ΔN30 overexpressors	*as1*-90-GUS plant containing the Alligator2/GRX480-ΔN30 (deletion in 20 N terminal amino acids) plasmid with a 3x HA-GRX480 construct under the control of 2x CaMV 35S promoter, $strp^R$.	This work
GRX370 OverExpressors	*as1*-90-GUS plant containing the Alligator2/GRX370 plasmid with a 3x HA-At5g40370 construct under the control of 2x CaMV 35S promoter, $strp^R$.	This work
grx480 mutant	A cross between Nossen and Landsberg erecta, with a transposon insertion upstream in the promoter region of At1g28480, resulting in a null mutation.	This work Ito *et al.* (2002) Kuromori *et al.* 2004) Ito *et al.* (2005)
GRX480 RNAi	*as1*-90-GUS plant containing pFGC5941/GRX480 binary vector with an At1g28480 RNAi cassette under the control of CaMV 35S promoter, km^R PPT^R.	Ayed Abdallat, 2004
GRX480prom-Luc-plants	*as1*-90-GUS plant containing the Luciferase gene under the control of the At1g28480 promoter.	This work
Ler	*Arabidopsis thaliana* Landsberg erecta	The European *Arabidopsis* Stock Centre
Nos	*Arabidopsis thaliana* Nossen-0	The European *Arabidopsis* Stock Centre
npr1-1 mutant	*A. thaliana* Col-O plants with point mutation in NPR1 gene (At1g64280)	Cao *et al*., (1994)

Plant	Description	Reference
npr1-1:grx480 double mutant:	A cross between the *Arabidopsis npr1-1* mutant and the grx480 double mutant, in progress	In progress
npr1-1mut: GRX480-ΔN30 overexpressors	*npr1-1* mutant plants, containing the Alligator2/GRX480-ΔN30 plasmid with the 3x HA- under the control of 2x CaMV 35S promoter, $strp^R$	This work
*npr1-1mut:*GRX480-DM OverExpressors	*npr1-1* mutant plants, containing the Alligator2/GRX480 DM plasmid with the 3x HA- under the control of 2x CaMV 35S promoter, $strp^R$	This work
npr1-1mut: GRX480-OhneHA OverExpressors	*npr1-1* mutant plants, containing the Alligator2/GRX480 Ohne HA plasmid without the 3x HA- under the control of 2x CaMV 35S promoter, $strp^R$	This work
npr1-1mut: GRX480-OverExpressors	*npr1-1* mutant plants, containing the Alligator2/GRX480 plasmid with a 3x HA-GRX480 construct under the control of 2x CaMV 35S promoter, $strp^R$	This work
tga2tga5tga6 mutant	*A. thaliana* plants with triple mutation in the TGA transcription factor genes coding for TGA2, TGA5 and TGA6.	Zhang *et al.*(2003)
tga2tga5tga6: GRX480	The *Arabidopsis tga* triple mutant over expressing the GRX gene At1g28480, generation in progress	This work

4.2 Media and Additives

4.2.1 Bacteria 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
LB medium	10 g/l Tryptone 5 g/l Yeast Extract 10 g/l NaCl Adjust pH to 7.0 with NaOH

Medium	Components and Concentrations
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
SD-(Met, Leu, Trp)	6.7g/L YNB (w/o amino acids) 12g/L select agar (For solid medium) 847.5mL H_2O Autoclave 40mL sterile 50% Glucose 100mL sterile 10x DI stock-(MLTH) 12.5mL sterile 80x HIS
SD-(Leu, Trp)	6.7g/L YNB (w/o amino acids) CSM-H-L-W-A Glucose 12g/L select agar (For solid medium) mL H_2O Autoclave Histidine stock 12.5mL sterile 80x HIS

4.2.3 BY2 Protoplast 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

4.3.1 Plasmids

Plasmid	Description	Reference
BT10 4X Gal4	It contains the Gal4 DNA promoter sequence, upstream of a GUS reporter construct.	Thurow, 2001
HBT-2.2VP16	HBT derivative contains the TGA2.2-VP16 coding sequence under the control of the chimeric HBT promoter; amp^r.	Thurow, 2001
HBT-L	pUC18 derivative contains the chimeric HBT promoter and a nos termination signal; amp^r.	Nickolov, 2003
HBTL/ GRX480	HBT-L derivative with At1g28480 cds under the chimeric HBT promoter; amp^r.	This work
HBTL/ GRX480-DM	HBT-L derivative contains the GRX480 cds mutated in the sequences coding for the two active site cysteines, and isdriven by the chimeric HBT promoter; amp^r.	This work
HBTL/ GAL4BD-GRX480	HBT-L derivative contains the GAL4BD-At1g28480 cds under the control of the chimeric HBT promoter; amp^r.	This work
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
pAlligator/ GRX370	Alligator2 derivative for constitutive expression of a 3x HA-At5g40370 fusion protein; $strp^r$.	This work

Plasmid	Description	Reference
pAlligator/ GRX480 OhneHA	Alligator2 derivative for constitutive expression of GRX480 fusion protein without the HA tag; strp^r.	This work
pAlligator/ GRX-DM	Alligator2 derivative for constitutive expression of a 3x HA-GRX480-double mutant- fusion protein; strp^r.	This work
pAlligator/ GRXΔN30	Alligator2 derivative for constitutive expression of a 3x HA-fused to N-terminal deleted form of GRX480; strp^r.	This work
pAlligator2	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/
pAlligator2/ GRX480	Alligator2 derivative for constitutive expression of a 3x HA-GRX480 fusion protein; strp^r.	Ayed Abdallat, 2004
pas-1-GUS	pUC18 derivative. Contains the cds for the uidA (GUS) reporter construct under the control of a -90 CaMC 35S promoter containing the *as-1* element as the only regulatory sequence. amp^r.	Thurow, 2002
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-/ GRX480	pBD derivative, contains HA-NLS-At1g28480 coding sequence under the control of the Met25 promoter; TRP1, amp^r.	Ayed Abdallat, 2004
pDEST-GAD-HA	Gateway expression vector for yeast, modified from pGADT7-Rec with the gateway sequence integrated within the *sma*I site. It includes a LEU and an AMP^R marker. It is designed to produce a fusion protein including a N-terminal yeast Gal4 binding domain and HA tag, driven by an ADH promoter.	CLONETECH, Barry Causier (unpublished)
pDEST-GAD-HA/TGA2	Derived from pDEST-GAD-HA, and containes full length cds of *atTGA2* gene in frame upstream with and HA-tag coding sequence.	This work
pDEST-GBKT7-myc	Gateway expression vector for yeast, modified from pGBKT7 with the gateway sequence integrated within the *sma*I site. It includes a TRP1 and a KAN^R marker. It is designed to produce a fusion protein including a N-terminal yeast Gal4 binding domain and myc tag, driven by an ADH promoter.	CLONETECH, Barry Causier (unpublished)
pDEST-GBKT7-myc/GRX370	Derived from pDEST-GB-myc, and containes full length cds of GRX370 gene.	This work
pDEST-GBKT7-myc/GRX480	Derived from pDEST-GB-myc, and containes full length cds of At1g28480 gene.	This work

Plasmid	Description	Reference
pDEST-GBKT7-myc/GRX-DM	Derived from pDEST-GB-myc, and contains full length cds of GRX480 -DM (-double mutant version) gene.	This work
pDEST-GBKT7-myc/GRX-dN30	Derived from pDEST-GB-myc, and containes N-terminal deletion version of At1g28480 gene.	This work
pDONR207	The Gateway donor vector; Gm^r, Cm^r.	GIBCOBRL
pDONR207/ GRX480	pDONER207™ derivative contains the GRX480 full-length coding sequence as a gateway construct; Gm^r.	This work
pDONR207/ GRX370	Derived from the Gateway donor vector pDONR207, contains full length cds for At5g40370. ; Gm^r, Cm^r.	This work
pDONR™223/ GRX-DM	Derived from the Gateway donor vector pDONR™223, contains coding sequence of At1g28480, with coding sequence for two active site cysteines mutated by site directed mutagenesis. ; Gm^r, Cm^r.	This work
pDONR™223/ GRXΔN30	Derived from the Gateway donor vector pDONR™223, contains cds of At1g28480, lacking the coding sequence for the first 30 amino acids. ; Gm^r, Cm^r.	This work
pFGC594	Gateway binary vector for RNAi constructs under the control of the 35S promoter; km^r, PPT^r.	http://ag.arizona.edu/chromatin/fgc5941.html
pGBT9/ GRX480	pGBT9 derivative contains the At1g28480 coding sequence in-frame with GAL4BD; TRP1, amp^r.	This work
pGBT9/TGA2	pGBT9 derivative contains the TGA2 coding sequence in-frame with GAL4BD; TRP1, amp^r.	R. Weigel, unpublished
pGWB233/ GRXpromoter	A binary vector containing the luciferase gene, expressed under the control of the At1g28480 promoter; KanR selectable marker.	This work
pUC18ENTRY/ GRXPromoter	Derived from pUC18ENTRY2 and contains the GRX promoter within the *Sal*I/*Xba*I restriction sites.	This work
pUC18ENTRY2	Gateway vector with cloning sites for convenient cloning of genes for expression or integration of sequences into an expression vector.	Marco H., Unpublished

4.3.2 Primers

Designation	Base Sequence (5'-3')	T_m (ºC)
35SI	ATTGATGTGATATCTCCACTGAC	57.1
Actin18 antisense	GCTGGATTCGCTGGAGATGA	55.0
Actin18 sense	AGGTCTCCATCTCTTGCTCG	50.8
At1g28480 C60-C63 C	GGAGAGGATCTTGCATGTCTCATGTGG	62.8
At1g28480 C60-C63 N	TCACCACATGAGACATGCAAGATCCTCTC	64.2
At1g28480 C63 C	AGGATGTTGCATGTCTCATGTGGTGAGG	64.8
At1g28480 C63 N	TCACCACATGAGACATGCAACATCCTCTC	64.9
At1g28480 gateway bck	GGGGACCACTTTGTACAAGAAAGCTGGGTCA TTAATTTACAATCACAACC	76.1
At1g28480 gateway fwd	GGGGACAAGTTTGTACAAAAAAGCAGGCTCC ATGCAAGGAACGATTTCTTG	80.3
At1g28480 GFP bck	GGTACCCAACCACAGAGAGCCCCAACTTC	67.7
At1g28480 transient bck	***CTGCAG***TCACAACCACAGAGCCCCAA	66.8
At1g28480 transient fwd	***GGTACC***ATGCAAGGAACGATTTCTTG	60.7
At5g40370 FWD	GCGAATTCACGAAGATGGCGATGCAGA	69.0
At5g40370 REV	GGGAATTCTCACATATGGATGTGCTCTT	60.6
DS3-2a	CCGGATCGTATCGGTTTTCG	49.0
GAD lower	GATGCACAGTTGAAGTGAACTTGCGGGG	67.7
GAD upper	TTCGATGATGAAGATACCCCACCAAACCC	66.8
GBTterm-primer	ATCATAAATCATAAGAAATTCGCCCG	59.7
GRX370 GatewayFWD	GGGGACAAGTTTGTACAAAAAAGCAGGCTCC ATGGCGATGC	78.6
GRX370 GatewayREV	GGGGACCACTTTGTACAAGAAAGCTGGGTCA TATGGATGTG	74.7
Grx480 ΔN30 FWD	GGA***GAATTC***ATGGAGAGAGTTCGGATGGTGG TGG	62.0
GRX480pstREV	CAC***CTGCAG***TCACAACCACAGAGCCCCAAC	62.0
GRXPROM1059 REV	GATTATGGGAATTGCATTTC	46.4
GRXPROM1507 REV	TGACATTATATTCTTAGTGATCA	41.6

Designation	Base Sequence (5'-3')	T_m (ºC)
GRXPROM1507 REV (23mer)	TGACATTATATTCTTAGTGATCA	41.6
GRXPROM607FWD	CCGATGCTACTATAAATGATTG	46.7
GRXPROMFWD	CAG**GTCGAC**TCCAGAGAAATTCCGATTAAAT C	65.3
GRXPROMREV	GTC**TCTAGA**TTTCAAGTATGTTTTTAAAGATA	52.4
LBa1 primer	TGGTTCACGTAGTGGGCCATCG	55.0
LP-GX480	GATTTTTGATCACTAAGAATATAATGTC	55.4
LP-SALK63GX370	TTTTTGTCAATCCATATCTTATATC	53.7
LP-SALK76GX370	GATTACAAGTCCTCACTCCGC	58.8
Nos	CATCGCAAGACCGGCAACAGG	63.7
NPR1FWD	ACGGATCCCCATGGACACCACCATTGATGG	73.4
NPR1BCK	GTGTCGACCGACGACGATGAGAGAG	62.9
PBD2	TCATCGGAAGAGAGTAGTAAC	55.9
SEQL1-pDONR	TCGCGTTAACGCTAGCATGGATCTC	62.3
SEQL1-pDONR	GTAACATCAGAGATTTTGAGACAC	46.9
PR1FWD	GGGGATCCATATGAATTTTACTGGC	57.8
PR1REV	CTGAGCTCTTAGTATGGCTTCTCG	53.7
REV24	TTCACACAGGAAACAGCTATGAC	61
RP-GX480	CCTCATCAATCTCAAGGACCG	61.9
RP-SALK63GX370	TGAAAACAACGACTGATTCGC	61.2
RP-SALK76GX370	CGCTTTTGTGTACCAATGGATG	62.5
UNI24	ACGACGTTGTAAAACGACGGCCAG	64.4
VSP2 ANTI	GCTTCAATATGAGATGCTTCCAGTAGG	57.8
VSP2 SENSE	TCCTCTCACTTTCACTTCTCTTGCTC	56.5

4.3.3 Hybridization probes

Probe	Source	Reference
GRX480	A 500 kb At1g28480 PCR product	This work
ERF1	PCR product from cDNA clone	This work
GST6 from *Arabidopsis*	A 600 bp SalI fragment from pGEM-GST6	Brenner, 2002

Probe	Source	Reference
GUS probe	1874bp purified fragment for an *Nco*I/*Pst*I digestion of a pHBTGUS-Nco vector	Thurow *et al.,* 2005
HEL1	PCR product from cDNA clone	Herde and Gärtner
LOX2	1100bp PCR product from cDNA clone	This work
PDF1.2	A 500bp PCR product from REGIA clone	This work
PR-1 from *Arabidopsis*	A 500 bp EcoRI fragment from pSK-AtPR1	Brenner, 2002
VSP2	580bp PCR product from cDNA	This work

4.3.4 DNA Standards

DNA Standard	Manufacturer
λ EcoRI, HindIII	Self made
MassRuler™ DNA Ladder Mix	MBI Fermentas

4.4 Proteins

4.4.1 Antibodies

Antibody	Specificity	Properties	Reference
α-myc	Myc-epitope tag	Mouse monoclonal	Amersham
α-HA	HA-Epitope tag	Mouse monoclonal	Santa Cruz
α-rabbit IgG	Rabbit immunoglobulin G	Donkey polyclonal	Amersham
α-mouse IgG	Mouse immunoglobulin G	Sheep polyclonal	Amersham

4.4.2 Enzymes

Enzyme	Manufacturer
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

Enzyme	Manufacturer
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
Mol tag	Molzym GmbH

4.4.3 Protein standards

Protein Standard	Manufacturer
Prestained Protein Ladder	MBI Fermentas

4.5 Chemicals

Chemical	Manufacturer
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
Alamecithin from *Trichoderma viridae*	Sigma

Chemical	Manufacturer
Ammonium persulfate (APS)	Biometra
Ampicillin (Amp)	AGS
ß-mercaptoethanol (ß-ME)	Roth
Basta®	AgrEvo
Bicine	Serva
Boric acid	Serva
Bovine Serum Albumin (BSA)	Serva
Bradford reagent	Roth
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

Chemical	Manufacturer
Imidazol	Sigma
Isoamyl alcohol	Roth
Isopropanol	Roth
Isopropylthiogalactoside (IPTG)	BioTech Grade
Jasmonic Acid Methy Ester (meJA)	Aldrich
Kanamycin (Km)	Sigma
Leucin	Sigma
Lithium acetate	Sigma
Lycine	Sigma
Magnesium acetate	Merck
Magnesium Chloride ($MgCl_2$)	AppliChem
Magnesium sulfat-Heptahydrat ($MgSO_4.7H_2O$)	AppliChem
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
Salicylic Acid (Natrium SA)	Sigma
Select Agar	Life Technologies
Select Sarcosyl	Sigma
Selected yeast Extract	GIBCO BRL
Sephadex G-50	Pharmacia

Chemical	Manufacturer
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
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

4.6 Consumables

Product	Manufacture
3MM paper	Whatman
Aluminum folia	Roth
Electroporation cuvettes	BioRad
Glass material	Brand, Schott

Product	Manufacture
Glutathione Sepharose 4B	Amersham Pharmacia
Greiner, Biozym	Roth
Micro Spin™ G25 Column	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
Whatmann paper	Machery-Nagel GmbH
X-ray films Cronex 10T, Cronex 4	DuPont

4.7 Devices

Device	Model	Manufacturer
Autoclave	3870 ELV	Tuttnauer
Automatic pipettes		Gilson
Balance	SPO51, SAC62 1207 MP2	Scaltec 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 V 15-17	AGS Gibco BRL
Handheld radiation monitor	Contamat	Eberline
Heating blocks	HB-130	Unitek
Heating shaker	Thermo mixer 5436	Eppendorf
Heating stirrer	RCT basic	IKA Labortechnik

Device	Model	Manufacturer
Hybridization ovens	Bachhofer	
Hybridization shaking water bath	The Belly Dancer®	Stovall
Ice machine	AF-20	Scotman
Incubation chambers	WTC binder Memmert	
Microtiter plate Spectrophotometer	MRX Plate Reader	Dynex
MiniCycler	PTC-150	MJ Research
PCR thermocycler	ProGene	Techne
Peristaltic pump	Cyclo1	Roth
pH-meter	HI 9321	Hanna Instruments
Phosphoimager	BAS-1000	Fuji
Photometer	Unikon 720 LC	Kontron
Plant growth chambers	I-37L4VL	Weiss Technik Percival Scientific
Polyacrylamide Gel Chamber	AGS	
RNA-/DNA-calculator	GeneQuant II	Pharmacia
Shaker	ST 5	M. Zipperer GmbH
Scales	A 120 S	Sartorius Analytic
Scanner	ScanJet 4c	Hewlett Packard
Sequencing station	ABI PRISM™ 310	Perkin Elmer
Spectrophotometer	Novaspek Biochrom	
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 bath	1086	GFL
Water deionization system	Option 4, Maxima	ELGA
Wet Blotting Apparatus	EB10	Fonds der chemischen industrie Frankfurt

4.8 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

4.9 Buffers and Solutions

Buffer or Solution	Components and Concentrations
Binding buffer for EMSA (5x)	125 mM Hepes, Adjust pH to 7.5with 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
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

Buffer or Solution	Components and Concentrations
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
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
Drop-in -Leucine -Tryptophan -Histindine solution (10x)	200 mg/l L-Adenine /Hemisulfat 300 mg/l L-Lysine 300 mg/l L-Tyrosine
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
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
KGB buffer (10x)	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
Klenow buffer (10x)	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

Buffer or Solution	Components and Concentrations
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 mM $MgCl_2$
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
PBS buffer (1x)	68 mM NaCl 58 mM Na_2HPO_4 17 mM NaH_2PO_4 pH 7.4 (NaOH)
PBS-T (1x)	1x PBS 0.1% (v/v) Tween 20
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
RNA loading buffer (3x)	50% Formamid 10% 10x MOPS 0.45% Formaldehyd 7% Glycerin 0.5% Bromphenolblau
SDS-PAGE running buffer (10x)	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

Buffer or Solution	Components and Concentrations
Solution 1 for small-scale plasmid DNA isolation	50 mM Tris-HCl, pH 8.0 10 mM EDTA
Solution 2 for small-scale plasmid DNA isolation	0.2 M NaOH 1% SDS
Solution 3 for small-scale plasmid DNA isolation	29.4 g Potassium acetate 5 ml Glacial acetic acid H_2O up to 100 ml
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
SSC (20x)	2 M NaCl 0.3 M Sodium citrate Adjust pH to 7.0 with HCl
TAE (20x)	800 mM Tris 20 mM EDTA 2.3% (v/v) Glacial acetic acid
TBE (5x)	450 mM Tris 450 mM Boric acid 1 mM EDTA Adjust pH to 8.0
TE (100x)	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

4.10 Software

Program	***Company***
Acrobat Reader 6.0	Adobe
BLAST and Bioinformatics	NCBI, MIPS, TAIR and TIGER
Chromas	Technelysium Pty Ltd
Clone Manager for Windows version 4.01 © 1995	Scientific and Educational Software
Color View Soft Imaging System	Olympus
CorelDRAW	Corel
Office	Microsoft
Oligo, DOS based version	MBI
PCBAS® Reader 2.09	Raytest GmbH
PhotoImpression 3	ArcSoft
PhotoPaint	Corel
PhotoShop	Adobe
TINA® 2.0	Raytest GmbH
Vector NTI 9.1.0	Invitrogen

5 Supplementary Methods

5.1 Cloning of recombinant DNA constructs

5.1.1 Cloning Procedures

5.1.1.1 Ligation of DNA Fragments

The conventional method of cloning a DNA fragment into a selected plasmid was carried out 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 5X 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.

5.1.1.2 Restriction Enzyme Analysis

For restriction enzyme digests of minipreps, 2µg of DNA was used per digestion reaction, assuming DNA concentration of mini-preps was 2µg/µl. For DNA to be purified by gel extractions, a greater amount of DNA of up to 20µg was used. For an overnight digestion, 1unit of enzyme was used per microgram of DNA per number of sites, relative to the number of the restriction sites in question present in λ -DNA and size of λ-DNA relative to the size of DNA to be digested.
In cloning strategies, the amount of DNA to be digested is calculated, and the amount of enzyme calculated respectively.
For 1 µg of DNA, the amount of enzyme used was in the range of:

(Size of λ-DNA) X (Nr. of RE sites on DNA to be digested)/ (Size of DNA to be digested) X (NR ofRE sites on λ-DNA)

The total amount of enzymes used (always kept at –20°C) did not exceed one tenth of the total volume of the reaction mixture. The recommended buffers were used for each enzyme, and water was used to make up to the total volume. Incubation of the digestion mixture was done at 37°C, for between 2hours and 16hrs. Analysis was carried out on 1-2% agarose gel, depending on the fragment sizes. 2µl of BOX loading buffer with marker (Saccharose color marker: 67% Saccharose, 0.5%(w/v) Bromophenol blue, 0.5%(w/v) Xylene cyanol FF, 0.5%(w/v) Orange G, 50% EDTA, pH 8.0 in water) was mixed with 10µl of digestion reaction, loaded unto the gel and ran at 120V for 45minutes. After soaking in an Ethidium Bromide tank (1mg/L EtBr in water) for 5minutes, the DNA band patterns were observed in the dark, above uv light. For fragments used for cloning, light of longer wavelength was used, and the bands cut out, for gel extraction purification and subsequent cloning.

5.1.1.3 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, with fusion tags that also fascilitate biochemical analysis of the protein expressed.

The first step in this cloning is the amplification of a PCR-specific product with specific flanking recombination sites (attB) predesigned with the primer sequence. IN a second step, the attB-PCR product is cloned into a pDONR207 plasmid. This cloning step, known as the BP recombination reaction, is catalyzed by the BP Clonase enzyme mix, and involved recombination between the attB and the attP sites of the PCR product and the pDONR vector respectively. The final product of this reaction is known as the pDONR/entry clone plasmid. The pDONR/entry clone plasmid is used in a second step LR recombination reaction to deliver the entry clone (the attB-PCR product) into a destination expressiong vector, creating the pENTR/expression clone plasmid. This is catalysed by the LR Clonase enzyme mix.
The BP recombination reaction contained a 29-50 fmol of the attB-PCR product, 150ng pDONR™ vector, 2µl 5x BP Clonase reaction buffer and TE buffer, pH 8.0, making up to 8 µl before adding 2µl of BP Clonase enzyme mix. The reaction was incubated at 25 °C for 2 hours. The reaction was stopped by adding 1µl of 2µg/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 pDONR™ were replaced by the pDONR/entry clone (linearized before use, for optimal results) and pDONER/entry clone plasmid, respectively.

5.1.1.4 Polymerase Chain Reaction (PCR)

Fragments of DNA were amplified using the polymerase chain reaction (PCR) (Mullis and Faloona, 1987). For each PCR reaction, the primers were designed using computer software (OLIGO, Vector NTI) taking into consideration the annealing temperature and that the GC content at the 5’ and 3’ends should be suitable for primer annealing. The most suitable primers were chosen to give identical annealing temperatures, and which characteristics which excluded the possibilities to form secondary structures and annealing at non-specific priming sites. PCR reactions consisted of a 94°C denaturation step, for 1minute, a primer-annealing step, which was variable depending on the specific primers, and an elongation step at 72°C, for a time depending on the length of the DNA to be amplified, and the rate of polymerization of the DNA polymerase used. Relatively error free *Pfu*-polymerase was used for overlapping PCR and polymerizes at 500 nucleotides per minute, while Taq polymerase polymerase, which polymerizes at 2000-4000 nucleotides per minute, was used otherwise.

The reaction mixture included 40ng of DNA template, 10mM of dNTPs, 5units of Taq polymerase (or 1.25units of pfu polymerase), 10pmols each of forward and reverse primers,5µl of PCR buffer, 4mM of Mg^{2+} ($MgSO_4$ used when Taq polymerase was used, and $MgCl_2$ used when *pfu* polymerse was used) and water, up to a final volume of 50µl. The products of the Tag polymerise reaction could be cloned into the pGEM-T vector system(Promega), due to it’s ability to generate a tag of dA-overhangs at the end of polymerization.

5.1.1.5 DNA Sequencing

The DNA sequencing was done using the RR-Kit (DyeTM Terminator Cycle sequencing Ready Reaction with Ampli Taq ®DNA polymerase, by Applied Biosystems) 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 RR sequencing mix and H2O 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.
9.5µl of water was added and the DNA product was precipitated using 30,5µl of 100% absolute ethanol (non denatured) at RT, and vortexed properly. After incubating at RT for 1Hr, the reaction mixture was centrifuged at RT for 20mins at a maximum speed of 13000rpm. Immediately after, the supernatant was carefully removed by pipetting and the pellet retained. To this was added 125µl of 70% absolute (non-denatured) ethanol, and vortexed. This was then centrifuged at maximum speed for 10mins, at RT. The ethanol was immediately removed using a yellow pipette tip and the pellet dried at 95°C for 1minute. To this was added 20 µl of TSR (Template suppresor reagent), vortexed and spun down. After incubation at 95°C for 2minutes to denature the DNA, it was placed directly on ice for 5 minutes, vortexed to mix and spun down. The samples were then transferred to sequencing tubes, avoiding air bubbles, and then loaded on an ABI-Prism™ 310 capillary electrophoresis sequencing station (Perkin- Elmer) for analysis.

5.1.2 Plasmid gateway constructs

5.1.2.1 pDONR207/GRX370

GRX370 was previously amplified from cDNA using gateway specific primers and inserted into the pDONR207 plasmid by the gateway BP reaction. (Ayed Abdallat, 2004). DNA sequence integrity was confirmed by sequencing with pDON-L1 and pDON-L2.

5.1.2.2 pDONR207/GRX480

At1g28480 was previously amplified from genomic DNA template using gateway specific primers *At1g28480gatewayFWD* and REV respectively. This was inserted into the pDONR207 plasmid by the gateway BP reaction. (Abdallat, 2004). DNA sequence integrity was confirmed by sequencing with pDON-L1 and pDON-L2.

5.1.2.3 pDONR223/GRX480-DM

The gateway primers, At1g28480 fwd and At1g28480 REV were used to amplify cDNA of At1g28480 from the template plasmid pBD-GRX480-DM. The PCR product was used in the first step gateway recombination reaction with pDONR223 according to the manufacturers' instructions. This was then transformed to electro- competent *E.coli DB3.1* and selected on LB spectinomycin plates. Positive colonies were controlled using restriction analysis with *SspBI* (Bsp1407I), and the sequence integrity verified by sequencing using the primers UNI24 and T7 respectively.

5.1.2.4 pDNR223(spec)/GRX480-ΔN30

A gateway primer was designed following the gateway cloning instructions, to amplify the AT1g28480 gene without the first 30 N-terminal amino acids. The gateway primers, GRX480-ΔN30 fwd and At1g28480 REV were used to amplify cDNA of At1g28480 from genomic DNA template.
The PCR reaction contained 10pmoles each of primers, 3pmoles of dNTPs, 0.5µl of Advantage Tag polymerase, 50ng of genomic DNA and 1X Tag polymerase buffer in a total volume of 25µl.

The two independent PCR products were used in the first step gateway recombination reaction with pDONR223 according to the manufacturers' instructions. This was then transformed to electro- competent *E. coli DB3.1* and selected on LB spectinomycin plates. Positive colonies were controlled using restriction analysis with *SspBI* (Bsp1407I), and the sequence integrity verified by sequencing using the primers *UNI24* and *T7* respectively.

5.1.2.5 pENTR201/TGA2

The bacteria clone containing this plasmid was picked up from the REGIA array plate, and amplified in gentamycin selective medium. Control plasmid digestion using *SspB*I gave characteristic fragments of 2225 and 1152 basepairs respectively. Two independent clones were sequenced using the SeqL1 and SeqL2 primers, and the sequence obtained was blasted on http://www.arabidopsis.org/Blast/. The hit obtained corresponded to At5g06950, coding for the transcription factor TGA2.

5.1.2.6 pDEST-GAD-HA

This destination vector contained an HA tag and a yeast Gal4 activation domain, designed to be fused in frame to any gene of interest, after recombination by the LR reaction, within the recombination sites attR1 and attR2.
The plasmid construct also contained the LEU2 gene, for the biosynthesis of leucine, important for selection in yeast, grown in leucine deficient medium.
After transformation into *E.coli DB3.1* and selection on ampicillin medium, positive clones were confirmed by restriction digestion using the enzyme *SspB*I producing characteristic fragments, 6090, 1221, 702 and 402bp respectively.

5.1.2.7 pDEST-GBKT7-myc

This destination vector contained a c-myc tag and yeast Gal4 DNA binding domain, designed to be fused in frame to any gene of interest, after recombination by the LR reaction within the recombination sites attR1 and attR2.
The plasmid construct also contained the TRYP1 gene, for the biosynthesis of tryptophan, important for selection in yeast, grown in tryptophan deficient medium.
After transformation into *E.coli DB3.1* and selection on kanomycin medium, positive clones were confirmed by restriction digestion using the enzyme *SspB*I giving characteristic fragments, 6090, 1283, 402 and 260 basepairs respectively.

5.1.3 Plasmid constructs for stable transformation into *Arabidopsis thaliana*

5.1.3.1 pAlligator/ GRX480

The previously generated pAlligator/GRX480 (Ayed Abdallat, 2004) was controlled by restriction digest with *SspB*I, producing characteristic fragments 8795, 525 and 459 base pairs respectively. The sequence integrity of the DNA was confirmed by sequencing using NOS and 35SI primers.

5.1.3.2 pAlligator/ GRX370

The pDONR207/GRX370 plasmid was linearised using restriction enzyme *EcoR*V. In a subsequent LR gateway recombination reaction with pAlligator2 following the gateway reaction manual, the product was obtained containing the glutaredoxin gene At5g40370, fused in frame at its N-terminus to a 3X-HA tag and driven by a 2X-CaMV 35S

promoter. The plasmid construct also contained the GFP gene, expressed under the control of a seed specific promoter, to facilitate selection of positive seeds.
After transformation into *E.coli DH5a* and selection on spectinomycin medium, positive clones were confirmed by restriction digest with *SspB*I, producing characteristic fragments 8815, 525 and 366 base pairs respectively. The sequence integrity of the DNA was confirmed by sequencing using NOS and 35SI primers.
The construct was transformed into electro-competent GV3101 *Agrobacteria tumefaciens* cells and selected on YEB medium containing spectinomycin, rifampicin and gentamycin, before infecting plants for stable transformation.

5.1.3.3 pAlligator/ GRX480-DM

The pDONR223/GRX480-DM plasmid was linearised using restriction enzyme *Pvu*I. In a subsequent LR gateway recombination reaction with pAlligator2 following the gateway reaction manual, the product was obtained containing the glutaredoxin gene At1g28480, mutated in its two active site cysteines (exchanged for serines) and fused in frame at its N-terminus to a 3X-HA tag and driven by a 2X-CaMV 35S promoter. The plasmid construct also contained the GFP gene, expressed under the control of a seed specific promoter, to facilitate selection of positive seeds.
After transformation into *E.coli DH5a* and selection on spectinomycin medium, positive clones were confirmed by restriction digest with *SspB*I, producing characteristic fragments 8795, 525 and 459 base pairs respectively. The sequence integrity of the DNA was also confirmed by sequencing using NOS and 35SI primers.
The construct was transformed into electro-competent GV3101 *Agrobacteria tumefaciens* cells and selected on YEB medium containing spectinomycin, rifampicin and gentamycin, before infecting plants for stable transformation.

5.1.3.4 pAlligator/ GRX480-ΔN30

The pDONR223/GRX480-ΔN30 plasmid was linearised using restriction enzyme *Pvu*I. In a subsequent LR gateway recombination reaction with pAlligator2 following the gateway reaction manual, the product was obtained containing the glutaredoxin gene At1g28480, lacking its first 30 N-terminal amino acids and fused in frame at its N-terminus to a 3X-HA tag and driven by a 2X-CaMV 35S promoter. The expressionclone also contained the GFP gene, expressed under the control of a seed specific promoter, to facilitate selection of positive seeds.
After transformation into *E.coli DH5a* and selection on spectinomycin medium, positive clones were confirmed by restriction digest with *SspB*I, producing characteristic fragments 8815, 525 and 351 base pairs respectively. The sequence integrity of the DNA was also confirmed by sequencing using NOS and 35SI primers.
The construct was transformed into electro-competent GV3101 *Agrobacteria tumefaciens* cells and selected on YEB medium containing spectinomycin, Rifampicin and Gentamycin, before infecting plants for stable transformation.

5.1.3.5 pAlligator/ GRX480-ohneHA

A version of the pAlligator vector containing the GRX gene At1g28480 without the HA tag (pAlligatorGRXOhneHA) was generated using an overlapping fragment PCR protocol. (described in manuscript).

After transformation into *E.coli DH5a* and selection on spectinomycin medium, positive clones were confirmed by digestion with restriction enzymes *Pst*I *and Nde*I, producing characteristic fragments 4252, 2489, 1733 and 1113 base pairs respectively. The sequence integrity of the DNA was also confirmed by sequencing using NOS and 35SI primers.
The construct was transformed into electrocompetent GV3101 *Agrobacteria tumefaciens* cells and selected on YEB medium containing spectinomycin, rifampicin and gentamycin, before infecting *Arabidopsis* plants for stable transformation.

5.1.3.6 pGWB233/GRXpromoter

The GRX promoter fragment, 2194 basepairs upstream of the start codon was amplified from genomic DNA using the primers GRXPROMFWD/GRXPROMREV, which were designed to contain the *Sal*I and *Xba*I sites respectively. At least three independent PCR reactions were done and the products were used. The bioXact enzyme was used. The PCR product was cloned into the Bluescript vector (pBS) and selected on Xgal/IPTG/Ampicillin plates. After control digestion with *Sal*I/*Xba*I restriction enzymes, the positive candidates were sequenced using the UNI and REV primers. Other primers, GRXPROM1059FWD/ GRXPROM1059REV/ LPGX480/ GRXPROM1507REV/ LP2GRX480/ LP1GRX480 were used to sequence and cover the entire region of the promoter.
The GRXpromoter was then subcloned into the gateway vector pUC18Entry2 within the *Sal*I and *Xba*I sites, and transformed into electro-competent *E.coli DB3.1.* This was controlled using the *Pvu*I restriction enzyme digest, with characteristic 4160 and 896 base pair products.
After linearising the pUC18Entry2/GRXpromoter with *Nde*I, it was used in a gateway LR reaction with the destination vector pGWB235 using the manufacturers instructions for the gateway reaction. The product was transformed into *E. coli* DH5α cells and selected for kanomycin resistance.

5.1.4 Plasmid constructs for transformation and expression in yeast

5.1.4.1 pDEST-GAD-HA/TGA2

The pENTRY201/TGA2 plasmid was linearised using restriction enzyme *Pst*I. The linear plasmid was purified by gel elution and used in a subsequent LR gateway recombination reaction with pDEST/GAD-HA following the gateway reaction instruction manual. The product obtained contained TGA2 fused in frame to an N-terminal HA tag and Gal4 activation domain. The expression was driven by an ADH1 promoter. The plasmid construct also contained the LEU2 gene, for the biosynthesis of leucine, important for selection in yeast, grown in leucine deficient medium.
After transformation into *E.coli DH5a* and selection on ampicillin medium, positive clones were confirmed by restriction digest with *SspB*I producing a characteristic band pattern of 6090, 1220, 1023 and 701 base pairs respectively.

5.1.4.2 pDEST-GBKT7-myc/GRX480

The pDONR207/GRX480 plasmid was linearised with the restriction enzyme *pvu*I. The linear plasmid was purified by gel elution and used in a subsequent LR gateway recombination reaction with pDEST/GBKT7-myc following the gateway reaction

instruction manual. The product obtained contained the GRX480 gene fused in frame to an N-terminal myc tag and Gal4 binding domain (GB). The expression was driven by an ADH1 promoter. The plasmid construct also contained the TRP1 gene, for the biosynthesis of tryptophan, important for selection in yeast, grown in tryptophan deficient medium.
After transformation into *E.coli DH5a* and selection on kanomycin medium, positive clones were confirmed by restriction digest with *SspB*I producing a characteristic band pattern of 6060, 1002, 444 and 265 base pairs respectively.

5.1.4.3 pDEST-GBKT7-myc/GRX480-DM

The pDONR223/GRX480DM plasmid was linearised with the restriction enzyme *pvu*I. The linear plasmid was purified by gel elution and used in a subsequent LR gateway recombination reaction with pDEST/GBKT7-myc following the gateway reaction instruction manual. The product obtained contained the GRX480, mutated in its active site cyteine residues (replaced by serine) fused in frame to an N-terminal myc tag and Gal4 binding domain (GB). The expression was driven by an ADH1 promoter. The plasmid construct also contained the TRP1 gene, for the biosynthesis of tryptophan, important for selection in yeast, grown in tryptophan deficient medium.
After transformation into *E.coli DH5a* and selection on kanomycin medium, positive clones were confirmed by restriction digest with *SspB*I producing a characteristic band pattern of 6060, 1002, 444 and 265 base pairs respectively.

5.1.4.4 pDEST-GBKT7-myc/GRX480-ΔN30

The pDONR223/GRX480-ΔN30 plasmid was linearised with the restriction enzyme *Xba*I. The linear plasmid was purified by gel elution and used in a subsequent LR gateway recombination reaction with pDEST/GBKT7-myc following the gateway reaction instruction manual. The product obtained contained the GRX480 gene deleted in its 30 N-terminal amino acids and fused in frame at its N-terminus to a myc tag and Gal4 binding domain (GB). The expression was driven by an ADH1 promoter. The plasmid construct also contained the TRP1 gene, for the biosynthesis of tryptophan, important for selection in yeast, grown in tryptophan deficient medium.
After transformation into *E.coli DH5a* and selection on kanomycin medium, positive clones were confirmed by restriction digest with *SspB*I producing a characteristic band pattern of 6060, 1002, 444 and 265 base pairs respectively.

5.1.4.5 pDEST-GBKT7-myc/GRX370

The pDONR207/GRX370 plasmid was linearised with the restriction enzyme *Bgl*II. The linear plasmid was purified by gel elution and used in a subsequent LR gateway recombination reaction with pDEST/GBKT7-myc following the gateway reaction instruction manual. The product obtained contained the GRX370 gene fused in frame at its N-terminus to a myc tag and Gal4 binding domain (GB). The expression was driven upstream by an ADH1 promoter. The plasmid construct also contained the TRP1 gene, for the biosynthesis of tryptophan, important for selection in yeast, grown in tryptophan deficient medium.
After transformation into *E.coli DH5a* and selection on kanomycin medium, positive clones were confirmed by restriction digest with *SspB*I producing a characteristic band pattern of 6060, 1002, 366 and 265 base pairs respectively.

5.1.5 Plasmid constructs for transient assays in protoplast

5.1.5.1 HBT/GRX480DM

This was prepared in two steps by a three fragment ligation method. First, HBTGBDGRX480DM was digested with NotI and SspBI, and the 200 basepair fragment eluted from the gel. The HBT/GRX480 plasmid (Abdallat, 2004) was also digested independently with NotI/EcoRI and SspBI/EcoRI, and the 3285 and 432 base pair fragments respectively eluted. The three fragments were then ligated within NotI/SspBI/EcoRI sites respectively into the HBT vector to yield HBT/GRX480DM.
The integrity of the product was controlled by digestion with the enzymes *Ssp*BI /*Eco*RI to produce fragments 2053, 1432 and 432 base pairs long respectively. This was further verified by sequencing using the Nos and 35SI primers.

5.1.5.2 HBTL-Gal4BD/GRX-DM

This was prepared by digesting pHBTGal4BD/GRX480DM (with pA tail) with *Ssp*BI, and cloning the GRXDM fragment into the pHBTGal4BD/GRX480 vector, replacing the GRX480 with the GRX480DM within the *Ssp*BI sites.

5.2 Other methods

5.2.1 Gene transfer into Bacteria

The *E. coli* and *A. tumefaciens* are competent by 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 can be made competent using special conditions. 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). 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, in a rotor. 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. After thawing the cells stored at –80°C, 1µl of DNA was added to 100µl of cells, and mixed. After placing the mixture in an electroporator cuvette chamber, a single pulse of 25mA was applied, at a capacitance of 25mF, a resistance of 200Ω, and a potential difference of 2.5kV. This lasted for about 5seconds, until a beep signal indicating that it was sufficient. 500µl of DYT medium was immediately added and the cells were incubated at 30°C incubator for 2hours to allow for outgrowth, after which they were plated on YEP medium (5g/L Peptone beef extract, 5g/L yeast extract, 5g/L Trpton, 5g/L Saccharose, 2mM $MgSO_4$, and pH adjusted to 7.0 with NaOH) containing selective antibiotic. They were placed in a 30°C incubator for 3days, for growth to occur. Agrobacterial minipreps were prepared from 10ml cultures, of single colonies according to the QIAprep protocol for agrobacterial DNA. The DNA was analysed by restriction enzyme digest, for positively transformed colonies.

5.2.2 The *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis*

Agrobacterium tumefaciens, were transformed with the binary vector, into which had been cloned the gene of interest, and subsequently used to infect plants floral parts.

Stable transformation of *Arabidopsis* plants was done using the floral dip method of Clough and Bent (1998). Plants were grown at a density of up to 10plants/pot, and when they reached the primary flowering stage, the inflorescence above 5cm was cut off. The secondary flowers were allowed to develop until they reached about 10 cm, and before self fertilization and seed maturation occurred. *Agrobacterium tumefaciens* strain GV3101 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. The cells were harvested by centrifugation for 30 minutes at 3000 rpm. The cells pellet was re-suspended in 400 ml infiltration medium to a final OD600 of approximately 2.0 prior to use. For the floral dip procedure, the inoculum was placed in a 500 ml beaker. The pots were inverted into the inoculum such that all tissues aboveground were submerged. The 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 transparent lid to maintain humidity for 24hours. A plastic shield was placed around the pots to contain the developing seeds, so as to ease harvesting. Plants were further grown for a 2-3 weeks until siliques were mature and dry. Seeds were harvested.

5.2.3 Isolation of plasmid DNA from *E. coli*

Isolation of small amounts of plasmid DNA from *E. coli* for analytical purposes (enzyme digestion) was done using a modification of the alkaline lysis method (Le Gouill *et al*., 1994). Colony Selection was carried out as followes. A master mix was prepared for the number of cultures, of LB (10g/L of Tryptone, 10g/L of yeast extract, 10g/L of NaCl) or DYT and the respective antibiotic added to the recommended final concentration (100µg/µl for Ampicillin, and 50µg/µl for kanamycin). Glass tubes were filled with 5ml each of DYT/antibiotic medium. Using a sterile pipette tip, a master plate was simultaneously created and used to inoculate the medium. This was then incubated at 37°C for 16hrs (overnight), to allow for the growth of positive colonies in antibiotic selective medium. 1.5ml of overnight culture was poured into micro-fuge tubes, and the bacterial pellet harvested at 13000rpm for 3mins. 3ml of the rest were collected in another tube and the pellet stored at -20°C for spin-prep of purer DNA preparations and for subsequent sequence analysis of positive colonies. Pellets were re-suspended in 100µl each of ice-cold Solution I (50mM Glucose, 50mM Tris- HCl, 10mM EDTA, pH adjusted to 8.0, autoclaved and 1mg/100ml of RNase added before use) and vortexed. Next, 200µl of freshly prepared solution II (0.2M NaOH, 1% SDS), and 200µl of chloroform were added, vortexed and left to stand for 1 minute, for alkali lysis to occur. 150µl of ice cold solution III (29.4g/100ml Potassium Acetate and 5ml/100ml formate) were then added and the mixture vortexed gently for 3 seconds to disperse the viscous bacterial lysate.

The lysate was centrifuged at 15000rpm for 2mins at 4°C, and 400µl of supernatant transferred to a freshly prepared tube. Using a glass pipette the tubes were filled in with ice-cold absolute ethanol. After mixing, they were centrifuged at 15000rpm in a cooled

centrifuge for 2mins. The content of the supernatant was poured out leaving the pellet at the bottom. The tubes were then filled with 70% ethanol (at room temperature) and centrifuged at maximum speed for 5mins at 4°C. The supernatant was removed and the tubes centrifuged again for 5mins, in order to remove residual liquid supernatant with a pipette tip. The tubes were dried at 37°C for 10mins, and to the dried pellets were added 20µl each of re-suspension buffer (10mM Tris/HCl, pH 8,5) After warming for 5minutes with shaking at 60°C to re-suspend the DNA pellet, 2-3µl of each preparation was used for restriction enzyme digest.

5.2.4 Preparation of genomic DNA from *A. thaliana*

DNA preparation buffer was freshly prepared from 2.5ml/10mL of DNA extraction buffer for plants (350mM Sorbit, 100mM Tris, 5mM EDTA, pH to 7.5), 2.5ml/10mL of nuclear lysis buffer (200mM Tris Base, 50mM EDTA, 2M NaCl, 2% CetyltetramethylammoniumBromide) 1mL/10mL of 5% sarcosyl solution, and 30mg/10mL of Na_2SO_3 . To 50ug of tissue material, 750µl of Preparation buffer, vortex and incubate at 65°C for 30-120 minutes. 750µl of Chloroform:Isoamyalcohol (24:1) mix was added under the hood and vortexed for 15 minutes. After spinning down at 10,000rpm for 5 minutes, about 500ul of upper phase was collected into a new 1.5mL eppendorf tube and 400ul of ice cold isopropanol added and mixed. After spinning down at 10,000rpm for 5minutes, the supernatant was discarded and the pellet washed with 750ul of 70% Ethanol. After spin down for 5minutes at 10,000 rpm, and removing supernatant, the pellet was dried at 65°C for 5 minutes, and re-suspended in 50-100ul of TE buffer.

5.2.5 Transient assay in protoplasts

In order to express proteins, study their interaction properties with promoter regulatory elements or with other proteins, BY2 protoplast were transformed with DNA using the PEG-mediated method of chemical transformation, adapted from the methods of Merkle *et al.* (1996) and of Haasen *et al*., (1999). A three day old cell culture was collected by centrifugation (400 x g, 5 min, with slow acceleration and decceleration using Eppendorf Centrifuge 5403 with Rotor 16A4-44). After re-suspension and washing in 20ml of Osmotic solution (0,5 % BSA; 0,01 % β-Mercaptoethanol; 0.05 M $CaCl_2$; 0.01 M Na-Acetat; 0.25 M Mannitol, pH 5,8) the cell suspension was re-suspended in 40 ml of iso-osmotic enzyme solution (1 % Cellulase Onozuka RS; 0.5 % Macerozym Onozuka R 10; 0.1 % Pectinase; 0.5 % BSA; 0.01 % β-Mercaptoethanol; 0.05 M CaCl2; 0.01 M Na-Acetate; 0.25 M Mannitol, pH 5.8) and incubated in the dark for overnight digestion. The protoplasts were then collected by centrifugation at 100 x g for 5 min at room temperature and washed in 20 ml Osmotic solution. They were then re-suspended in 10ml solution W5 (154 mM NaCl; 125 mM $CaCl_2$; 5 mM KCl; 5 mM Glucose, pH 5,8-6.0), centrifuged (100 x g, 5 min slow start and stop, RT) and re-suspended in 5 ml solution W5. The cells were then incubated in the dark for one hour at 4°C while the cell number was determined using a Fuchs-Rosenthal-counting chamber. The protoplasts were again centrifuged (100 x g, 5 min with slow acceleration and deceleration, RT) and re-suspended to a dilution of 2×10^6 cells per ml in solution MMM

(15 mM MgCl2; 0,1 % MES; 0,5 M Mannitol, pH 5,8).

Protoplast transformation was mediated by PEG-4000: 300µl of protoplasts were added to a total of 25ug of effector DNA/7ug of reporter DNA and to the mixture, 300µl PEG-

solution (40 % PEG-4000; 0.4 M Mannitol; 0.1 M Ca $(NO_3)_2$, pH 8-9) was added, and incubated for 20minutes. To stop, 10 ml of solution W5 was added. The cells were collected (100 x g, 5 min slow start and stop, RT) and re-suspended in 700µl of MS-Medium with Sucrose (MS Medium, 0.4 M Sucrose). This was then incubated overnight at 25°C in the dark, to allow for expression of proteins. The cells were either subjected to microscopic analysis or used for a MUG assay**.**

For MUG assay of protoplast transformed cells after overnight expression, 700µl of Solution 2 (250mM Manitol, 250mM Sorbitol, 50mM $CaCl_2$, 1mM MES, pH 5.8) were carefully added to the cells and centrifuged (400 x g,10 min with slow start and stop, RT). After carefully removing the supernatant, 1 ml of Solution 2 was added to the cell suspension and transfered to a 1.5ml ependorf tube. The cells were collected by centrifugation for 10 min at 5000 rpm. The pellet was resuspended in 100 µl GUS-EX-Puffer (50 mM Sodium Phosphate buffer; 10 mM EDTA; 0,1 % Triton X-100; 0.1 % Sarkosyl; 0.05 % ß- Mercaptoethanol, pH 7.5). After vigorous vortexing, the cells were broken by two freeze thaw cycles in liquid Nitrogen and 37°C. The protein concentration of the supernatant after centrifugation (15000 rpm, 20 min, 4°C) was determined using the Bradford method. 5-20ug of the total protein was used for MUG-Assay (see manuscript).

5.2.6 Total RNA preparation from *A. thaliana* and gel blot analysis

Total RNA was extracted from 14-21-day-old plants using the TRIZOL method (Invitrogen life technologies, Chomczynski and Mackey, 1995). It was optimized as follows: 150-300mg of crushed and frozen plant material in a 2ml ependorf tube was allowed to thaw and 1ml of Trizol reagent (38% Phenol saturated in citrate buffer, 0.8M Guanidinium thiocyanate, 0.4M ammonium thiocyanate, 0.1M Sodium Acetate or 33.4mL of3M pH5.2 stock and 5% glycerol) was added. After vigorous agitation for 10 minutes, 200µl of Chloroform was added and the mixture vortexed for 5minutes, and allowed to settle for 1minute. After phase separation at 14000rpm at 4°C for 10minutes, the aqueous supernatant was transferred to a mixture of 250µl isopropanol and 250µl of high salt precipitation buffer (0.8M Sodium Citrate/1.2M NaCl). After precipitation for 15 minutes, the precipitated RNA was collected by centrifugation at 14000rpm at 4°C for 15 minutes and the supernatant discarded. The pellet was washed in 75% Ethanol, and dried by spinning down and removing the residual liquid, as well as drying the pellet at 65°C for 3 minutes. The Pellet was re suspended in 200µl of RNAse free water and by further heating at 65°C for 5 minutes, with intermittent vortexing. After clarification by spinning down at 14000rpm at 4°C for 5minutes, the RNA suspension was transferred to fresh tubes and stored at -80°C.

After determining the RNA concentration (GeneQuant II, Pharmacia), 10-20ug of extracted RNAs in loading buffer (3X loading buffer contained 50% Formamid, 10% 10X MOPS, 0.45% Formaldehyde, 7% Glycerin and 0.5% Bromophenol Blue) were subjected to electrophoresis on 1% formaldehyde/agarose gels and capillary-blotted in 10X SSC buffer (1M NaCl, 0.15M Sa citrate, pH 7.0) to Hybond N+ membranes (Amersham Pharmacia). The ethidium bromide photographs of the gels were taken to verify equal loading of RNA samples. The membranes were cross-linked at 80°C for 2 hours.

RNA blots were pre-hybridized for 1hour at 65°C in Church buffer (250 mM Sodium phosphate, pH 7.2 , 1 mM EDTA, pH 8.0, 7% (w/v) SDS). Hybridization was performed

in the same solution with 100mg/ml salmon sperm carrier DNA and [α-^{32}P]- dATP labeled cDNA probe for 14–16 h at 65°C (modified from Church and Gilbert, 1984). The probe was made by the random-priming method (Feinberg and Vogelstein, 1983) with the Mega-prime DNA labeling system (Amersham). Washing was performed with 2X SSC, 0.1% SDS at 65 °C for 20-30 min. Quantification of RNA levels was accomplished by Bioimager analysis (Fuji Bas 1000; Fuji, Tokyo) after exposure for 24 h on a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA).

5.2.7 Radioactive labeling of DNA fragments for northern blots

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, 1.4µl 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), 5µl of [α-32P]-dATP (800 Ci/mmol) and 1 Unit of klenow Exo$^-$ were added. The reaction mix was incubated for 2 hours at 37 °C.
The radiolabeled probe was precipitated for at least two hours at -20 °C, in 50µl of STE and 250 µl of absolute ethanol. After collecting the pellet at 13000rpm for 20 minutes at room temperature, the pellet was washed under the same conditions in 70% Ethanol. The pellet was re-suspended in 100ul of TE or elution buffer, and the counts of radioactivity measured using a GM counter.

5.2.8 Radioactive labeling of total cDNA for dot (macro-array) blots

In order to study how an array of genes is regulated in response to different conditions, REGIA array filters (REGIA version 2.0) were used, on which a cDNA library of genes is spotted on membranes. In order to obtain radioactively labeled total cDNA as a probe for the hybridization of the REGIA Filters, total RNA was prepared from different sample treatments and controls. An oligo$(dT)_{20}$-primer which anneals to the poly adenosine tail of mRNA molecules is used as a starting point for the transcriptional activity of the enzyme reverse transcriptase. Only mRNA is transcribed into cDNA.
A reaction mixture was prepared in a 1.5ml safe-lock ependorf tube containing 30µg of total RNA and 2µg of oligo(dT)-primer (3.3µl of 100µM) up to a final volume of 20µl. After pipetting up and down, the mixture was incubated at 70°C for 2minutes to denature the RNA, after which it was immediately placed on ice. To the mixture was finally added 5µl of 5X first strand buffer (M-MULVRT from MBI fermentas), 1µl of dNTP mix (10mM each of dATP, dGTP, dTTP and 0.1mM of dCTP), 0.5µl of RNase inhibitor, 5µl of ^{33}P-dCTP (10µCi/µl), 1µl of reverse transcriptase H$^-$(Reverse Aid H-Minus M-MULVRT from MBI) and H_2O up to a final volume of 50µl.
After mixing, the reaction was incubated at 42°C for primer extension and polymerization to take place for 1hour. The reaction was stopped at 70°C for 10 minutes, and filled up to 100µl with water. The radioactivity of the test and control reactions was measured using a hand counter.
The labeled probe was cleared of free radioactive nucleotides by centrifugation through a sephadex column at 2700 rpm for 2 mins. The residual radioactivity was measured, 100µl of HSP added to the probe and boiled in a water bath for 10mins.
The REGIA filters used for hybridization were treated in exactly the same way. They were pre-hybridized with 10mL of Church Buffer for 60mins at 65°C, after which after

which radioactive-labeled probe in pre-boiled Church buffer was applied to the membrane.
After an overnight incubation, the membranes were washed first with 2X SSC in 0.1% SDS for 30 mins, and then with 1X SSC in 0.1% SDS for 30 mins. The filters were then covered with a thin transparent paper film, and exposed to a phosphor imager screen for between 2-14days.

5.2.9 ONPG assays in yeast

The yeast *Saccharomyces cerevisiae* strain MAV203 (Vidal, 1997; Invitrogen) was used for protein expression and ONPG interaction assays. It contained the ß-galactosidase gene, downstream of a Gal4 promoter. Protein-protein interaction assay were generally performed with GRX coding regions fused to a myc-tag and to the GAL4-DNA binding domain, while the TGA2 transcription factor was fused in frame to an HA tag and to the GAL4 activation domain.
The combination of these constructs, alongside control plasmids lacking either of the GRX or TGA2 interacting partners or both, were transformed into MAV203 yeast cells.

Competent cells were prepared (adapted from Dohmen *et al.,* 1991) by inoculating 10mL of -80°C stock of cells in YPED medium (20g/L bactopeptone, 10g/L yeast extract, 20g/L Glucose) overnight, and then a 100mL culture until it attained an OD of 0.6. The cells were collected by centrifugation at 3000rpm for 5 minutes and washed in 10mL of solution A (10mM Bicine, 1M sorbitol, 3%(v/v) ethyleneglycol, pH 8.35). After re-suspending in 2mL of solution A, 100µl aliquots were frozen for subsequent transformation purposes.

For yeast transformation, 50µg of denatured HSP was mixed with 2µg of plasmid DNA and pre-incubated at 37°C for 5 minutes. 1mL of solution B (200mM Bicine, and 40% (w/v) PEG1000, pH 8.35) was added and after gently mixing, was incubated for 1hour at 30°C. The cells pellet was washed in 800µl of solution C (10mM Bicine, 150mM NaCl, pH8.35) and re-suspended with 100µl of the same, before streaking on SD selective plates (6.7g/L yeast nitrogen base without amino acids, 20g/L glucose, 0.6g/L CSM[-W-L-A-H], 11g/L select agar, 10mg/L adenine and 19.2mg/L Histidine).

The activity of β-Galactosidase reporter gene in yeast cells was quantified using ONPG (o-Nitrophenyl-β-D-Galactopyranoside) assay. The enzyme β-Gal catalyzes the conversion of the colorless ONPG substrate into the fluorescent o-Nitrophenol product.

Single colonies from plates of at most 2week old transformation were inoculated in 5mL of SD selective medium (lacking Leucine and Tryptophan), and grown for 16 hours until an OD of between 0.6 and 1.3 was attained. 4mL of yeast culture was collected by centrifugation and resuspended in 2mL of Z-buffer (16.1g/L of $Na_2HPO_4.7H_2O$, 5.5g/L $NaH_2PO_4.H_2O$ 0.75g/L of KCL and .246g/L of $MgSO_4.7H_2O$). The OD of each culture was measured, and the concentration factor determined.

After washing and re-suspending the cells in 300ul of Z-buffer, 100µl of cell suspension were broken by three sequential freeze thaw cycles in liquid Nitrogen and 37°C. After adding 700ul of Z-buffer with 0.27% of ß-Mercaptoethanol, 160ul of 4mg/ml ONPG substrate was added to the cell lysate. After color formation, the reaction was stopped using 1M Na_2CO_3. The OD at a wavelength of 420 was measured for the supernatant of each sample, and the ß-galactosidase units calculated as the amount which hydrolyses 1µmol of ONPG to o-nitrophenol per min per cell (Miller, 1972).

To study the interaction of TGA2 with GRX under reducing or oxidizing conditions, yeast cells were inoculated in 10ml of selective SD medium and grown for 16 hours. The cultures were then split into two equal volumes and 5ml of YPD medium added. They were then grown with and without 1mM Diamide (SIGMA) for 5 hours, after which the cells were harvested and used for ONPG assay as above.

5.2.10 Growth Assays With Salt Stress

In order to investigate the effect of salt stress on germination of *A. thaliana* plants, knock-out lines, over-expressing lines as well as wild type control background lines in each case were germinated in 2X-MS (4.4g/l MS-medium, 20g/l sucrose, 8g/L of agar, pH 5.7) including varying concentrations of NaCl (0mM, 50mM, 100mM, 150mM, 200mM, and 250mM). The seeds of the following lines :- Col-O, Landsberg erecta, Nössen, GRXEx#1, GRXEx#3, *grx480knock#1* and *grx480knock#1*, *npr1-1* and *npr*GRX#8 and #10 – were collected, seeds were surface sterilized for 6 hours with hypochloride gas.

For surface sterilization, the seeds in open ependorf tubes were placed in an epi-holder and placed on a beaker. 100ml of Natriumhypochloride stored at 4°C was placed in the beaker and to it, was added 5ml of 32% conc. HCl in a vacuum hood standing in an exicator. A vacuum of 800mbar was generated and the seeds incubated for the time period until they were transferred to a sterile bench, where the hood was cleaned with Ethanol and opened up.

The seeds were sown on 2XMS plates (4.4g/L Murashige & Stoog medium, 20g/L sucose, pH 5.8 with KOH, 6.4g/L select agar) with different concentrations of NaCl. The plates were sealed with parafilm under sterile conditions. After vernalization at 4°C for 2 days, they were placed under environmental growth conditions (21/19°C, 150 $\mu E/m^2/S^2$ brightness- 16-hrs-light/8-hrs-dark cycle, and 60% relative humidity), for 3 weeks. The phenotype of the plants was observed during germination, and pictures were taken in effect.

6 Supplementary Results

6.1 Structural analysis of GRX480 and related genes

The At1g2840 (GRX480) gene encodes a glutaredoxin protein which is 137 amino acids long with a molecular weight of 14.75kD. It has an isoelectric point of 5.72, with a charge of -2.07 at pH 7.0.

The amino acid sequence of the At1g2840 gene was subjected to a BLAST search on the *Arabidopsis* Information Resource Center (*http://www.arabidopsis.org/Blast/*), to search for other protein sequences from the *A. thaliana* data base with significant sequence similarity to At1g2840. The BLATp program was used for amino acid query from AGI protein datasets (Altschul *et al.,* 1997). A total of 39 sequences were identified, with expectation values ranging from 6e-16 to 8.2 and scores ranging from 79.3bits to 25.8bits. The 39 gene hits were classified based on known information about their interpro domains and based on these, 2 were identified as thioredoxin family-like proteins (At3g15660 and At4g04950), one as an auxin transport protein (At3g02260), two as cation exchanger (CAX) interacting proteins (At2g38270 and At3g54900) and 36 of them were identified as glutaredoxin family proteins. Previous bioinformatic analyses reports that there exist at least 30 glutaredoxins in the *Arabidopsis* genome (Lemaire, 2004).

In order to study possible gene duplication patterns or possible gene identities in tandem on the *Arabidopsis* physical map, the distribution of these genes in the *Arabidopsis* chromosomes was observed using the chromosome map tool (http://www.arabidopsis.org/jsp/ChromosomeMap/tool.jsp). The genes identical to GRX480 were distributed randomly in all the 5 chromosomes of *A. thaliana* (Figure 6.1A). Some glutaredoxins however exist as clusters in tandem, such as on chromosome 3 (At3g62930, At3g62950, and At3g62930) and chromosome 4 (At4g15660, At4g15670, At4g15680, At4g15690 and At4g15700).

The amino acid sequences of all the 39 glutaredoxin and glutaredoxin-like genes obtained from the BLAST search were aligned against the profile of GRX480 using the vector NTi software, and used to generate a phylogenetic tree (Figure 6.1B). At1g28480 clustered with the CC type glutaredoxins, which are a class specific only among higher plants.

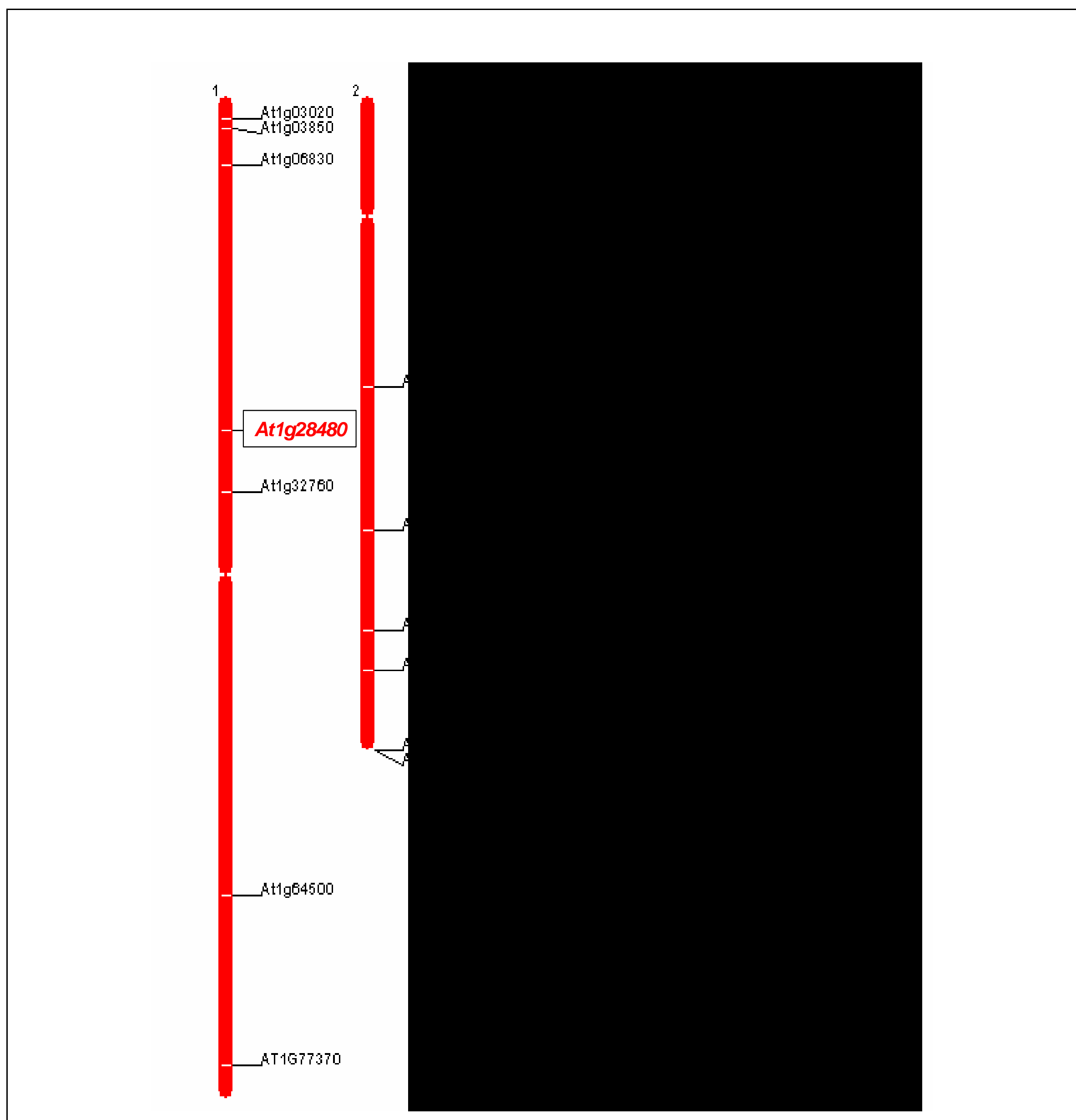


Figure 6.1A: Physical map showing distribution of genes with sequences producing significant alignments to At1g28480. *The amino acid sequence of At1g28480 (boxed in red italics) was used to search for sequences producing significant alignment to it, on http://www.arabidopsis.org/Blast/. The AGI codes were used in the chromosome map tool (http://www.arabidopsis.org/jsp/ChromosomeMap/tool.jsp) to produce the physical map of their distribution on all 5 Arabidopsis chromosomes.*

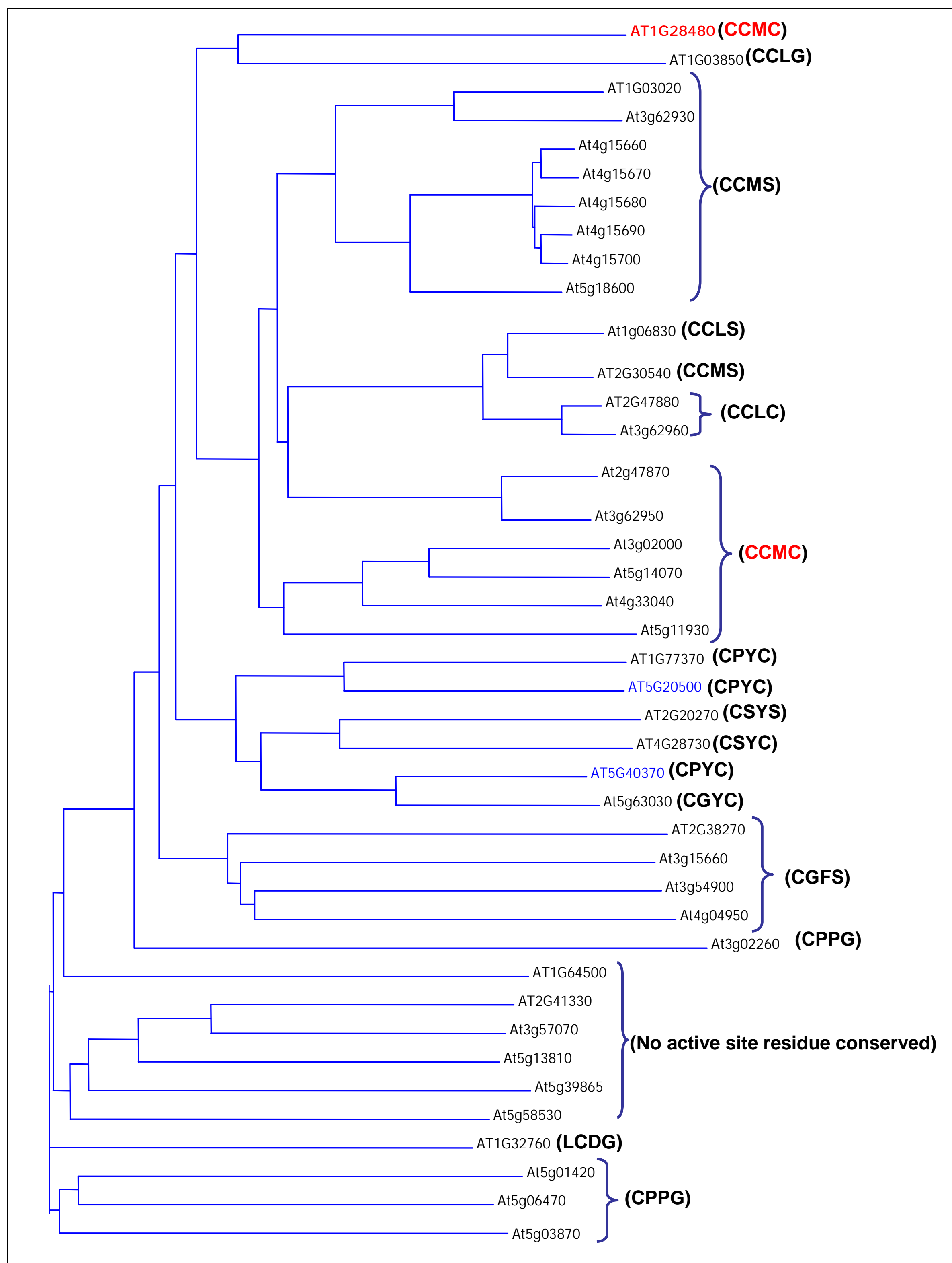


Figure 6.1B. Tree diagram showing At1g28480 protein, in the glutaredoxin family of proteins. *The At1g28480 protein sequence (red) was aligned as a profile against 39 glutaredoxin/glutaredoxin-like proteins whose amino acid sequences produced a significant alignment to At1g28480. A tree diagram was generated using the vector NTi software. Distances between lines show how significant the alignment is. At the end of each line is the AGI code for the gene, and 4 amino acid residues of the glutaredoxin active site.*

```
                         44                                               93
At1g28480      (1)  MQGTISCARNYNMTTTVGESLRPLSLKTQGNGERVRMVVEENAVIVIG-R
At1g03850     (21)  IFLLKNEDKPSSSSSSLSWLTSGSPKPTSISNKRSSNLVVMENAVVVFAR
At1g03020      (1)  -------------------------------MEKISNLLEDKPVVIFS-K
At3g62930      (1)  -------------------------------MESVRSLVEDKPVVIFS-K
At4g15660      (1)  -------------------------------MEKIQKMISEKSVVIFS-N
At4g15670      (1)  -------------------------------MEKLQKMTSEKSLVIFS-K
At4g15680      (1)  -------------------------------MDKLQKMISEKSVVIFS-K
At4g15690      (1)  -------------------------------MENLQKMISEKSVVIFS-K
At4g15700      (1)  -------------------------------MENLQKMISEKSVVIFS-K
At5g18600      (1)  -------------------------------MDMITKMVMERPVVIYS-K
At1g06830      (1)  -------------------------------MDKVMRMSSEKGVVIFT-K
At2g30540      (1)  -------------------------------MDKVVRMSSEKGVVIFS-K
At2g47880      (1)  -------------------------------MDKVMRMSSEKGVVIFT-K
At3g62960      (1)  -------------------------------MDKVMRMSSEKGVVIFT-K
At2g47870      (1)  -------------------------------MERVRDLASEKAAVIFT-K
At3g62950      (1)  -------------------------------MERIRDLSSKKAAVIFT-K
At3g02000      (1)  ---MQYQTESWGSYKMSSLGFGGLGMVADTGLLRIESLASESAVVIFS-V
At5g14070      (1)  -MQYKTETRGSLSYNNNSKVMNNMNVFPSETLAKIESMAAENAVVIFS-V
At5g11930     (24)  PPPPPLPPPAPSTVSSSTASTSLSFDEEETSESKIGRLISEHPVIIFTRF
At1g77370      (3)  DQSPRRVVVAALLLFVVLCDLSNSAGAANSVSAFVQNAILSNKIVIFS-K
At5g20500      (1)  MTMFRSISMVMLLVALVTFISMVSSAASSPEADFVKKTISSHKIVIFS-K
At2g20270     (44)  PTVIGIASWPPLRCSSVKAMSSSSSSSGSTLEETVKTTVAENPVVVYS-K
At4g28730     (39)  KRCLKQSCSVRAMTSSSSAASSSSSSFGSRMEESIRKTVTENTVVIYS-K
At5g40370      (1)  -----------------------------MAMQKAKEIVNSESVVVFS-K
At5g63030      (1)  -------------MGSMFSGNRMSKEEMEVVVNKAKEIVSAYPVVVFS-K
Consensus     (44)                                 MEKV KMVSEK VVIFS K


                         94                                              143
At1g28480     (50)  RGCCMCHVVRRLLLGLGVNPAVLEIDEEREDEVLSELENIGVQGGGGT--
At1g03850     (71)  RGCCLGHVAKRLLLTHGVNPVVVEIGEEDN---------NNYDNIVSDK-
At1g03020     (19)  TSCCMSHSIKSLISGYGANSTVYELDEMSN----GPEIERALVELGCK--
At3g62930     (19)  SSCCMSHSIQTLISGFGAKMTVYELDQFSN----GQEIEKALVQMGCK--
At4g15660     (19)  NSCCMSHTIKTLFLDLGVNPTIYELDEINR----GKEIEYALAQLGCS--
At4g15670     (19)  NSCCMSHTIKTLFLDLGVNPTIYELDEINR----GKEIEQALAQLGCS--
At4g15680     (19)  NSCCMSHTIKTLFIDFGVNPTIYELDEINR----GKEIEQALAQLGCS--
At4g15690     (19)  NSCCMSHTIKTLFLDFGVNPTIYELDEINIG----REIEQALAQLGCS--
At4g15700     (19)  NSCCMSHTIKTLFLDLGVNPTIYELDEISR----GKEIEHALAQLGCS--
At5g18600     (19)  SSCCMSHTIKTLLCDFGANPAVYELDEISR----GREIEQALLRLGCS--
At1g06830     (19)  SSCCLSYAVQVLFQDLGVNPKIHEIDKDPE----CREIEKALMRLGCS--
At2g30540     (19)  SSCCMSYAVQVLFQDLGVHPTVHEIDKDPE----CREIEKALMRLGCS--
At2g47880     (19)  SSCCLCYAVQILFRDLRVQPTIHEIDNDPD----CREIEKALLRLGCS--
At3g62960     (19)  SSCCLCYAVQILFRDLRVQPTIHEIDNDPD----CREIEKALVRLGCA--
At2g47870     (19)  SSCCMCHSIKTLFYELGASPAIHELDKDPQ----GPDMERALFRVFGSN-
At3g62950     (19)  SSCCMCHSIKTLFYELGASPAIHELDKDPE----GREMERALRALGSSN-
At3g02000     (47)  STCCMCHAVKGLFRGMGVSPAVHELDLHPY----GGDIQRALIRLLGCS-
At5g14070     (49)  STCCMCHAIKRLFRGMGVSPAVHELDLLPY----GVEIHRALLRLLGCSS
At5g11930     (74)  SSCCMCHVMKKLLSTVGVHPTVIEIDDGEI----------AYLAVEAA--
At1g77370     (52)  SYCPYCLRSKRIFSQLKEEPFVVELDQRED----GDQIQYELLEFVGRR-
At5g20500     (50)  SYCPYCKKAKSVFRELDQVPYVVELDERED----GWSIQTALGEIVGRR-
At2g20270     (93)  TWCSYSSQVKSLFKSLQVEPLVVELDQLGSE---GSQLQNVLEKITGQY-
At4g28730     (88)  TWCSYCTEVKTLFKRLGVQPLVVELDQLGPQ---GPQLQKVLERLTGQH-
At5g40370     (21)  TYCPYCVRVKELLQQLGAKFKAVELDTESD----GSQIQSGLAEWTGQR-
At5g63030     (37)  TYCGYCQRVKQLLTQLGATFKVLELDEMSD----GGEIQSALSEWTGQT-
Consensus     (94)  SSCCMCH IKTLF DLGVNP VHELD        G EIE AL  LG S
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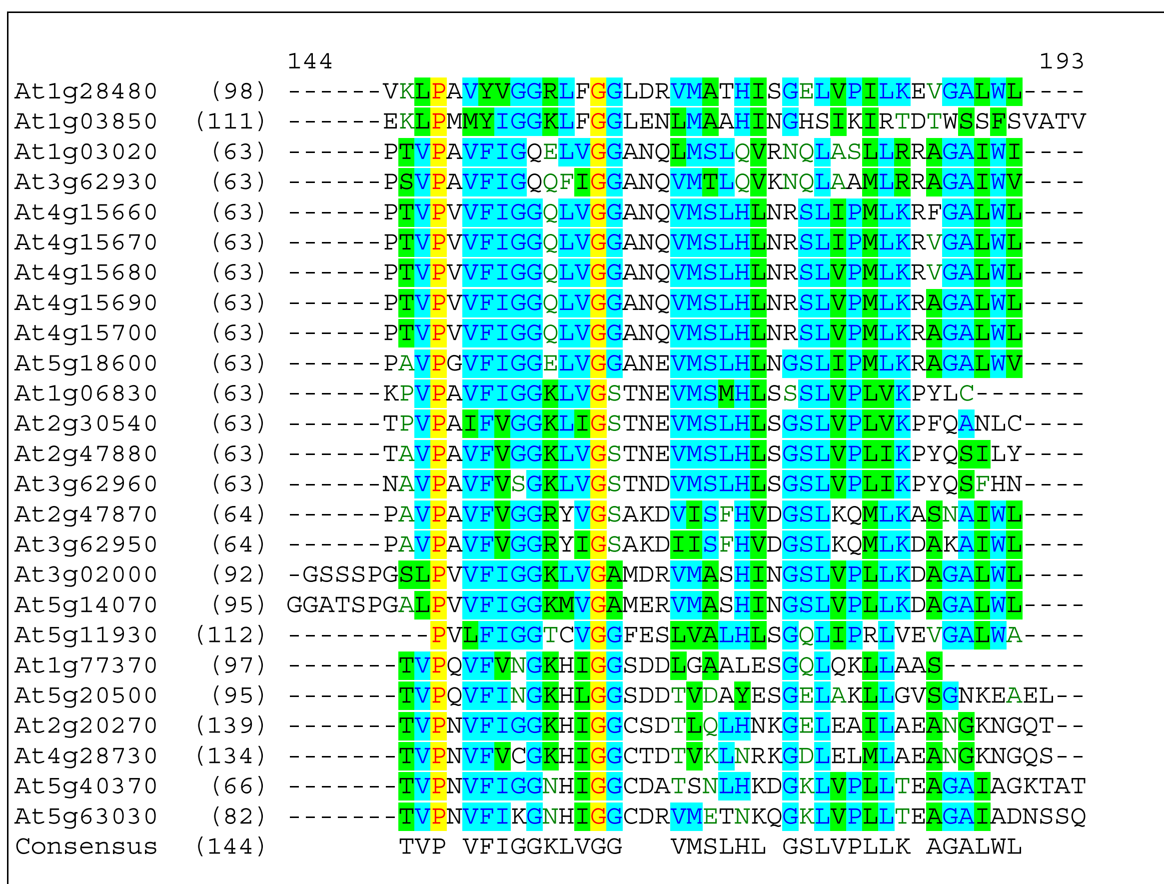


Figure 6.1C: Sequence Alignment of At1g28480 protein with related glutaredoxin family of proteins. *The protein sequence of At1g28480 along with 24 other glutaredoxin protein sequences were aligned using Vector NTi software. The numbering of the consensus sequence of the aligned residues takes into consideration the amino acid sequence with the longest N-terminal extension as position 1 (At2g20270).The CGFS class of glutaredoxins is not included in this alignment. The color code with respect to the consensus sequence is as follows: Non-similar alignment (black letters, white background); conservative alignment (deep blue letters, light blue background); block of similar alignment (black letters and green background); identical alignment (red letters, yellow background) and weakly similar alignment (green letters and white background).*

The CC class of glutaredoxins is not found in lower plant types like *Chlamydomonas* or in *Synechocystis* (Lémaire, 2004). At1g03850 has the closest homology to At1g28480. It is predicted to contain both chloroplast and cytoplasmic localization sequences.

The glutaredoxins generally have a conserved active site (Figure 6.1C, position 96-99). Unlike most glutaredoxins of the CC class which have the second cysteine of their active site replaced by a serine residue (CCMS and CCLS) and posses an additional cysteine at position 140 further downstream (see alignment Figure 6.1C, position based on consensus sequence), At1g28480 contains a CCMC configuration like other glutaredoxins At2g47870, At3g02000, At5g14070, At4g33040, and At5g11930. Only 3 glutaredoxins from this group contain the classical CPYC active site: the At5g40370 (GRX370),

At5g20500 and the At1g77370. The glutaredoxins, At5g63063 and At4g28730 have also been classified to belong to the same family as the CPYC class of glutaredoxins (Lemaire, 2004).

Another unique feature about GRX480 which is seen in the alignment is that it contains a unique N-terminal extension of about 30 amino acids, which is lacking in some CC-type glutaredoxins such as the CCMS and the CCLC groups of glutaredoxins. It is also missing in the classical CPYC glutaredoxin such as GRX370.

The coding sequence of GRX480 was then analyzed (Appendix 10.2.1) in order to generate different mutant derivatives. We wanted to map the residues and domains responsible for its ability to interact with TGA2 transcription factor, as well as its physiological role in the cell.

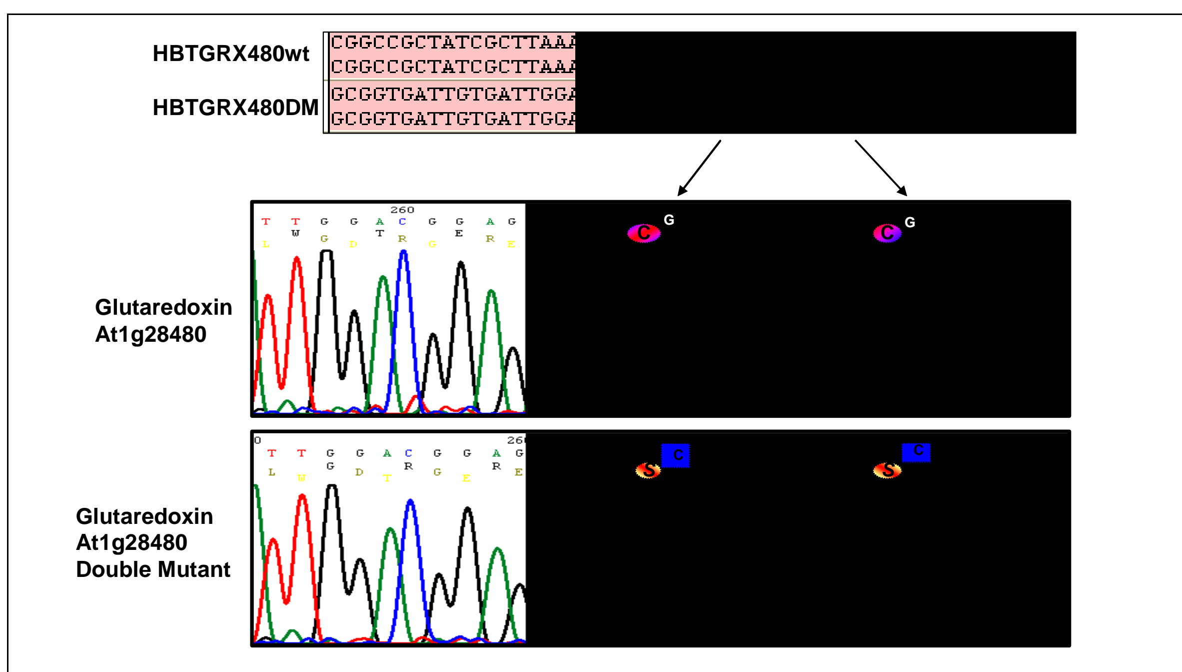


Figure 6.1D. Chromatogram of region coding for active site residues in double cyteine mutant of GRX480 (GRXDM).

After performing a PCR-site directed mutagenesis using specific primers, the mutant and wild-type versions were cloned into the pHBT vector and subsequently subcloned into the pDONR vectors using gateway technology. Sequencing using specific primers confirmed base pair substitutions of the Guanine residues (black chromatogram signal) at positions 155 and 164 in the wild type coding sequence by cytosine residues (blue chromatogram signal in the mutant coding sequence), thereby replacing the cysteine codons by serine codons.

Above is a sequence alignment of the sequenced obtained after sequencing.

In the chromatograms below correspond to the sequences obtained respectively.

The letters in the first row above each chromatogram indicate the coding sequence as read, and the letters in the second row indicate the amino acid sequence of two reading frames. The chromatogram signals have the following color codes: Adenine (Green), Thymine (Red), Guanine (Black) and Cytosine (Blue).

First of all, a mutant version of GRX480 was generated using a PCR site directed mutagenesis method (Abdallat, 2004). The product contained a mutated version of the enzymatically active site, in which both cysteines are replaced by a serine residue. This double mutant version (GRX480-DM) was confirmed by sequencing both the wild type and the mutant derivatives and comparing the sequences using the Chromas software (Figure 6.1D).

Secondly, a mutant derivative, lacking the first 30 N-terminal amino acids (GRXΔN30) was generated using sequence specific primers with gateway sequences (Figure 6.1E). The sequence integrity was confirmed to be correct by sequencing from upstream and downstream of the points of insertion in the destination vectors.

These mutant versions were analyzed for their ability to interact with TGA2 transcription factor in yeast, and for their effect on gene expression both *in vivo* and *in vitro*, using protoplast assays, and plants stably expressing these constructs driven by the 35S CaMV promoter.

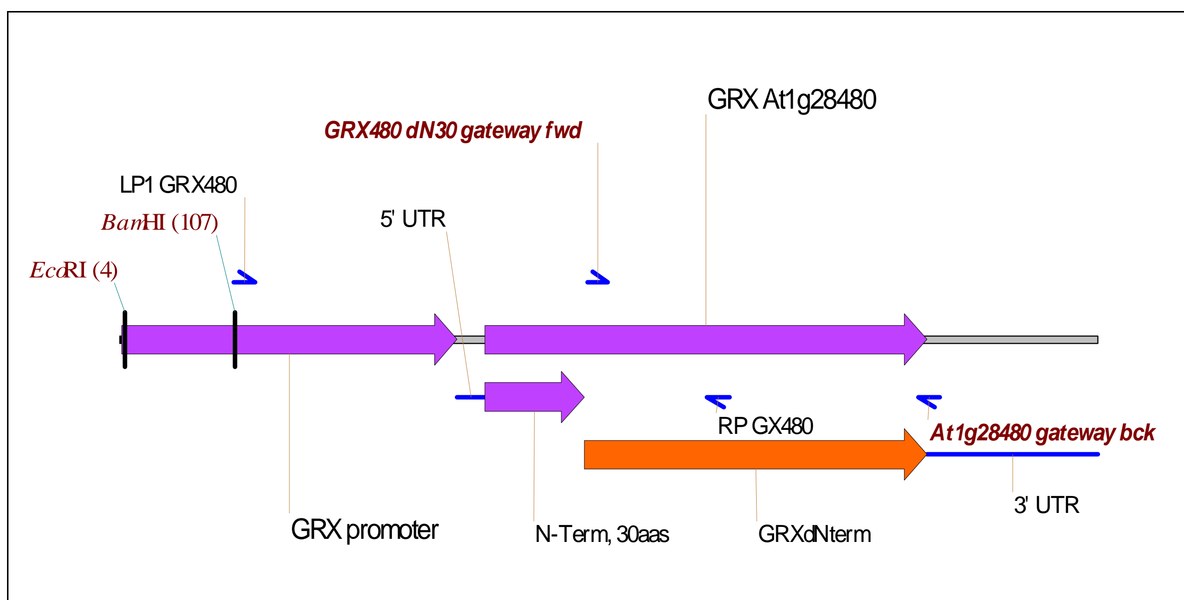


Figure 6.1E. Map outline for the generation of GRX480 version with deletion of 30 N-terminal amino acids (GRX480dN30).
Gateway primers were designed with gateway specific sequences (see materials and methods). Blue arrows indicate primer binding sites for the primers indicated..GRX480dN30gateway forward and back primers amplify GRXdN30 from genomic DNA. Other primers indicated were used to verify sequence integrity. The promoter indicated is truncated.

6.2 Differential expression of glutaredoxins

The expression pattern of GRX480 and related glutaredoxins was analyzed based on information available from the *Arabidopsis* microarray database. The GENVESTIGATOR toolkit was used to compare the expression patterns under different conditions of biotic/abiotic stress, during different stages of development as well as expression patterns occurring in specific tissues (Zimmermann *et al*., 2004; https://www.genevestigator.ethz.ch/at/index.php?page=home).

6.2.1 Stress induced differential expression of GRX480 and related glutaredoxins

The AGI codes for genes producing significant alignment to At1g28480 were input into the Meta-Analyzer program. The following were not available in the GeneExpress database: At4g15670, At2g47870, At4g28730, At5g01420 and At5g06470. The genes coding for proteins whose active site residues were not conserved (see Figure 6.1B) were also left out in the analysis.

It is interesting to note that At1g03850 which is most closely related to GRX480 is also inducible by pathogens, cyclohexamide and SA, in a similar pattern as GRX480. GRX480 is more differentially induced by salt stress in the roots.

At4g15690 is not inducible by pathogen *P. syringae*, but is SA inducible. GRX480 has 1.8 and 1.5 fold stronger inducibility by SA than At1g03850 and At4g15690 respectively. Pathogen induction by GRX480 is also 1.3 fold stronger than by At1g03850.

Even though these array data show a similar induction of GRX480 by SA and JA, northern blot data show weak induction in the presence of JA, stronger induction in the presence of SA (Figure 6.2), and synergistically stronger induction in the presence of both SA and JA (see Figure 5 of manuscript). The induction by SA is nevertheless 5-11 fold weaker than that by cyclohexamide (also reported in northern analysis by Thurow, unpublished) in wild type Columbia plants. Both SA and JA pathways seem to be important in inducing the accumulation of GRX480 transcript, especially as induction is also dependent on the *COI* protein (Thurow, unpublished).

It is also worth noting that GRX480 is uniquely regulated by cyclohexamide, unlike At1g03850 and At4g15690 which are between 40 and 60 times less inducible than GRX480 after cyclohexamide treatment.

Table 6.2A Differential expression of GRX480 and related glutaredoxins under biotic and abiotic stress induced conditions.

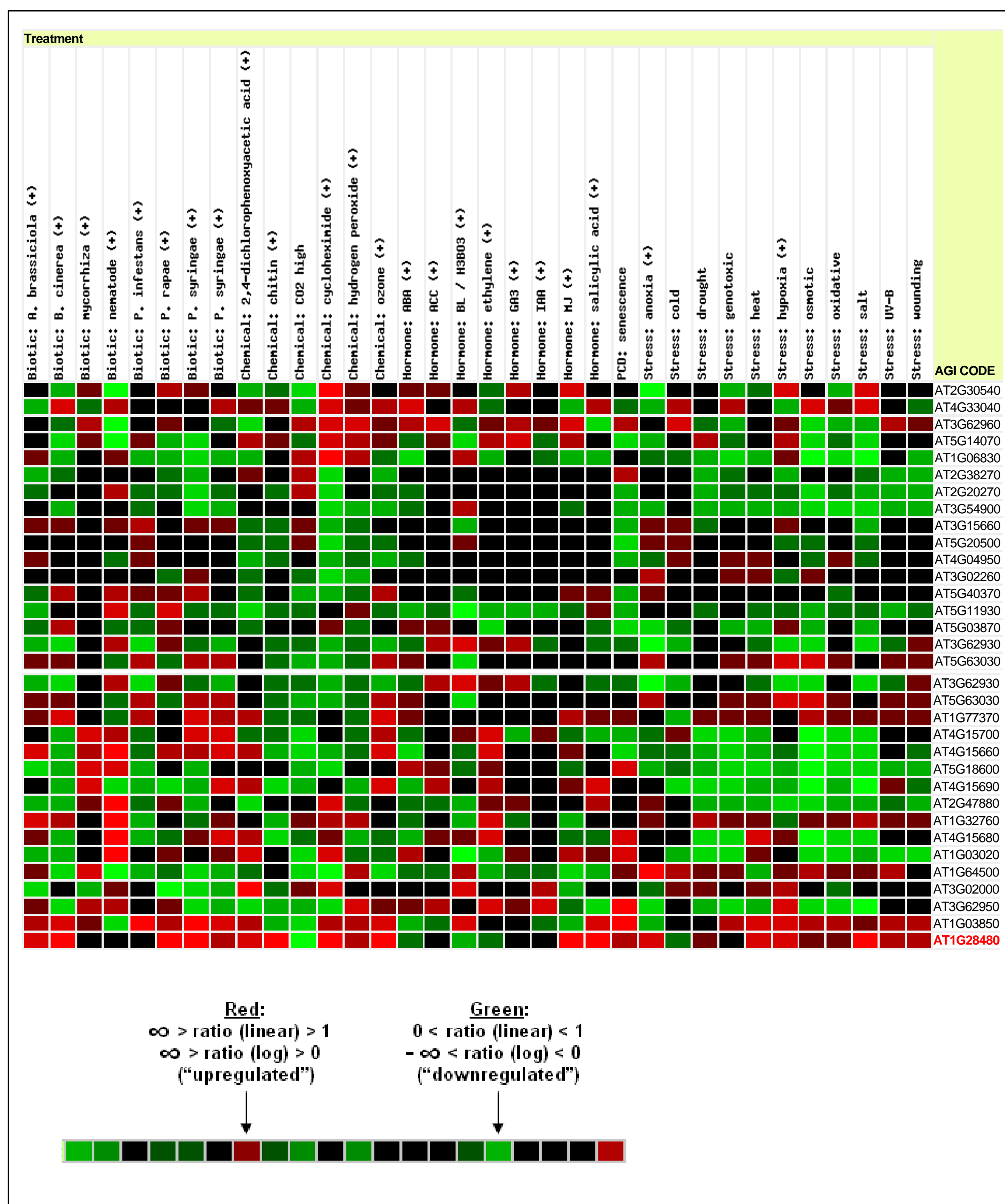


Table shows the regulation of different genes (In rows with their AGI codes indicated on the last column to the right) by different stress situations (columns, with the biotic and abiotic stress types indicated on the first row on top).

Key(Below): For the red-green scheme, red indicates that "the signal intensity of the treatment is higher than signal intensity of the corresponding control", and green means that "the signal intensity of the treatment is lower than signal intensity of the corresponding control".

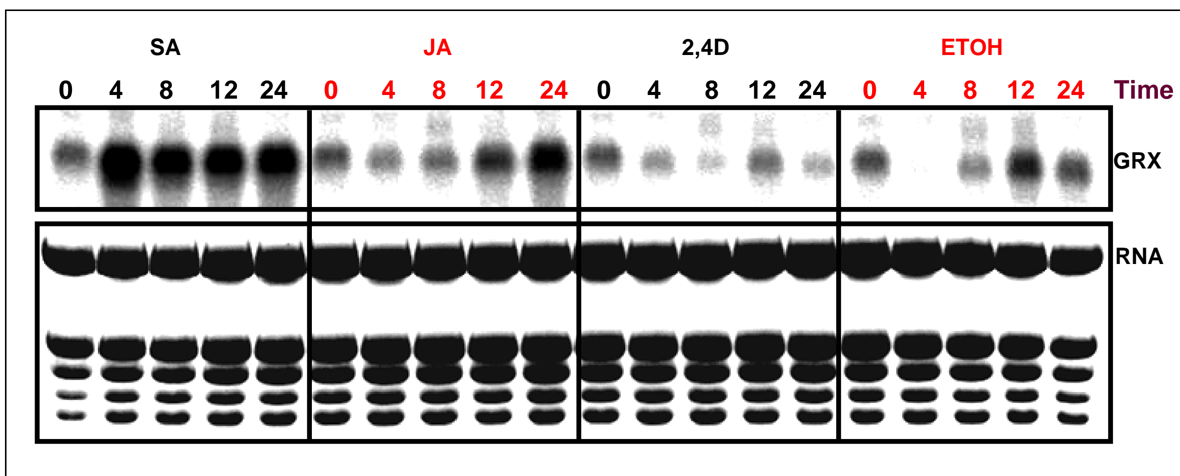


Figure 6.2: Expression analysis of GRX480
Three weeks old A. thaliana Col-O plants were treated with either 1mM SA, 20µM meJA, 0.1mM 2,D or 0.01% Ethanol control for the time periods indicated above the lanes. 20 µg of RNA was loaded and the blot was hybridized with probes for GRX480 (GRX). Ethidium bromide stained RNA is shown to document equal loading.

6.2.2 Developmental stage specific expression of GRX480 and related glutaredoxins

The AGI codes for genes producing significant alignment to At1g28480 were input into the Meta-Analyzer program except for At4g15670, At2g47870, At4g28730, At5g01420, At5g06470 and the genes coding for proteins whose active site residues were not conserved.

The developmental stage specific expression pattern of the input genes was displayed (see Table 6.2B). GRX480 basal expression peaks during *Arabidopsis* development occur during stages 1.03-1.05 (16-18 days old) when there are 3-5 rosette leaves; and stage 3.7 (26-28 days old) when the rosette leaves are about 70% of their final size. The amounts accumulating in the later are higher and might explain the reason why *as-1::GUS* plants which are 4 weeks old and above are unresponsive to auxin induced expression of the GUS transcript (results not shown), since high amounts of GRX480 repress *as-1* mediated expression in *as-1::GUS* plants. This is nevertheless only speculative.

Table 6.2B. Differential expression of GRX480 and related glutaredoxins during development.

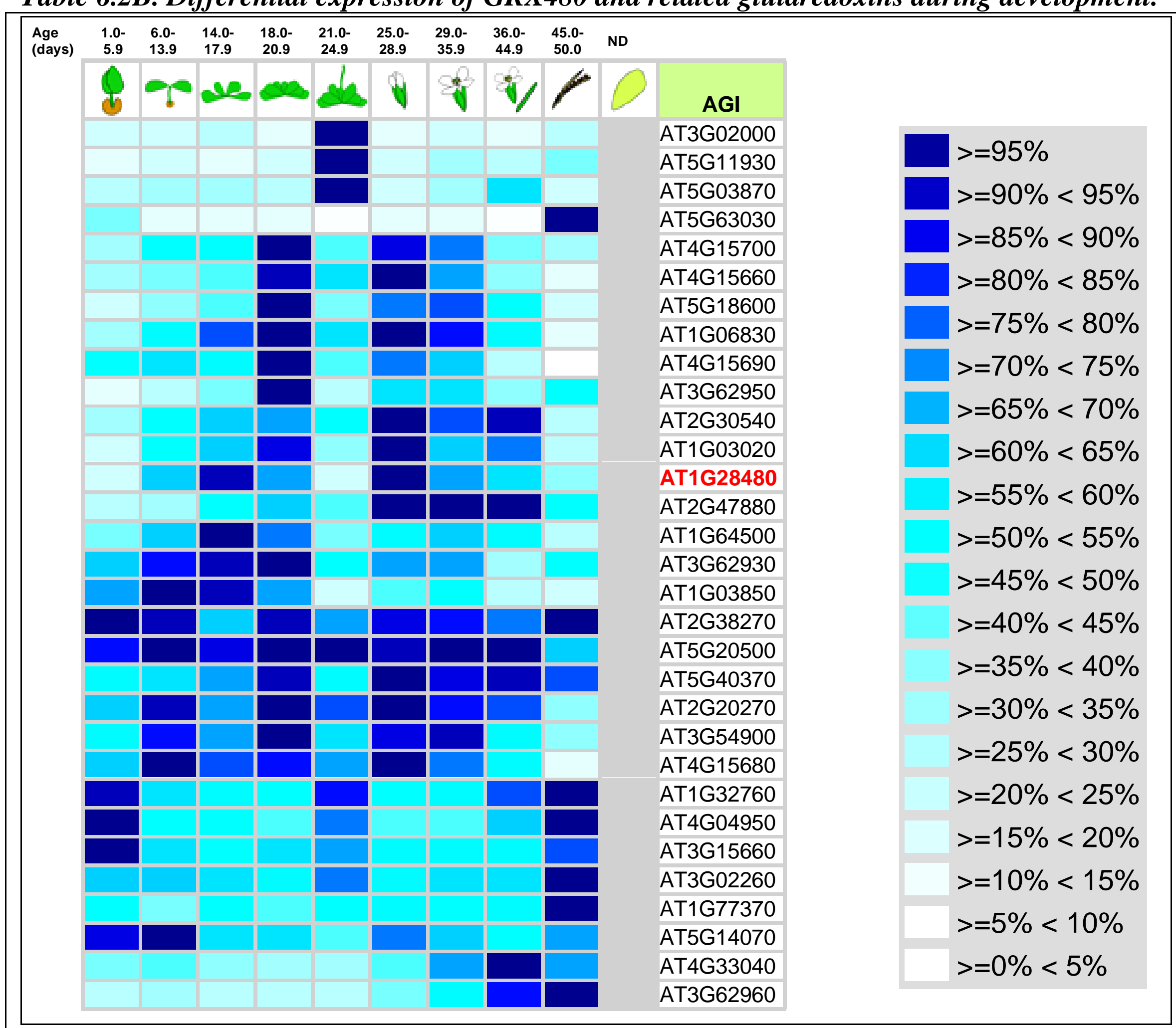


Table shows the regulation of different genes (In rows with their AGI codes indicated on the last column to the right) during different developmental stages (columns, with age in days indicated on the first row on top). GRX480 is indicated in red.

Color Key(Right): For the blue-white scheme, all gene-level profiles were normalized for coloring such that for each gene the highest signal intensity obtains value 100% (dark blue) and absence of signal obtains value 0 % (white).

Even though the glutaredoxins are regulated in expression during diverse developmental stages, a few cluster during specific developmental stages. At3g02000 (ROXY1), known for regulating flower development clusters along with others around days 21-25 of development.

6.2.3 Tissue specific expression of GRX480 and related glutaredoxins

AGI codes for genes producing significant alignment to At1g28480 were input into the Meta-Analyzer as in section 6.2.1 above. The expression of GRX480 as well as other glutaredoxins in different tissue types is indicated on Table 6.2C below.

Table 6.2C. Differential expression of GRX480 and related glutaredoxins in different plant tissues.

0 callus		2 seedling	21 cotyledons	22 hypocotyl	23 radicle	3 inflorescence	31 flower	311 carpel	3111 ovary	3112 stigma	312 petal	313 sepal	314 stamen	3141 pollen	315 pedicel	32 silique	33 seed	34 stem	35 node	36 shoot apex	37 cauline leaf	4 rosette		42 adult leaf	43 petiole		5 roots	52 lateral root		AGI CODE
																														AT3G15660
																														AT4G04950
																														AT1G32760
																														AT3G02260
																														AT1G77370
																														AT5G40370
																														AT5G20500
																														AT2G20270
																														AT3G54900
																														AT4G33040
																														AT5G63030
																														AT2G47880
																														AT1G64500
																														AT3G62960
																														AT5G18600
																														AT4G15690
																														AT4G15680
																														AT4G15700
																														AT1G06830
																														AT3G62950
																														AT1G03020
																														AT4G15660
																														AT3G62930
																														AT5G14070
																														AT2G30540
																														AT2G38270
																														AT3G02000
																														AT5G03870
																														AT5G11930
																														AT1G03850
																														AT1G28480

The table above shows the regulation of different genes (In rows with their AGI codes indicated on the last column to the right) in different plant tissue types (columns, with tissue type indicated on the first row on top). GRX480 is indicated in red.

Key: For the blue-white scheme, all gene-level profiles were normalized for coloring such that for each gene the highest signal intensity obtains value 100% (dark blue) and absence of signal obtains value 0 % (white). (See Key of Table 6.2B above).

The highest expression levels of GRX480 are found in the sepals and in the ridicule. These are stronger, compared to expression levels in the roots and adult leaves. This is somewhat different from the expression pattern of a classic glutaredoxin like At5g20455 which is widely and highly expressed in most tissues.

6.3 Interaction assays of glutaredoxins with TGA2 in yeast

In order to map out the interaction domains of the GRX480 protein, DNA coding for different mutant versions of the wild type protein were generated using PCR specific primers and co-expressed in the yeast strain MAV203, with the transcription factor TGA2. The yeast MAV203 strain contained the ß-galactosidase gene reporter gene, downstream of a Gal4 promoter.

The glutaredoxin mutant versions included the double mutant and a version containing a deletion of the first 30 amino acids in the N-terminus (see Figure 6.1E). As a control, GRX370 was also co-expressed with TGA2 in the same yeast system. The glutaredoxins were expressed as fusion proteins to the yeast Gal4-DNA binding domain and a myc tag, while the transcription factor TGA2 was expressed as a fusion protein to the yeast Gal4-activation domain, and an HA tag. Control transformations were performed using plasmids lacking either TGA2 or glutaredoxin or both (Figure 6.3AI). After transformation, the yeasts were grown on selective medium lacking leucine and tryptophan. Individual colonies were analyzed for ß-galactosidase activity and protein expression respectively (Figure 6.3AII).

The double mutant version of GRX480 (GRX-DM) still interacted with TGA2. Of all the clones tested in 5 independent experiments (3-5 clones analyzed each time), they showed significant ONPG activity above the controls where there was no interaction occurring. When compared with the wild type protein, only in about 32% of the clones was interaction with TGA2 less than in the wild type. In most cases, the strength of the interaction was within the range of the double mutant.

The deletion of the 30 N-terminal amino acids of GRX480 did not result in a loss in its ability to interact with TGA2. Interestingly, of the 17 clones tested, about 52% showed significantly stronger interaction than the double mutant protein, while in the rest, the interaction was within the range of the double mutant. In 3 independent experiments, they showed average ONPG activity higher than in the wild type.

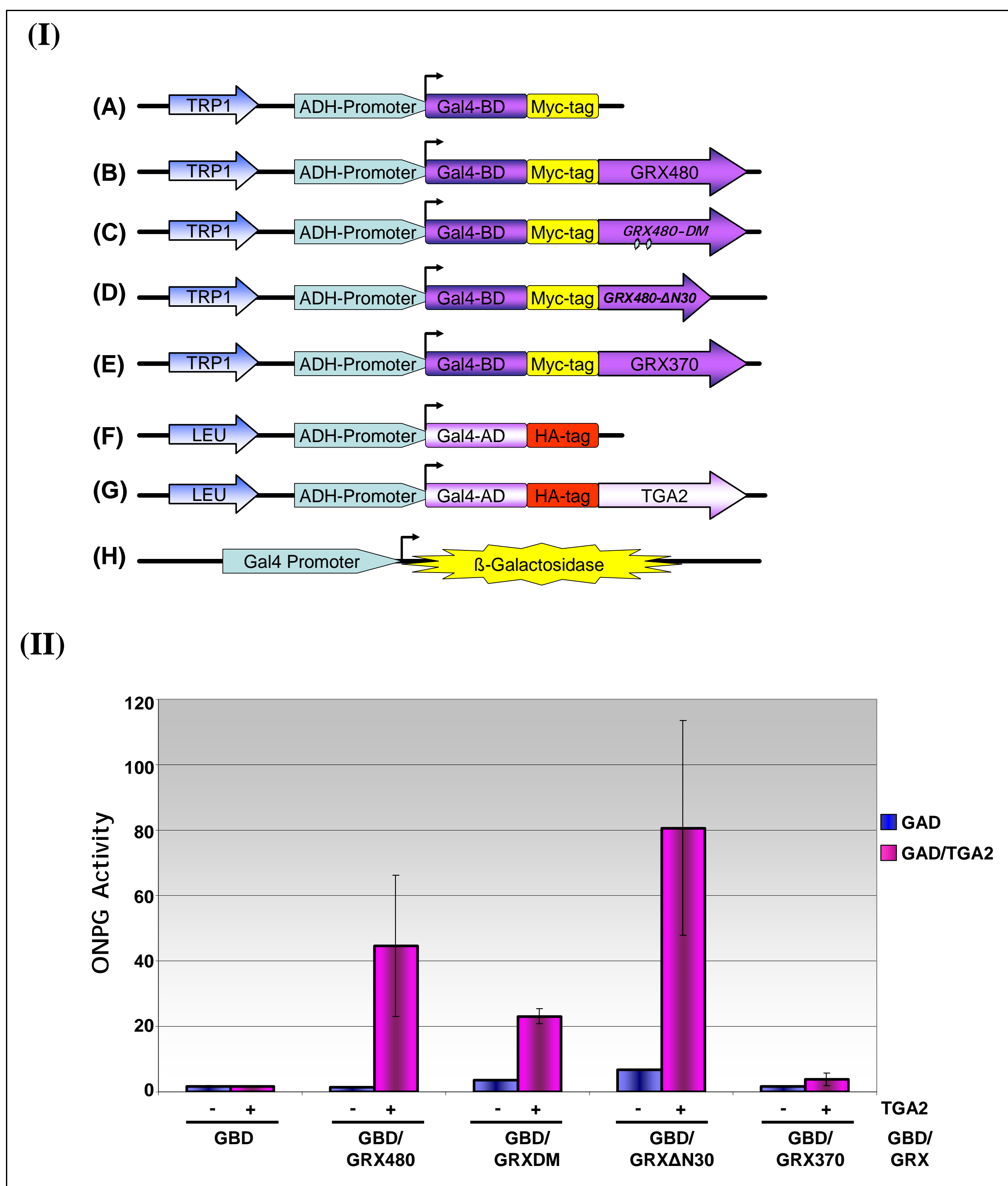


Figure 6.3A: ONPG Assay for interaction between TGA2 and glutaredoxins.
I: *Plasmid constructs used. Plasmids A (control lacking any glutaredoxin), B (with WT At1g28480 as GRX480), C (with double cysteine mutant of At1g28480 as GRX480-DM), D (with N-terminal deletion mutant of At1g28480 as GRXΔN30) and E (with GRX At5g40370 as GRX-370) contain the tryptophan selectable marker for yeast selection. The ADH promoter drives the expression of a fusion protein with the and N-terminal yeast Gal4-binding domain (Gal4BD) and myc tag. The plasmid constructs F (control lacking TGA2) and E (with TGA2) contain the Leucine selectable marker for yeast selection. The ADH promoter drives the expression of a fusion protein with the and N-terminal yeast Gal4-activating domain (Gal4AD) and HA tag. H, is the Gal4 promoter reporter construct present in the yeast MAV203 strain.*
(II): *ONPG Assay, showing average activity from clones measured. Yeast cells were co-transformed with the different GRX types or their control plasmid (GBD) together with TGA2 or the control plasmid (GAD) indicated as +/-. Individual clones showed similar behavior as above when the ONPG assay was repeated.*

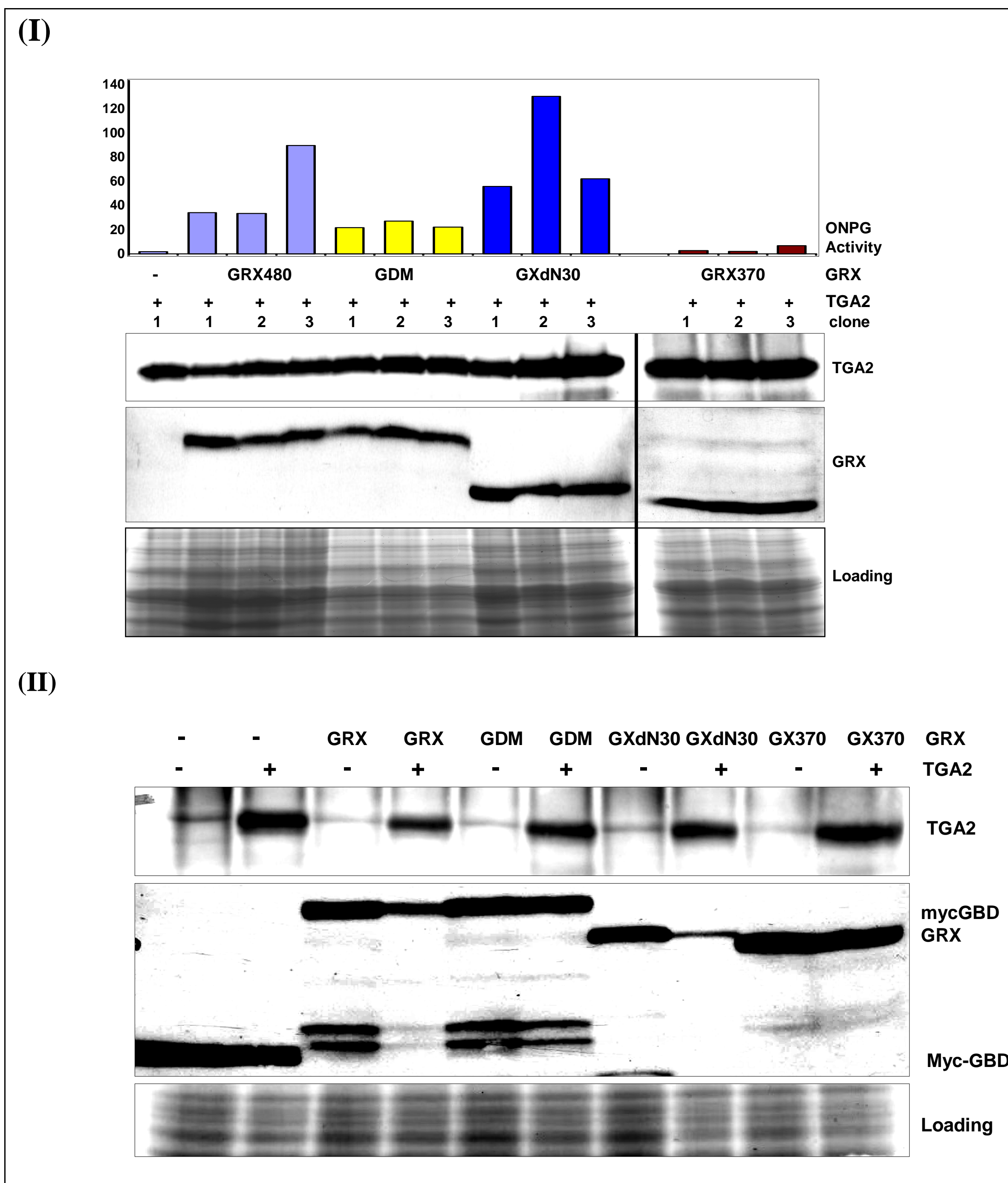


Figure 6.3B: Expression of TGA and interacting glutaredoxin types in yeast.

The TGA2 was detected using antiserum raised against the C-terminal part of TGA2. GRX was detected using monoclonal antibody against the myc-tag.

(I): *Protein extracts were prepared from individual clones from the experiment in Figure 6.3A(II) above which were used before for the ONPG assay. In the first lane, is the protein extract from a colony expressing TGA alone. Three colonies were analyzed in each case. Clone #3 containing WT At1g28480 (GRX480), and clone #2 containing the N-terminal deletion mutant (GXdN30) had two-fold stronger signals in the ONPG interaction assay.*

(II): *An independent experiment showing the expression of TGA2 and all the glutaredoxin types when co-transformed into yeast cells. In the control experiments, where one of the interacting proteins is absent, the TGA2 or GRX is also detected.*

The wild type and N-terminal deletion mutant of At1g28480 showed more variation in their interaction with TGA2 (ONPG activity) than the GRX double mutant.
The question arose as to whether the variation of ONPG activity is proportional to the levels of expression of any of the interacting proteins. To answer this question, protein extracts from three individual clones with varying ONPG activity of the wild type, double mutant and N-terminal deletion mutant were separated on SDS PAGE and analyzed for their expression of GRX or TGA2 (Figure 6.3BI). Clone #3 of the wild type GRX480 and clone #2 of the N terminal deletion mutant which showed the strongest ONPG activity of up to 2 fold higher than the other two clones nevertheless expressed both GRX and TGA2 within the same range (Figure 6.3BI).

To investigate whether the redox active state of the wild type has any influence in its ability to interact with TGA2, yeast cells were subjected to oxidative stress in the presence of 1mM diamide for 5 hours. They nevertheless did not show a difference in their ONPG activity under these conditions (results not shown). It could very well be that an already oxidized state within the cells may have made no difference after diamide treatment, in terms of the activity of GRX480

.

There appears therefore to have been two populations of cells, with some showing interaction stronger than others, but it is not clear why they showed this behavior. Even though the wild type and N-terminal deletion mutant clones also had variable sizes on selective plates, it did not seem to correlate with their ONPG activity. The clones of all the other transformations had regular and comparable sizes.

The classical glutaredoxin GRX370 did not interact at all with the transcription factor TGA2. The ONPG activity was in the same range as all the negative controls (Figure 6.3AII).
All the glutaredoxin types tested in all the experiments and in controls were expressed in the yeast cells. The TGA2 was also expressed. This was the case in either the presence or absence of GRX or TGA (Figure 6.3BII).

6.4 Transient assays in BY2 protoplast cells

6.4.1 The *in vivo* interaction between TGA2.2 and GRX480 in protoplasts

A modified two hybrid interaction assay was carried out in BY2 protoplasts, to determine the levels of interaction between TGA2.2 and GRX or GDM. The TGA2.2 (a homologue of TGA2 from *N. tabacum*) was expressed as a fusion protein with the *Herpes simplex* virus protein derived VP16 activation domain, while both GRX and GRX-DM were expressed as fusion proteins to the yeast derived Gal4 binding domain (Gal4BD). The expression of the effector proteins was driven by the HBT promoter. These interacting partners were co-transformed with a promoter-reporter construct containing the *uidA* gene (ß-glucoronidase or GUS) driven by a 4x-Gal4 promoter derived from the yeast system. In a control transformation, only the GRX/Gal4BD and reporter constructs were transformed into BY2 protoplasts. The strength of the interaction could be measured quantitatively by the GUS assay. (Figure 6.4A)

On the average, the reporter assay measurements showed that GRX and TGA2.2 interact, with the GUS activity being 5 times more than the background due to GRX alone. GRX-DM on the other hand showed about a 2-fold weaker interaction with TGA2.2 compared to the wild type GRX. This result was repeated at least twice. In other control assays, TGA2.2VP/reporter alone, Gal4BD/reporter alone and G4BD-GRX/reporter alone showed only background activity, compared with the activity due to the interaction between GRX and TGA2.2 in the same assay (Abdallat, 2004). These results gave a hint that the active site cysteine residues of GRX may be important in their ability to interact with TGA transcription factors.

These results nevertheless assume that the levels of expression of both GRX and GRX-DM proteins are the same in the protoplast cells. This could nevertheless not be verified here, since there was no available antibody against the GRX.

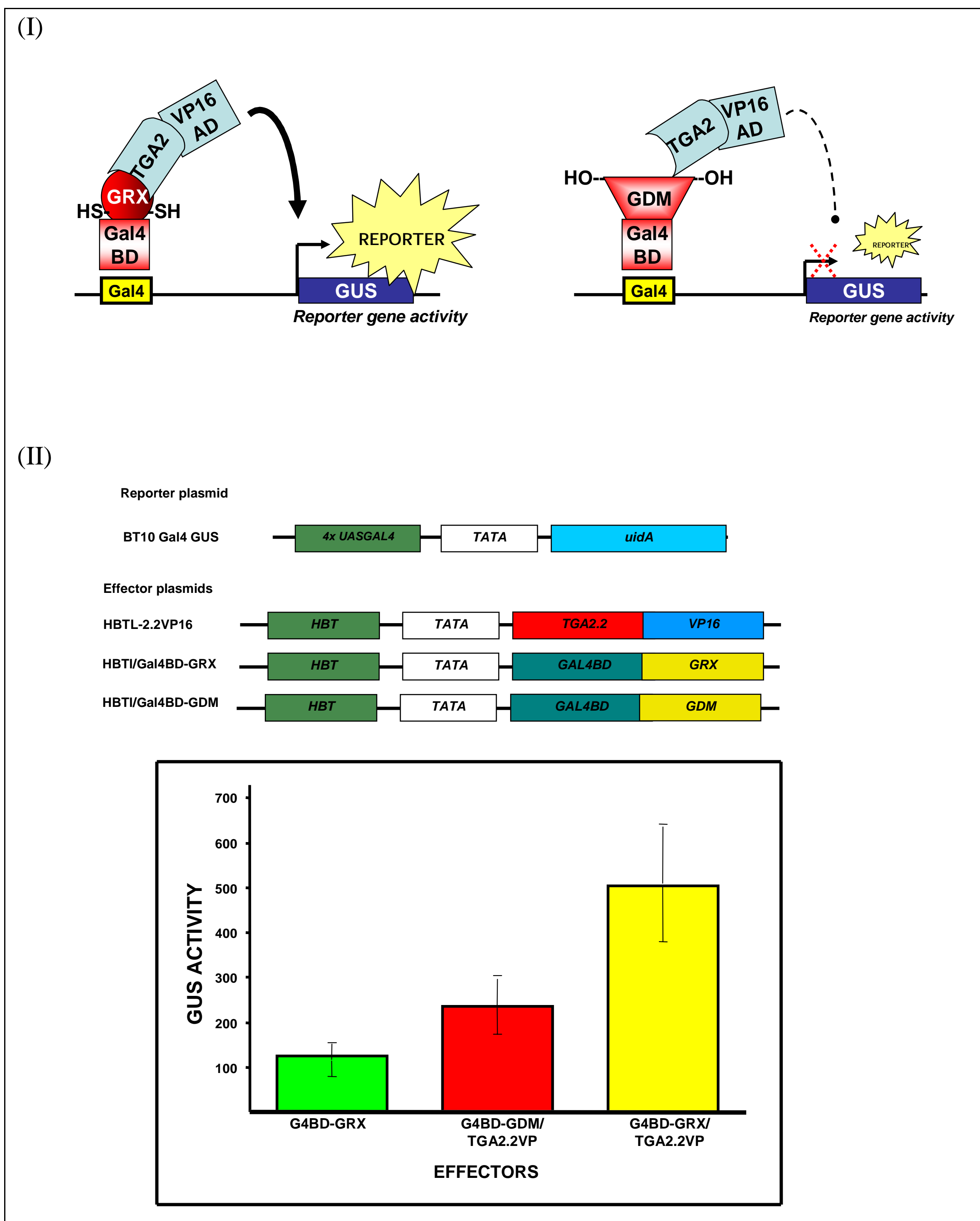


Figure 6.4A. Interaction between TGA2.2 and GRX480 in protoplasts.
(I)An illustration of the two hybrid assay in protoplasts, where interaction between VP16AD-TGA2.2 and Gal4BD-GRX, is a prerequisite for transactivation, and GUS reporter gene expression in this assay.
(II)Plasmid constructs (above) and GUS activity (below) as a result of the interaction between the effectors. 10 µg of BT10 Gal4 GUS reporter gene plasmid were co-transfected with a total of 25 µg of effector plasmids (i.e. 12.5µg HBTL/GAL4BD-At1g28480 + 12.5 µg HBT-TGA2.2VP16 or 12.5µg HBTL/GAL4BD-GDM + 12.5 µg HBT-TGA2.2VP16 effector plasmids) into BY-2 protoplasts. After an overnight expression, the cells were assayed for ß-glucuronidase enzyme activity measured in [pmole/min/µg protein]. The standard deviation in each case is a result of three independent transformations.

6.4.2 GRX480 suppresses transcription from promoters containing the *as-1* element in protoplasts

The effect of GRX and GRX-DM (GDM) *on as-1::GUS* inducible gene expression was also investigated in BY-2 protoplasts. The effector proteins GRX and GRX-DM were expressed in protoplasts, driven upstream by the HBT chimeric promoter. These were co-transformed with a promoter-GUS construct, which contains the *as-1* element as the only regulatory element. A control plasmid containing only the *HBTL* promoter was also co-transformed with the *as-1::GUS* reporter construct. The relative GUS activity was measured after expression.

In every case observed over three times, and with independent plasmid preparations (also reported by Abdallat, 2004), the *as-1::GUS* reporter activity was always repressed in the presence of GRX. This was nevertheless not the case in the presence of GRX-DM, where the *as-1::GUS* reporter activity remained normal. (Figure 6.4B).

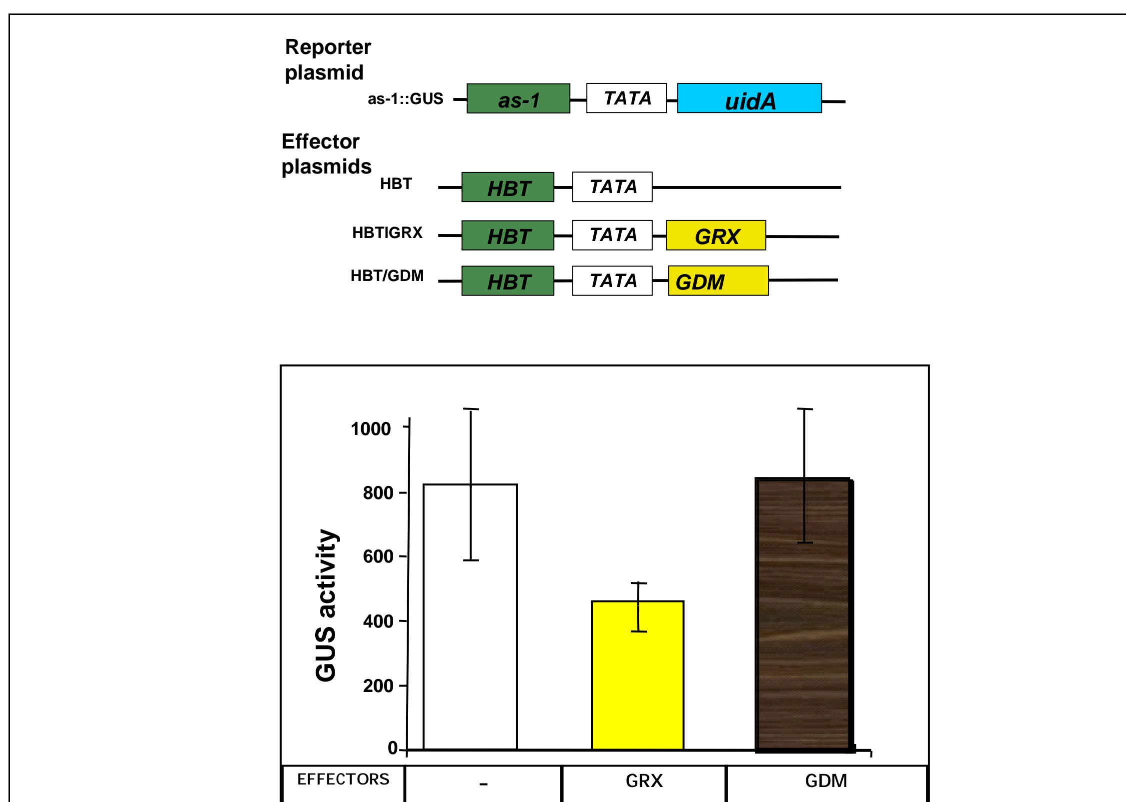


Figure 6.4B. GRX480 but not the double mutant represses the activity of the as-1 element in protoplasts. *Plasmid constructs are shown (above) and GUS reporter activity (below) as a result of the interaction between the effectors.*
10 µg of as-1::GUS reporter plasmid were co-transfected with either 25 µg HBTL, 25 µg HBTL/GRX480 or 25 µg HBTL/GDM effector plasmids into BY-2 protoplasts. After overnight expression, the cells were assayed for β-glucuronidase enzyme activity measured in [pmole/min/µg protein].
The standard deviation in each case is a result from 3 independent transformations.

The effect observed on the *as-1::GUS* reporter activity was further investigated to find out how specific it was. Was it a general effect which GRX480 had on all promoters or was this a specific effect on a promoter containing the *as-1* element as the only regulatory element. For this purpose, a control experiment was carried out using a construct containing the yeast *Gal4* promoter, driving a downstream GUS reporter. These were co-transformed into BY-2 protoplasts, with constructs which lack or express the GRX, driven by the *HBT* promoter. The *Gal4::GUS* promoter reporter activity remained unchanged in the presence of GRX (Figure 6.4C), further solidifying the observation that the GRX protein represses only *as-1::GUS* mediated expression in protoplast.

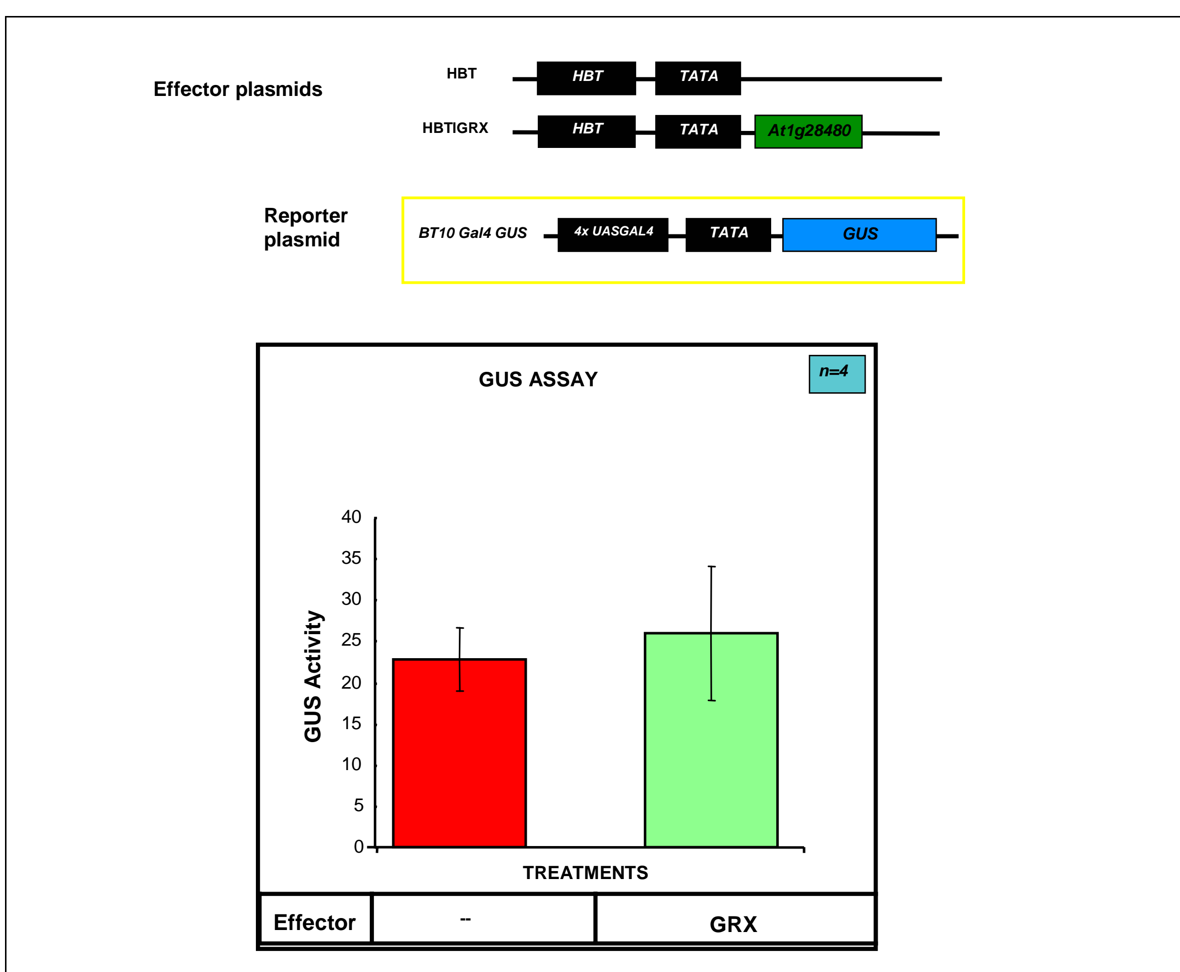


Figure 6.4C. GRX480 does not repress promoters that lack the as-1 element.
Plasmid constructs are shown (above) and GUS reporter activity (below) as a result of the interaction between the expressed GRX480 effector and the promoter reporter construct, after co-transformation into BY2protoplast cells.
10 µg of BT10 Gal4 GUS reporter gene plasmid were co-transfected with 25 µg HBTL or 25 µg HBTL/GRX480 effector plasmids into BY-2 protoplasts. After an overnight expression, the cells were assayed for ß-glucuronidase enzyme activity measured in [pmole/min/µg protein]. The standard deviation in each case is a result of four independent transformations.

6.5 Analysis of Stably Expressing GRX480 lines *in planta*

6.5.1 GRX480 suppresses *as-1* mediated transcription in *A. thaliana*

In order to verify if the effect of GRX observed in protoplasts was also functional in plant systems, the GRX480 was stably expressed in a background of *A. thaliana* plants, containing the *as-1::GUS* transgene (Abdallat, 2004). The *as-1* element is inducible by several stresses including SA, 2,4D, and oxidative stress. The reporter gene activity was measured by both observable histochemical staining and quantitative GUS assay measurements.

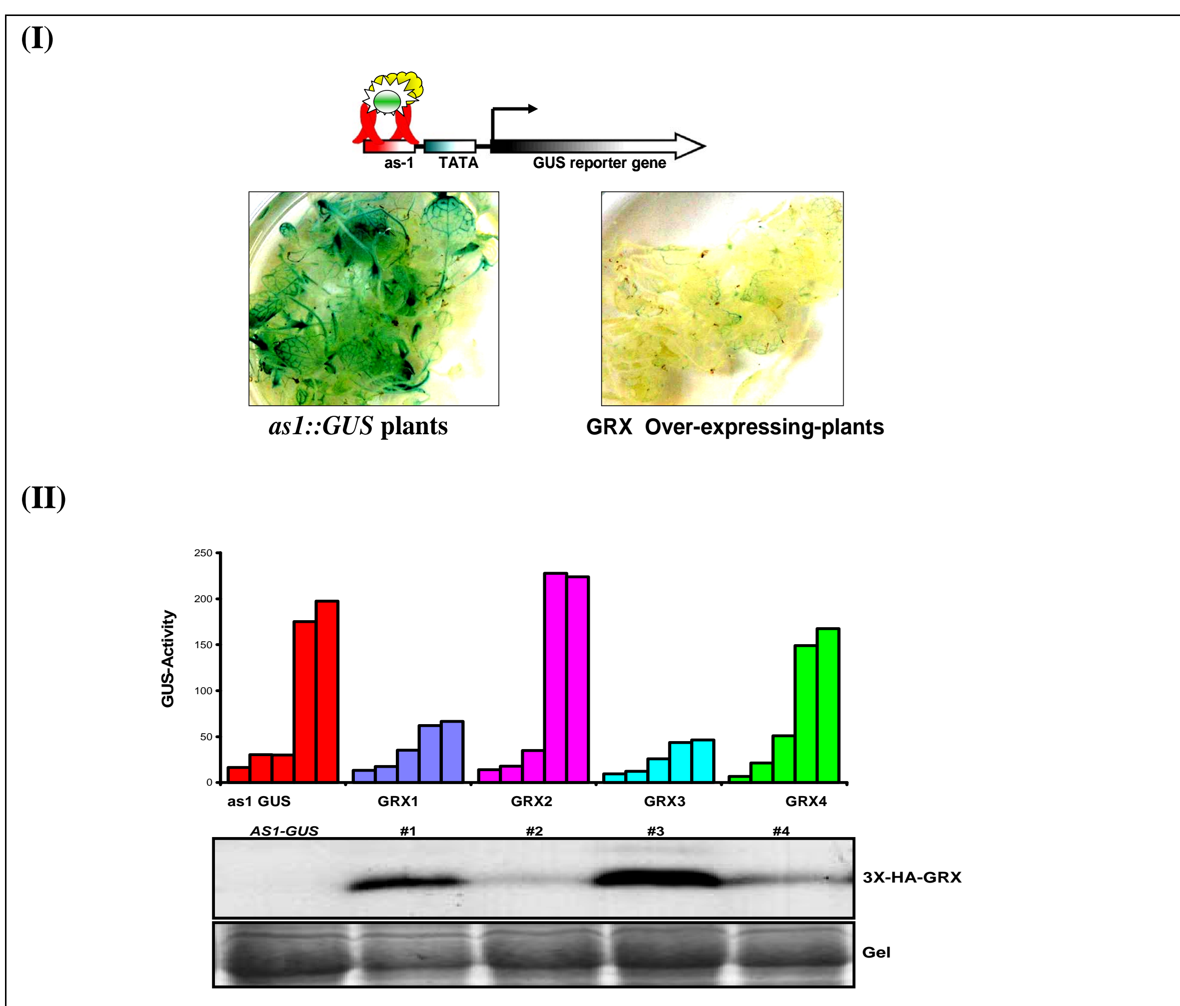


Figure 6.5A. GRX480 represses the activity of as-1 mediated transcription in vivo.
Two week old soil grown WT A. thaliana Col-O plants, containing the as-1 element upstream of a GUS reporter (I), and lines stably expressing GRX480 in the same the same background were induced by floating in phosophate buffer containing 100 µM 2,4 D (auxin). Samples were collected at 0, 2, 4, 8 and 12 hours. ***β-glucuronidase enzyme activity*** *was quantified by histochemical GUS staining of a 12hr sample (I) and GUS assay (II above). Some sample was used for RNA preparation. High protein expression of GRX480 (detected by anti HA antibody in II below, by Abdallat, 2004) correlated with significant repression of GUS activity, also seen in the northern blot (Manuscript, figure 3A).*

After induction with 2,4D for 12 hours, plants that strongly and stably over-expressed the GRX480 protein had significantly reduced GUS reporter activity than control plants which had detectibly significant amounts of GUS activity (Fig. 6.5A). This correlated with the amounts of GRX480 and GUS transcripts accumulating (manuscript, Fig 3A).

6.5.2 Another glutaredoxin, GRX370 does not suppress *as-1* mediated transcription in plants.

In order to determine whether the effect of the repression on the *as-1* element containing promoter was due to a general activity of glutaredoxins or specific for GRX480, it was necessary to also stably express a classical glutaredoxin in *A. thaliana*. The GRX At5g40370 coding sequence was therefore expressed in the same background of plants (*as-1::GUS*) driven by the 35S CaMV promoter as an N-terminal 3X-HA-tagged fusion protein. The plasmid construct contained a GFP construct expressed under the control of an At2S3 seed specific promoter, as a marker for selecting transgenic seeds (Figure 6.5B)

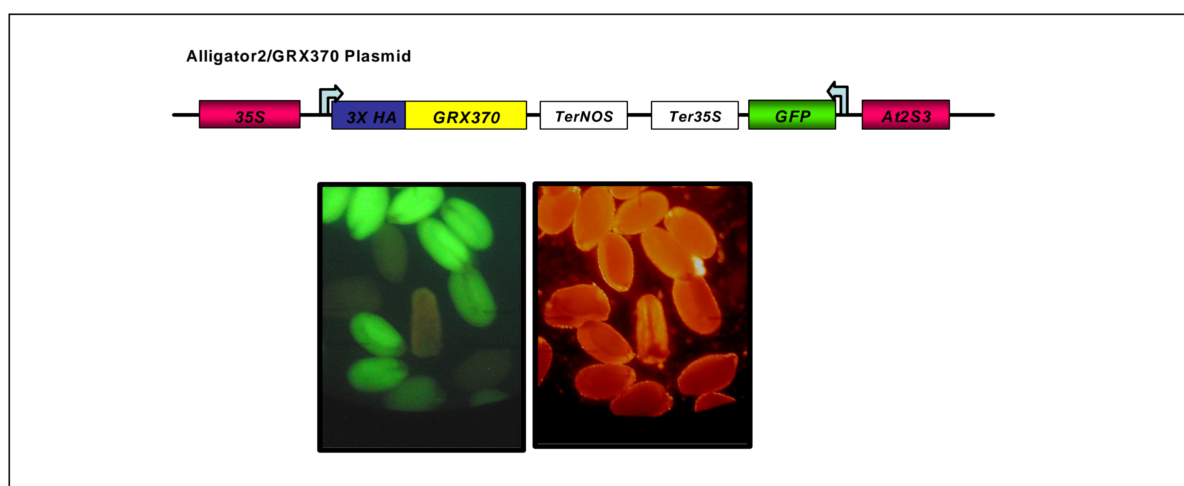


Figure 6.5B. Selection of transgenic seeds, expressing A5g40370.
A pAlligator construct (above) was transformed into wildtype as-1::GUS plants using the agro-bacterial mediated method. The seeds containing the construct were selected under blue light, and could be distinguished from those that did not glow (picture on the left). The control seen under white light is shown on the right.

F2 progeny of 6 different transgenic lines were induced with 2,4D and the GUS activity for the plants measured over a time course. GRX370 lines showed GUS activity at levels comparable to that induced in control plants (Figure 6.5C-I). This was confirmed by western blotting experiments using the HA antibody, that is able to detect high levels of expression of the glutaredoxin proteins in the over-expressing lines (Figure 6.5C-II).

GRX370 therefore does not reduce *as1-GUS* mediated transcription. The effect therefore observed due to GRX480 over-expression is not a general effect of glutaredoxins but is a specific effect due to GRX480 protein accumulation.

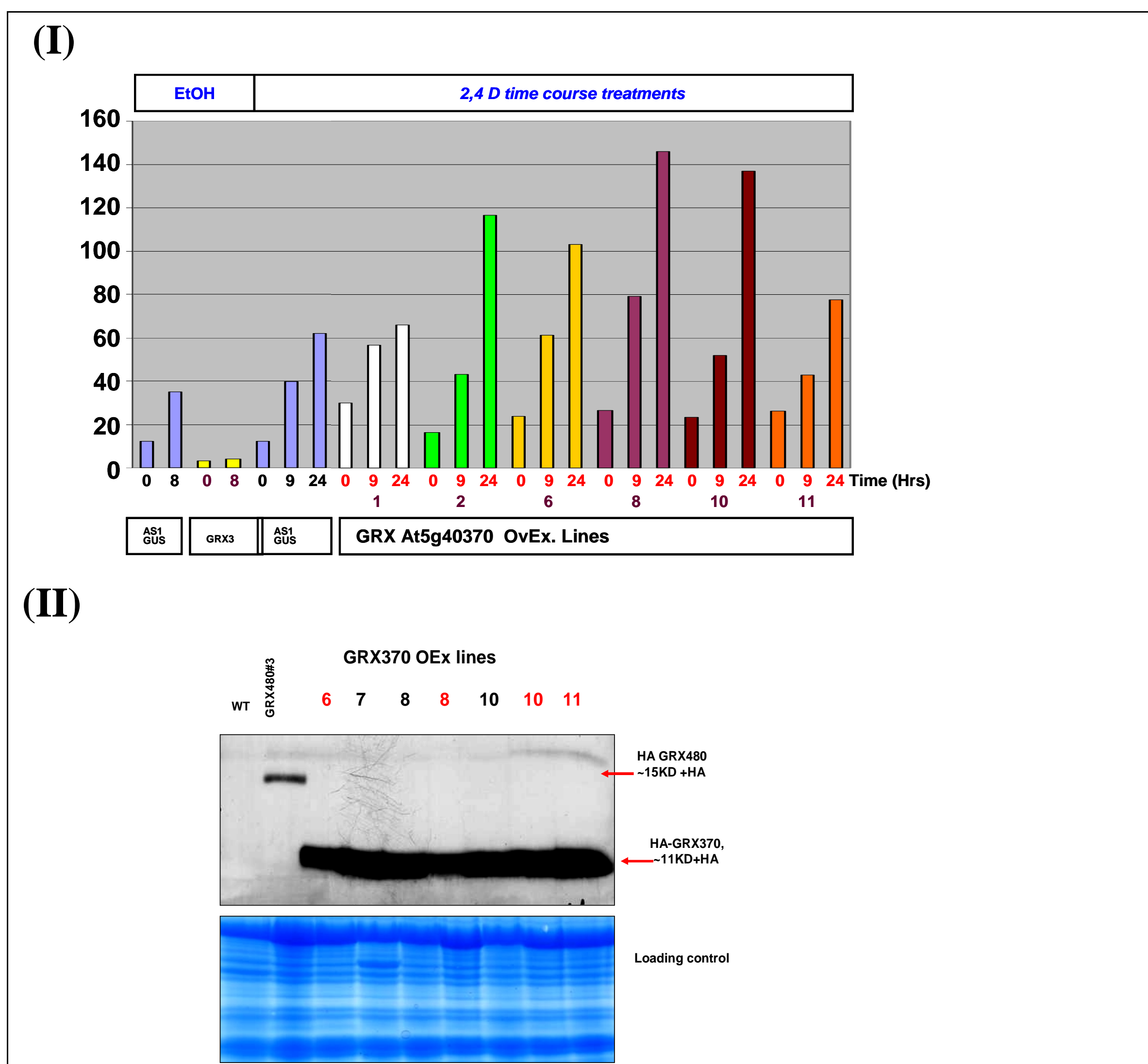


Figure 6.5C. Auxin induced as-1 element mediated GUS expression is not repressed in stably expressing GRX370 lines.

(I)Two weeks old Arabidopsis plants grown on soil were treated with 100µM 2,4 D (auxin) and samples of 5 plants per time point were collected over time points 0hr, 9hrs, and 24hrs. Crude native protein extracts were prepared from these plants and the β-glucuronidase (GUS) enzyme activity of the overexpressing lines #1, #2, #6, #8, #10 and #11 were compared with the background as1::GUS line. (Above) Background induction by ethanol is also compared between as1::GUS and GRX480 overexpressing line (GRX3) at 8Hrs.

(II) Samples of the same uninduced plant lines used in experiment (I) i.e. #6, #8, #10 and #11 shown in red; as well as samples from other F2 progeny (shown in black) were used to prepare denaturing protein extracts. Equivalent amounts were loaded on an SDS PA-gel and blotted unto nitrocellulose membrane. The HA-tagged glutaredoxins were detected using the anti-HA monoclonal antibody in the overexpressing lines but not in the as-1::GUS wild-type control line.

Since GRX370 does not interact with TGA2 transcription factor (see manuscript Figure 2 and Figure 6.3A), this would likely be a specific effect mediated by its interaction with TGA transcription factors, that bind to the *as-1* element.

6.5.3 Effect of GRX480 over-expression on *Arabidopsis* defense genes.

The accumulation of the anti-fungal protein PDF1.2, which is under normal circumstances induced by jasmonic acid, was compromised in the presence of constitutively high levels of GRX480 (Figure 4A, manuscript) which is also inducible by SA (Figure 1B of manuscript). In order to find out the array of possible transcription factors whose expression may be regulated by Atg28480 and possibly influence the expression of PDF1.2 and other target genes, a macro-array experiment was carried out on a REGIA array, on which different transcription factors which regulate diverse stress responses were spotted.

The RNA used as a probe was taken from an experiment where wild-type control plants and plants stably expressing the GRX480 gene under the influence of the 35SCaMV promoter were treated under the same conditions with 20µM meJA. RNA from the sample time point 4 hours post induction was used for the array experiment (see manuscript, Figure 4A).

The candidate genes down regulated by GRX480 over-expression among the confirmed plant defensin protein PDF1.2a, included a Transcription factor II homolog, a bHLH protein, and a bZIP protein BZ02H2. Though these were not as significantly reduced on the stress array as PDF1.2, their relevance still has to be investigated by northern blotting.

The candidate genes upregulated by GRX480 over-expression included a salt-tolerance zinc finger protein, a CONSTANS B-box zinc finger family protein, a jasmonic acid regulatory protein, a disease resistance protein EDS1, a flavanone 3-hydroxylase (FH3), PAD4, a NAM-like Protein (At4g27410), two WRKY family transcription factors, a heat shock transcription factor HSF4, zinc finger protein Zat12 and a MYB96TF-like protein. (See Table 6.5A).

Their relevance also has to be further investigated by northern blotting.

The GENVESTIGATOR toolkit (Zimmermann *et al.*, 2004) was nevertheless used to compare the expression pattern of At1g2840 and the genes differentially regulated as observed in the array. Expression patterns during different conditions of biotic/abiotic

stress as well as tissue specific expression patterns from results of different microarray experiments were used for comparison. The results are illustrated on Tables 6.5B-E.

Table 6.5A. Genes which are differentially regulated by over expression of GRX480 in REGIA macro array of stress related transcription factors.

Genes Downregulated by overexpressing GRX480	
AGI Code	**Annotation/Description**
At4g31720	Transcription initiation factor IID protein
At5g24800	bZIP transcription factor family protein
At5g44420	Plant defensin protein, PDF1.2a, Encodes an ethylene- and jasmonate-responsive plant defensin. mRNA levels are not responsive to salicylic acid treatment.
At5g65640	Basic helix-loop-helix (bHLH) family protein

Genes Upregulated by overexpressing GRX480	
AGI Code	**Annotation/Description**
At1g27730	Salt tolerance zinc finger (C2H2-type) protein (ZAT10) responsive to chitin oligomers.
At1g62300	Wrky6 transcription factor
At1g78600	Zinc finger (B-box type) family protein
At3g15500	Jasmonic acid regulatory protein
At3g48090	Disease resistance protein EDS1 Component of R gene-mediated disease resistance in *Arabidopsis thaliana* with homology to eukaryotic lipases.
At3g51240	Flavanone 3-hydroxylase (FH3)/ Naringenin 3-dioxygenase Encodes flavanone 3-hydroxylase that is coordinately expressed with chalcone synthase and chalcone isomerases. Regulates flavonoid biosynthesis.
At3g52430	Phytoalexin-deficient 4 protein (PAD4), Encodes a lipase-like gene that is important for salicylic acid signaling and function in resistance (R) gene-mediated and basal plant disease resistance. PAD4 can interact directly with EDS1, another disease resistance signaling protein. Expressed at elevated level in response to green peach aphid (GPA) feeding, and modulates the GPA feeding-induced leaf senescence through a mechanism that doesn't require camalexin synthesis and salicylic acid (SA) signaling.
At4g27410	No apical meristem (NAM) family protein (RD26)
At4g31800	WRKY family transcription factor (WRKY18)
At4g36990	Heat shock transcription factor HSF4 Encodes a protein whose sequence is similar to heat shock factors that regulate the expression of heat shock proteins. Transcript level is increased in response to heat shock. However, over-expression of this gene did not result in the increase or decrease of heat shock proteins.
At5g59820	Zinc finger (C2H2 type) family protein (ZAT12) Encodes a zinc finger protein involved in high light and cold acclimation
At5g62470	myb family transcription factor (MYB96)

Table 6.5B Differential expression of genes down-regulated by the stable expression of GRX480, after 20uM JA induction: How they are normally regulated under biotic and abiotic stress induced conditions.

Treatment

Biotic: A. brassicicola (+)	Biotic: B. cinerea (+)	Biotic: mycorrhiza (+)	Biotic: nematode (+)	Biotic: P. infestans (+)	Biotic: P. rapae (+)	Biotic: P. syringae (+)	Biotic: P. syringae (+)	Chemical: 2,4-dichlorophenoxyacetic acid (+)	Chemical: CO2 high	Chemical: cycloheximide (+)	Chemical: hydrogen peroxide (+)	Chemical: ozone (+)	Hormone: ABA (+)	Hormone: ACC (+)	Hormone: ethylene (+)	Hormone: GA3 (+)	Hormone: IAA (+)	Hormone: MJ (+)	Hormone: salicylic acid (+)	Stress: anoxia (+)	Stress: cold	Stress: drought	Stress: genotoxic	Stress: heat	Stress: hypoxia (+)	Stress: osmotic	Stress: oxidative	Stress: salt	Stress: UV-B	Stress: wounding	
																															AT4G31720
																															AT5G65640
																															AT5G24800
																															AT5G44420
																															AT1G28480

Table shows the regulation of different genes (In rows with their AGI codes indicated on the last column to the right) by different stress situations (columns, with the biotic and abiotic stress types indicated on the first row on top).
See color key below.

Table 6.5C. Differential expression of genes down-regulated by the stable expression of GRX480, after 20uM JA induction: How they are normally expressed in different tissues.

0 callus	[illegible]	2 seedling	21 cotyledons	22 hypocotyl	23 radicle	3 inflorescence	31 flower	311 carpel	3111 ovary	3112 stigma	312 petal	313 sepal	314 stamen	3141 pollen	315 pedicel	32 silique	33 seed	34 stem	35 node	36 shoot apex	37 cauline leaf	4 rosette	[illegible]	42 adult leaf	43 petiole	[illegible]	5 roots	52 lateral root	[illegible]	AGI/Links/Annotation
																														AT4G31720 (TFIID)
																														AT5G24800 bZIP transcription factor
																														AT5G65640 (bHLH)
																														AT1G28480]glutaredoxin
																														AT5G44420 (PDF1.2a)

Table shows the regulation of different genes (In rows with their AGI codes indicated on the last column to the right) in different plant tissue types (columns, with tissue type indicated on the first row on top).
See Color Key below.

Table 6.5D. Differential expression of genes up-regulated by the stable expression of GRX480, after 20uM JA induction: How they are normally regulated under biotic and abiotic stress inducible conditions.

Treatment																												
Biotic: nematode (+)	Biotic: P. infestans (+)	Biotic: P. rapae (+)	Biotic: P. syringae (+)	Biotic: P. syringae (+)	Chemical: 2,4-dichlorophenoxyacetic acid (+)	Chemical: CO2 high	Chemical: cycloheximide (+)	Chemical: hydrogen peroxide (+)	Chemical: ozone (+)	Hormone: ABA (+)	Hormone: ACC (+)	Hormone: ethylene (+)	Hormone: GA3 (+)	Hormone: IAA (+)	Hormone: MJ (+)	Hormone: salicylic acid (+)	Stress: anoxia (+)	Stress: cold	Stress: drought	Stress: genotoxic	Stress: heat	Stress: hypoxia (+)	Stress: osmotic	Stress: oxidative	Stress: salt	Stress: UV-B	Stress: wounding	
																												At1g27730
																												AT1G28480
																												AT5G59820
																												AT1G78600
																												AT5G62470
																												AT3G51240
																												AT3G48090
																												AT4G27410
																												AT4G31800
																												AT3G52430
																												AT3G15500
																												AT4G36990
																												AT1G62300

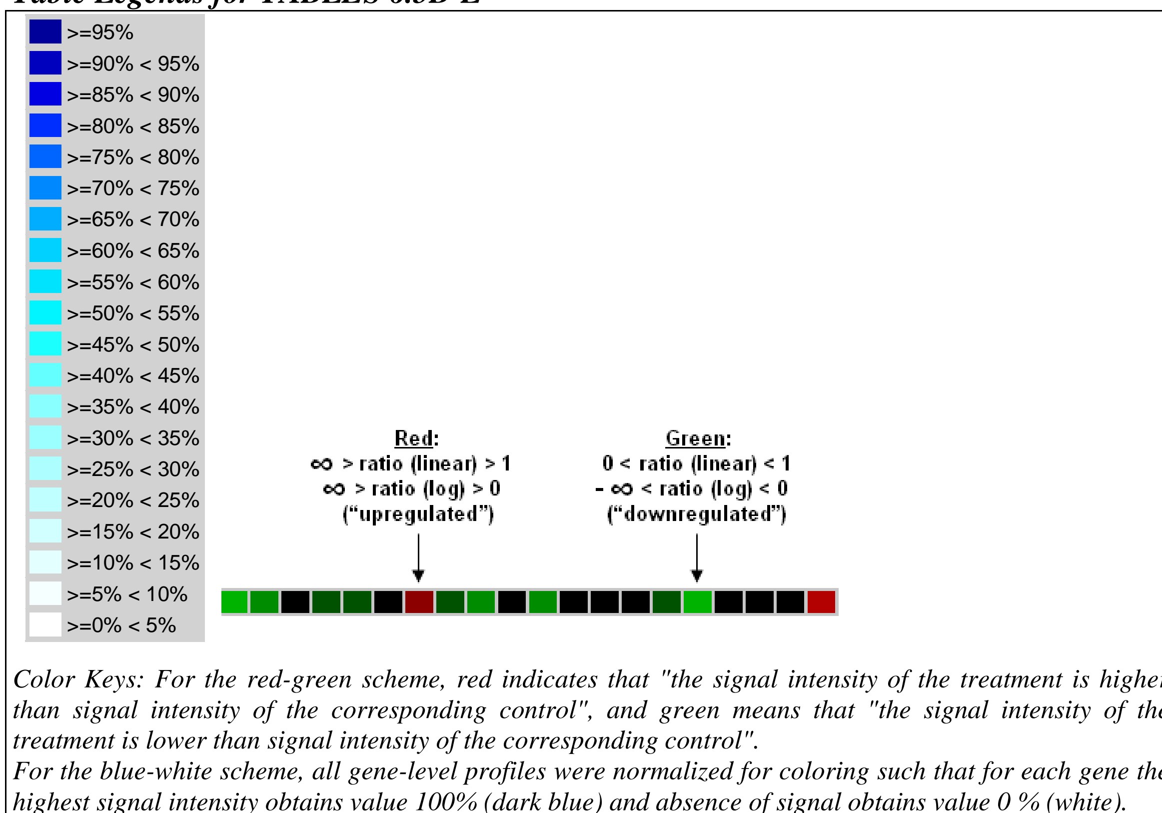
Table shows the regulation of different genes (In rows with their AGI codes indicated on the last column to the right) by different stress situations (columns, with the biotic and abiotic stress types indicated on the first row on top).
See color key below.

Table 6.5E. Differential expression of genes up-regulated by the stable expression of GRX480, after 20uM JA induction: How they are normally expressed in different tissues.

[illegible]	2 seedling	21 cotyledons	22 hypocotyl	23 radicle	3 inflorescence	31 flower	311 carpel	3111 ovary	3112 stigma	312 petal	313 sepal	314 stamen	3141 pollen	315 pedicel	32 silique	33 seed	34 stem	35 node	36 shoot apex	37 cauline leaf	4 rosette	[illegible]	42 adult leaf	43 petiole	[illegible]	5 roots	52 lateral root	[illegible]	
																													AT3G15500 (NAC3)
																													AT4G27410, (RD26)
																													AT5G59820, (ZAT12)
																													AT1G27730, (ZAT10)
																													AT4G31800, WRKY family transcription factor
																													AT1G28480, glutaredoxin family protein
																													AT1G62300, WRKY family transcription factor
																													AT4G36990, (HSF4)
																													AT3G48090, (EDS1)
																													AT3G52430, (PAD4)
																													AT1G78600, zinc finger (B-box type) family protein
																													AT3G51240, (F3H)/ N3D
																													AT5G62470, (MYB96)

Table shows the regulation of different genes (In rows with their AGI codes indicated on the last column to the right) in different plant tissue types (columns, with tissue type indicated on the first row on top).
See Color Key below.

Table Legends for TABLES 6.5B-E



Color Keys: For the red-green scheme, red indicates that "the signal intensity of the treatment is higher than signal intensity of the corresponding control", and green means that "the signal intensity of the treatment is lower than signal intensity of the corresponding control".
For the blue-white scheme, all gene-level profiles were normalized for coloring such that for each gene the highest signal intensity obtains value 100% (dark blue) and absence of signal obtains value 0 % (white).

6.5.4 Generation of lines stably over-expressing mutant derivatives of GRX480

Col-O *(as-1::GUS*) and *npr1-1* mutant plants were transformed with pAlligator constructs containing the following:

i. GRX480 without the 3X-HA tag fusion, i.e. the pAlligator construct was engineered to exclude the HA tag. It was annotated, GRXOhneHA.

ii. GRX480 lacking the 30 N-terminal amino acids, annotated GRXΔN30.

iii. GRX480 with a cysteine double mutant, annotated GRXDM.

The transgenic plants were selected for the GFP seed specific florescence marker. After growth for 4 weeks, leaves of plants from individual lines were cut out and induced with meJA for 6hours. The RNA transcripts of these plants were analyzed by northern blotting for both their accumulation of transgenic glutaredoxin transcript, and their transcriptional regulation of the JA inducible protein defensin PDF1.2.

The HA tag had not effect on the behavior of the glutaredoxin, in its ability to repress the accumulation of PDF1.2 transcript (Figure 6.5D, GXoHA line#2). This was also

observed in other lines and a similar behavior observed in the *npr1-1* mutant background (results not shown).

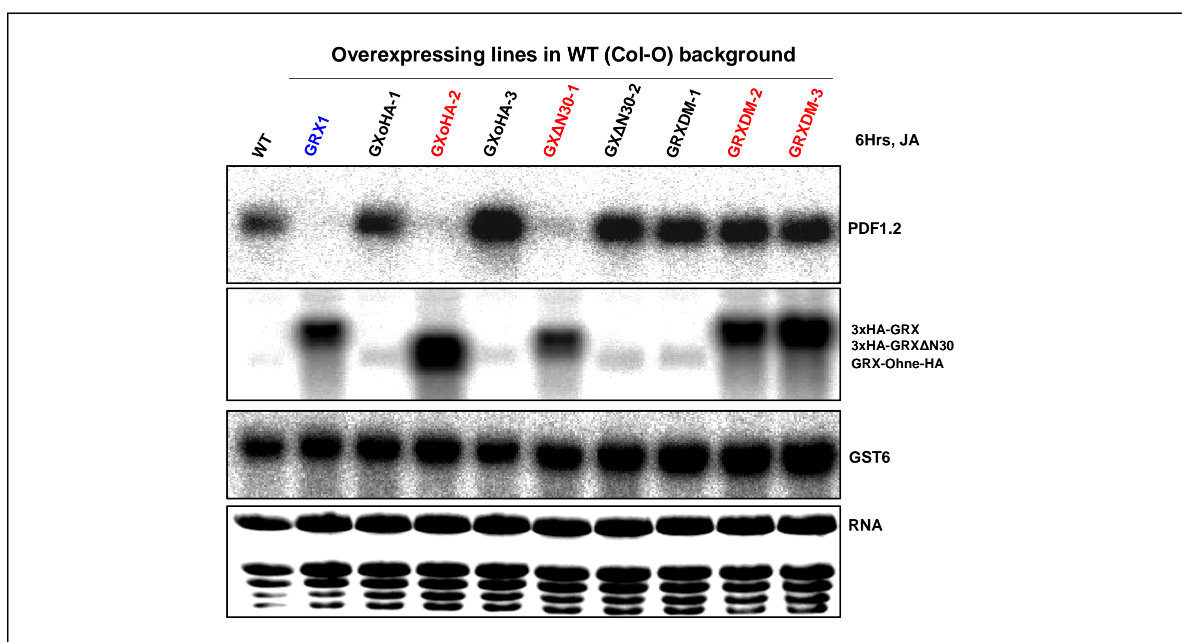


Figure 6.5D: Northern blot showing the effect of over-expressing GRX480 and mutant derivatives on PDF1.2 transcript accumulation.

2-3 leaves each (200mg) from four-week old wild type Col-O (as::1 GUS line); transgenic plants GRX480 over-expressing line #1 with a 3X HA tag (GRX1); lines over-expressing GRX480 without an HA tag (GXoHA); lines over-expressing GRX480 without N-terminal deletion and including an HA tag (GXΔN30) and lines over-expressing GRX480 double cysteine mutant with an HA tag (GRXDM) were floated in 20µM meJA solution for 6hrs. After sample collection and freezing, samples were used for RNA preparation. 20µg each of RNA was equally loaded unto two independent gels and separated on a denaturing agarose gel. After northern blotting, the membranes were hybridized with radioactively labeled probes for detecting the PDF1.2 and GRX480 (GRX) transcripts. The membrane hybridized with GRX was later on hybridized with GST6. The EtBr stained RNA loading control of one of the representative gels (used for GRX and GST6) is shown.

The expression of the N-terminal deletion derivative of GRX480 did not compromise its ability to repress PDF1.2 transcript accumulation (GXΔN30 line #1), probably relating to its ability to interact with TGA2 as observed in yeast (Figure 6.3AII). Other lines in both the Col-O and *npr1-1* mutant background showed the same behavior (results not shown).

Two lines over expressing the double mutant of GRX480 at comparable levels were compromised in their ability to repress PDF1.2 transcript accumulation (Figure 6.5D, GRXDM lines #2 and 3). Similar results were observed for lines over-expressing the same construct in the *npr1-1* mutant background (results not shown).

6.5.5 Generation of GRX480 over-expressing lines in the *npr1-1* background for epistasis analysis

The observation that the over-expression of the SA-inducible gene-GRX480 significantly repressed the JA-inducible expression of PDF1.2 prompted the investigation of the involvement of GRX480 in the cross-talk between the SA and the JA pathways. It had been previously reported that SA and its functional analogues INA and BTH suppress JA-dependent defense gene expression (Peña-Cortés *et al.*, 1995; van Wees *et al.*, 1999). Furthermore, Spoel *et al*., in 2003 reported that NPR1 modulates the cross-talk between the SA and JA dependent pathways in the cytoplasm, as a requirement by SA in repressing the JA synthesis and JA response genes.

Yeast data indicate that TGA2.2, NPR1 and At1g2840 are able to form a ternary complex. (Abdallat, 2004, manuscript table1). In order to find out if GRX480 is required for this cross-talk mechanism involving NPR1, over-expressing lines were generated in a background of the *npr1-1* mutant plants. Two approaches were carried out.

In the first approach, two GRX480 over-expressing lines (#1 and #3) were crossed with *npr1-1* mutant plants, and the F1 progeny which contained the GFP marker for the presence of GRX480 were selected. This was confirmed in a northern blot experiment by their accumulation of GRX480 transcript (Figure 6.5E). Only the F2 progeny of lines 8, 10, 13, 14 and 15 which will be homozygous for *npr1-1* (unable to accumulate PR1 after SA induction) and contain at least one copy of the GRX480 gene would be suitable for use.

In a second and faster approach, the pAlligator/GRX480 construct was transformed by *Agrobacteria*-mediated transformation into the *npr1-1* mutant plants. Seeds of the F1 progeny which expressed the GFP marker for the presence of GRX480 were selected, and after growth, were analyzed for their inability to induce PR1. Lines which accumulated 3XHA-GRX480 transcript (runs higher than the endogenous transcript) and were unable to accumulate PR1 transcript after SA induction were selected for use (Figure 6.5EI). Lines 8 and 10 were used in subsequent experiments, since they contain levels of over expressing GRX480 transcript comparable to that of GRXOEx #3.

Epistasis analysis showed that in the absence of NPR1, GRX480 is still able to repress JA induced PDF1.2, showing that it functions either downstream of or independent of NPR1.

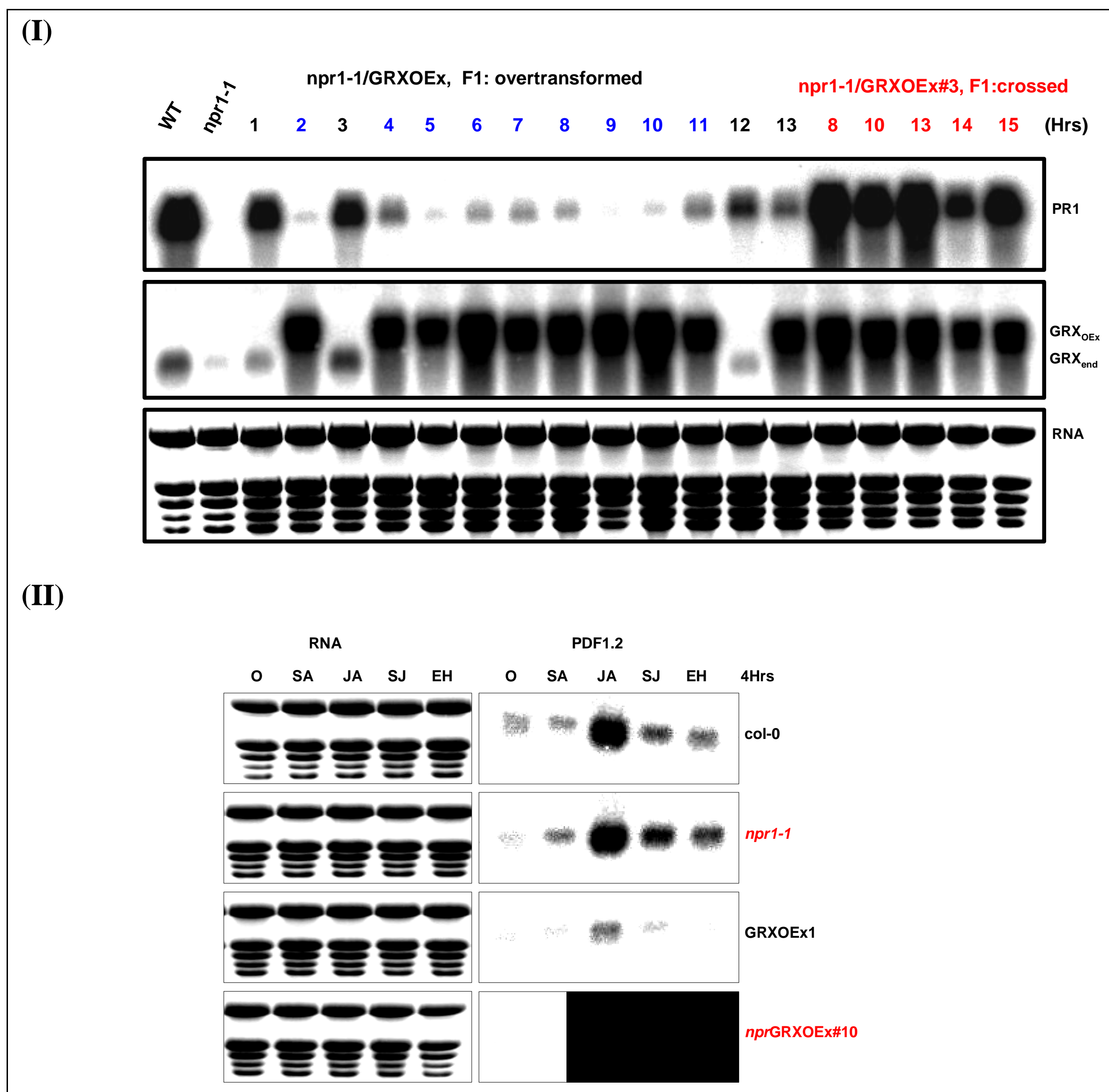


Figure 6.5E. Epistasis analysis for GRX480 relative to NPR1, in SA/JA crosstalk
(I) Selection of lines over-expressing GRX480 on an npr1-1 mutant background by two approaches. *npr1-1 mutant plants were over-transformed with the pAlligator/GRX480 construct. npr1-1 mutant plants were also crossed with GRX480OEx line#3. F1 progeny containing GRX480 were selected in each case by the GFP seed specific marker. After growth for 4weeks side by side, WT and npr1-1 mutant controls, plants were treated with SA for 2hrs, 100mg of leaf sample collected. After RNA preparation and northern blotting, the membrane was hybridized with a radioactively labeled probe for detecting the PDF1.2 and GRX480 transcripts respectively.*
The F1 Lines from the "transformation approach" marked in blue were positive and suitable for use. The F1-Lines from the "cross approach" (in red) have to be analysed to obtain plants in the F2 generation that are homozygous for the npr1-1 mutation and contain at least one copy of the 35S::GRX480 construct.
(II) F2 analysis shows that GRX480 represses PDF1.2 independent of or downstream of NPR1.
F2 plants from over transformed line #10 above (nprGRX480#10) and controls i.e. WT Col-O, npr1-1 and GRXOEx#1 were induced with either 1mM SA/Ethanol (SA), 20µM meJA (JA), SA/JA (SJ) or 0.02% Ethanol(EH) for 4 hours. Un-induced plants (0) were also collected. After pooling and collecting an average of 10 plants per time point, 20µg each of prepared RNA was separated on a denaturing agarose gel. After northern blotting, the membrane was hybridized with radioactively labeled probes for detecting the transcripts of PDF1.2. JA induced PDF1.2 is still repressed in npr1-1 mutant plants expressing GRX480 and in the absence of SA. The RNA loading control in each case is based on EtBr staining.

6.6 Characterization of GRX480 knockout lines

6.6.1 Analysis of GRX RNAi lines

The gene expression of the GRX480 was suppressed in plants using the anti-sense (RNAi) approach by transforming plants with the full length cDNA of GRX480 cloned into a pFGC vector in the sense and anti-sense orientation (Abdallat, 2004). Four of these lines were analyzed to determine whether the ability of these plants to suppress the JA induced gene PDF1.2 would be compromised either in the absence of SA or in the presence of SA induction. These plants showed incomplete but reduced amounts of the accumulation of GRX480 transcript after SA induction (Figure 6.6A), consistent with the fact that the RNA construct was functional in the plants.

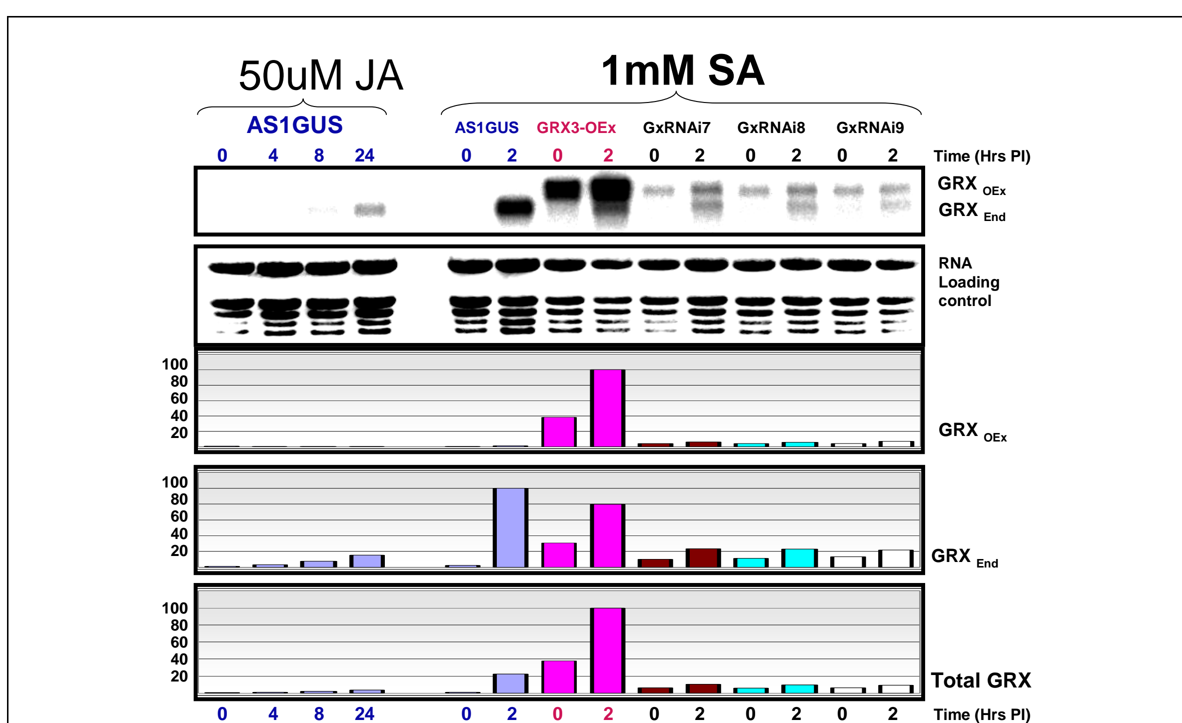


Figure 6.6A. Analysis of GRX480 RNAi line, by inability of SA to induce transcript accumulation. *Three week old GRX480 RNAi lines #7, #8 and #9, control line (as-1::GUS) as well as over-expressing line #3 were treated for 2hrs with 1mM SA by spraying. Control as-1::GUS plants were also treated with 50µM JA by floating in a solution of phosphate buffer, and samples collected at time points 0, 4, 8 and 24hrs. 20µg each of prepared RNA was separated on a denaturing agarose gel. After northern blotting, the membrane was hybridized with a radioactively labeled probe for detecting the At1g28480 (GRX) transcript (above). The RNA loading control is based on EtBr staining. The transcript was also quantified using the TINA software for the relative levels of expression per amount of RNA blotted, for the over-expressing (GRX_{OEx}), endogenous (GRX_{End}) or total amount of GRX accumulating (graphs below).*

Since constitutive expression of GRX480 led to a repression at the PDF1.2 promoter, we assumed that the absence of the same in RNAi lines might super-induce the PDF1.2

promoter activity. Based on this hypothesis, the RNAi lines were induced with meJA, but unlike expected, they were unable to super-induce the induction of PDF1.2 relative to wild type plants (Figure 6.6B).

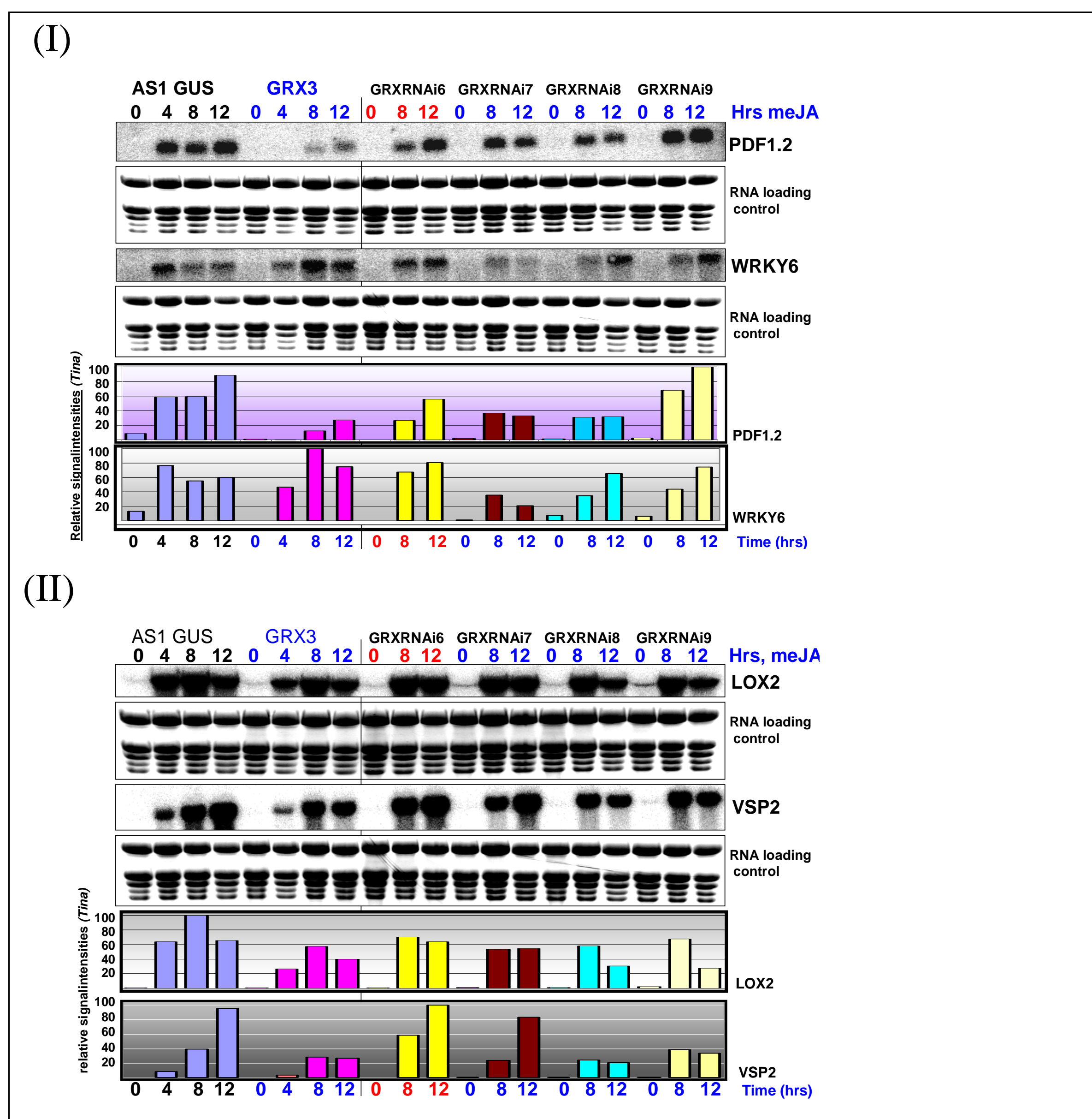


Figure 6.6B. The induction kinetics of JA-inducible genes in GRX480 RNAi lines.
Three week old GRX480 RNAi lines#6, #7, #8 and #9; wild type control line (as-1::GUS) as well GRX480 over-expressing line #3 were induced with 20µM meJA by floatation in buffer. Samples were collected over time points 0, 8 and 12hrs. Total cell RNA from the respective lines was prepared and 20ug loaded and separated on denaturing gels. After northern blotting, membranes were consecutively hybridized with radioactively labeled probes for detecting the transcripts. The loading controls are indicated for each case.
(I)Expression of PDF1.2 and WRKY6 in the respective lines.
(II)Expression of LOX2 and VSP2 in the respective lines.

Other JA response genes (LOX2 and VSP2) were also not affected in the RNAi lines. The WRKY6 gene, which contains an *as-1* like element in its promoter was also not significantly affected.

In the presence of inducible amounts of SA, RNAi lines were still able to some extent to suppress the accumulation of PDF1.2 in the presence of SA (Figure 6.6C).

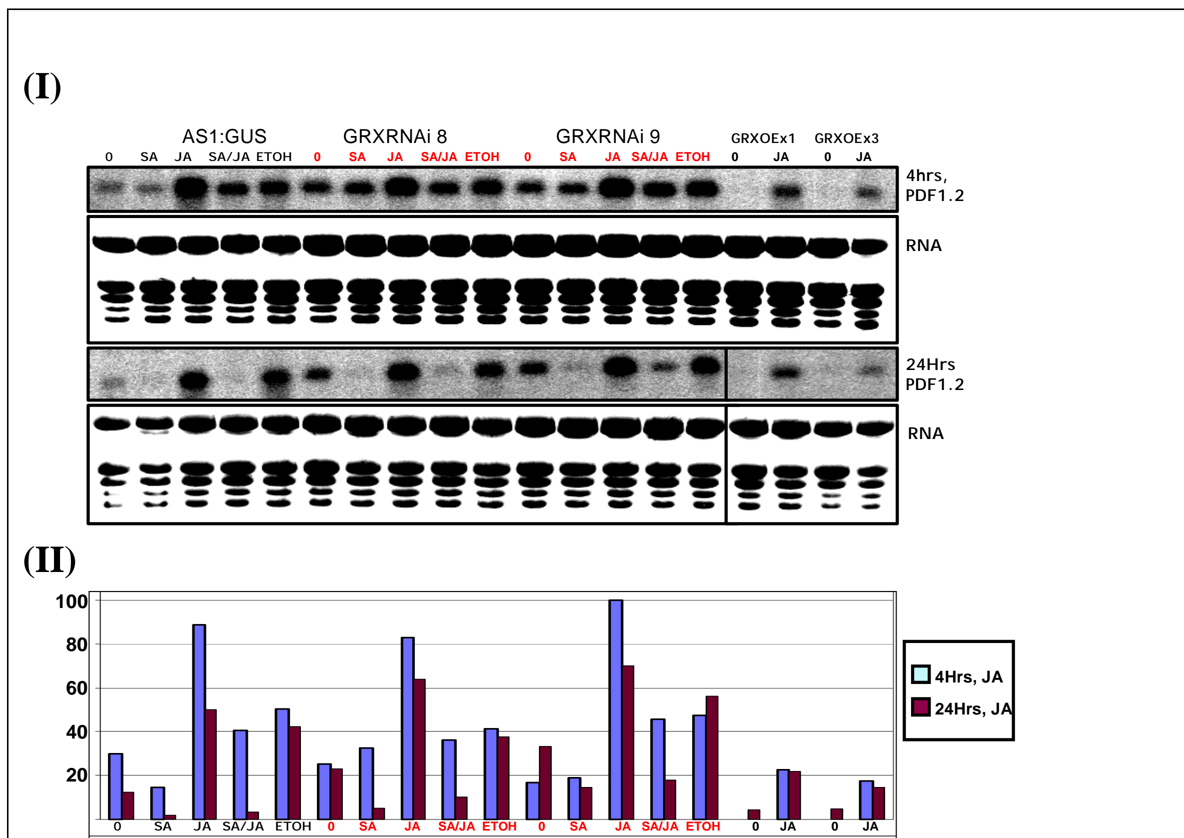


Figure 6.6C. Analysis of GRX480 dependent cross talk in RNAi lines.

Three week old GRX480 RNAi lines #8 and #9; wild type control line (as-1::GUS) as well GRX480 over-expressing lines #1 and #3 were induced with either 1mM SA/Ethanol (SA), 20µM meJA (JA), SA/JA (SJ) or 0.02% Ethanol(EH) for 4 and 24 hours. Un-induced plants (0) were also collected. After pooling and collecting an average of 10 plants per time point, 20µg each of prepared RNA was separated on a denaturing agarose gel. After northern blotting, the membrane was hybridized with a radioactively labeled probe for detecting the PDF1.2 transcript (I - above). The RNA loading control is based on EtBr staining.

The signals were quantified using TINA software, and normalized based on the loading/blotting controls, for all the gels. The relative signal intensities are indicated for each time point in the graph below (II).

Since the RNAi lines did not completely knock out the GRX480 transcript, it can be assumed that the basal amounts of transcript induced after SA treatment in the RNAi lines could still repress the induction of PDF1.2 by JA. When the amounts of residual GRX transcript was quantified using TINA software, it could be observed that they still accumulate up to 5 fold increased levels after SA induction compared to untreated plants,

and to about 10fold increased levels compared to meJA treated plants at 4hours after induction (Figure 6.6A). It was nevertheless observed that the RNAi line #9 with seemingly the strongest RNAi effect was least able to suppress the induction of PDF1.2 by JA in the presence of SA after 4hrs (Figure 6.6C).

The RNAi line #9 was also able to induce PDF1.2 stronger that wild type and other RNAi lines, at comparable time points. Though this was observed 3 times, it is not statistically significant (Figure 6.6B). It was therefore necessary to analyze knockout lines, which are completely compromised in their ability to induce GRX480, in order to better understand their contribution to the cross-talk on the JA pathway.

6.6.2 Analysis of Transposon Insertion Lines

The reverse genetic approach was used to study the function of the GRX480. A "RIKEN *Arabidopsis* Transposon mutant" line, in which a *Ds* transposon was inserted 45bp upstream of the start codon in an *Arabidopsis thaliana* Nössen/Landsberg cross- ecotype was used (Fedoroff *et al.*, 1993, Smith *et al.,* 1996). Sequence analysis downstream from the H (19S-Hyg)-edge of the Ds transposon insertion gave a hit for the GRX480 coding sequence comparable with that known for *A. thaliana* Col-O ecotype. The sequence upstream from the G (GUS)-edge of the Ds-transposon insertion was also comparable to the promoter of the Colombia ecotype, except for a few base pair insertions and exchanges which differ from the sequence published from the Colombia ecotype (see appendix 10.29). The *as-1* like elements (*TGAGC* motives), found in the promoter are nevertheless conserved.

A combination of primers that anneal on the Ds-transposon (*Ds3-2a* anneals 150bp upstream of the D-edge), primers annealing upstream of the transposon insertion site (*LP2GRX480* and *LP1GRX480* annealing 409 and 193bp upstream respectively) and a primer annealing downstream of the Ds-insertion site (*RP480* annealing 272bp downstream) were used to analyze the transposon insertion lines, in order to distinguish wild-type from heterozygous and homozygous mutant lines (Figure 6.6D).

In WT plants (Columbia, Nössen and Landsberg erecta), product sizes of 680bp and 464bp were expected from using upstream/downstream primers *LP2GRX480*/*RPGX480* and *LP1GRX480/RPGX480* respectively. In each case, no product was expected if the plant line was homozygous for the transposon insertion mutation. In case the plants are

heterozygous, two products of 680bp and 422bp were expected when the primer combination of *LP2GRX480/DS3-2a/RPGX480* were used

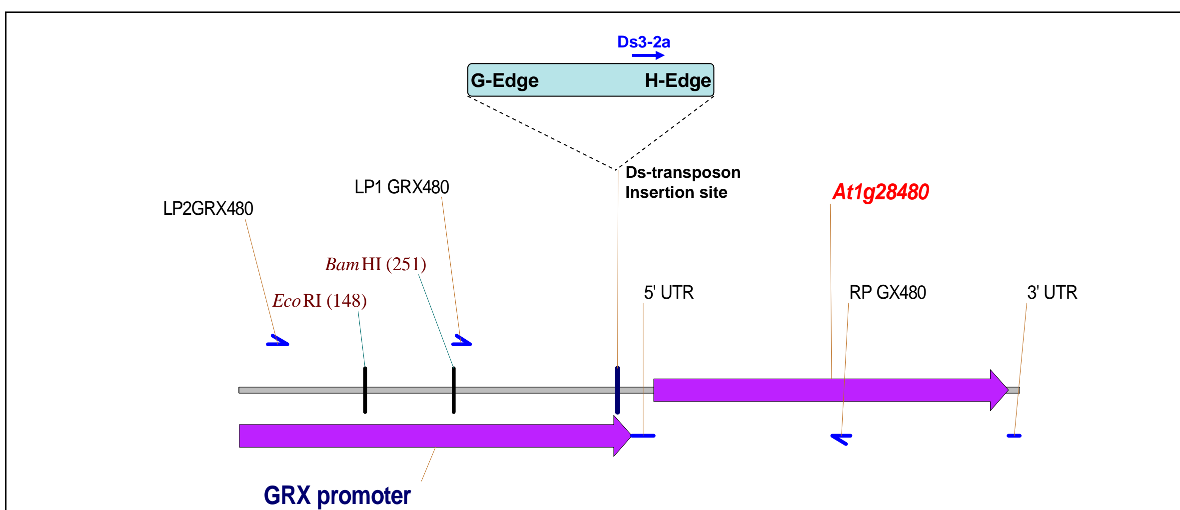


Figure 6.6D. Schematic diagram showing primer design for the analysis of At1g28480 transposon insertion mutants.

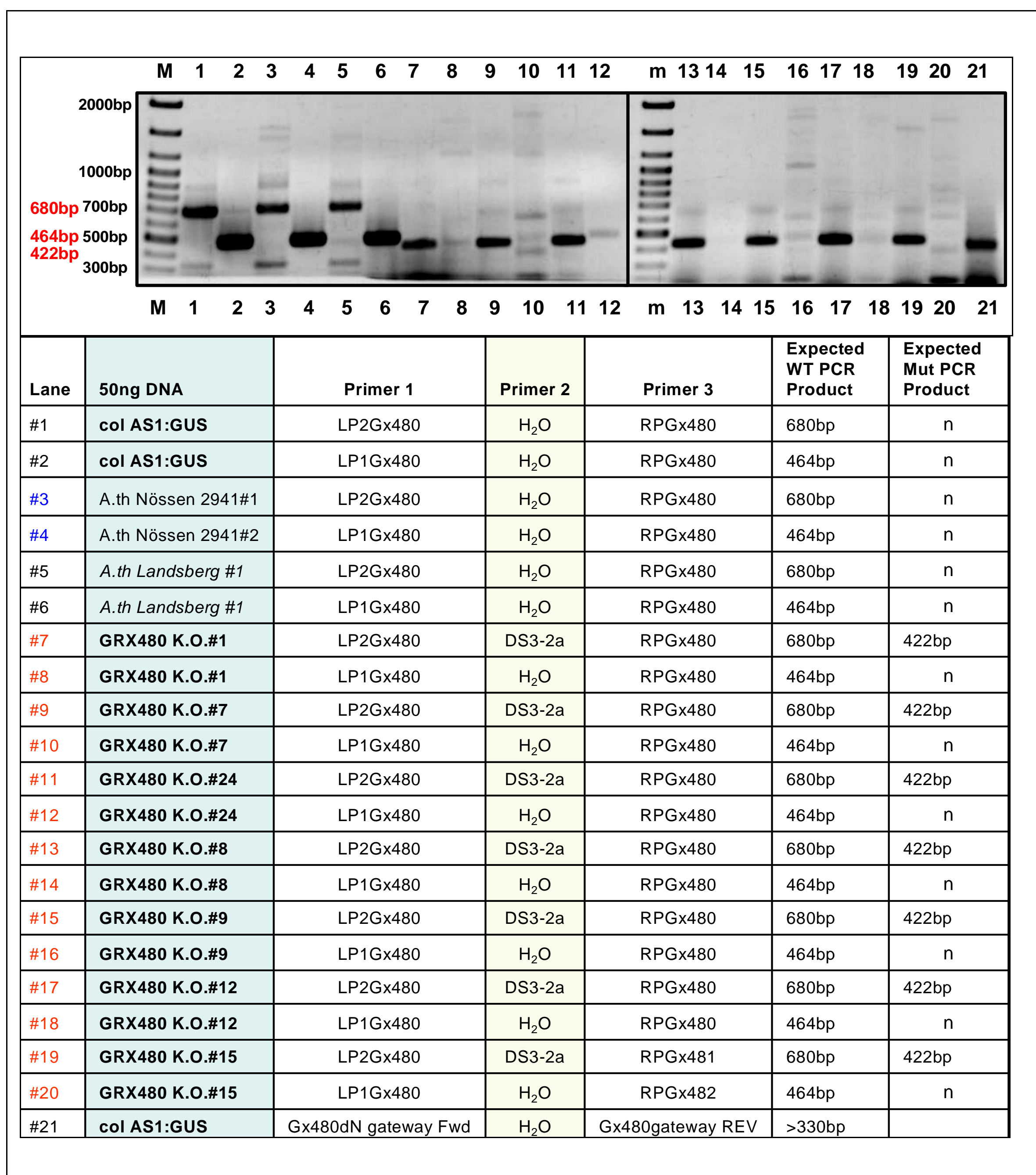
The transposon contains a 5´G-edge and a 3´ H-edge. Primers were designed (blue arrows) to analyze the genomic DNA of the plants, so that by the size of the PCR product, a distinction can be made between WT, homozygous and heterozygous mutant lines. Primers flanking the transposon insertion site would not produce a product in a homozygous mutant, due to the large size of the transposon insertion. The illustrated promoter is truncated. The illustrated transposon insertion is not to scale.

.

Seven lines analyzed were detected to be homozygous for containing the Ds-transposon insertion, and therefore produced no PCR product when primer combinations flanking the transposon insertion site were used, unlike in the controls (Colombia, Nössen and Landsberg erecta) where PCR products were obtained. A product was only detectible with the transposon insertion mutants when a primer annealing on the transposon was used in combination with a downstream primer downstream of the insertion site. (Figure 6.6E)

Mutant lines were further confirmed by their inability to accumulate GRX480 transcripts even after induction with salicylic acid. (Figure 6.6F)

The knockout lines were further characterized to determine if there was any phenotype observed in the ability for the plants to repress JA induced PDF1.2 in the presence of SA (Figure 6.6G). The plants were still able to repress the accumulation of PDF1.2 after induction with JA in the presence of exogenously applied SA. The amounts of PDF1.2 transcript levels accumulating in the knockout lines after JA induction were about 3-fold less in the presence of SA, compared to about 7-15 fold reduction in the control lines.



Lane	50ng DNA	Primer 1	Primer 2	Primer 3	Expected WT PCR Product	Expected Mut PCR Product
#1	**col AS1:GUS**	LP2Gx480	H_2O	RPGx480	680bp	n
#2	**col AS1:GUS**	LP1Gx480	H_2O	RPGx480	464bp	n
#3	A.th Nössen 2941#1	LP2Gx480	H_2O	RPGx480	680bp	n
#4	A.th Nössen 2941#2	LP1Gx480	H_2O	RPGx480	464bp	n
#5	*A.th Landsberg #1*	LP2Gx480	H_2O	RPGx480	680bp	n
#6	*A.th Landsberg #1*	LP1Gx480	H_2O	RPGx480	464bp	n
#7	**GRX480 K.O.#1**	LP2Gx480	DS3-2a	RPGx480	680bp	422bp
#8	**GRX480 K.O.#1**	LP1Gx480	H_2O	RPGx480	464bp	n
#9	**GRX480 K.O.#7**	LP2Gx480	DS3-2a	RPGx480	680bp	422bp
#10	**GRX480 K.O.#7**	LP1Gx480	H_2O	RPGx480	464bp	n
#11	**GRX480 K.O.#24**	LP2Gx480	DS3-2a	RPGx480	680bp	422bp
#12	**GRX480 K.O.#24**	LP1Gx480	H_2O	RPGx480	464bp	n
#13	**GRX480 K.O.#8**	LP2Gx480	DS3-2a	RPGx480	680bp	422bp
#14	**GRX480 K.O.#8**	LP1Gx480	H_2O	RPGx480	464bp	n
#15	**GRX480 K.O.#9**	LP2Gx480	DS3-2a	RPGx480	680bp	422bp
#16	**GRX480 K.O.#9**	LP1Gx480	H_2O	RPGx480	464bp	n
#17	**GRX480 K.O.#12**	LP2Gx480	DS3-2a	RPGx480	680bp	422bp
#18	**GRX480 K.O.#12**	LP1Gx480	H_2O	RPGx480	464bp	n
#19	**GRX480 K.O.#15**	LP2Gx480	DS3-2a	RPGx481	680bp	422bp
#20	**GRX480 K.O.#15**	LP1Gx480	H_2O	RPGx482	464bp	n
#21	**col AS1:GUS**	Gx480dN gateway Fwd	H_2O	Gx480gateway REV	>330bp	

Figure 6.6E. PCR-analysis of GRX480 transposon insertion mutant lines.

Genomic DNA was prepared from leaves of WT plants (Col-O, Nös and Ler) and seven "RIKEN transposon insertion lines". These were used as template for 21 PCR reactions. The primer combinations and the expected outcome are illustrated in the table. All the reaction products separated on a 1% agarose gel, after loading in the slots indicated 1-21. PCR 21 was used as a control. A DNA ladder (M) was also loaded to access the band sizes of the products obtained.

This amount was nevertheless about 2 fold more than in the Nössen ecotype, and about 8-fold more than in the Colombia and Landsberg ecotypes under the same conditions. These results were reproducible when individual induction experiments were compared side by side between the knockout lines and Landsberg lines at the same time point.

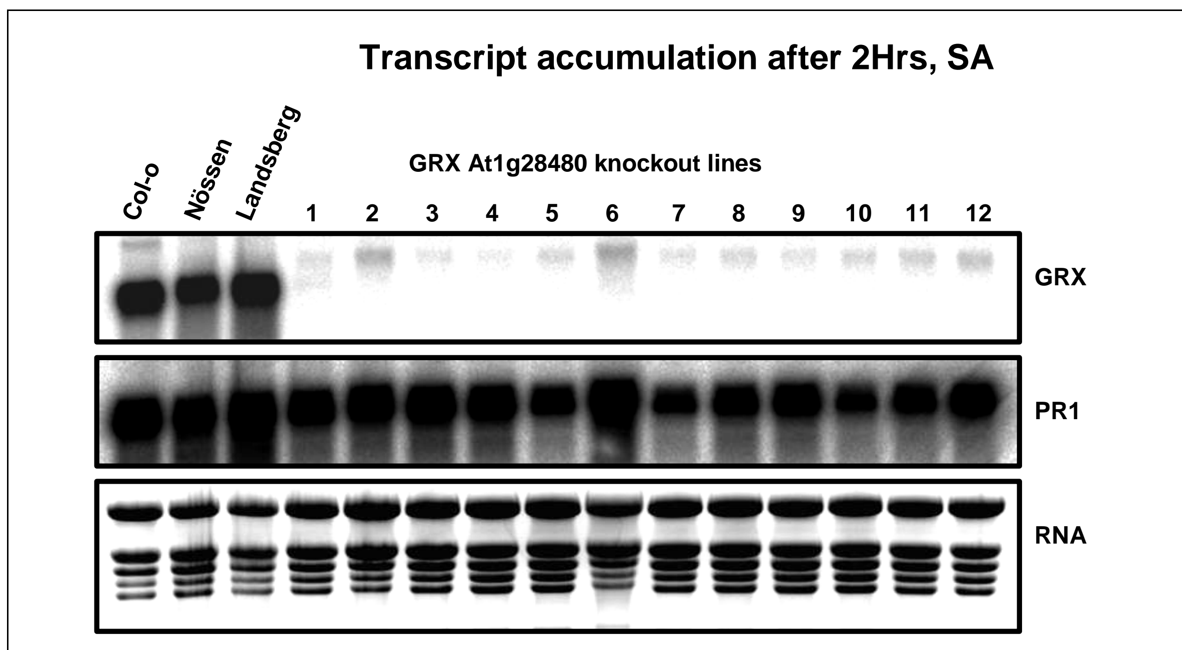


Figure 6.6F. Northern blot analysis of GRX480 transposon insertion mutant lines.

Four-week old GRX480 transposon insertion lines and WT control lines (Col-O, Nös and Ler) were treated for 2hrs with 1mM SA by spraying. 100mg of leaf material (1-2 leaves) were harvested and used for RNA preparation. 20µg each of RNA was separated on a denaturing agarose gel. After northern blotting, the membrane was hybridized with radioactively labeled probes for detecting the At1g28480 (GRX) and PR1 transcripts. The RNA loading control is based on EtBr staining.

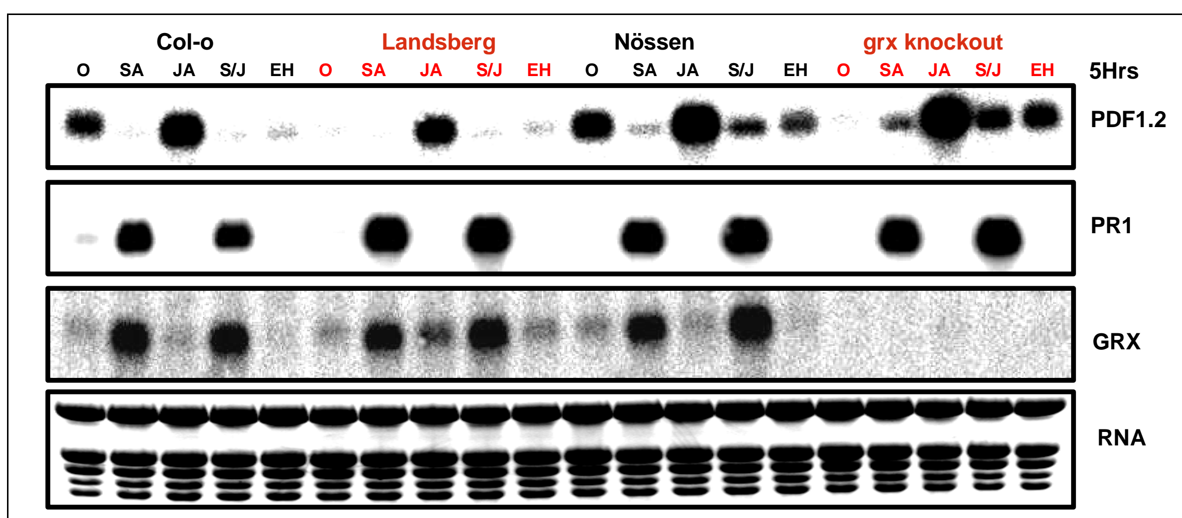


Figure 6.6G. Analysis of GRX480 dependent cross talk in GRX480 transposon insertion lines.

Three week old GRX480 transposon insertion lines #1 and wild type control lines (Col-O, Nös and Ler) were induced by floating in phosphate buffer with either 1mM SA/Ethanol (SA), 20µM meJA (JA), SA/JA (SJ) or 0.01% Ethanol (EH) for 5hours. Un-induced plants (0) were also collected. After pooling and collecting an average of 15 plants per time point, 20µg each of prepared RNA was separated on a denaturing agarose gel. After northern blotting, the membrane was hybridized with radioactively labeled probes for detecting the At1g28480 (GRX), PR1 and PDF1.2 transcripts. The RNA loading control is based on EtBr staining.

The repression of the JA pathway in the presence of SA at later time points (after 12 hours) was comparably the same in the wild type and the controls (results not shown).

It could be concluded from the above observations that GRX480 is necessary but not sufficient on its own in repressing the JA pathway. The above results suggest the fact that there might be a GRX-independent pathway actively complementing the absence of GRX480, possibly involving NPR1. The generation of a double mutant between *npr1-1* and *grx480* by crossing, and analyzing these for their phenotype would give a better idea about the function of GRX480. The two proteins might function in parallel pathways to repress parts of the JA pathway, since in the *npr1-1* mutant which is compromised in its ability to repress the JA pathway, there still occurs about a 2 fold repression of the PDF1.2 transcript in the presence of SA.

Alternatively, these results might be due to a redundancy in GRX480 protein by other GRX480-like proteins. The glutaredoxin coded by the gene AT1G03850, which also has the closest homology to GRX480 (Figure 6.1B) and is also pathogen inducible (Table 6.2A) might be a good candidate worth investigating for its ability to interact with TGA2 transcription factor, its effect after over-expression and its phenotype in a double mutant with GRX480.

The interpretation of results was also made difficult by the fact that the mutant is present in a background of a cross between two ecotypes. Some factors and characteristics which are randomly inherited from the different ecotype-backgrounds make it hard to compare with accuracy, the effects observed in the mutant lines with that observed in the controls. As an example, the induction of PDF1.2 by JA was always weaker in the Landsberg ecotype, while the nössen ecotype in many cases had more background of PDF1.2.

6.7 The role of TGA factors in GRX480 induction and function

The fact that several TGACG motives are present in the promoter of the GRX480 gene prompted the investigation of the role of TGA factors in its induction and expression. Four of such motives are located -192, -218, -252 and -1092 base pairs up stream of the start codon respectively.

The presence of a TGACG motif in the promoter of PDF1.2 (-398bp upstream of the start codon) also prompted us to investigate if TGA factors could play a role in mediating the repression of PDF1.2 expression by GRX480.

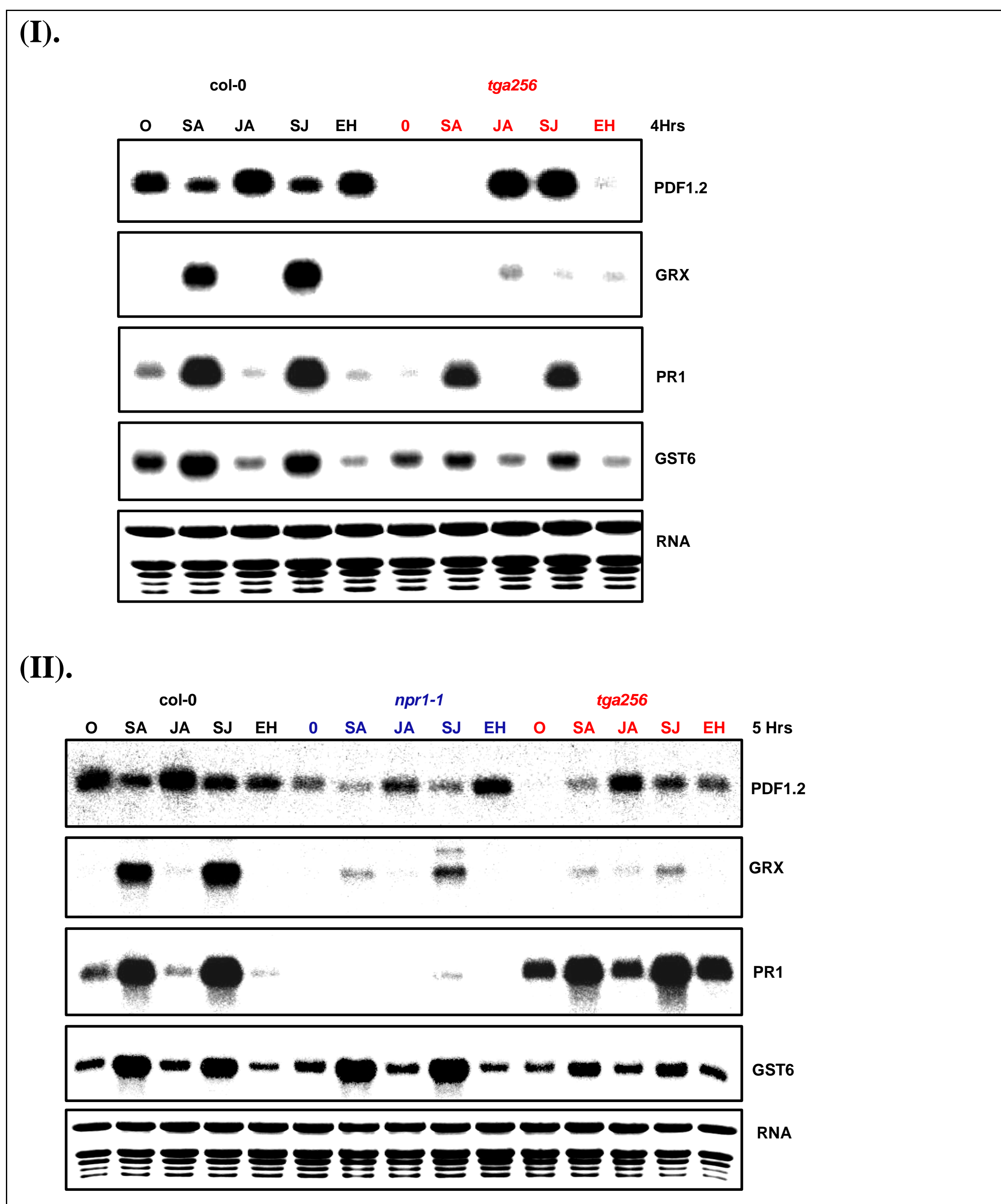


Figure 6.7: The influence of TGA factors on GRX480 expression and function.

Three week old plants were induced by floating in phosphate buffer with either 1mM SA/Ethanol (SA), 20µM meJA (JA), SA/JA (SJ) or 0.01% Ethanol (EH) for 4 - 5 hours, at an average density of 15 plants per treatment. Un-induced plants (0) were also collected. After collection and freezing, 20µg each of prepared RNA was separated on a denaturing agarose gel. After northern blotting, the membrane was hybridized with radioactively labeled probes for detecting the At1g28480 (GRX), PR1, GST6 and PDF1.2 transcripts. The RNA loading controls are based on EtBr staining.

I: Wild type (Col-O) plants and tga2tga5tga6 triple mutant plants (tga256) were treated in the same way.

II: Wild type (Col-O) plants, npr1-2 mutant plants and tga2tga5tga6 triple mutant plants (tga256) were compared by the same treatment in an independent experiment.

In two independent experiments, it could be observed that the induction of GRX480 transcript accumulation is severely compromised in the *tga256* triple mutant. This is even twice as much more severe than in the *npr1-1* mutant.

Secondly, it was observed that the cross-talk of SA on the JA inducible PDF1.2 transcription was not affected in the experiment which still showed pretty strong induction of PDF1.2 in the presence of amounts of SA that normally repress PDF1.2 expression (Figure 6.7I, 4hrs). There was nevertheless a reduced amount of accumulating transcript under similar conditions in an independent experiment (Figure 6.7II, 5hrs). Other factors that might contribute to NPR1 independent crosstalk. It could be that in the plants under these conditions, high amounts of ethylene might be playing a role in contributing to the cross talk. It is also worth noting that the plants under these conditions seem to have higher SA levels, which induce the PR1 transcript. Higher background PR1 levels under uninducible conditions were also reported when the *tga256* triple mutant was grown in INA inducible conditions (Zhang *et al.,* 2003). The experiment nevertheless has to be conducted over a time course. The early SA inducible gene GST6 is reduced in the *tga256* triple mutant as expected.

The synergistic effect of SA and JA are again observed here. GRX480 transcript is at least 2 fold induced in the presence of both hormones. This gives a hint that its optimal function might require the presence of both pathways.

6.8 The role of GRX480 in salt stress resistance

The analysis of At1g2840 on micro array database experiments from Genevestigator (https://www.genevestigator.ethz.ch/at/) revealed that the At1g2840 transcript is not only inducible by pathogen (Abdallat, 2004) but by cyclohexamid, and by salt stress in the roots, among other abiotic stresses.

This was further confirmed by the observation that in the REGIA array experiment, the salt stress tolerance gene ZAT10 is also induced in GRX480 over expressing plants after JA treatment (Table 6.4A).

The significance of this observation was investigated by finding out how well the plants can tolerate salt stress by germinating under differing concentrations of salt.

Plants over-expressing the Atg28480 in both the *npr1-1* and Col-O background, knockout plants as well their respective controls were used in the study. After surface sterilizing the seeds, they were germinated on 2X MS plates containing concentrations of NaCl, ranging from 50mM to 250mM.

After growth for 4weeks, it could be clearly distinguishable to see that plants that stably expressed the GRX480 transcript were more tolerant to salt stress. They could germinate more healthily; grow bigger and faster compared to their background controls. Representative pictures of plants on 100mM and 150mM MS are shown on Figure 6.8A.

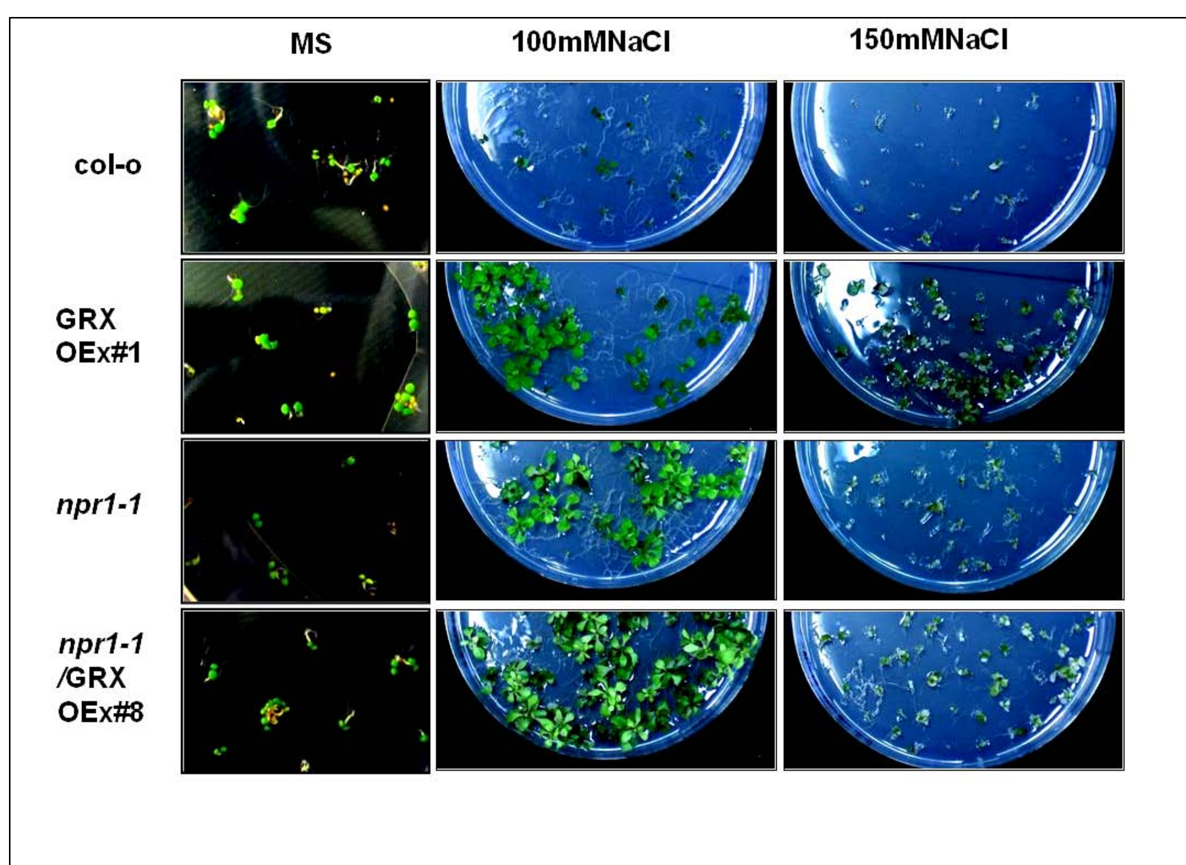


Figure 6.8A. Salt stress tolerance in GRX480 stably expressing plants.

Seeds from GRX480 over-expressing plants in both Col-O and npr1-1 background (GRXOEx#1 and npr1-1/GRXOEx#8) as well as their controls were surface sterilized and germinated on MS plates containing different concentrations of NaCl. After a week of germination, a picture of the seedlings on MS plates was taken. Three weeks after germination, a picture of seedlings on plates containing high salt concentrations was also taken. At high concentrations of 100-150mM NaCl, the seedlings containing stably expressing GRX are able to thrive better.

On the other hand, knockout lines for GRX480 (*grx ko*) showed no significant difference to wild type lines especially at lower salt concentrations (Figure 6.8B). At a salt concentration of 250mM, the plants did not grow at all.

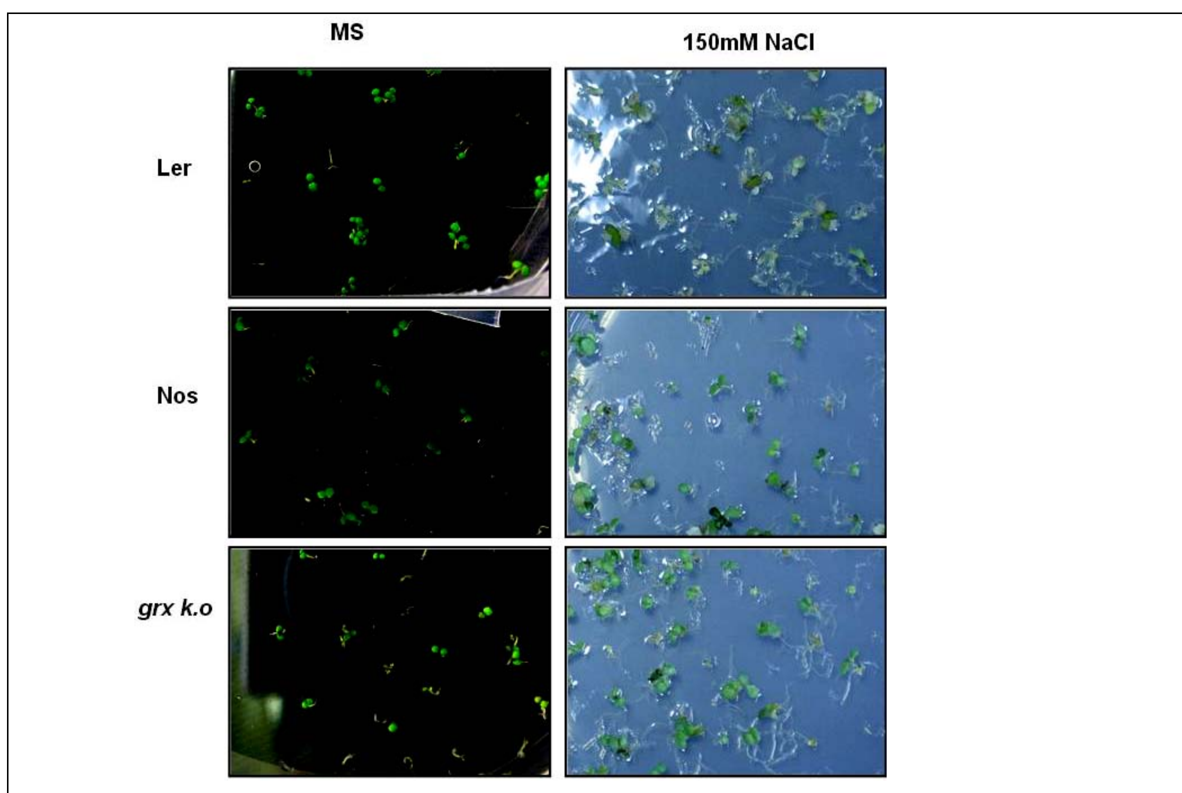


Figure 6.8B. Salt stress tolerance in GRX480 knockout plants.
Seeds from GRX480 knockout plants (grxko#1) as well as their controls (Ler and Nös) were surface sterilized and germinated on MS plates containing different concentrations of NaCl. After a week of germination, a picture of the seedlings on MS plates was taken. Three weeks after germination, a picture of seedlings on plates containing high salt concentrations was also taken.

7 Discussion

7.1 The expression pattern of GRX GRX480 is highly regulated and reveals its involvement in defense mechanisms

When plants are challenged by pathogen infection, they undergo tightly regulated transcriptional reprogramming which aims at focusing the cells and plants resources in defense responses, which are critical for the survival of the plant. Responses involving the SA, JA and ethylene pathways are among the well characterized signaling pathways associated with defense responses in plants. In general, during infection, necrotic pathogens lead to the activation of pathways involving JA and ethylene signaling, while biotic pathogens activate SA mediated defense pathways (Glazebrook, 2005). The fact that GRX480 is distinctively induced during infection by *Pseudomonas syringae* and treatment with SA shows that it favors SA mediated defense processes. It is only mildly induced by JA and necrotrophic fungi such as *Alternaria brassicicola* and *Botrytis cinerea* which induce 5-10 time less efficiently than *P. syringae*. The hypersensitive response which triggers the onset of SA mediated gene expression and causes local cell death also facilitates plant infection by necrotrophic pathogens which activate the JA pathway (Govrin and Levine, 2000). Conditions could occur in the cells during which both pathways are functional and active, but they need to be regulated. Our observation that the accumulation of the GRX480 transcript under both JA and SA inducible conditions is always about two fold higher when compared to only SA inducible conditions gives hints that both pathways need to be synergistically active for the full expression and function of the protein. The importance of the JA pathway in the expression of this gene is further confirmed by the fact that in the *coi1* mutant plants which are critical for induction of JA inducible genes (Xie *et al.,* 1998), the induction of GRX is also compromised, and reduced by about three fold (Thurow, unpublished data).
COI1 (for Coronatine Insensitive 1), which is an F-box, leucine rich repeat (LRR) protein, forms part of a functional E3-type ubiquitin ligase complex, otherwise known as SCF^{COI} (skip-cullin-F-box) complex. It functions in targeting repressors or JA response genes for ubiquitin mediated degradation (Feys *et al*., 1994).
The COI1 protein might therefore function in a similar pathway, in targeting repressors of GRX480 expression for degradation. This would also be very likely since cyclohexamide, which represses *de novo* protein synthesis also induces the accumulation of GRX480

transcript (Table 6.2A). The induction of GRX480 by cyclohexamide is up to 10 fold higher than induction by SA and it is NPR1 independent (unpublished results by C. Thurow).

Genes which are expressed in association with defense have been generally grouped into either early or late defense genes (Uquillas et al., 2004, Zhang *et al*., 1999). Like most late SAR inducible genes, the induction of GRX480 also requires NPR1, since this is compromised in the *npr1-1* mutant plants. Though GRX480 is quickly inducible by SA as early as 2 hours post induction, and could be thought of as an early inducible gene, there might be exceptions to the general classification as some genes which require NPR1 for their induction have been reported to be induced early (Blanco *et al.,* 2006).

NPR1 mediates its effects in SA mediated gene expression through TGA factors, which bind to *as-1* elements of promoters of target genes. Our results show that the induction of GRX480 is also severely compromised in the absence of the TGA factors 2, 5 and 6 respectively (the *tga2tga5tga6* triple mutant, Zhang *et al.,* 2003). This further confirms that the *as-1* elements located at positions -192, -218, -252 and -1092bp respectively upstream of the start codon (see manuscript) may play a functional role in the induction of the gene. Since these have been proven binding sites for TGA transcription factors (Niggeweg *et al.,* 2000a, Despres *et al.,* 2000), they certainly bind to these sites on the promoter, and under SA inducible conditions, NPR1 possibly binds to and modulates transcription factor activity, mediating transcription and expression of the GRX480 gene (Despres et al., 2000; Kinkema et al., 2000; Johnson et al., 2003).

It nevertheless remains to be proven through promoter deletion and subsequently gel retardation assays how the promoter activity varies with the alteration of these motives. Transgenic plants containing the GRX480 promoter luciferase construct will also be important in studying the kinetics of the regulation of the promoter under different conditions and in different tissues.

7.2 GRX480 might function in a redox regulated manner

Redox regulated proteins have been shown to play an important role in the regulation of gene expression during defense responses. Among those well characterized, are TGA1, TGA4 and NPR1. NPR1 exits in the cytoplasm in an uninduced state as oligomers, formed through intermolecular disulfide bridges. In 1999, Zhang and colleagues were

able to show through a series of experiments that a reducing environment is necessary to activate NPR1. In natural circumstances where pathogen infection occurs, a hypersensitive response reaction results in the generation of reactive oxygen species (ROS) and a rapid oxidative burst. A change in cellular reduction potential occurs, resulting in a reduced environment and the reduction of NPR1 to a monomer form, which relocates from the cytoplasm to the nucleus. Mutations in the conserved cysteines which are important in the oligomer formation lead to a constitutive localization in the nucleus, and a constitutive activation of PR1 gene (Mou *et al.,* 2003).

In a similar way, Despres *et al.,* showed in 2003 that in an uninduced state, two conserved cysteine residues in TGA1 and TGA4 are oxidized, forming intramolecular disulfide bonds, which are neither able to interact with NPR1 or activate transcription. Upon SA induction, the disulfide bond is broken and NPR-TGA1 interaction occurs to mediate gene expression.

The question that was left to be answered was whether specific redox mediators are involved in controlling NPR1, TGA1 and TGA4 oxidation/reduction exchange.

It was clear to see that the circumstances that activate TGA1, and NPR1 are the same circumstances that lead to an induction of the glutaredoxin GRX480. Both situations correlate with a rise in levels of SA, which subsequently leads to a reducing environment. Above all, GRX480 interacts with all the known identified TGA factors (Abdallat, 2004). It also bridges the interaction between TGA2 and NPR1 in yeast (Abdallat, 2004). This gives a hint that its association with these critical proteins *in vitro* would certainly have a significant role i*n vivo*.

Glutaredoxins have been described to play a unique role in the reactivation of proteins altered by post-translation modifications after oxidative stress conditions (Rouhier *et al*.,2003). They do this by deglutathionylation of cysteine residues, a function which cannot be carried out by thioredoxins (Lemaire, 2004). Furthermore, they contain a glutathione binding site, absent in thioredoxins.

Glutaredoxins have been described to function either through a monothiol or a dithiol redox mechanism, depending on the presence of either one or two conserved cysteines in its active site (Vlamis-Gardikas and Holmgren, 2002; *Qin et al.,* 2000).

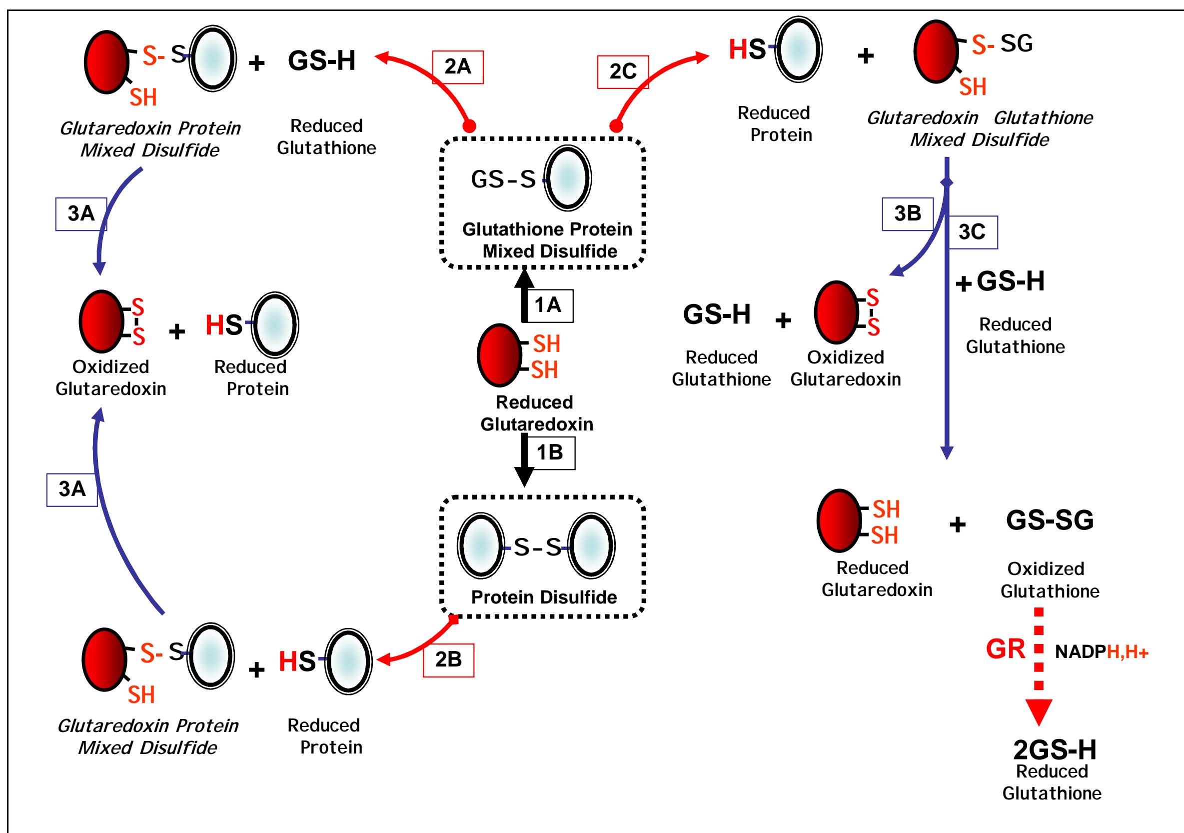


Figure 7.2: Redox modification mechanisms mediated by glutaredoxins.
Glutaredoxins can reduce disulfide bonds of either protein disulfides or glutathione protein mixed disulfides (Reactions 1A and 1B, black arrows; target proteins are in boxes with dotted borders). Following a nucleophilic attack of the modified disulphide by the most N-terminal cysteine of the active site glutaredoxin, the reaction can proceed through a transiently formed disulfide bond between the most N-terminal cysteine and either target protein (2A or 2B, red arrows) or glutathione (2C). In a third step reaction, the second cysteine of the dithiol glutaredoxin serves as an electron donor, and reduces the protein, resulting in an oxidized glutaredoxin (3A and 3B). In the case of monothiol glutaredoxins, a glutathione molecule serves as an electron donor (3C). This also occurs in dithiols. The reaction is driven downstream by glutathione reductase (GR, red dotted arrow) which needs NADPH as the source of reducing power.

Dithiol glutaredoxins like GRX480 can therefore reduce both protein disulfides and GSH-thiol disulfides, as shown in the scheme below on Figure 7.2.

During oxidative stress, redox modifying proteins form transient disulfide bonds with proteins to keep them stable from the harsh or otherwise destructive effects of ROS. The reversible modifications by glutaredoxins are thus important in target protein structure and function by reversibly oxidation and reduction, or otherwise activation and inactivation.

7.3 The function of GRX480 may be linked to redox modification in yeasts, protoplasts, and plants

When first identified as a NtTGA2.2 interacting protein in 2004, Abdallat observed in histidine prototrophy growth assays that GAL4BD-TGA2.2 and GAL4AD-GDM (the glutaredoxin double mutant) do not interact. When the domains were switched, there was only a weak interaction in the double mutant of GRX480 compared to that between GRX480 and TGA2.2. This gave first hints of a possibility that redox changes could modulate the interaction between these proteins.

In our current study, the interaction was more quantitatively studied using the ONPG assays. In addition, the interacting proteins were expressed fused to tags that could be used for immunological detection of the expressing proteins in the yeast (Section 6.3). The interaction between AtTGA2 and At1g28480 was compared to the interaction with a classical plant glutaredoxin GRX370. GRX480 always interacted with TGA2, and up to 20 fold higher, when compared to the interaction with GRX370, where there was no interaction occurring at all. It could therefore be ruled out that the absence of interaction with the glutaredoxin GRX370 had anything to do with a poorly expressed protein, since western data confirmed that they were stably and strongly expressed (Fig 6.3B).

It could also be said at this point that a general glutaredoxin activity was not responsible in this case for the interaction between TGA2 and GRX480.

Secondly, the interaction between the double mutant of GRX480 (GDM) and TGA2 was investigated. There was still an interaction occurring in this case, detectible by ONPG activity. It was on the average 10 fold higher when compared to negative controls. It was nevertheless difficult to quantify the difference in interaction between TGA2 and GRX480 or the GDM. This was because in a few cases (about 32%), the strength of the interaction between the wild type protein and TGA2 was up to 4 fold higher than in the double mutant protein. In most of the cases, the interaction in the wild-type protein was within the same range as that of the double mutant. We can speculate that there appears to conditions of growth or within the individual clones of yeast cells, which change the structure or behavior of the glutaredoxin GRX480 protein, such that they interact in varying affinities with the TGA2 transcription factor. There would certainly be conditions that change the glutaredoxin protein conformation to resemble the double mutant, which itself mimics an oxidized state. To our surprise, when the yeast cells were grown under

oxidative stress conditions, the interaction of the wild type GRX480 with TGA2 did not show any significant difference when compared to cultures grown under normal growth conditions.

The strength of the interaction quantified by the ONPG assays did not also correlate with the levels of expression of the protein, since both colonies showing strong or weak interaction between partners showed the same range of expression of the interacting proteins (Fig 6.3BI). Domain switch experiments may give more stable interactions which may be easier to quantify.

We also investigated the interaction in Tobacco BY2 protoplast wells (section 6.4) where the TGA2.2 and GRX480 proteins were still able to show an interaction in a two hybrid assay. This was on an average, 5 fold more, when compared to a negative control. Interestingly, the double mutant of GRX480 interacted two fold weaker with TGA2. Unfortunately, the limitation of antibody availability for detecting GRX did not permit us to provide expression data for the interacting proteins in protoplasts. We cannot rule out the fact that a weaker interaction could also result from an unstable or weakly expressed protein. The data nevertheless do not contradict the observations made in yeasts so far.

Furthermore, the interaction between GRX480 and TGA factors which likely mediate the repression of JA inducible gene PDF1.2 might be relevant in wild type situations in *A. thaliana* plants. On the contrary, when the double mutant of GRX480 is stably over expressed to comparable levels as the wild type in plants, the ability to repress the PDF1.2 transcript accumulation under JA inducible conditions is compromised. This observation truly validates the fact that the active site cysteine residues of the GRX480 protein are critical for its function *in vivo*. We nevertheless need to show in the F1 progeny of the tested lines that the protein is stably expressed within levels comparable to the over expressed wild type protein which cause a repression in the JA inducible induction of PDF1.2. Taken together, these results highlight the importance of protein disulfide bond formation in maintaining, stabilizing or modifying protein-protein interactions, as previously reported for NPR1 and TGA1. (Despres *et al.,* 2003, Mou *et al.,* 2003).

Several other glutaredoxins have been reported to play a role in regulating the function of transcription factors such as OxyR (Aslund *et al.,* 1999) and NF-kappa B (anti-apoptotic activity exerted by human GRX and *E. coli* GRX2, Daily *et al.,* 2001). The glutaredoxins

influence their structure, function, intracellular localization and binding to DNA, through direct or indirect mechanisms.

7.4 GRX480 mediates its function by a domain other than the N-terminus

Apart from the activity of the active site cysteines in GRX480 that might account for its ability to interact with TGA factors and mediate repression of *as-1* mediated transcription, other structural domains may be important in this interaction and function. This is especially true because in yeast, the ability of GRX480 to interact with TGA2 is not completely abolished.

GRX480 contains a unique N-terminus of 30 amino acid residues which is not a plastid signal peptide (Lemaire, 2004). Inspite of the fact that they are lacking in most of the CC-type glutaredoxins, a deletion of these residues neither destabilizes the interaction with TGA2 in yeast nor its ability to repress JA inducible PDF1.2 expression. On the contrary, the effects appear to be even stronger than the wild type GRX480. This indicates that the presence of this domain may rather negatively interfere with the functional domain of this protein. The function might be mapped to a C-terminal domain. This nevertheless has to be investigated.

7.5 GRX480 specifically represses *as-1* mediated transcription

The transcriptional activation from a promoter containing an *as-1* element is repressed in the presence of GRX480 as observed both in BY2 protoplasts and in *as-1::GUS* containing plants.

On one hand, this was specific for a promoter containing an *as-1* element, since the same effect of transcriptional repression was not observed from a *Gal 4* promoter.

On another hand, the repression is specific, and not based on a general glutaredoxin activity as transgenic plants which over-express the glutaredoxin GRX370 do not repress transcriptional activation from a promoter containing an *as-1* element. This agrees with the observation that this protein does not also interact with TGA2.

The promoter of PDF1.2 contains a conserved *as-1* element. Even though Spoel *et al.,* in 2003 showed through truncated promoter - reporter constructs that the *as-1* element is not required for activating JA inducible transcription, we do not rule out the possibility that it

might be required by GRX480, perhaps with a combination of other elements, to repress JA inducible transcription.

Gel mobility shifts show that TGA2 specifically binds to the *as-1* element also present on the promoter of the PDF1.2 gene (Zhang *et al.,* 1999, Spoel *et al.,* 2003). Since TGA2 also interacts with GRX480, it may mediate its function through TGA2.

Preliminary chromatin immunoprecipitation (CHIP) assays using antibodies against TGA2 and promoter specific primers suggest that TGA2 factors may bind to the PDF1.2 promoter *in vivo*. It remains to be shown using an antibody that pulls down GRX480 that it is also directly or indirectly associated with the PDF1.2 promoter.

7.6 GRX480 mediates an NPR1-independent negative cross talk on the JA pathway

The cross talk between the SA and ET/JA signaling pathways has been widely described (Reviewed by Beckers and Spoel, 2005). We showed from our results of epistasis analysis that the repression of PDF1.2 by GRX480 might occur either independent of NPR1 (NPR1 independent pathway) or downstream of NPR1 (NPR dependent pathway). The ERF1 transcription factor has been described to regulate PDF1.2 transcription (Lorenzo *et al*., 2003). It has not been previously described whether NPR1 mediated cross talk depends on ERF1. We show that this is the case, as the induction of ERF1 by JA is repressed in the presence of SA, but compromised in *npr1-1* mutant plants. GRX480 over-expression does not also repress ERF1 transcription. Taken together, GRX480 mediated repression of PDF1.2 would act downstream or independent of ERF1.

Transposon insertion mutant and RNAi plants of GRX480 show no strong phenotype with respect to cross talk on the JA pathway. Unlike expected, some considerable amount of cross talk still occurs in these plant lines (Figures 6.6C, 6.6G).

These could be explained by the fact that an NPR1 dependent but GRX480 independent mechanism compensates for the absence of GRX480. Further on, we can remark that inspite of the fact that the cross talk is compromised in *npr1-1* mutants, there is still a considerable amount of cross talk occurring (Figure 6.7II), which may be accounted for by GRX480 also induced by SA. Another alternative explanation which cannot be ruled out could be the presence of other glutaredoxins, which have a redundant function in the absence of GRX480. Other unidentified or uncharacterized pathways might also be involved in the cross talk under these conditions.

7.7 GRX480 may have family members with redundant functions

GRX480 represents a member of a CC class of glutaredoxins only present among higher plants (Lemaire, 2004). As described in the structural analysis, other potential members of this group need to be analyzed. Among them, are the At1g03850 which is also SA and pathogen inducible. At4g15690 is SA inducible and not pathogen inducible. If these family members are able to interact with TGA2 transcription factor, they would be potentially redundant in their function. Mapping the interacting domain in the C terminus of GRX480 would give a clearer idea about the conserved residues and which family members are potentially able to use them.

ROXY1 (At3g02000), which is a CC- type *Arabidopsis* glutaredoxin has also been reported to regulate the development of flower primordia, by inhibiting the AGAMOUS gene. (Xing *et al*. 2005). Xing and colleagues reported that the N-terminal Cysteine 49 in the CXXC motif is crucial for the ROXY1 protein to properly exert its function as an oxidoreductase during petal development. Other domains within this protein might be important in its function in repressing the promoters of target genes.

It cannot be ruled out that thioredoxins, could also have redundant functions to glutaredoxins. Redundancy in the case of GRX may be partial since deglutathionylation is clearly GRX specific, a function which cannot be efficiently fulfilled by TRX.

It has been reported that the expression of thioredoxin (TRX) h5 gene, is closely related to pathogen attack, as well as other stresses like wounding, abscission, senescence and oxidative stress (Laloi *et al*, 2004). It was also reported to contain *as-1* elements, W-boxes and to be regulated by the WRKY6. Nevertheless T-DNA insertion mutant plants of *trxh5*, in which the gene was inactivated, showed no particular visible phenotype under both stress conditions and standard growth conditions, suggesting that other members of the thioredoxin or glutaredoxin families may compensate for the lack of TRXh5.

7.8 Other possible functions of GRX480

Glutaredoxins have been reported to be abundantly present in phloem (Ishiwatari *et al.,* 1995), which is a critical site for plant pathogen interacting during infection and disease. Given the conditions under which it is induced, it is possible that GRX480 functions as a mobile signal during SA mediated gene expression.

It might also regulate other genes associated with different pathways. The abscissic acid (ABA) pathway important in germination and tolerance to salt stress might be regulated by GRX480, especially as plants overexpressing this protein are more tolerant to high salt concentrations of up to 150mM NaCl (Figure 6.7A). This correlates with the high expression of this protein in roots after salt induction (Zimmermann *et al.,* 2004), and the expression of salt tolerance gene ZAT10 as seen in the transcription factor array (Table 6.5A). The relevance of this phenotype nevertheless has to be further investigated.

8 Conclusions

A model showing what is understood so far about the function of the GRX480 protein can be summarized in the model below (Figure 8.0)

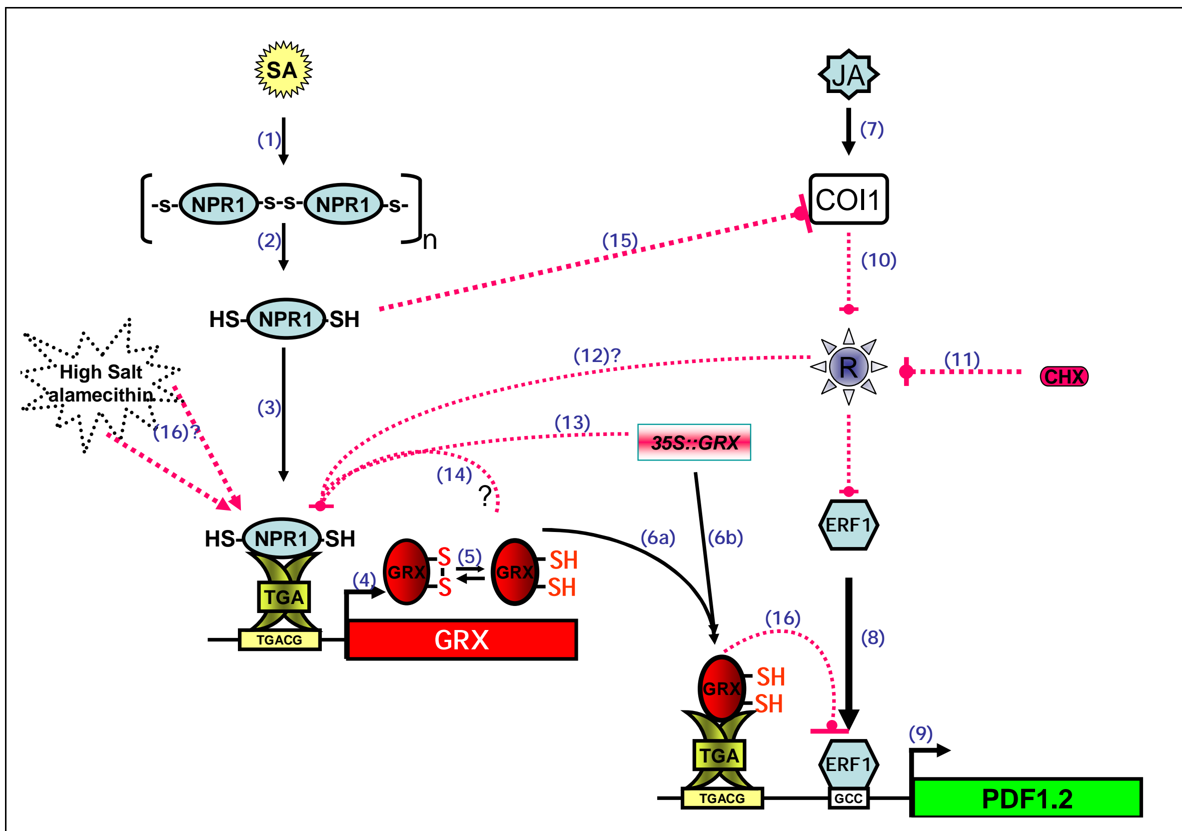


Figure 8.0: Model for the regulation and function of At1g28480 (GRX).
Negative repression is shown as red dotted lines with a barred end Arrows show processes of steps from upstream to downstream.
Upon abiotic and biotic stress conditions, JA and SA pathways can be activated (1, 7). Rise in SA levels lead to activation of NPR1 (2) and its nuclear localization where it interacts with TGA factors which bind as-1 elements (TGACG) on the GRX promoter (3). This mediates transcription of GRX (4) which is active in the reduced state (5). Upon induction or constitutive expression, GRX can interact in the nucleus with TGA factors which bind to the as-1 element on the promoter of PDF1.2(6a, 6b), and mediate repression of JA inducible expression (16, 9). GRX can also possibly down regulate its expression (13, 14). NPR1 can also mediate repression of expression by repressing COI, upstream of ERF1 (15) which normally activates JA inducible transcription by binding to GCC box on the promoter (8).
Simultaneous induction by SA and JA can enhance GRX levels. This occurs by COI1 mediated degradation of a repressor of GRX accumulation (R, 12). COI1, and F-box protein in the SCF-COI complex is inducible by JA (7). It targets repressors of JA inducible genets for degradation (10).This effect is mimicked by cyclohexamide treatment (CHX, 11) which induces GRX accumulation (11-12). High salt and alamecithin (derived from Trichoderma viridae;Herde, 2002) also induce GRX accumulation by mechanisms that are yet unclear.

In the future outlook, other glutaredoxins of the same family as GRX480 which show both significant structural similarity and inducible patterns will be tested for their ability

to interact with TGA2. Potential interacting partners will be studied for their ability to interfere with *as-1* mediated gene transcription.

RNAi interference strategy would possibly be helpful in impairing the expression of homologous genes by transfecting constructs containing them into the *GRX480* knockout plants.

The phenotype of salt tolerance, root expression and growth due to At1t28480 has to be studied with respect to the ABA pathway. It will also be worth investigating the auxin inducible repression of the WRKY6 promoter in roots (Silke Robatzek *et al,* 2001) using hydroponic plants, for a possible function, mediated by GRX480 in under conditions.

The induction kinetics of GRX480 will be studied using the promoter luciferase construct.

The dependence of TGA factors on GRX mediated repression of PDF1.2 will be studied in *tga256* mutant plants over-expressing GRX480.

Protein expression assays for the double mutant expression in protoplasts and in plants will be carried out.

It will be will be interesting to investigate alternative target genes using the microarray approach, and both GRX480 knockouts and over-expressors as samples.

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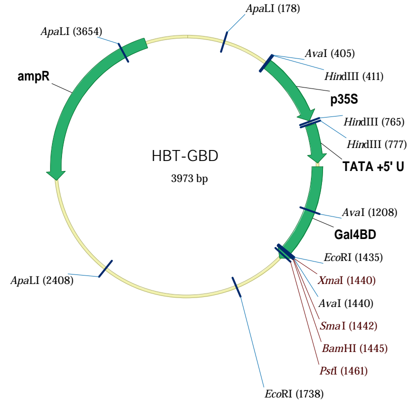
10 Appendix

10.1 List of plasmid and gene maps

10.1.1 HBT-GBD



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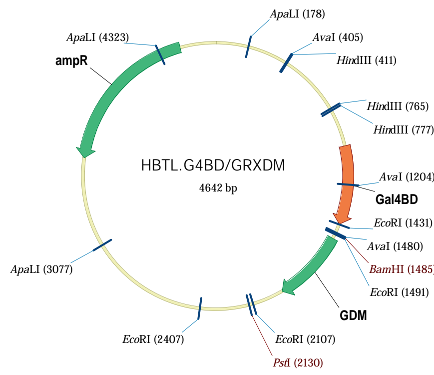

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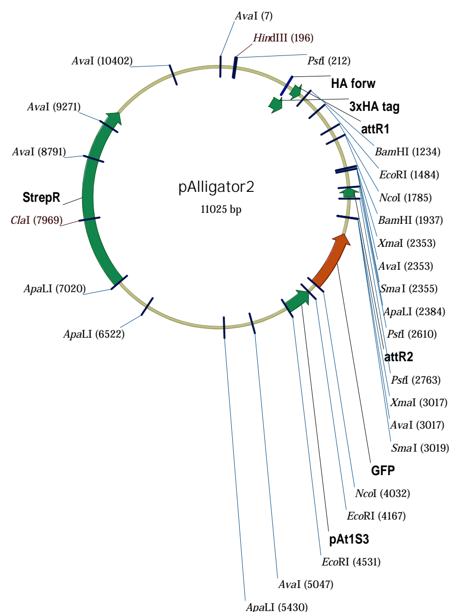

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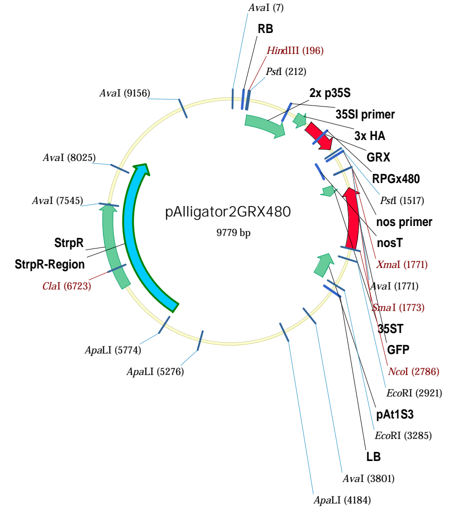

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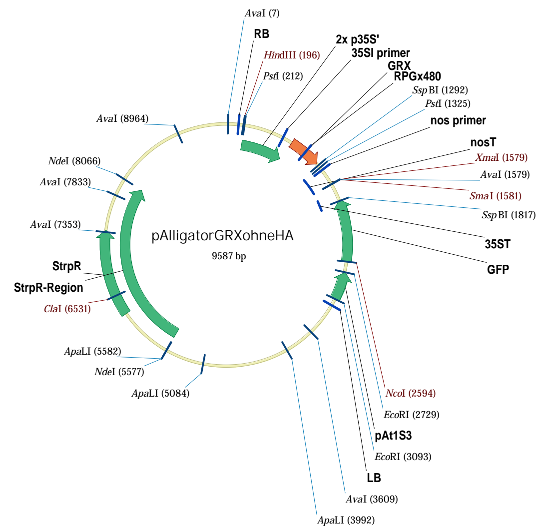

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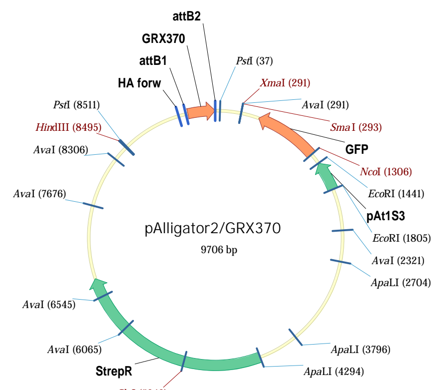

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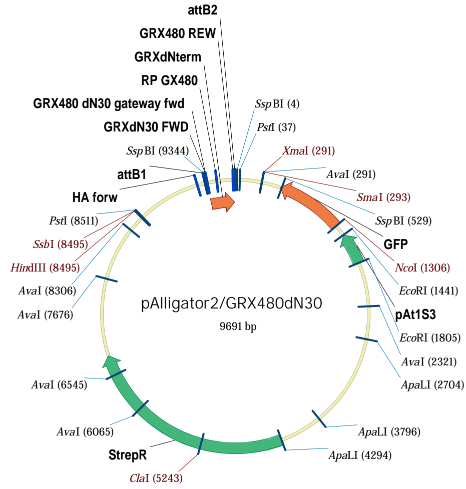

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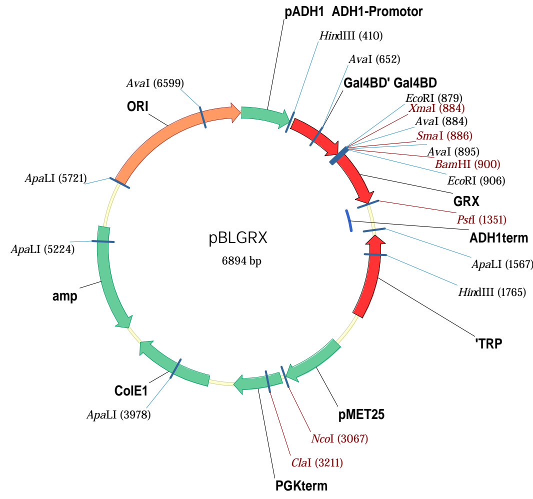

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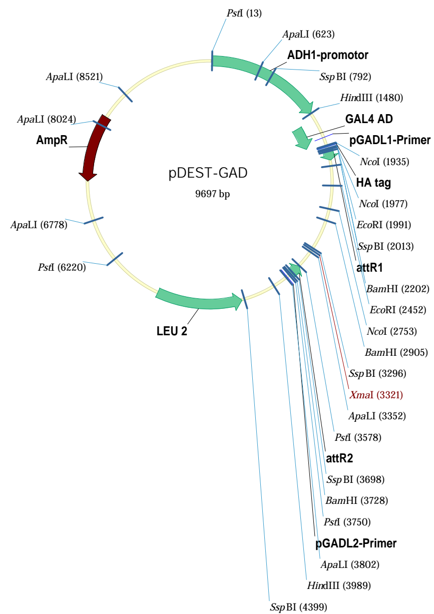

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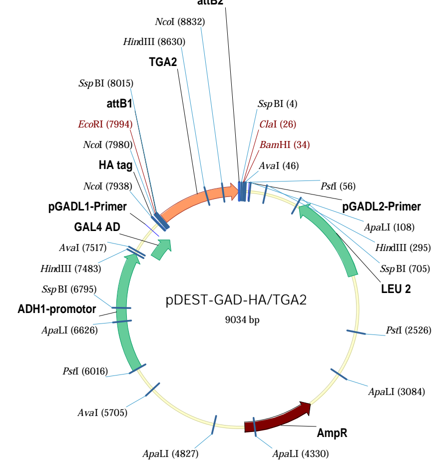

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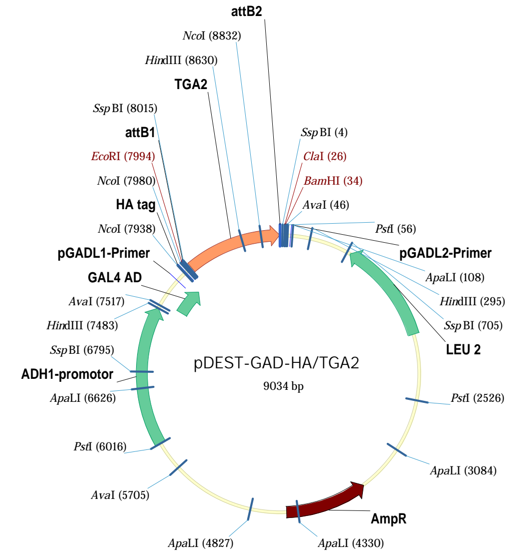

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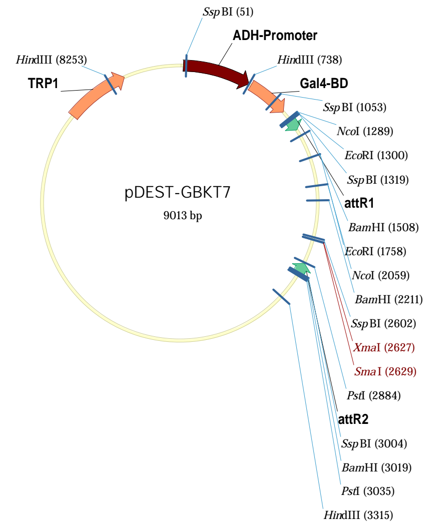

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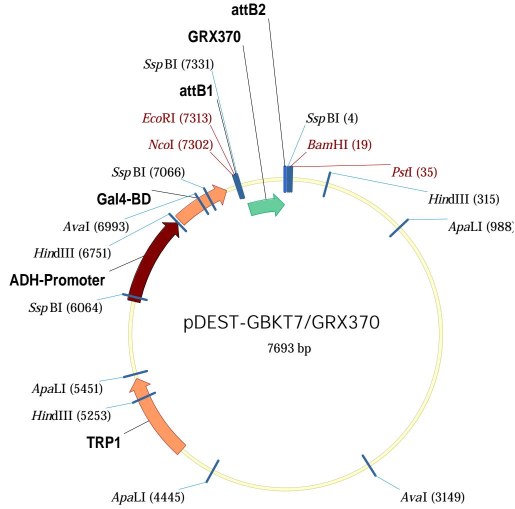

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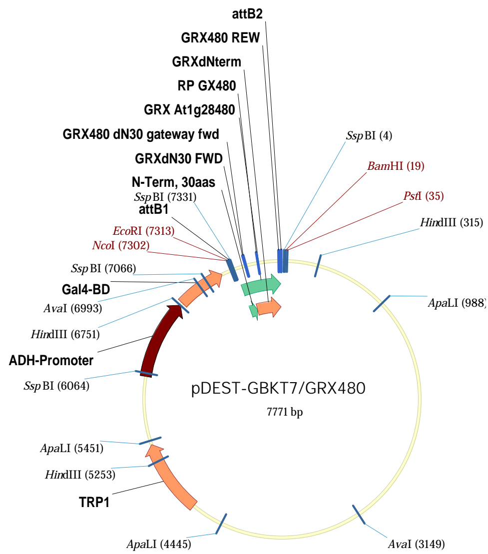

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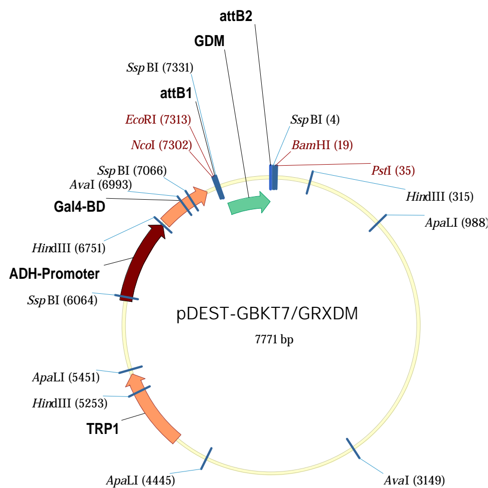

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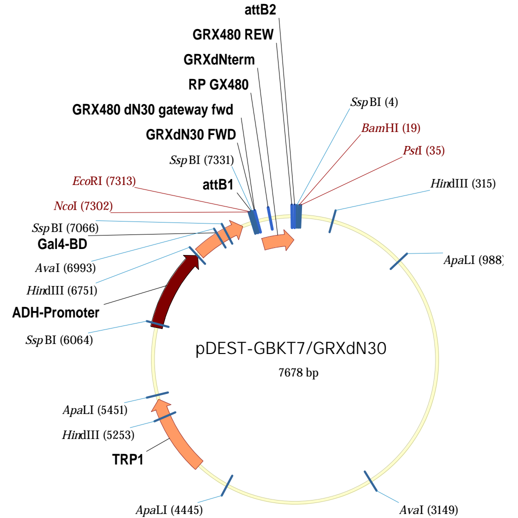

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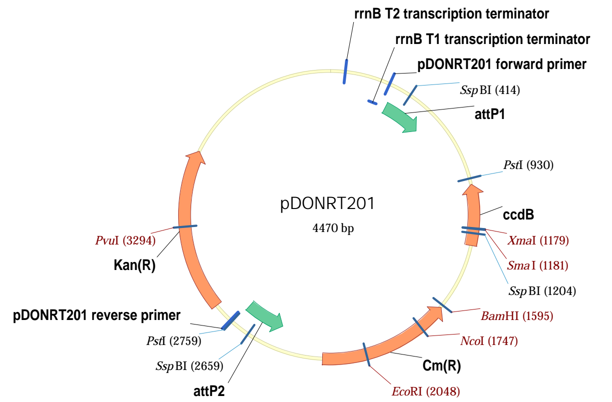

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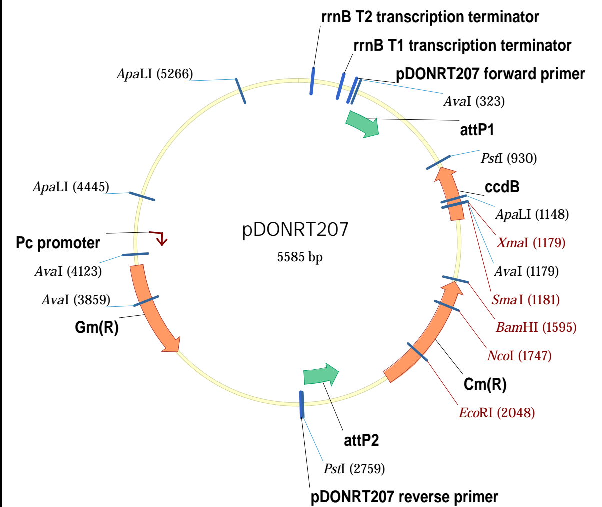

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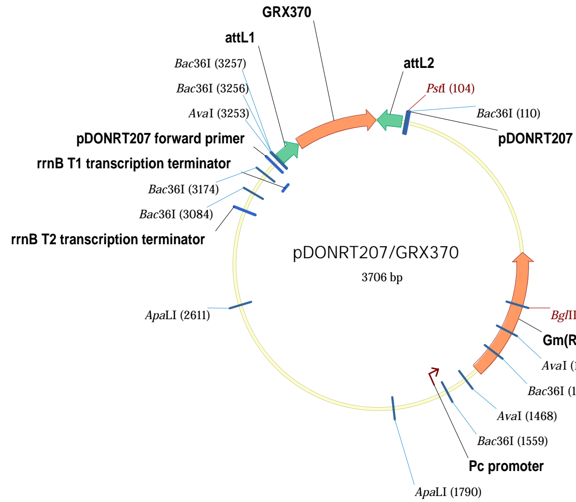

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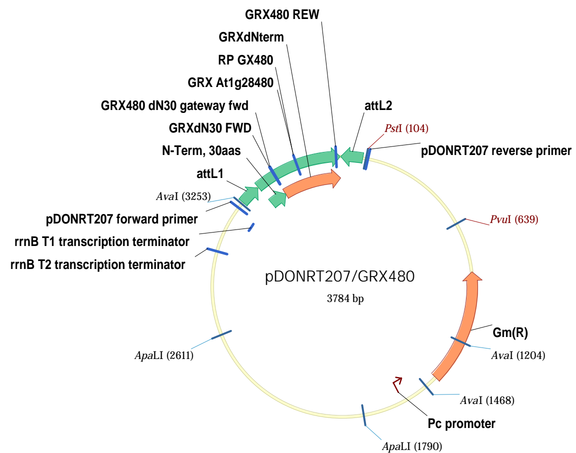

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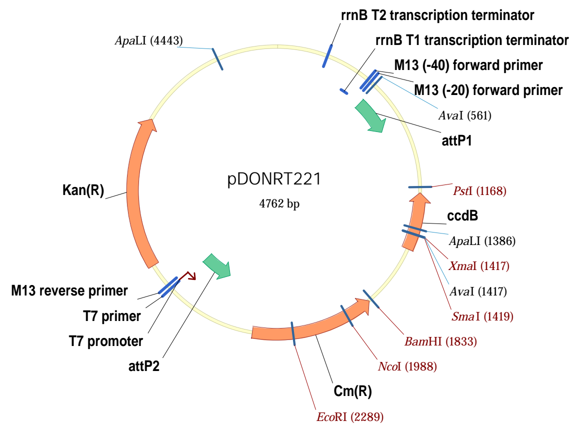

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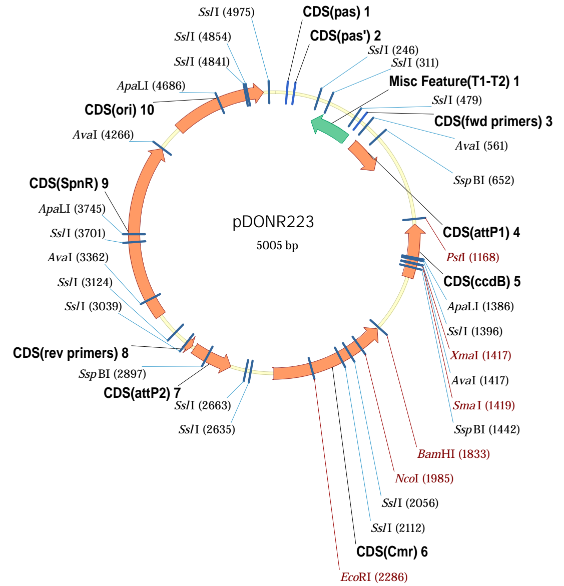

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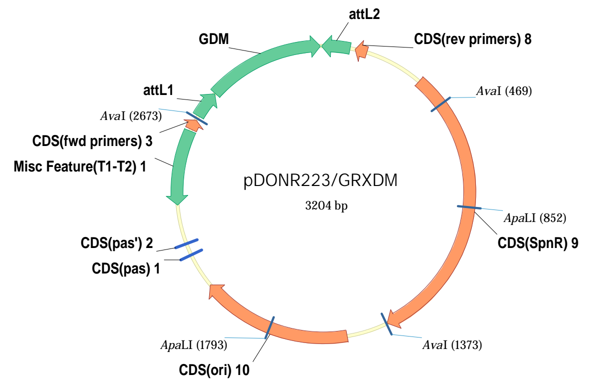

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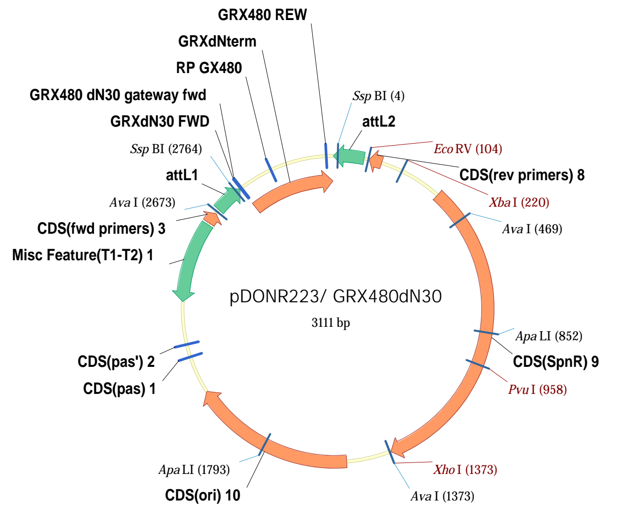
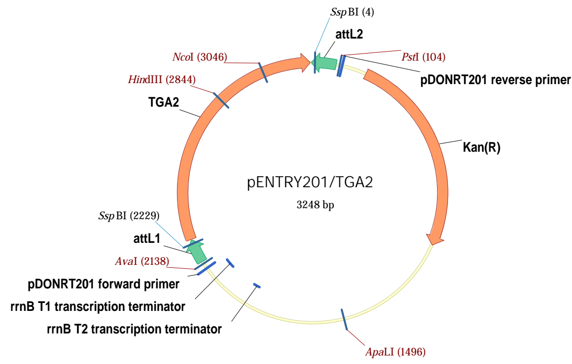
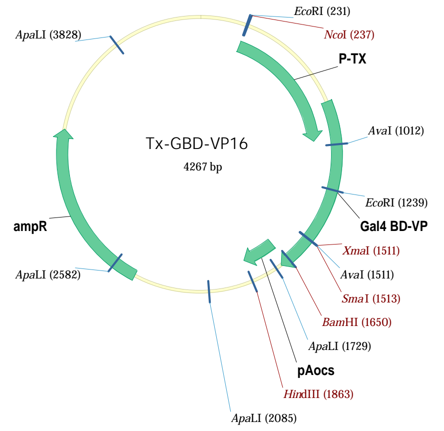


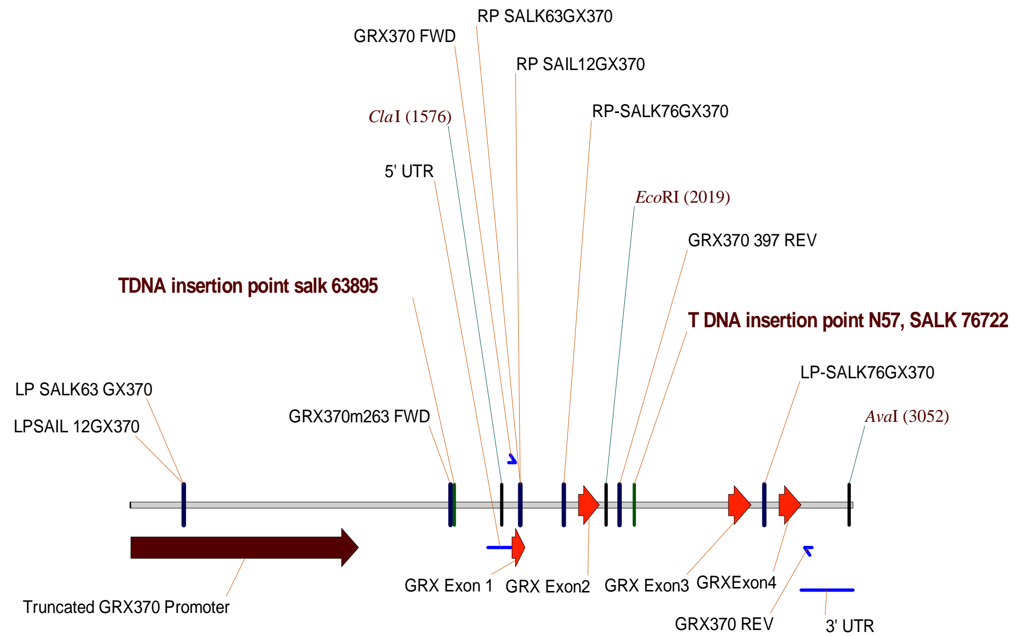
10.1.25 pENTRY201/TGA2



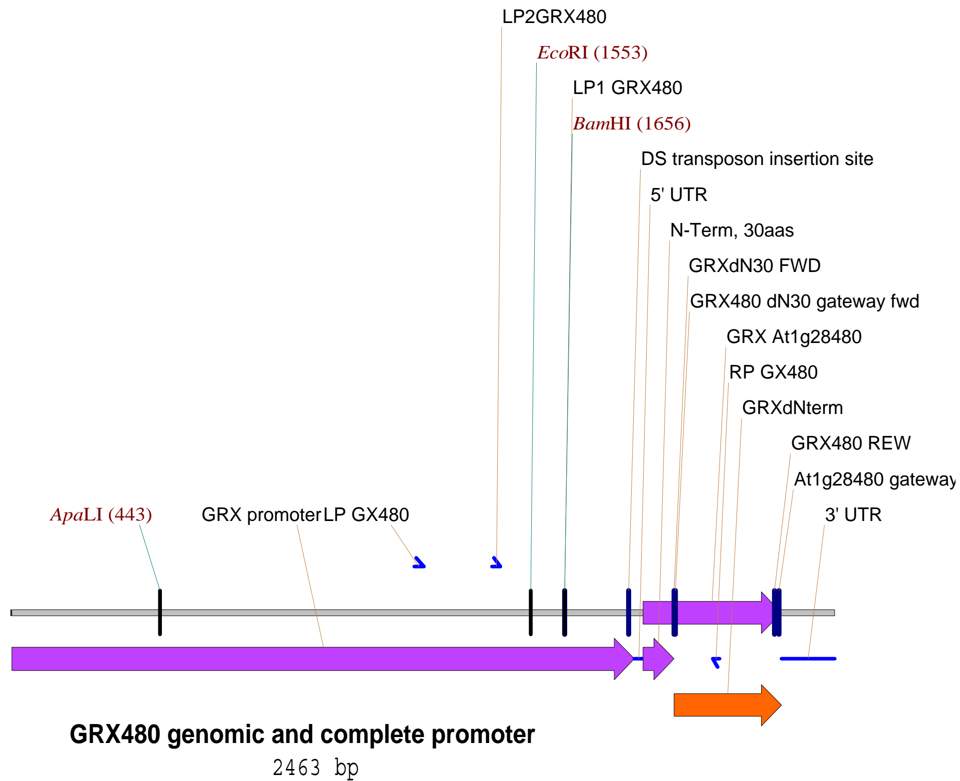
10.1.26 Tx-Gal4BD-VP16AD



10.1.27 GRX370 Full Length Genomic Sequence Map with Promoter, Introns, Exons, and Primers for Analysis of Knockout Lines



10.1.28 GRX480 Genomic Sequence Map of promoter and coding sequence.



10.2 Sequences and alignments.

10.2.1 GRX480 nucleotide sequence of promoter and coding sequence, showing primer binding sites.

```
   1  ATAGCAAAAC GCATCACCTG CGGTTGGCTT TGAGATTCGC TGAAAGTTTA TTTTTGGGCT ACTGGAGAAT CTTATAAAGC
  81  CCAATAAGAG ACCACAGAGC CCAATAAGTT ATTACAAATC TACGACAGTA TTAATAAAGA CCATTTTCTA CGTTACAAAA
 161  TTTTGATTAT TAACTGTTAT AGAAAATGGT TATATTGAAA CGCATATTTG GCATTTGTTA TAGAAGAAAG AAACCACTCT
 241  TGCTGAGTTT TTGTTGGTTA AAGCGTGACC TAAGTCTGTC TATTGAGAAG ATTCATAGTC CATGAAAAAT CCGATGCTAC
 321  TATAAATGAT TGTTTACTAA TCCTAGAAAC CAAAAACAAA TAATTGTTTT CTCAATTCAA AAATTGTATG GTAAAATTTT
                                                 ApaLI
                                                 ~~~~~~
 401  AGGGTTTACA CATTTAAGAT TTTAAGTGTT ATAGATTTCC GGTGCACAGA AGAATGGTAT ATATTGTGAG TTTGTGATTA
 481  GTTTTGTTAT GCAAACTTCT CACGTTAGGC AACTGTAAAT ATAATATCTC CAACAAAGCG GTCATGCAAT TTGTTTTGTT
 561  GAAAATTTGT ATATTTTACT AGCGGTCATG AATTTTTAAA TCCCCCACAC TCTATTTTTT AAAATATTTT TAACGTTTTG
 641  GGACCATGAT GGATGTTGAA CTTTCATTTC AGGATTTGGT GGAACGATAA AATTGGAAAG GCTTTGGAGT TTGGAACCTG
 721  AAGAACTTAT AAAGTTAAAG GATGACACGT GACTTTAATA AAGAAATGCA ATTCCCATAA TCATATAATA GTCCAAATTT
 801  AGGAAGAAAT GGGTGACATG AGTTTTAAAG AAGAAAGACG TCATGATTCC ATTAAAACAA ACCAAATTAG TTGGTGGCTT
 881  GGTGCTGACG TCTACCGACT TTTCCACCGT CCATCCTTCG ACGATGGTTC TTTCTAAATG ACCTTCTCAG TTTTTTCCTG
 961  TCATGCGTAT TATTATTATT AGTTAATAAG GCTCCACTAC TACTCGGAAA GTCCGAGTAC AAATAATATG ACAATAATGT
1041  CAAACTTATT CTTAGTATTG GTTTAAAAGA AGGATTCAAC AAGAGTACTT AATCACGTCT CATAATTACC ACTTTCATCC
1121  AAACATGGTC ATTACTATAT GACATGATCG TGTTACATTA ATTTGGAAAG TAGTATATGT CTTTTTCCTA AAAGTTTATG
                      LP GX480
          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~>
1201  TTTAGATTTT TGATCACTAA GAATATAATG TCATATATAT TATTAACCTT TACACCTAAA TGTTATATTA ATTACTTGAA
1281  TGATAAAACG GAAAATATCG AAAGTTTAGG CTAGGTCTCT CTAGCATCTC TAGTGTATTT ATTTAGCAAT CCCAAAACAA
                                                                                        ~~
1361  AATTGTGTTT TCTACAATTT TAGTATTAGA TTACCAAAAT TGTTGTATAT AGTCTGGTGA TTCCGTTTTG ATGGTCGAGA
            LP2GRX480
      ~~~~~~~~~~~~~~~~~~~~~~~~>
1441  TCGAAAGAAG CAAGCATCCA CTATGTGGTC GTCACATGCG AATATTTCTA AGTATACAAC CATCTATCGA AAGTTTCGAA
                                       EcoRI
                                       ~~~~~~
1521  ACAACCCAAA TATAATAGTA ATAAATTCTG AGAATTCTCC TATTTTTTAT TGATATGAAA AAAGCAGTAA AATATTATGT
                                                                           LP1 GRX480
                                                                      ~~~~~~~~~~~~~~~~~~~~~~~>
                                                                      BamHI
                                                                      ~~~~~~~
1601  AAAGTAGAGA GGAACCAAAA TTAAAACGTG CAGATTTATT AAAAGAATAA AGTGGGATCC AAAAAGTCAG CGTGAAACAT
1681  GTGATACGAT ATAACGACAC GGTCCTATGA CTAAGTCCAC TCCAATCCAG TGACGTAAAC AGCACCATCA CCCATAGCTT
1761  CCTGTGACGC ACATCCTTAC GTAACCATCG TTGACGCTAG ACTTTCCTCT CTGATCTCTC TTTCTTCATG TATATATAAC
            DS transposon insertion site
            ~
                                          5' UTR
                            ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                                         M   Q  G  T   I  S  C   A  R  N  Y
      .
1841  AAAACCTTCC TTTCCTAATT GGTATCTATC TTTAAAAACA TACTTGAAAA TGCAAGGAAC GATTTCTTGT GCAAGAAATT
                                                                           GRX480 dN30 gateway fwd
                                                                                 ~~~~~~~~~~~~~~~~
                                                                                 GRXdN30 FWD
                                                                              ~~~~~~~~~~~~~~~~~~~~
      Y  N  M  T   T  T  V   G  E  S  L   R  P  L   S  L  K   T  Q  G  N   G  E  R   V  R  M
1921  ATAACATGAC GACAACCGTC GGGGAATCTC TGCGGCCGCT ATCGCTTAAA ACGCAGGGAA ACGGCGAGAG AGTTCGGATG
      GRX480 dN30 gateway fwd
      ~~~~>
      GRXdN30 FWD
      ~>
       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
2001  GTGGTGGAGG AGAACGCGGT GATTGTGATT GGACGGAGAG GATGTTGCAT GTGTCATGTG GTGAGGAGGC TGCTTCTTGG
      G 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
2081  ACTTGGAGTG AATCCGGCGG TCCTTGAGAT TGATGAGGAG AGGGAAGATG AAGTTTTGAG TGAGTTGGAG AATATTGGAG
                        ~~~~~~~~~~~~~~~~~~~~~~~~>
                              RP GX480
      V  Q  G  G   G  G  T   V  K  L  P   A  V  Y   V  G  G   R  L  F  G   G  L  D   R  V  M
2161  TTCAAGGCGG CGGAGGTACG GTGAAGTTAC CGGCGGTTTA TGTAGGAGGG AGGTTGTTTG GAGGGTTAGA TAGGGTTATG
       A  T  H  I   S  G  E   L  V  P   I  L  K  E   V  G  A   L  W  L   *
2241  GCTACTCATA TCTCCGGTGA GTTAGTTCCA ATTCTTAAGG AAGTTGGGGC TCTGTGGTTG TGATTGTAAA TTAATAATTT
                                                   <-~~~~~~~~~~~~~~~~~~~~~~
                                                         GRX480 REW
                                                                <-~~~~~~~~~~~~~~~~~~~~~
                                                                At1g28480 gateway bck
2321  AAAATTATTT TTTTTTCTTT TAATTAAGAA TCTTGATTGG TAATTGTTGT TTACGGTTTA TAATTGAATC GTTTCATATA
2401  TATGTATATA AAGAAATAAA TAAAAGAAAA GTCTCAAGTT GAAATTTGCT AGAGATTGTA CCC
```

10.2.2 GRX480 double mutant full length coding sequence

```
          M  Q  G  T   I  S  C   A  R  N   Y  N  M  T   T  T  V   G  E  S   L  R  P  L ·
   1     ATGCAAGGAA CGATTTCTTG TGCAAGAAAT TATAACATGA CGACAACCGT CGGGGAATCT CTGCGGCCGC
         TACGTTCCTT GCTAAAGAAC ACGTTCTTTA ATATTGTACT GCTGTTGGCA GCCCCTTAGA GACGCCGGCG
         ·  S  L  K   T  Q  G   N  G  E  R   V  R  M   V  V  E   E  N  A  V   I  V  I ·
  71     TATCGCTTAA AACGCAGGGA AACGGCGAGA GAGTTCGGAT GGTGGTGGAG GAGAACGCGG TGATTGTGAT
         ATAGCGAATT TTGCGTCCCT TTGCCGCTCT CTCAAGCCTA CCACCACCTC CTCTTGCGCC ACTAACACTA
         · G  R  R   G  S  C  M   S  H  V   V  R  R   L  L  L  G   L  G  V   N  P  A
 141     TGGACGGAGA GGATCTTGCA TGTCTCATGT GGTGAGGAGG CTGCTTCTTG GACTTGGAGT GAATCCGGCG
         ACCTGCCTCT CCTAGAACGT ACAGAGTACA CCACTCCTCC GACGAAGAAC CTGAACCTCA CTTAGGCCGC
          V  L  E  I   D  E  E   R  E  D   E  V  L  S   E  L  E   N  I  G   V  Q  G  G ·
 211     GTCCTTGAGA TTGATGAGGA GAGGGAAGAT GAAGTTTTGA GTGAGTTGGA GAATATTGGA GTTCAAGGCG
         CAGGAACTCT AACTACTCCT CTCCCTTCTA CTTCAAAACT CACTCAACCT CTTATAACCT CAAGTTCCGC
         ·  G  G  T   V  K  L   P  A  V  Y   V  G  G   R  L  F   G  G  L  D   R  V  M ·
 281     GCGGAGGTAC GGTGAAGTTA CCGGCGGTTT ATGTAGGAGG GAGGTTGTTT GGAGGGTTAG ATAGGGTTAT
         CGCCTCCATG CCACTTCAAT GGCCGCCAAA TACATCCTCC CTCCAACAAA CCTCCCAATC TATCCCAATA
         · A  T  H   I  S  G  E   L  V  P   I  L  K   E  V  G  A   L  W  L   *
 351     GGCTACTCAT ATCTCCGGTG AGTTAGTTCC AATTCTTAAG GAAGTTGGGG CTCTGTGGTT GTGA
         CCGATGAGTA TAGAGGCCAC TCAATCAAGG TTAAGAATTC CTTCAACCCC GAGACACCAA CACT
```

10.2.3 GRX480 ΔN30 mutant (N-terminal deletion derivative) coding sequence

```
          M  G  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 ·
   1     ATGGGCGAGA GAGTTCGGAT GGTGGTGGAG GAGAACGCGG TGATTGTGAT TGGACGGAGA GGATGTTGCA TGTGTCATGT
         TACCCGCTCT CTCAAGCCTA CCACCACCTC CTCTTGCGCC ACTAACACTA ACCTGCCTCT CCTACAACGT ACACAGTACA
         · V  R  R   L  L  L  G   L  G  V   N  P  A   V  L  E  I   D  E  E   R  E  D   E  V  L  S ·
  81     GGTGAGGAGG CTGCTTCTTG GACTTGGAGT GAATCCGGCG GTCCTTGAGA TTGATGAGGA GAGGGAAGAT GAAGTTTTGA
         CCACTCCTCC GACGAAGAAC CTGAACCTCA CTTAGGCCGC CAGGAACTCT AACTACTCCT CTCCCTTCTA CTTCAAAACT
         ·  E  L  E   N  I  G   V  Q  G  G   G  G  T   V  K  L   P  A  V  Y   V  G  G   R  L  F
 161     GTGAGTTGGA GAATATTGGA GTTCAAGGCG GCGGAGGTAC GGTGAAGTTA CCGGCGGTTT ATGTAGGAGG GAGGTTGTTT
         CACTCAACCT CTTATAACCT CAAGTTCCGC CGCCTCCATG CCACTTCAAT GGCCGCCAAA TACATCCTCC CTCCAACAAA
          G  G  L  D   R  V  M   A  T  H   I  S  G  E   L  V  P   I  L  K   E  V  G  A   L  W  L ·
 241     GGAGGGTTAG ATAGGGTTAT GGCTACTCAT ATCTCCGGTG AGTTAGTTCC AATTCTTAAG GAAGTTGGGG CTCTGTGGTT
         CCTCCCAATC TATCCCAATA CCGATGAGTA TAGAGGCCAC TCAATCAAGG TTAAGAATTC CTTCAACCCC GAGACACCAA
         · *
 321     GTGA
         CACT
```

10.2.4 GRX370 coding sequence of cDNA

```
          M  A  M  Q   K  A  K   E  I  V   N  S  E  S   V  V  V   F  S  K
   1     ATGGCGATGC AGAAAGCTAA GGAGATCGTT AACAGCGAAT CAGTCGTTGT TTTCAGCAAG
         TACCGCTACG TCTTTCGATT CCTCTAGCAA TTGTCGCTTA GTCAGCAACA AAAGTCGTTC
          T  Y  C  P   Y  C   V   R  V  K   E  L  L  Q   Q  L  G   A  K  F
  61     ACTTATTGTC CATATTGCGT GAGAGTGAAG GAGCTTTTGC AACAATTGGG AGCTAAGTTC
         TGAATAACAG GTATAACGCA CTCTCACTTC CTCGAAAACG TTGTTAACCC TCGATTCAAG
          K  A  V  E   L  D  T   E  S  D   G  S  Q  I   Q  S  G   L  A  E
 121     AAGGCCGTTG AGCTCGACAC CGAAAGTGAT GGTAGCCAAA TTCAATCAGG TCTCGCAGAA
         TTCCGGCAAC TCGAGCTGTG GCTTTCACTA CCATCGGTTT AAGTTAGTCC AGAGCGTCTT
          W  T  G  Q   R  T  V   P  N  V   F  I  G  G   N  H  I   G  G  C
 181     TGGACAGGAC AACGTACCGT GCCTAATGTG TTTATAGGAG GAAATCACAT CGGTGGCTGT
         ACCTGTCCTG TTGCATGGCA CGGATTACAC AAATATCCTC CTTTAGTGTA GCCACCGACA
          D  A  T  S   N  L  H   K  D  G   K  L  V  P   L  L  T   E  A  G
 241     GATGCAACAT CAAACTTGCA TAAAGATGGG AAGTTGGTTC CGCTGTTAAC TGAAGCTGGA
         CTACGTTGTA GTTTGAACGT ATTTCTACCC TTCAACCAAG GCGACAATTG ACTTCGACCT
          A  I  A  G   K  T  A   T  T  S   A  *
 301     GCGATCGCAG GAAAGACTGC AACAACTTCT GCTTAA
         CGCTAGCGTC CTTTCTGACG TTGTTGAAGA CGAATT
```

10.2.5 Alligator/GRX480 Essential Regions

```
            AvaI
           ~~~~~~~
   1     TTGATCCCGA GGGGAACCCT GTGGTTGGCA TGCACATACA AATGGACGAA CGGATAAACC TTTTCACGCC CTTTTAAATA
                                                                              RB
                                                                              ~
  81     TCCGTTATTC TAATAAACGC TCTTTTCTCT TAGGTTTACC CGCCAATATA TCCTGTCAAA CACTGATAGT TTAAACTGAA
                                                                                   2x p35S
                                                                   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                                  HindIII       PstI
                                                  ~~~~~~~     ~~~~~~~
 161     GGCGGGAAAC GACAATCTGA TCCAAGCTCA AGCTAAGCTT GCATGCCTGC AGGTCCGATT GAGACTTTTC AACAAAGGGT
                                                2x p35S
         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
 241     AATATCCGGA AACCTCCTCG GATTCCATTG CCCAGCTATC TGTCACTTTA TTGTGAAGAT AGTGGAAAAG GAAGGTGGCT
                                                2x p35S
         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
```

```
 321  CCTACAAATG CCATCATTGC GATAAAGGAA AGGCCATCGT TGAAGATGCC TCTGCCGACA GTGGTCCCAA AGATGGACCC
                                      2x p35S
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
 401  CCACCCACGA GGAGCATCGT GGAAAAAGAA GACGTTCCAA CCACGTCTTC AAAGCAAGTG GATTGATGTG ATGGTCCGAT
                                      2x p35S
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
 481  TGAGACTTTT CAACAAAGGG TAATATCCGG AAACCTCCTC GGATTCCATT GCCCAGCTAT CTGTCACTTT ATTGTGAAGA
                                      2x p35S
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
 561  TAGTGGAAAA GGAAGGTGGC TCCTACAAAT GCCATCATTG CGATAAAGGA AAGGCCATCG TTGAAGATGC CTCTGCCGAC
                                      2x p35S
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
 641  AGTGGTCCCA AAGATGGACC CCCACCCACG AGGAGCATCG TGGAAAAAGA AGACGTTCCA ACCACGTCTT CAAAGCAAGT
              35SI primer
        ~~~~~~~~~~~~~~~~~~~~~~~~~
                                      2x p35S
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
 721  GGATTGATGT GATATCTCCA CTGACGTAAG GGATGACGCA CAATCCCACT ATCCTTCGCA AGACCCTTCC TCTATATAAG
                                                                               3x HA
                                                         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                        2x p35S
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                                          M  A  Y   P  Y  D  V   P  D  Y ·
 801  GAAGTTCATT TCATTTGGAG AGGACACGCT GACAAGCTGA CTCTAGTAAA AATGGCATAC CCATACGACG TTCCGGACTA
                                       3x HA
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
      · A  S  L   G  G  S  S   P  S  S   E  L  H   R  G  G  G   R  I  F   Y  P  Y   D  V  P  D ·
 881  CGCTTCTTTG GGTGGTTCTA GCCCAAGCTC AGAGCTCCAC CGCGGTGGCG GCCGCATCTT TTACCCATAC GATGTTCCTG
                         3x HA
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                                                                   SspBI
                                                                                   ~~~~~~
      ·  Y  A  G   Y  P  Y   D  V  P  D   Y  A  D   I  S  R   Q  I  T  S   L  Y  K   K  A  G
 961  ACTATGCGGG CTATCCCTAT GACGTCCCGG ACTATGCAGA TATCTCTAGG CAGATCACAA GTTTGTACAA AAAAGCAGGC
                                        GRX
         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       S  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 ·
1041  TCCATGCAAG GAACGATTTC TTGTGCAAGA AATTATAACA TGACGACAAC CGTCGGGGAA TCTCTGCGGC CGCTATCGCT
                                      GRX
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
      · K  T  Q   G  N  G  E   R  V  R   M  V  V   E  E  N  A   V  I  V   I  G  R   R  G  C  C ·
1121  TAAAACGCAG GGAAACGGCG AGAGAGTTCG GATGGTGGTG GAGGAGAACG CGGTGATTGT GATTGGACGG AGAGGATGTT
                                      GRX
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
      ·  M  C  H   V  V  R   R  L  L  L   G  L  G   V  N  P   A  V  L  E   I  D  E   E  R  E
1201  GCATGTGTCA TGTGGTGAGG AGGCTGCTTC TTGGACTTGG AGTGAATCCG GCGGTCCTTG AGATTGATGA GGAGAGGGAA
                                                         ~~~~~~~~~~~~~~~~~~~~~~~
                                                                RPGx480
                                      GRX
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                SspI
                               ~~~~~~~
       D  E  V  L   S  E  L   E  N  I   G  V  Q  G   G  G  G   T  V  K   L  P  A  V   Y  V  G ·
1281  GATGAAGTTT TGAGTGAGTT GGAGAATATT GGAGTTCAAG GCGGCGGAGG TACGGTGAAG TTACCGGCGG TTTATGTAGG
                                      GRX
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
      · G  R  L   F  G  G  L   D  R  V   M  A  T   H  I  S  G   E  L  V   P  I  L   K  E  V  G ·
1361  AGGGAGGTTG TTTGGAGGGT TAGATAGGGT TATGGCTACT CATATCTCCG GTGAGTTAGT TCCAATTCTT AAGGAAGTTG
              GRX                                                                        nosT
      ~~~~~~~~~~~~~~~~~~                                                                 ~~~~
                                                 SspBI                            PstI
                                                 ~~~~~~                           ~~~~~~
      ·  A  L  W   L  *
1441  GGGCTCTGTG GTTGTGATTG TAAATTAATG ACCCAGCTTT CTTGTACAAA GTGGTGATCT GACGCCTCGA CCTGCAGATC
                                                 nos primer
                                              ~~~~~~~~~~~~~~~~~~~~~~~
                                       nosT
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
1521  GTTCAAACAT TTGGCAATAA AGTTTCTTAA GATTGAATCC TGTTGCCGGT CTTGCGATGA TTATCATATA ATTTCTGTTG
                                       nosT
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
1601  AATTACGTTA AGCATGTAAT AATTAACATG TAATGCATGA CGTTATTTAT GAGATGGGTT TTTATGATTA GAGTCCCGCA
                                       nosT
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
1681  ATTATACATT TAATACGCGA TAGAAAACAA AATATAGCGC GCAAACTAGG ATAAATTATC GCGCGCGGTG TCATCTATGT
          nosT
      ~~~~~~~~~~~~~
                SmaI
               ~~~~~~~
                XmaI
               ~~~~~~~
                AvaI
               ~~~~~~~
1761  TACTAGATCC CCGGGTACCA CTGGATTTTT TGGTTTTAGG AATTAGAAAT TTTATTGATA GAAGTATTTT ACAAATACAA
                                                                                  35ST
                                                                        ~~~~~~~~~~~~~~~~~~~~~~
1841  ATACATACTA AGGGTTTCTT ATATGCTCAA CACATGAGCG AAACCCTATA AGAACCCTAA TTCCCTTATC TGGGAACTAC
                                       35ST
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
1921  TCACACATTA TTATAGAGAG AGATAGATTT GTAGAGAGAG ACTGGTGATT TCAGCGATGC AGCCGGGCGG CGCTTACAGC
                                                                                           GFP
```

```
                                  35ST
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
             SspBI
             ~~~~~~~
2001  TCGTCCTTGT ACAGCTCGTC CATGCCGAGA GTGATCCCGG CGGCGGTCAC GAACTCCAGC AGGACCATGT GATCGCGCTT
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                           GFP
2081  CTCGTTGGGG TCTTTGCTCA GGGCGGACTG GGTGCTCAGG TAGTGGTTGT CGGGCAGCAG CACGGGGCCG TCGCCGATGG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                           GFP
2161  GGGTGTTCTG CTGGTAGTGG TCGGCGAGCT GCACGCTGCC GTCCTCGATG TTGTGGCGGA TCTTGAAGTT CACCTTGATG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                           GFP
2241  CCGTTCTTCT GCTTGTCGGC CATGATATAG ACGTTGTGGC TGTTGTAGTT GTACTCCAGC TTGTGCCCCA GGATGTTGCC
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                           GFP
2321  GTCCTCCTTG AAGTCGATGC CCTTCAGCTC GATGCGGTTC ACCAGGGTGT CGCCCTCGAA CTTCACCTCG GCGCGGGTCT
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                           GFP
2401  TGTAGTTGCC GTCGTCCTTG AAGAAGATGG TGCGCTCCTG GACGTAGCCT TCGGGCATGG CGGACTTGAA GAAGTCGTGC
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                           GFP
2481  TGCTTCATGT GGTCGGGGTA GCGGCTGAAG CACTGCACGC CGTAGGTCAG GGTGGTCACG AGGGTGGGCC AGGGCACGGG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                           GFP
2561  CAGCTTGCCG GTGGTGCAGA TGAACTTCAG GGTCAGCTTG CCGTAGGTGG CATCGCCCTC GCCCTCGCCG GACACGCTGA
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                           GFP
2641  ACTTGTGGCC GTTTACGTCG CCGTCCAGCT CGACCAGGAT GGGCACCACC CCGGTGAACA GCTCCTCGCC CTTGCTCACC
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                           GFP
                                                                          NcoI
                                                                         ~~~~~~~
2721  ATGTCGGCCG AGGATAATGA TAGGAGAAGT GAAAAGATGA AAAAGAGAAA AAGATTAGTC TTCACCATGG CTATCGTTCG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                     GFP
2801  TAAATGGTGA AAATTTTCAG AAAATAGCTT TTGCTTTAAA AGAAATGATT TAAATTGCTG CAATAGAAGT AGAATGCTTG
                                                EcoRI
                                                ~~~~~~~
2881  ATTGCTTGAG ATTCGTTTGT TTTGTATATG TTGTGTTGAG AATTCGCCCT TGTTTTGCTA TTTGTGTATG TTTTCTTGTT
                                                  ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                                                   pAt1S3
2961  TTGGTTTGAG TGAAGAGTGT AGTGAAGGGT TGGTTTGTAT TTATAGGTGA GAAAGGAGAT TTGCATGGCA ATCACGTGTA
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                           pAt1S3
3041  AGAATGCATG CATGCATGAG TGTGTGTGGA GAGAGAGAGA TGCTCTTACG TTGTTGTCGG GATTATGTGT TGGAAAAGAG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                           pAt1S3
3121  TGACAAGTGA CGAACAAAAC AATACCACTC ATCATAGCTC CGCTTATGCA TGCTATGTGT GATTTGTAGC TCTTGTTCTG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                           pAt1S3
3201  ACATGACACT ATGAACAATT TTTTAAAAAG AAGGTTAAAC TTTTATTTAA ATAAAAACCC TATGTTAATT TGGTTTCAAG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                           pAt1S3
         EcoRI
         ~~~~~~
3281  GGCGAATTCA ATTCGGCGTT AATTCAGTAC ATTAAAAACG TCCGCAATGT GTTATTAAGT TGTCTAAGCG TCAATTTGTT
      ~~~~
      ....
                  LB
                  ~
3361  TACACCACAA TATATCCTGC CACCAGCCAG CCAACAGCTC CCCGACCGGC AGCTCGGCAC AAAATCACCA CTCGATACAG
```

10.2.6 Alligator/GRX480 Ohne HA (Without HA) - Essential Regions

```
               2x p35S'                                                   GRX
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~                        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                                            M  Q  G   T  I  S  C   A  R  N ·
 801  GAAGTTCATT TCATTTGGAG AGGACACGCT GACAAGCTGA CTCTAGTAAA AATGCAAGGA ACGATTTCTT GTGCAAGAAA
                                           GRX
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
      · Y  N  M   T  T  T  V   G  E  S   L  R  P   L  S  L  K   T  Q  G   N  G  E   R  V  R  M ·
 881  TTATAACATG ACGACAACCG TCGGGGAATC TCTGCGGCCG CTATCGCTTA AAACGCAGGG AAACGGCGAG AGAGTTCGGA
                                           GRX
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
      ·  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
 961  TGGTGGTGGA GGAGAACGCG GTGATTGTGA TTGGACGGAG AGGATGTTGC ATGTGTCATG TGGTGAGGAG GCTGCTTCTT
                                           GRX
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       G  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 ·
1041  GGACTTGGAG TGAATCCGGC GGTCCTTGAG ATTGATGAGG AGAGGGAAGA TGAAGTTTTG AGTGAGTTGG AGAATATTGG
                                ~~~~~~~~~~~~~~~~~~~~~
                                      RPGx480
                                           GRX
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
```

```
      ·V  Q  G   G  G  G  T   V  K  L   P  A  V   Y  V  G  G   R  L  F   G  G  L   D  R  V  M·
1121  AGTTCAAGGC GGCGGAGGTA CGGTGAAGTT ACCGGCGGTT TATGTAGGAG GGAGGTTGTT TGGAGGGTTA GATAGGGTTA
                                  GRX
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
      · A  T  H   I  S  G   E  L  V  P   I  L  K   E  V  G   A  L  W  L   *
1201  TGGCTACTCA TATCTCCGGT GAGTTAGTTC CAATTCTTAA GGAAGTTGGG GCTCTGTGGT TGTGATTGTA AATTAATGAC
                                                                   nosT
                                                      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                 SspBI                            PstI
                 ~~~~~~                          ~~~~~~~
1281  CCAGCTTTCT TGTACAAAGT GGTGATCTGA CGCCTCGACC TGCAGATCGT TCAAACATTT GGCAATAAAG TTTCTTAAGA
```

10.2.7 pDEST-GBKT7-myc/GRX370 - Essential Regions

```
         SspBI            BamHI          PstI
         ~~~~~~           ~~~~~~~        ~~~~~~~
   1  CTTGTACAAA GTGGTGGGGA TCCGTCGACC TGCAGCGGCC GCATAACTAG CATAACCCCT TGGGGCCTCT AAACGGGTCT
      ~~~~~~~~~~~~~~~
          attB2
                                                 TRP1
                          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                           M   S  V  I   N  F  T   G  S  S  G   P  L  V   K  V  C   G  L  Q  S·
4721                      A TGTCTGTTAT TAATTTCACA GGTAGTTCTG GTCCATTGGT GAAAGTTTGC GGCTTGCAGA
                                        TRP1
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
      · T  E  A   A  E  C   A  L  D  S   D  A  D   L  L  G   I  I  C  V   P  N  R   K  R  T
4801  GCACAGAGGC CGCAGAATGT GCTCTAGATT CCGATGCTGA CTTGCTGGGT ATTATATGTG TGCCCAATAG AAAGAGAACA
                                        TRP1
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       I  D  P  V   I  A  R   K  I  S   S  L  V  K   A  Y  K   N  S  S   G  T  P  K   Y  L  V·
4881  ATTGACCCGG TTATTGCAAG GAAAATTTCA AGTCTTGTAA AAGCATATAA AAATAGTTCA GGCACTCCGA AATACTTGGT
                                        TRP1
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
      ·G  V  F   R  N  Q  P   K  E  D   V  L  A   L  V  N  D   Y  G  I   D  I  V   Q  L  H  G·
4961  TGGCGTGTTT CGTAATCAAC CTAAGGAGGA TGTTTTGGCT CTGGTCAATG ATTACGGCAT TGATATCGTC CAACTGCATG
                                        TRP1
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
      · D  E  S   W  Q  E   Y  Q  E  F   L  G  L   P  V  I   K  R  L  V   F  P  K   D  C  N
5041  GAGATGAGTC GTGGCAAGAA TACCAAGAGT TCCTCGGTTT GCCAGTTATT AAAAGACTCG TATTTCCAAA AGACTGCAAC
                                        TRP1
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       I  L  L  S   A  A  S   Q  K  P   H  S  F  I   P  L  F   D  S  E   A  G  G  T   G  E  L·
5121  ATACTACTCA GTGCAGCTTC ACAGAAACCT CATTCGTTTA TTCCCTTGTT TGATTCAGAA GCAGGTGGGA CAGGTGAACT
                                        TRP1
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                                                 HindIII
                                                                 ~~~~~~
      ·L  D  W   N  S  I  S   D  W  V   G  R  Q   E  S  P  E   S  L  H   F  M  L   A  G  G  L·
5201  TTTGGATTGG AACTCGATTT CTGACTGGGT TGGAAGGCAA GAGAGCCCCG AAAGCTTACA TTTTATGTTA GCTGGTGGAC
                                        TRP1
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
      · T  P  E   N  V  G   D  A  L  R   L  N  G   V  I  G   V  D  V  S   G  G  V   E  T  N
5281  TGACGCCAGA AAATGTTGGT GATGCGCTTA GATTAAATGG CGTTATTGGT GTTGATGTAA GCGGAGGTGT GGAGACAAAT
                                TRP1
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       G  V  K  D   S  N  K   I  A  N   F  V  K  N   A  K  K   *
5361  GGTGTAAAAG ACTCTAACAA AATAGCAAAT TTCGTCAAAA ATGCTAAGAA ATAGGTTATT ACTGAGTAGT ATTTATTTAA
              ApaLI
              ~~~~~~~
5441  GTATTGTTTG TGCACTTGCC GATCTATGCG GTGTGAAATA CCGCACAGAT GCGTAAGGAG AAAATACCGC ATCAGGAAAT
5521  TGTAAACGTT AATATTTTGT TAAAATTCGC GTTAAATTTT TGTTAAATCA GCTCATTTTT TAACCAATAG GCCGAAATCG
5601  GCAAAATCCC TTATAAATCA AAAGAATAGA CCGAGATAGG GTTGAGTGTT GTTCCAGTTT GGAACAAGAG TCCACTATTA
5681  AAGAACGTGG ACTCCAACGT CAAAGGGCGA AAAACCGTCT ATCAGGGCGA TGGCCCACTA CGTGAACCAT CACCCTAATC
5761  AAGTTTTTTG GGGTCGAGGT GCCGTAAAGC ACTAAATCGG AACCCTAAAG GGAGCCCCCG ATTTAGAGCT TGACGGGGAA
5841  AGCCGGCGAA CGTGGCGAGA AAGGAAGGGA AGAAAGCGAA AGGAGCGGGC GCTAGGGCGC TGGCAAGTGT AGCGGTCACG
5921  CTGCGCGTAA CCACCACACC CGCCGCGCTT AATGCGCCGC TACAGGGCGC GTCCATTCGC CATTCAGGCT GCGCAACTGT
                                                              ADH-Promoter
                                                ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                                                          SspBI
                                                                          ~~~~~~
6001  TGGGAAGGGC GATCGGTGCG GGCCTCTTCG CTATTACGCC AGATCCTTTT GTTGTTTCCG GGTGTACAAT ATGGACTTCC
                                       ADH-Promoter
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
6081  TCTTTTCTGG CAACCAAACC CATACATCGG GATTCCTATA ATACCTTCGT TGGTCTCCCT AACATGTAGG TGGCGGAGGG
                                       ADH-Promoter
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
6161  GAGATATACA ATAGAACAGA TACCAGACAA GACATAATGG GCTAAACAAG ACTACACCAA TTACACTGCC TCATTGATGG
                                       ADH-Promoter
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
6241  TGGTACATAA CGAACTAATA CTGTAGCCCT AGACTTGATA GCCATCATCA TATCGAAGTT TCACTACCCT TTTTCCATTT
                                       ADH-Promoter
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
6321  GCCATCTATT GAAGTAATAA TAGGCGCATG CAACTTCTTT TCTTTTTTTT TCTTTTCTCT CTCCCCCGTT GTTGTCTCAC
                                       ADH-Promoter
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
6401  CATATCCGCA ATGACAAAAA AAATGATGGA AGACACTAAA GGAAAAAATT AACGACAAAG ACAGCACCAA CAGATGTCGT
                                       ADH-Promoter
```

```
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
6481   TGTTCCAGAG CTGATGAGGG GTATCTCGAA GCACACGAAA CTTTTTCCTT CCTTCATTCA CGCACACTAC TCTCTAATGA
                                         ADH-Promoter
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
6561   GCAACGGTAT ACGGCCTTCC TTCCAGTTAC TTGAATTTGA AATAAAAAAA GTTTGCTGTC TTGCTATCAA GTATAAATAG
                                         ADH-Promoter
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
6641   ACCTGCAATT ATTAATCTTT TGTTTCCTCG TCATTGTTCT CGTTCCCTTT CTTCCTTGTT TCTTTTTCTG CACAATATTT
                ADH-Promoter                                                Gal4-BD
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~                          ~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                      HindIII
                                      ~~~~~~~
                                                                  M  K   L  L  S  S   I  E  Q ·
6721   CAAGCTATAC CAAGCATACA ATCAACTCCA AGCTTGAAGC AAGCCTCCTG AAAGATGAAG CTACTGTCTT CTATCGAACA
                                           Gal4-BD
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       · A  C  D   I  C  R  L   K  K  L   K  C  S   K  E  K  P   K  C  A   K  C  L   K  N  N  W ·
6801   AGCATGCGAT ATTTGCCGAC TTAAAAAGCT CAAGTGCTCC AAAGAAAAAC CGAAGTGCGC CAAGTGTCTG AAGAACAACT
                                           Gal4-BD
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       ·  E  C  R   Y  S  P   K  T  K  R   S  P  L   T  R  A   H  L  T  E   V  E  S   R  L  E
6881   GGGAGTGTCG CTACTCTCCC AAAACCAAAA GGTCTCCGCT GACTAGGGCA CATCTGACAG AAGTGGAATC AAGGCTAGAA
                                           Gal4-BD
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                         AvaI
                                         ~~~~~~
        R  L  E  Q   L  F  L   L  I  F   P  R  E  D   L  D  M   I  L  K   M  D  S  L   Q  D  I ·
6961   AGACTGGAAC AGCTATTTCT ACTGATTTTT CCTCGAGAAG ACCTTGACAT GATTTTGAAA ATGGATTCTT TACAGGATAT
                                           Gal4-BD
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                 SspBI
                                 ~~~~~~~
       · K  A  L   L  T  G  L   F  V  Q   D  N  V   N  K  D  A   V  T  D   R  L  A   S  V  E  T ·
7041   AAAAGCATTG TTAACAGGAT TATTTGTACA AGATAATGTG AATAAAGATG CCGTCACAGA TAGATTGGCT TCAGTGGAGA
                                           Gal4-BD
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       ·  D  M  P   L  T  L   R  Q  H  R   I  S  A   T  S  S   S  E  E  S   S  N  K   G  Q  R
7121   CTGATATGCC TCTAACATTG AGACAGCATA GAATAAGTGC GACATCATCA TCGGAAGAGA GTAGTAACAA AGGTCAAAGA
           Gal4-BD
       ~~~~~~~~~~~~~~~
        Q  L  T  V   S  P  E   F  V  I   R  L  T  I   G  R  A   A  I  M   E  E  Q  K   L  I  S ·
7201   CAGTTGACTG TATCGCCGGA ATTTGTAATA CGACTCACTA TAGGGCGAGC CGCCATCATG GAGGAGCAGA AGCTGATCTC
                                                                    attB1               GRX370
                                                     ~~~~~~~~~~~~~~~~~~~~~~~   ~~~~~~~~~~~~~~
                              NcoI        EcoRI               SspBI
                             ~~~~~~      ~~~~~~              ~~~~~~~
       · E  E  D   L  H  M  A   M  E  A   E  F  P   S  Q  V  C   T  K  K   Q  A  M   A  M  Q  K ·
7281   AGAGGAGGAC CTGCATATGG CCATGGAGGC CGAATTCCCA TCACAAGTTT GTACAAAAAA GCAGGCTATG GCGATGCAGA
                                            GRX370
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       ·  A  K  E   I  V  N   S  E  S  V   V  V  F   S  K  T   Y  C  P  Y   C  V  R   V  K  E
7361   AAGCTAAGGA GATCGTTAAC AGCGAATCAG TCGTTGTTTT CAGCAAGACT TATTGTCCAT ATTGCGTGAG AGTGAAGGAG
                                            GRX370
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
        L  L  Q  Q   L  G  A   K  F  K   A  V  E  L   D  T  E   S  D  G   S  Q  I  Q   S  G  L ·
7441   CTTTTGCAAC AATTGGGAGC TAAGTTCAAG GCCGTTGAGC TCGACACCGA AAGTGATGGT AGCCAAATTC AATCAGGTCT
                                            GRX370
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       · A  E  W   T  G  Q  R   T  V  P   N  V  F   I  G  G  N   H  I  G   G  C  D   A  T  S  N ·
7521   CGCAGAATGG ACAGGACAAC GTACCGTGCC TAATGTGTTT ATAGGAGGAA ATCACATCGG TGGCTGTGAT GCAACATCAA
                                            GRX370
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       ·  L  H  K   D  G  K   L  V  P  L   L  T  E   A  G  A   I  A  G  K   T  A  T   T  S  A
7601   ACTTGCATAA AGATGGGAAG TTGGTTCCGC TGTTAACTGA AGCTGGAGCG ATCGCAGGAA AGACTGCAAC AACTTCTGCT
       GRX370
       ~~~
        *
7681   TAAACCCAGC TTT
             ~~~~~~~~
              attB2
```

10.2.8 pDEST-GAD-HA/TGA2 – Essential Regions

```
         SspBI                     ClaI      BamHI                  PstI
         ~~~~~~                   ~~~~~~    ~~~~~~                 ~~~~~~
   1   CTTGTACAAA GTGGTGGGTG GGCATCGATA CGGGATCCAT CGAGCTCGAG CTGCAGATGA ATCGTAGATA CTGAAAAACC
       ~~~~~~~~~~~~~~~
           attB2
                      pGADL2-Primer
                  ~~~~~~~~~~~~~~~~~~~~~~~~
  81   CCGCAAGTTC ACTTCAACTG TGCATCGTGC ACCATCTCAA TTTCTTTCAT TTATACATCG TTTTGCCTTC TTTTATGTAA
 161   CTATACTCCT CTAAGTTTCA ATCTTGGCCA TGTAACCTCT GATCTATAGA ATTTTTTAAA TGACTAGAAT TAATGCCCAT
                                                                HindIII
                                                                ~~~~~~
 241   CTTTTTTTTG GACCTAAATT CTTCATGAAA ATATATTACG AGGGCTTATT CAGAAGCTTT GGACTTCTTC GCCAGAGGTT
 321   TGGTCAAGTC TCCAATCAAG GTTGTCGGCT TGTCTACCTT GCCAGAAATT TACGAAAAGA TGGAAAAGGG TCAAATCGTT
```

```
 401   GGTAGATACG TTGTTGACAC TTCTAAATAA GCGAATTTCT TATGATTTAT GATTTTTATT ATTAAATAAG TTATAAAAAA
 481   AATAAGTGTA TACAAATTTT AAAGTGACTC TTAGGTTTTA AAACGAAAAT TCTTATTCTT GAGTAACTCT TTCCTGTAGG
 561   TCAGGTTGCT TTCTCAGGTA TAGCATGAGG TCGCTCTTAT TGACCACACC TCTACCGGCC GGTCGAAATT CCCCTACCCT
                                                                                SspBI
                                                                                ~~~~~~
 641   ATGAACATAT TCCATTTTGT AATTTCGTGT CGTTTCTATT ATGAATTTCA TTTATAAAGT TTATGTACAA ATATCATAAA
 721   AAAAGAGAAT CTTTTTAAGC AAGGATTTTC TTAACTTCTT CGGCGACAGC ATCACCGACT TCGGTGGTAC TGTTGGAACC
                                ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                                         LEU 2
 801   ACCTAAATCA CCAGTTCTGA TACCTGCATC CAAAACCTTT TTAACTGCAT CTTCAATGGC CTTACCTTCT TCAGGCAAGT
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                                LEU 2
 881   TCAATGACAA TTTCAACATC ATTGCAGCAG ACAAGATAGT GGCGATAGGG TTGACCTTAT TCTTTGGCAA ATCTGGAGCA
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                                LEU 2
 961   GAACCGTGGC ATGGTTCGTA CAAACCAAAT GCGGTGTTCT TGTCTGGCAA AGAGGCCAAG GACGCAGATG GCAACAAACC
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                                LEU 2
1041   CAAGGAACCT GGGATAACGG AGGCTTCATC GGAGATGATA TCACCAAACA TGTTGCTGGT GATTATAATA CCATTTAGGT
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                                LEU 2
1121   GGGTTGGGTT CTTAACTAGG ATCATGGCGG CAGAATCAAT CAATTGATGT TGAACCTTCA ATGTAGGAAA TTCGTTCTTG
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                                LEU 2
1201   ATGGTTTCCT CCACAGTTTT TCTCCATAAT CTTGAAGAGG CCAAAACATT AGCTTTATCC AAGGACCAAA TAGGCAATGG
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                                LEU 2
1281   TGGCTCATGT TGTAGGGCCA TGAAAGCGGC CATTCTTGTG ATTCTTTGCA CTTCTGGAAC GGTGTATTGT TCACTATCCC
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                                LEU 2
1361   AAGCGACACC ATCACCATCG TCTTCCTTTC TCTTACCAAA GTAAATACCT CCCACTAATT CTCTGACAAC AACGAAGTCA
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                                LEU 2
1441   GTACCTTTAG CAAATTGTGG CTTGATTGGA GATAAGTCTA AAAGAGAGTC GGATGCAAAG TTACATGGTC TTAAGTTGGC
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                                LEU 2
1521   GTACAATTGA AGTTCTTTAC GGATTTTTAG TAAACCTTGT TCAGGTCTAA CACTACCTGT ACCCCATTTA GGACCACCCA
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                                LEU 2
1601   CAGCACCTAA CAAAACGGCA TCAACCTTCT TGGAGGCTTC CAGCGCCTCA TCTGGAAGTG GGACACCTGT AGCGTCGATA
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                                LEU 2
1681   GCAGCACCAC CAATTAAATG ATTTTCGAAA TCGAACTTGA CATTGGAACG AACATCAGAA ATAGCTTTAA GAACCTTAAT
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                                LEU 2
1761   GGCTTCGGCT GTGATTTCTT GACCAACGTG GTCACCTGGC AAAACGACGA TCTTCTTAGG GGCAGACATT AGAATGGTAT
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                           LEU 2
1841   ATCCTTGAAA TATATATATA TATTGCTGAA ATGTAAAAGG TAAGAAAAGT TAGAAAGTAA GACGATTGCT AACCACCTAT

                                             ADH1-promotor
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
7361   AAGTATAAAT AGACCTGCAA TTATTAATCT TTTGTTTCCT CGTCATTGTT CTCGTTCCCT TTCTTCCTTG TTTCTTTTTC
                        ADH1-promotor                                          GAL4 AD
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~            ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                                    HindIII
                                                    ~~~~~~
                                                                   M  D   K  A  E  L   I  P  E ·
7441   TGCACAATAT TTCAAGCTAT ACCAAGCATA CAATCAACTC CAAGCTTTGC AAAGATGGAT AAAGCGGAAT TAATTCCCGA
                                               GAL4 AD
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       · P  P  K   K  K  R  K   V  E  L   G  T  A   A  N  F  N   Q  S  G   N  I  A   D  S  S  L ·
7521   GCCTCCAAAA AAGAAGAGAA AGGTCGAATT GGGTACCGCC GCCAATTTTA ATCAAAGTGG GAATATTGCT GATAGCTCAT
                                               GAL4 AD
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       · S  F  T   F  T  N   S  S  N  G   P  N  L   I  T  T   Q  T  N  S   Q  A  L   S  Q  P
7601   TGTCCTTCAC TTTCACTAAC AGTAGCAACG GTCCGAACCT CATAACAACT CAAACAAATT CTCAAGCGCT TTCACAACCA
                                               GAL4 AD
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
        I  A  S  S   N  V  H   D  N  F   M  N  N  E   I  T  A   S  K  I   D  D  G  N   N  S  K ·
7681   ATTGCCTCCT CTAACGTTCA TGATAACTTC ATGAATAATG AAATCACGGC TAGTAAAATT GATGATGGTA ATAATTCAAA
                                                                                  pGADL1-Primer
                                                                                  ~~~~~~~~~
                                               GAL4 AD
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       · P  L  S   P  G  W  T   D  Q  T   A  Y  N   A  F  G  I   T  T  G   M  F  N   T  T  T  M ·
7761   ACCACTGTCA CCTGGTTGGA CGGACCAAAC TGCGTATAAC GCGTTTGGAA TCACTACAGG GATGTTTAAT ACCACTACAA
       pGADL1-Primer
       ~~~~~~~~~~~~~~
                                      GAL4 AD
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       ·  D  D  V   Y  N  Y   L  F  D  D   E  D  T   P  P  N   P  K  K  E   I  F  N   T  T  H
7841   TGGATGATGT ATATAACTAT CTATTCGATG ATGAAGATAC CCCACCAAAC CCAAAAAAAG AGATCTTTAA TACGACTCAC
                                               HA tag
                                     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                         NcoI                                                NcoI          EcoRI
                        ~~~~~~~                                             ~~~~~~~        ~~~~~~
        Y  R  A  S   A  A  M   E  Y  P   Y  D  V  P   D  Y  A   H  M  A   M  E  A  S   E  F  H ·
7921   TATAGGGCGA GCGCCGCCAT GGAGTACCCA TACGACGTAC CAGATTACGC TCATATGGCC ATGGAGGCCA GTGAATTCCA
                       attB1                                            TGA2
               ~~~~~~~~~~~~~~~~~~~~~~~~    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
```

```
                     SspBI
                     ~~~~~~~
                                       M  A  D   T  S  P   R  T  D  V   S  T  D   D  D  T
       · P  S  T   S  L  Y  K   K  A  G
8001   CCCATCAACA AGTTTGTACA AAAAAGCAGG CTATGGCTGA TACCAGTCCG AGAACTGATG TCTCAACAGA TGACGACACA
                                             TGA2
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
        D  H  P  D   L  G  S   E  G  A   L  V  N  T   A  A  S   D  S  S   D  R  S  K   G  K  M ·
8081   GATCATCCTG ATCTTGGGTC GGAGGGAGCA CTAGTGAATA CTGCTGCTTC TGATTCGAGT GACCGATCGA AGGGAAAGAT
                                             TGA2
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       · 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  Q ·
8161   GGATCAAAAG ACTCTTCGTA GGCTTGCTCA AAACCGTGAG GCAGCAAGGA AAAGCAGATT GAGGAAGAAG GCTTATGTTC
                                             TGA2
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       ·  Q  L  E   N  S  R   L  K  L  T   Q  L  E   Q  E  L   Q  R  A  R   Q  Q  G   V  F  I
8241   AGCAGCTAGA GAACAGCCGC TTGAAACTAA CCCAGCTTGA GCAGGAGCTG CAAAGAGCAA GACAGCAGGG CGTCTTCATT
                                             TGA2
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
        S  G  T  G   D  Q  A   H  S  T   G  G  N  G   A  L  A   F  D  A   E  H  S  R   W  L  E ·
8321   TCAGGCACAG GAGACCAGGC CCATTCTACT GGTGGAAATG GTGCTTTGGC GTTTGATGCT GAACATTCAC GGTGGTTGGA
                                             TGA2
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       · E  K  N   K  Q  M  N   E  L  R   S  A  L   N  A  H  A   G  D  S   E  L  R   I  I  V  D ·
8401   AGAAAAGAAC AAGCAAATGA ACGAGCTGAG GTCTGCTCTG AATGCGCATG CAGGTGATTC TGAGCTTCGA ATAATAGTCG
                                             TGA2
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       ·  G  V  M   A  H  Y   E  E  L  F   R  I  K   S  N  A   A  K  N  D   V  F  H   L  L  S
8481   ATGGTGTGAT GGCTCACTAT GAGGAGCTTT TCAGGATAAA GAGCAATGCA GCTAAGAATG ATGTCTTTCA CTTGCTATCT
                                             TGA2
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                                                             HindIII
                                                                             ~~~~~~~
        G  M  W  K   T  P  A   E  R  C   F  L  W  L   G  G  F   R  S  S   E  L  L  K   L  L  A ·
8561   GGCATGTGGA AAACACCAGC TGAGAGATGT TTCTTGTGGC TCGGTGGATT TCGTTCATCC GAACTTCTAA AGCTTCTGGC
                                             TGA2
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       · N  Q  L   E  P  M  T   E  R  Q   L  M  G   I  N  N  L   Q  Q  T   S  Q  Q   A  E  D  A ·
8641   GAATCAGTTG GAGCCAATGA CAGAGAGACA GTTGATGGGC ATAAATAACC TGCAACAGAC ATCGCAGCAG GCTGAAGATG
                                             TGA2
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       ·  L  S  Q   G  M  E   S  L  Q  Q   S  L  A   D  T  L   S  S  G  T   L  G  S   S  S  S
8721   CTTTGTCTCA AGGGATGGAG AGCTTACAAC AGTCACTAGC TGATACTTTA TCGAGCGGGA CTCTTGGTTC AAGTTCATCA
                                             TGA2
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                        NcoI
                                       ~~~~~~
        G  N  V  A   S  Y  M   G  Q  M   A  M  A  M   G  K  L   G  T  L   E  G  F  I   R  Q  A ·
8801   GGGAATGTCG CAAGCTACAT GGGTCAGATG GCCATGGCAA TGGGAAAGTT AGGTACACTC GAAGGATTTA TCCGCCAGGC
                                             TGA2
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       · D  N  L   R  L  Q  T   L  Q  Q   M  I  R   V  L  T  T   R  Q  S   A  R  A   L  L  A  I ·
8881   TGATAATTTG AGACTACAAA CATTGCAACA GATGATAAGA GTATTAACAA CGAGACAGTC AGCACGTGCT CTACTTGCAA
                                     TGA2
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       ·  H  D  Y   F  S  R   L  R  A  L   S  S  L   W  L  A   R  P  R  E   *
8961   TACACGATTA CTTCTCACGG CTACGAGCTC TAAGCTCCTT ATGGCTTGCT CGACCCAGAG AGTGAACCCA GCTTT
                                                                            ~
                                                                             attB2
```

10.2.9 The sequence upstream and downstream of the Ds-transposon insertion in the promoter of GRX480

```
                          1465                                                                       1539
GRX480 gen.seq   (1465)  GTGGTCGTCACATGCGAATATTTCTAAGTATACAACCATCTATCGAAAGTTTCGAAACAACCCAAATATAATAGT
  Ds.G-Edge.up     (43)  GTGGTCA-CATAAGCGAATATTTNGAAGTATAAAAAGTTACAAACCATCAATCGAAAACAAC-AAAAATTATAGT
  Ds.H-Edge.dw      (1)  ---------------------------------------------------------------------------
     Consensus   (1465)  GTGGTC  CA A GCGAATATTT  AAGTATA AA    T  A     A     TCGAAA   A C AAA AT ATAGT
                          1540                                                                       1614
GRX480 gen.seq   (1540)  AATAAA---TTCTGAGAATTCT-CCTATTTTTTATTGATATGAAAAAAGCAGTAAAATATTATGTAAA-------
  Ds.G-Edge.up    (116)  AATAAACTATTCTGTTAATTTGACCCCAAAAAAAGAGATATGAAAAA-GCAGTAAAATATTATGTAACAACACGT
  Ds.H-Edge.dw      (1)  ---------------------------------------------------------------------------
     Consensus   (1540)  AATAAA    TTCTG  AATT    CC         A  GATATGAAAAA GCAGTAAAATATTATGTAA
                          1615                                                                       1689
GRX480 gen.seq   (1604)  --GTAGAGAGGAACCAAAATTAAAACGTGCAGATTTATTAAAAGAATAAAGTGGGATCCAAAAAGTCAGCGTGAA
  Ds.G-Edge.up    (190)  ACGTAGAAAGGAACCAAAATTAAAACGTGCAGATTTATTAAAAGAATAAAGTGGGATACAAAAAGTCAGCGTGAA
  Ds.H-Edge.dw      (1)  ---------------------------------------------------------------------------
     Consensus   (1615)    GTAGA AGGAACCAAAATTAAAACGTGCAGATTTATTAAAAGAATAAAGTGGGAT CAAAAAGTCAGCGTGAA
                          1690                                                                       1764
GRX480 gen.seq   (1677)  ACATGTGATACGATATAACGACACGGTCCTATGACTAAGTCCACTCCAATCCAG-TGACG-TAAACA-GCACCAT
  Ds.G-Edge.up    (265)  ACATGTGATACGATATAACGACACGGTCCTATGACTAAGTCCACTCCAATCCANGTGACNGTAAACAAGCACCAT
  Ds.H-Edge.dw      (1)  ---------------------------------------------------------------------------
     Consensus   (1690)  ACATGTGATACGATATAACGACACGGTCCTATGACTAAGTCCACTCCAATCCA  TGAC   TAAACA GCACCAT
                          1765                                                                       1839
GRX480 gen.seq   (1749)  CACCCATAGCTTCCTGTGACGCACATCCTTACGTAACCATCGTTGACGCTAGACTTTCCTCTCTGATCTCTCTTT
```

```
  Ds.G-Edge.up    (340) CACCCATAGCTTCCTGTGACGCATATCCTTACGTAACCATCGTTGACGCTAGACTTTCCTCTCTGATCTCTCTTT
  Ds.H-Edge.dw      (1) ---------------------------------------------------------------------------
     Consensus   (1765) CACCCATAGCTTCCTGTGACGCA ATCCTTACGTAACCATCGTTGACGCTAGACTTTCCTCTCTGATCTCTCTTT
                        1840                                                                   1914
GRX480 gen.seq   (1824) CTTCATGTATATATAACAAAACCTTCCTTTCCTAATTGGTATCTATCTTTAAAAACATACTTGAAAATGCAAGGA
  Ds.G-Edge.up    (415) CTTCATGTATATATAACAAAAC-----------------------------------------------------
  Ds.H-Edge.dw      (1) --------------AACAAAACCTTCCTTTCCTANTTGGTNTCTATCTTTAAAAACATACTTGAAAATGCAAGGA
     Consensus   (1840) CTTCATGTATATATAACAAAACCTTCCTTTCCTA TTGGT TCTATCTTTAAAAACATACTTGAAAATGCAAGGA
                        1915                                                                   1989
GRX480 gen.seq   (1899) ACGATTTCTTGTGCAAGAAATTATAACATGACGACAACCGTCGGGGAATCTCTGCGGCCGCTATCGCTTAAAACG
  Ds.G-Edge.up    (437) ---------------------------------------------------------------------------
  Ds.H-Edge.dw     (62) ACGATTTCTTGTGCAAGAAATTATAACATGACGACAACCGTCGGGGAATCTCTGCGGCCGCTATCGCTTAAAACG
     Consensus   (1915) ACGATTTCTTGTGCAAGAAATTATAACATGACGACAACCGTCGGGGAATCTCTGCGGCCGCTATCGCTTAAAACG
                        1990                                                                   2064
GRX480 gen.seq   (1974) CAGGGAAACGGCGAGAGAGTTCGGATGGTGGTGGAGGAGAACGCGGTGATTGTGATTGGACGGAGAGGATGTTGC
  Ds.G-Edge.up    (437) ---------------------------------------------------------------------------
  Ds.H-Edge.dw    (137) CAGGGAAACGGCGAGAGAGTNCGGATGGTGGTGGAGGANAACGCGGTGATTGTGATTGGACGGAGAGGATGTTGC
     Consensus   (1990) CAGGGAAACGGCGAGAGAGT CGGATGGTGGTGGAGGA AACGCGGTGATTGTGATTGGACGGAGAGGATGTTGC
                        2065                                                                   2139
GRX480 gen.seq   (2049) ATGTGTCATGTGGTGAGGAGGCTGCTTCTTGGACTTGGAGTGAATCCGGCGGTCCTTGAGATTGATGAGGAGAGG
  Ds.G-Edge.up    (437) ---------------------------------------------------------------------------
  Ds.H-Edge.dw    (212) ATGTGTCATGTGGTGAGGAGGCTGCTTCTTGGACTTGGAGTGAATCCGGCGGTCCTTGAGATTGATGAGGAGAGG
     Consensus   (2065) ATGTGTCATGTGGTGAGGAGGCTGCTTCTTGGACTTGGAGTGAATCCGGCGGTCCTTGAGATTGATGAGGAGAGG
                        2140                                                                   2214
GRX480 gen.seq   (2124) GAAGATGAAGTTTTGAGTGAGTTGGAGAATATTGGAGTTCAAGGCGGCGGAGGTACGGTGAAGTTACCGGCGGTT
  Ds.G-Edge.up    (437) ---------------------------------------------------------------------------
  Ds.H-Edge.dw    (287) GAAGATGAAGTTTTGAGTGAGTTGGAGAATNTTGNAGTTCAAGGCGGCNGAGGTNCGGNGAAGTNACCGGCGGTT
     Consensus   (2140) GAAGATGAAGTTTTGAGTGAGTTGGAGAAT TTG AGTTCAAGGCGGC GAGGT CGG GAAGT ACCGGCGGTT
                        2215                                                                   2289
GRX480 gen.seq   (2199) TATGTAGGAGGGAGGTTGTTTGGAGGGTTAGATAGGGTTATGGCTACTCATATCT-CCGGTGAGTTAGTTCCAAT
  Ds.G-Edge.up    (437) ---------------------------------------------------------------------------
  Ds.H-Edge.dw    (362) TATGTNNGAGGGAGGNTGNTCGGAGGNTTNTATAGGGNCATCGCTACTCNTATCTNCCGGTNANCTAGTTTCCAT
     Consensus   (2215) TATGT  GAGGGAGG TG T GGAGG TT  ATAGGG  AT GCTACTC TATCT CCGGT A  TAGTT C AT
                        2290                                   2329
GRX480 gen.seq   (2273) TCTTAAGGAAGT-TGGGGCTCTGTGGTTGTGATTGTAAAT
  Ds.G-Edge.up    (437) ----------------------------------------
  Ds.H-Edge.dw    (437) TCTTAAGGAAGCATGGNGCTCTTGNGGTGGTCGNTTNCNT
     Consensus   (2290) TCTTAAGGAAG  TGG GCTCT   G TG       T   T
```

10.3 List of Abbreviations

% (v/v)	volume % (volume-per-volume)
% (w/v)	Weight % (weight-per-volume)
°C	Degree Celsius
2,4-D	2,4-dichlorphenoxyacetic acid
5'-UTR	5'-untranslated region
A	Ampere
A. thaliana	*Arabidopsis thaliana*
A. tumefaciens	Agrobacteruim tumefaciens
a.a.	Amino acid(s)
Ac	Acetate
Amp	Ampicillin
AmpR	Ampicillin resistance
approx.	approximately
APS	Ammonium persulfate
as-1	activating sequence-1
ASF-1	Activating sequence factor 1
ATP	Adenosine triphosphate
bp	basepairs
BSA	Bovine serum albumin
BY-2	Bright-Yellow-2 Nicotiana tabacum
bZIP	basic region / leucine-zipper domain
CaMV	Cauliflower Mosaic Virus
cDNA	copy-DNA from mRNA
Cds	Coding sequence
cpm	counts per minute
C-terminus	Carboxyl-terminus
cv.	Cultivar
dA	Deoxyadenine
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytosine -5'-triphosphate
DEPC	Diethylpyrocarbonate
dGTP	2'-deoxyguanosine-5'-triphosphate
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dNTPs	Deoxynucleotide -5'-triphosphates
dsDNA	Double-stranded DNA
dT	Deoxythymidine
DTT	Dithiothreitol
dTTP	2'-deoxythymidine-5'-triphosphate
dYT	Double Yeast extract and Tryptone medium
e.g.	exempli gratia (for example; for instance)
ECL+	Enhanced Chemiluminescence™+ kit
EDTA	Ethylenediamnotetraacetate
EMSA	Electrophoretic mobility shift assay
et al.	et alii (and others)
ET mix	Enhanced terminators sequencing ready-to-use mix
EtBr	Ethidium bromide
etc.	et cetera (and the rest; and all the others)
EtOH	Ethanol
F	Farad
g	gram
GFP	Green fluorescent protein
GST	Glutathione S-transferase

GTP	**Guanosine-5'-triphosphate**
GUS	**ß-glucuronidase**
hrs	**hours**
HBT	**"Hybrid Booster of Transcription" promoter**
Hepes	**N-[2-hydroxyethyl]-piperazine-N'-[2-ethanesulfonic acid]**
HR	**Hypersensitive response**
i.e.	**id est (which is to say; in other words)**
INA	**2,6-dichlorisonicotinic acid**
IPTG	**Isopropyl-1-thio-ß-D-galactoside**
k	**kilo (x 103)**
Kan	**Kanamycin**
Kanr	**Kanamycin resistance**
kb	**kilobases**
kDa	**kilodaltons**
l	**liter**
lacZ	**ß-galactosidase gene**
LB	**Luria-Bertrani medium**
m	**mili (x 10-3)**
M	**molar**
MES	**2-[N-morpholino]-ethanesulfonic acid**
min	**minutes**
Mol	**mole**
MOPS	**3-[N-morpholino]-propansulfonic acid**
mRNA	**messenger ribonucleic acid**
MS	**Murashige & Skoog medium**
MW	**molecular weight**
n	**nano (x 10-9)**
N. tabacum	***Nicotiana tabacum***
NLS	**Nuclear localization sequence**
nos	**Nopaline synthase**
NPR1, npr1	**Non-expressor of pathogenesis-**
nt	**nucleotide**
N-terminus	**Amino-terminus**
ocs	**Octopine synthase**
OD	**Optical density**
pA	**Polyadenylation signal**
PAA	**Polyacrylamide**
PCR	**Polymerase chain reaction**
PEG	**Polyethyleneglycol**
pH	**Negative decimal logarithm**
PMSF	**Phenylmethylsulfonylfluoride**
PR	**Pathogenesis-related**
PVDF	**Polyvinylendifluoride**
RNA	**Ribonucleic acid**
RNAse A	**Ribonuclease A**
ROS	**Reactive oxygen species**
rpm	**rotations per minute**
RT	**Room temperature**
SA	**Salicylic acid**
sai1	***salicylic-acid -insensitive 1***
SAR	**Systemic acquired resistance**
SDS	**Sodiumdodecylsulfate**
SDS-PAGE	**Denaturing SDS-polyacrylamide electrophoresis**
sec	**seconds**
sni1	***suppressor of npr-1, inducible***
SSC	**Strandard saline citrate buffer**

ssDNA	Single-stranded DNA
ß-Gal	ß-galactosidase
ß-ME	ß-mercaptoethanol
TAE	Tris-acetate-EDTA buffer
Taq	Thermophylus aquaticus
TBE	Tris-borate-EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
TMV	Tobacco Mosaic Virus
Tris	Tris-(hydroxymethyl)-aminomethane
TSR	Template suppress reagent
U	Unit (enzyme activity)
UV	Ultraviolet light
V	Volt
vol	volume
W	Watt
wt	Wild-type
X-Gal	5-chlor -4-brom-3-indolyl-ß-D-galactopyranoside
Y2H	Yeast two-hybrid system
α	Antiserum
λ	Wavelength, bacteriophage Lambda
µ	micro (x 10-6)
Ω	Ohms

Amino acid one- and three-letter codes:

A	Ala	Alanine	M	Met	Methionine
B	Asx	Aspartate/Asparagine	N	Asn	Asparagine
C	Cys	Cysteine	P	Pro	Proline
D	Asp	Aspartic acid	Q	Gln	Glutamine
E	Glu	Glutamic acid	R	Arg	Arginine
F	Phe	Phenylalanine	S	Ser	Serine
G	Gly	Glycine	T	Thr	Threonine
H	His	Histidine	V	Val	Valine
I	Ile	Isoleucine	W	Trp	Tryptophane
K	Lys	Lysine	Y	Tyr	Tyrosine
L	Leu	Leucine	Z	Glx	Glutamate/Glutamine

Nucleotide code:

A	Adenine	R	A or G (purine)
C	Cytidine	Y	C or T (pyrimidine)
G	Guanine	K	G or T (keto-base)
T	Thymidine	M	A or C (amino-base)
B	C, G or T	S	G or C
D	A, G or T	W	A or T
H	A, C or T	N	A, C, G or T
V	A, C or G		

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10.7 Curriculum Vitae

I. PERSONAL DATA

First Name	Ivan
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Birth date	November 7th, 1977
Place of Birth	Bamenda, Cameroon
Gender	Male
Family Status	Married

II. EDUCATION AND TRAINING

Dates	Institution	Qualifications/Experience obtained
Primary School Education		
1982-1989	Government School, Ngomgham, Bamenda	First School leaving certificate (FSLC)
Secondary School Education		
1989-1994	Cameroon Protestant College (CPC) Bali, NWP, Cameroon	GCE Ordinary Level in 11 Papers
High School Education		
1994-1996	Baptist High School (BHS) Buea, SWP, Cameroon	GCE Advanced Level, in 4 Papers, Bio(A), Chem(A), Math(B), Phys(B), GPA: 18/20
University Education		
1996-1999	University of Buea, Cameroon	BSc. Hons in Biochemistry, with Minor in Medical laboratory technology
1999-2000	University of Yaounde I, Cameroon	Course work(Maitrise) Biochemistry, Methods: Molecular Characterization of Malaria drug resistance by RFLP. Biotechnology for innovation and discovery (c/o the Fobang Foundation)
2000-2001	University of Buea, Cameroon/University of Lund, Sweden	First semester, MSc courses in Biochemistry, Bioinformatics course, phase I.
2001-2002	Max Plank Research School for Molecular Biology, Georg August University, Göttingen	MSc. Molecular Biology Title: Use of the Tandem Affinity Purification Tag to identify proteins that interact with transcription factor TGA2 – Cloning and expression of the chimeric protein

2002-Present date	Max Plank Research School for Molecular Biology, Georg August University, c/o Albrecht-von-Haller-Institut for plant research, Dep't of General and developmental physiology of plants.	PhD in Molecular Biology, in preparation, due Feb 2006. Title: Characterization of TGA transcription factor interacting partner, GRX480.

PRESENTATIONS OF RESEARCH DATA

DATE	EVENT/LOCATION	PRESENTATION
04-05/12/2003	International Max Planck Research School 1st International PhD Student Symposium, Decoding Nature: Horizons in Molecular Biology GZMB, Göttingen	Poster: The Tandem Affinity Purification (TAP) Tag approach in the study of the mechanism of systemic acquired resistance.
17-19/03/ 2005	International Max Planck Research School 2nd International PhD Student Symposium, Decoding Nature: Hierarchy of interactions MPI-bpc, Göttingen	Poster: The involvement of glutaredoxin At1g28480 in gene expression mediated by salicylic acid, auxin, jasmonic acid and oxidative stress.
17-23/07/2005	Arabidopsis meeting, Vienna XVII International Botanical Congress	Talk by Prof Christiane Gatz: An Arabidopsis glutaredoxin interacts with central components of the SA activated signal transduction network
22-24/08/2005	Arabidopsis Summer school: **"Environmental signaling: Arabidopsis as a model" ,** Utrecht University, the Netherlands	Poster: Characterization of TGA interacting protein glutaredoxin GRX1 with respect to its role in gene expression mediated by salicylic acid, auxin, jasmonic acid and oxidative stress.
24-27/08/2005	2nd Tri-National Arabidopsis Meeting 2005, Advancing the Genomics Frontier. Neuchâtel, Switzerland	Poster by Prof Christiane Gatz: A glutaredoxin interacts with TGA transcription factors involved in salicylic acid and jasmonic acid dependent transcription

Publications

May 2003	Use of the Tandem Affinity Purification Tag to identify proteins that interact with transcription factor TGA2 – Cloning and expression of the chimeric protein. MSc Thesis, Georg August University, Göttingen
March 2006	Submission of Manuscript for publication. Title: SA-induced Arabidopsis glutaredoxin interacts with TGA factors and suppresses JA-induced PDF1.2 transcription

LABORATORY SKILLS

General molecular biology/genetic skills Cloning, PCR, northern blotting, southern blotting, RFLP studies in Plasmodium, analysis of RNAi, generation of transgenic plants, epistasis analysis of signal transduction components using mutants and transgenic lines in *Arabidopsis thaliana*.

Biochemistry skills Histochemical staining, western blotting, Whole mount insitu hybridization in *Xenopus* developmental stages, yeast two hybrid assays, ONPG interaction assays in yeast, quantitative GUS assays in plant tissues, Gel shift assays (EMSA), TAP tag pull down experiments, protein expression and purification, ion exchange chromatography, crystallography trials, analysis of oxylipin signaling intermediates using hydroponic culture feeding and northern blot analysis.

Cell Biology Skills Protoplast transient expression assays of GFP fusion proteins, cell cultures,

Other lab skills General microbiology methods.

PERSONAL SKILLS

LANGUAGES	Reading Skills	Writing Skills	Verbal Skills
English	Excellent	Excellent	Excellent
French	Good	Good	Excellent
German	Good	Fair	Good

Communication Skills

- Quarterly oral presentations on progress report/literature review at Max plant research school for Molecular Biology, Plant Biochemistry PhD student seminars, and PhD thesis committee and at the Department of general and developmental plant physiology.
- Public interpretation/translation from German and French into English, at an international Christian community, Göttingen.
- Speaker at plenary session of Cameroonian Christians abroad (Europe) outreach meeting, on vision for nation building, November 2003 (Brauschweig, Germany) April 2005 (Berlin)
- Motivational speaker in several schools, colleges and youth assemblies of CYJ, in Buea, Yaounde, Ebolowa, Douala and Bamenda, with a focus on youth integrity, and their future as a hope for their nation - Cameroon.

Leadership skills

- Assistant head boy, class 6, GS. Ngomgham, 1988
- Class prefect, forms 1 through 3, CPC Bali, 1989-1992
- Dispensary/Health Assistant, CPC Bali, 1992-1993
- Vice president, CYJ Buea/Yaounde, 1998-2000
- Coordinator, Ev. Centre of CYJ, Molyko, Un- District, Buea.
- Keeper of stores/accounts at building site of a SOWEDA. building, June-August 1996, Buea, Cameroon.

Teaching Experience -Teaching tutorial on metabolic biochemistry.

-Practical projects with a student.

COMPUTER SKILLS Very well acquainted with the following programs:

MS Word, MS Excel, MS Power point, Vector NTI sequence analysis software, Clone, Chromas, spdbv, and other software in picture editing, video production and web development.

AWARDS AND HONOURS

1994 Prizes for best in Mathematics, Further-mathematics, Human-Biology and one of the best all-round students of the class and year, CPC Bali.

1996 Prizes for best behaved student of the year, best in Biology, Best in Chemistry and best all round science student of the year, Baptist High School, Buea.

1998 Government prize/scholarship for excellence, University of Buea, Cameroon.

2001 Scholarship award, International Max Plank Research school for molecular Biology

2002 Georg-christoph lichtenberg stipendium

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