Pontificia Universidad Católica de Chile
Facultad de Ciencias Biológicas
Programa de Doctorado en Ciencias Biológicas
Mención Genética Molecular y Microbiología

TESIS DOCTORAL:

TRANSCRIPTIONAL CONTROL OF GLUTAREDOXIN *GRXC9* BY STRESS IN *Arabidopsis*

Por

ARIEL ESTEBAN HERRERA VÁSQUEZ

Noviembre 2015

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Tesis presentada a la Pontificia Universidad Católica de Chile como parte de los requisitos para optar al grado de Doctor en Ciencias Biológicas mención Genética Molecular y Microbiología

Por

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ABREVIATIONS

2,4D:	2,4-dichlorophenoxyacetic acid
ANAP:	Arabidopsis Network Analysis Pipeline
ARR:	Arabidopsis Response Regulator
as-1:	activation sequence 1
ASC:	Ascorbate
BTH:	Benzothiadiazole
bZIP:	basic/leucine zipper
CamV 35S:	Cauliflower Mosaic Virus 35S
CBP60:	Calmodulin Binding Protein 60
ChIP:	Chromatin immunoprecipitation
$Co\text{-}R_A/Co\text{-}R_I$:	Active Co-regulator complex / Inactive Co-regulator complex
CYP:	Cytochrome P450
DHA:	Dehydroascorbate
EDS:	Enhanced Disease Susceptibility
ETI:	Effector trigger immunity
GRX:	Glutaredoxin
GSH:	Reduced glutathione
GSSG:	Oxidized glutathione
GST:	Glutathione *S*-transferases
GUS:	*β*-glucuronidase
HRP:	Horseradish peroxidase
ICS:	Isochorismate synthase
JA:	Jasmonic acid
MD/MP:	Distal mutation / Proximal mutation
MED:	Mediator complex
NADPH:	Nicotinamide adenine dinucleotide phosphate
NIMIN:	NPR1 Interacting
NPR:	Non-expressor of pathogenesis related genes
PAD:	Phytoalexin deficient
PAL:	Phenylalanine ammonia-lyase
PAN:	Perianthia
PR:	Pathogenesis related
Pst:	Pseudomonas syringae pv. tomato
PTI:	PAMP trigger immunity
RCD:	Radical-induced Cell Death
RNAPII:	RNA polymerase II
ROS:	Reactive oxygen species
SA:	Salicylic acid
SAIG:	Salicylic acid induced gene
SCL:	Scarecrow-like
TRX:	Thioredoxin
UGT:	UDP-glucosyl transferases
UTR:	Untranslated region
UV:	Ultraviolet
WT:	Wild type

RESUMEN

El Ácido Salicílico (SA) es una hormona que media la reprogramación transcripcional en el contexto de la respuesta de defensa a estrés en plantas. El gen *GRXC9*, que codifica para una glutarredoxina de Arabidopsis, es un gen inducido de forma temprana e independientemente del coactivador maestro NPR1. En esta tesis describimos el mecanismo de regulación transcripcional del gen modelo *GRXC9* por SA. Inicialmente establecimos que la expresión de *GRXC9* es inducida por exposición a luz UV-B a través en un mecanismo dependiente de SA e independiente de NPR1 validando su activación en una condición fisiológica. Análisis del promotor de *GRXC9* indican que SA controla la transcripción del gen a través de dos elementos tipo-*as-1* ubicados en la región proximal al inicio de la transcripción. Los factores de transcripción TGA2 y TGA3, están constitutivamente unidos al la región promotora de *GRXC9*. Concordantemente, el reclutamiento transitorio de la RNA polimerasa II al promotor de GRXC9 y la acumulación del transcrito detectados en plantas silvestres tratadas con SA, se pierde en plantas mutantes para los factores de transcripción TGA de la clase II (que incluyen a TGA2). A partir de esto, concluimos que la unión constitutiva de TGA2 es esencial para el control de la expresión de *GRXC9*. Finalmente, mediante el uso de plantas que sobre-expresan GRXC9 podemos concluir que GRXC9 regula negativamente su propia expresión formando parte del complejo unido a la región promotora que contiene los elementos tipo-*as-1*. Estos descubrimientos son integrados en un modelo que explica como SA controla la expresión de GRXC9 en el contexto de la respuesta de defensa a estrés.

ABSTRACT

Salicylic acid (SA) is a key hormone that mediates genes transcriptional reprogramming in the context of the defense response to stress in plants. *GRXC9*, coding for a glutaredoxin from *Arabidopsis*, is a SA-responsive gene induced early and transiently by a NPR1-independent pathway. Here, we address the mechanism involved in this SA-dependent pathway, using *GRXC9* as a model gene. We first established that *GRXC9* expression is induced by UVB exposure through this pathway, validating its activation in a physiological stress condition. SA controls the *GRXC9* gene transcription through two *as-1*-like elements located in its proximal promoter region. The transcription factors TGA2 and TGA3, are constitutively bound to this promoter region. Accordingly, the transient recruitment of RNA polymerase II to the *GRXC9* promoter, as well as the transient accumulation of gene transcripts detected in SA-treated WT plants, was abolished in a *knock out* mutant for the TGA class II factors. We conclude that constitutive binding of TGA2 is essential for controlling *GRXC9* expression. Finally, over-expression of *GRXC9* indicates that the GRXC9 protein negatively controls its own gene expression, forming part of the complex bound to the *as-1*-containing promoter region. These findings are integrated in a model that explains how SA controls transcription of *GRXC9* in the context of plant defense response to stress.

1. INTRODUCTION

1.1 Salicylic acid: a key hormone for plant stress responses

Salicylic acid (SA) is a key plant hormone involved in stress defense responses against a wide range of biotrophic and hemibiotrophic pathogens, and abiotic stress conditions such as UV, high light radiation, ozone exposure, salinity, osmotic and drought stress (Nawrath and Metraux, 1999; Borsani et al., 2001; Wildermuth et al., 2001; Ogawa et al., 2005; Mateo et al., 2006; Garcion et al., 2008; Lee et al., 2010; Miura et al., 2013).

In plants, SA is synthetized by two different pathways: the Phenylalanine ammonia-lyase (PAL) pathway and the Isochorismate synthase (ICS) pathway. The major contribution in SA production under stress conditions is given by the ICS pathway controlling the production of about 90% of total SA (Wildermuth et al., 2001; Garcion et al., 2008).

The mutation of *ICS1* gene decreases dramatically the SA accumulation in response to pathogens and UV light, pointing to this gene as key in SA production (Wildermuth et al., 2001; Garcion et al., 2008). The ICS1 is a plastid-localized, stromal enzyme that catalyzes the conversion of chorismate to isochorismate (Strawn et al., 2007). So far, the enzyme able to convert isochorismate to SA is still unknown in plants. Once synthetized in the chloroplast, the SA is transported to the cytoplasm by the transporter Enhanced Disease Susceptibility 5 (EDS5) located in the chloroplastic membrane (Serrano et al., 2013). On the other hand, the two lipase-like proteins Enhanced Disease Susceptibility 1 (EDS1) and Phytoalexin Deficient 4 (PAD4) trigger the accumulation of SA showing an important role as upstream components of the SA-

mediated pathway in the effector trigger immunity (ETI) and in the basal immunity (PTI) responses (Jirage et al., 1999; Feys et al., 2001; Shah, 2003).

The Ca^{2+} signature has been also postulated as an important signal that triggers SA production. This effect could be mediated by post-translational modification of the transcription factors Calmodulin Binding Protein 60g (CBP60g), CBP60a, WRKY8/28/48 and CAMTA3/SR1, which regulate the expression of *ICS1* and *EDS1* genes (Du et al., 2009; Gao et al., 2013; Truman et al., 2013).

In plant responses to biotic stress it is well reported that SA production increases (Tsuda et al., 2008; Blanco et al., 2009), and that plants unable to accumulate the hormone display susceptibility to biotrophic pathogens (Nawrath and Metraux, 1999; Feys et al., 2001; Wildermuth et al., 2001). Increases in SA levels have been also reported in responses to abiotic stress conditions such as UV-C and UV-B radiation and ozone exposure (Sharma et al., 1996; Surplus et al., 1998; Nawrath et al., 2002; Garcion et al., 2008). In response to water deficit produced by salt stress, osmotic stress or drought, despite there are no reports of increases in SA levels in Arabidopsis, the evidence indicates that exogenous treatments with SA trigger different effects depending on its concentration. Under physiological concentrations, the hormone has a protective function in seed germination under saline stress and improved growth under drought stress. In contrast, the use of high SA concentrations has an inhibitory effect on plant stress responses, which may be caused by ROS overproduction and the consequent oxidative stress (Lee et al., 2010; Miura and Tada, 2014).

In the context of the defense response to stress, SA triggers a global transcriptional reprogramming in the infected/damaged tissues, as well as in the neighboring cells, orchestrating local and systemic defense responses (Vlot et al., 2009;

Fu and Dong, 2013). These responses define the plant survival to the stress stimuli. The effect of SA over varies depending on the specie and the concentrations of SA.

Despite the importance of SA in the plant defense responses against stress is already established, the mechanisms by which the hormone exerts its effect are not fully understood.

1.2 The interplay between salicylic acid and reactive oxygen species

Recent reports support evidence that SA interplays with redox signals, such as H_2O_2 and glutathione, in the modulation of the defense responses (Foyer and Noctor, 2011; Dubreuil-Maurizi and Poinssot, 2012; Noshi et al., 2012; Han et al., 2013). ROS signaling is involved upstream and downstream of SA production (Herrera-Vasquez et al., 2015). Under stress produced by pathogens, UV-B and ozone, an apoplastic oxidative burst occurs. Particularly for stress produced by UV-B exposure, the H_2O_2 produced in the apoplast triggers the SA accumulation (Mackerness et al., 2001), although the mechanism remains unknown. A hypothesis is that the ROS production triggers modifications of transcription factors that control the expression of genes coding for components of the SA metabolic pathway as *ICS1* or *EDS1/PAD4* (Herrera-Vasquez et al., 2015).

Also, SA has a bi-functional role in the redox homeostasis controlling the ROS production and detoxification. In early stages of stress, the ROS production is essential for defense responses (Garreton et al., 2002; Lee et al., 2010). Furthermore, high concentrations of SA (>100µM) promote ROS production, inducing oxidative stress, and reducing tolerance to drought and salinity (Lee et al., 2010; Miura and Tada, 2014). This effect could be explained by the inhibition of ROS detoxifying enzymes such as catalase and cytosolic ascorbate peroxidase by SA (Chen et al., 1993; Durner and

Klessig, 1995). On the contrary, the available evidence supports that SA promotes ROS scavenging being essential for the antioxidant response that constrains ROS bursts in responses to avirulent bacteria, high light, ozone, salinity (Grant and Loake, 2000; Mateo et al., 2006; Yoshida et al., 2009; Lee and Park, 2010). Conversely, plants that over accumulate SA show increased GSH levels and reducing power (ratio GSH/GSSG) (Mateo et al., 2006) suggesting that the hormone can play its antioxidant role modulating the GSH production. Although this interplay is being increasingly recognized (Fu and Dong, 2013), the precise mechanisms that govern this relationship are still unknown.

SA is also able to modulate ROS detoxification through changes in gene expression (Mou et al., 2003; Koornneef et al., 2008; Tada et al., 2008). Supporting this idea, genes coding for detoxifying enzymes are overrepresented among the genes induced by SA treatments, compared to the Arabidopsis genome (Blanco et al., 2009). Accordingly, we previously reported that a subset of the early SA-inducible genes (early SAIGs) code for enzymes with GSH-dependent antioxidant and detoxifying activities, such as glutaredoxins (GRX) and glutathione *S*-transferases (GST) (Blanco et al., 2005; Blanco et al., 2009). Moreover, we showed that the expression of *GRXS13*, one of the GRXs coded by early SAIGs, is critical for limiting basal and high light stress-induced ROS production and for regulation of the ascorbate/dehydroascorbate (ASC/DHA) ratio after stress (Laporte et al., 2012). These data support the idea that these genes could be involved in the ROS-scavenging/antioxidant network that constrains the oxidative burst produced under stress conditions. Here, we studied *GRXC9* (also known as *GRX480*), a second *GRX* gene identified as an early SAIG (Blanco et al., 2009).

1.3 The role of the plant glutaredoxins

GRXs are small disulphide oxidoreductases that catalyze the reduction of disulphide bridges and protein–GSH adducts (*S*-glutathionylated proteins) using the reducing power of GSH and NADPH (Rouhier et al., 2008) (FIGURE 1). The covalent binding of GSH to a cysteine residue of a protein can protect the residue from irreversible oxidation and can also modify the biological activity of the protein (Meyer et al., 2009). GRXs catalyze the removal of GSH from proteins in a process called *S*-deglutathionylation (Meyer et al., 2009). The antioxidant role of GRXs has been characterized in bacteria, yeast and mammals (Meyer et al., 2009).

The Arabidopsis genome contains 50 putative *GRX* genes grouped in five classes, according to their sequence homology and the aminoacid motif present in the active site (Belin et al., 2014) (FIGURE 2). Only two of these *GRXs* are described as SA-responsive genes, *GRXC9* and *GRXS13*, which code for GRXs belonging to a plant-specific class called CC-type by the two consecutives cysteine residues present at the active site (Ndamukong et al., 2007; La Camera et al., 2011; Laporte et al., 2012).

The *GRXS13* gene is critical for ROS detoxification under basal conditions and after photooxidative stress (Laporte et al., 2012). Furthermore, *GRXS13* facilitates the *Botrytis cinerea* infection (La Camera et al., 2011).

The GRXC9 protein is located in the nucleus and the cytoplasm. In the nucleus it interacts with the bZIP transcription factor TGA2 (Ndamukong et al., 2007). This interaction is not exclusive for GRXC9 and it has been also described for ROXY1/2 (also called GRXC7/GRXC8) and GRXS13 (Li et al., 2009; Murmu et al., 2010; La Camera et al., 2011). On the other hand, the transcription factors TGA3 and PAN are able to interact with ROXY1-5 and ROXY18-20 (Li et al., 2011).

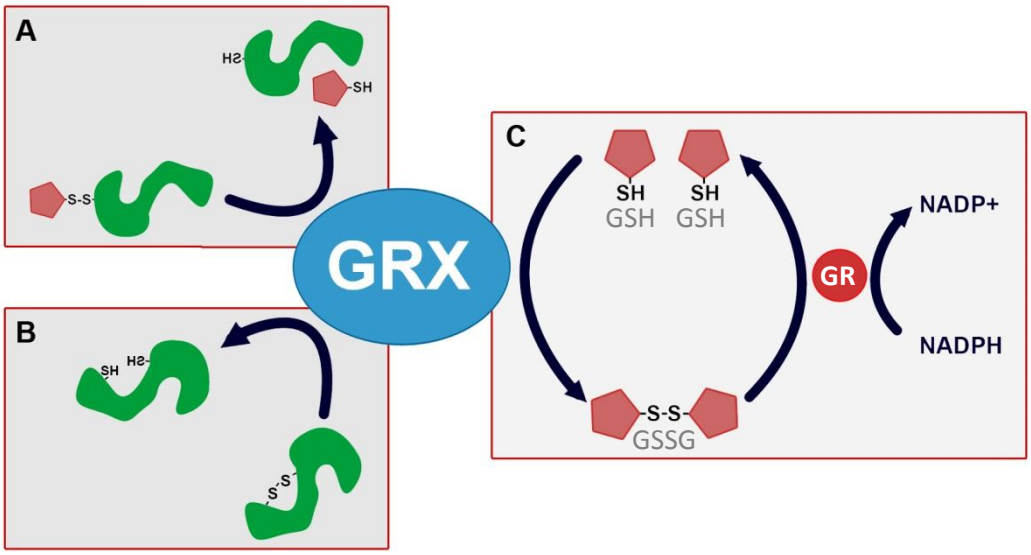

FIGURE 1. Reactions catalyzed by glutaredoxins

The GRXs can reduce glutathione-protein adducts in a reaction called deglutathionylation (A) or reduce inter or intramolecular disulfide bounds of proteins (B). These reactions are coupled to the conversion of two glutathione molecules (GSH) into the oxidized glutathione form (GSSG). Then, the GSSG form is reduced to GSH in a NADPH-dependent reaction catalyzed by the enzyme glutathione reductase (GR) (C).

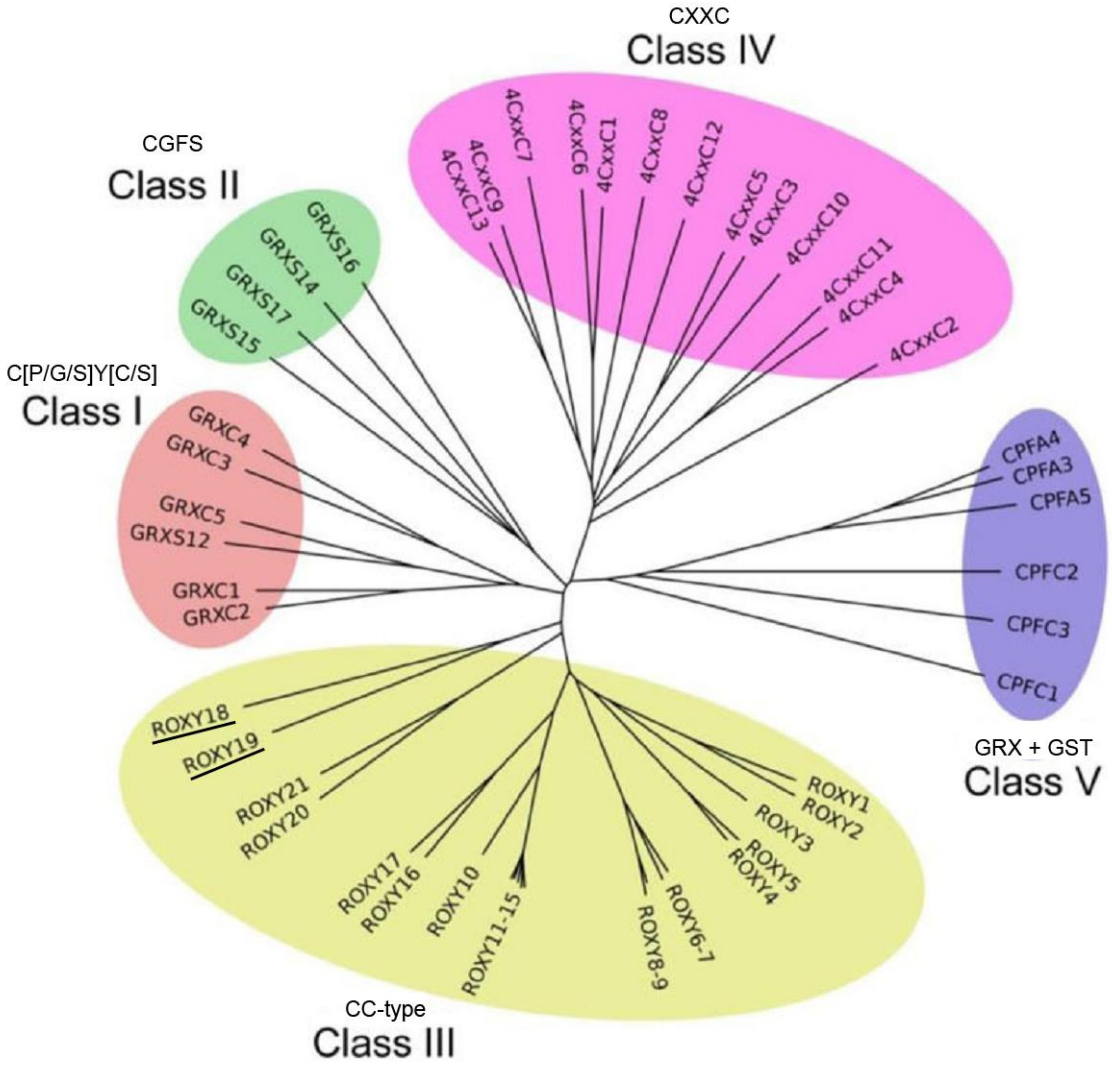

FIGURE 2. Phylogenetic tree of glutaredoxins from *Arabidopsis thaliana***.**

All putative Arabidopsis GRXs were clustered in five different subgroups. The Class I groups GRXs that harbor the C[P/G/S]Y[C/S] motif at the active site. The Class II groups the GRXs with the CGFS motif at the active site. The class III GRXs, that harbor a CC motif at the active site, is the most abundant group that is exclusive from plants. The fourth group is characterized by the motif CXXC at the active site and finally the class V contains six proteins that display both, a GRX domain and a glutathione S-transferase domain. The GRXs belonging to the class III are named according to the ROXY classification described by (Li et al., 2009). The underlined genes ROXY18 and ROXY19 are induced by SA and correspond to GRXS13 and GRXC9. Adapted from (Belin et al., 2014)

GRXC9 gene is early induced by SA showing a peak of induction at 2.5 h after initiated the treatment, decreasing thereafter to basal levels around 24 h ((Blanco et al., 2009). Functionally, GRXC9 plays a negative role on the pathway mediated by Jasmonic Acid (JA, a hormone that acts antagonistically to SA). *GRXC9* is able to suppress the expression of JA-responsive genes such as *PDF1.2*, a classical JA gene marker (Ndamukong et al., 2007) and the transcription factor Octadecanoid-responsive Arabidopsis AP2/ERF 59 (ORA59) a master regulator in JA response (Zander et al., 2012). For this reason, GRXC9 has been proposed as an important protein that could regulate the crosstalk between SA and JA in the defense response (Koornneef et al., 2008).

In this thesis work, we specifically focus in unrevealing the transcriptional control mechanisms of *GRXC9* by SA.

1.4 Transcriptional activation of genes by SA. What do we know?

The transcriptional activation of most of the SAIGs, including the *PATHOGENESIS RELATED 1* gene (*PR-1*, the marker gene for SA signaling) is mediated by the master co-activator NON-EXPRESSOR OF PATHOGENESIS RELATED GENES 1 (NPR1) (Dong, 2004; Fu and Dong, 2013). The NPR1 protein is highly regulated by SA controlling its redox state, localization, activity and stability. Basally NPR1 is located at the cytoplasm in an oligomeric form. When SA levels increase, the thioredoxin TRXh5 reduces NPR1 allowing its movement as a monomer from the cytoplasm to the nucleus (Mou et al., 2003; Tada et al., 2008). At the nuclear level, monomeric NPR1 interacts with the transcription factor TGA2 acting as a transcriptional co-activator (Zhou et al., 2000; Mou et al., 2003; Rochon et al., 2006; Tada et al., 2008; Fu et al., 2012). Recently, the NPR1 protein has been described as a

SA receptor. The binding of SA to NPR1 allows a conformational change in the protein releasing an auto-inhibitory domain activating its function as transcriptional coactivator (Wu et al., 2012) Also, the NPR1 paralogs NPR3 and NPR4 were identified as direct receptors of SA that regulate the NPR1 degradation, controlling the SA responses mediated by this co-activator (Fu et al., 2012).

Contradictory evidence has been shown concerning the role of NPR1 in the transcriptional activation of *GRXC9*. Ndamukong and colleagues report that *GRXC9* is induced by SA through an NPR1-dependent pathway (Ndamukong et al., 2007). In contrast, evidence from our group supports that *GRXC9* is activated by an NPR1-independent pathway (Blanco et al., 2009). More analysis is necessary to elucidate this point of discussion.

The existence of a pathway that leads to an early and transient activation of SAIGs through an NPR1-independent mechanism, has been reported by ours and other groups (Lieberherr et al., 2003; Uquillas et al., 2004; Blanco et al., 2005; Langlois-Meurinne et al., 2005; Fode et al., 2008; Blanco et al., 2009; Shearer et al., 2012). Looking for components of this pathway, the *Scarecrow-like 14* gene (*SCL14*) coding for a putative coactivator was identified (Fode et al., 2008). This gene is involved in the genetic activation of a group of genes belonging to the early NPR1-independent SAIGs. The SCL14 protein is able to interact with TGA2 transcription factor, an important component in the defense response. By gene ontology, the genes with decreased expression in *scl14* mutant plants compared to wild type plants (i.e. the SCL14-dependent genes) might be involved in detoxification of xenobiotics (Fode et al., 2008). The *GRXC9* and *GRXS13* genes are not included in the group of SCL14-dependent genes described by (Fode et al., 2008). Therefore, SCL14 does not explain the mechanism by which SA activates the expression of these early SAIGs. In this work we

assess this mechanism using *GRXC9* as a model for the SA-dependent and NPR1-independent pathway that controls defense gene expression.

Promoter analyses of NPR1-independent early SAIGs show over-representation of a *cis-acting* element with high identity to the *activating sequence-1 (as-1*) (Blanco et al., 2005; Blanco et al., 2009). The *as-1* sequence consists of two adjacent variants of the palindromic sequence TGAC/GTCA (TGA box), separated by 4 base pairs (Ellis et al., 1993; Krawczyk et al., 2002). The TGA boxes are recognized by basic/leucine zipper (bZIP) factors of the TGA family (Jakoby et al., 2002; Gatz, 2012). The *as-1* element was first identified in the Cauliflower Mosaic Virus 35S (CaMV 35S) promoter as an element that conferred basal expression in root tips (Benfey et al., 1989). Subsequent studies showed that the *as-1* element from the CaMV 35S promoter responds early and transiently to SA (Qin et al., 1994), to xenobiotic compounds like the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4D) (Johnson et al., 2001), as well as to H_2O_2 and methyl viologen (Garreton et al., 2002). Accordingly, this element has been found over-represented, not only in early SAIGs promoters (Blanco et al., 2005; Blanco et al., 2009), but also in the promoters of plant genes associated to chemical detoxification process induced by xenobiotics like 2,4-D and oxidized lipids (oxilipins) (Johnson et al., 2001; Fode et al., 2008; Köster et al., 2012).

The upstream intergenic region of *GRXC9* gene contains two sequences similar to the *as-1* element. These *as-1*-like elements are located between -80/-90 bp and -114/-133 bp upstream of the transcriptional start site and we call them proximal and distal *as-*

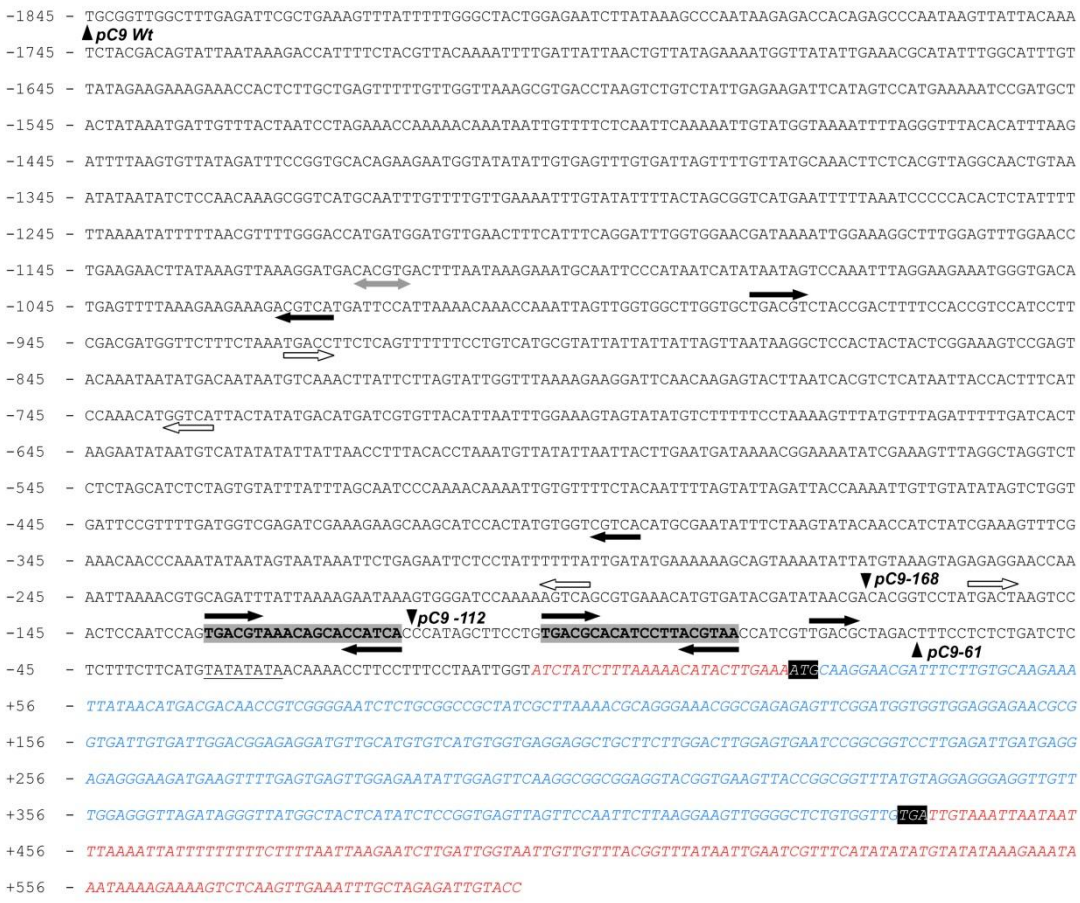

FIGURE 3. *GRXC9* gene sequence.

The complete *GRXC9* nucleotidic sequence is shown. The intergenic region, considered as the *GRXC9* promoter, is shown in black letters; the 5' and 3' UTR sequences are shown in red letters; and the GRXC9 coding sequence is shown in blue letters. The putative TATA box is underlined; the start and stop codons of the coding sequence are shown in black boxes; the *as-1-like* motifs are shown in bold and gray boxes. TGA boxes and W boxes are indicated with black and white arrows, respectively, as described in Ndamukong, et al., 2007. G boxes are indicated in gray arrows (Köster et al., 2012). Arrow-heads indicate the sequences included in the truncated versions of the *GRXC9* promotor analyzed in FIGURE 8.

1-like element respectively (FIGURE 3). We do not know if those elements are functionally active.

Interesting evidence shows that the overexpression of *GRXC9* negatively regulates genes controlled by the *as-1* element in chemical treatments (Ndamukong et al., 2007). These results link functionally the as-1-element and the GRXC9 protein.

The family of TGA transcription factors has 10 members in *Arabidopsis* (Jakoby et al., 2002; Gatz, 2012). Three of them, PERIANTHIA (PAN) or TGA8, TGA9 and TGA10 have been implied in developmental processes. PAN is involved in flower development while TGA9 and TGA10 are required for male gametogenesis (Chuang et al., 1999; Murmu et al., 2010). The other seven TGA factors have been related to the defense response and they are grouped by sequence similarity in three different classes. TGA class I includes TGA1 and TGA4. TGA1 has been described as a transcription factor regulated by redox conditions in SA response (Despres et al., 2003; Lindermayr et al., 2010). Also, TGA class I factors participate in basal defense responses against *Pseudomonas syringae* controlling the apoplastic oxidative burst, callose deposition and PR-1 accumulation by a NPR1-independent mechanism (Shearer et al., 2012; Wang and Fobert, 2013). Moreover, this class of TGA factors is involved in regulation of the nitrate response in Arabidopsis roots (Alvarez et al., 2014). TGA class II factors (TGA2, TGA5 and TGA6) are the most relevant for the SA pathway (Gatz, 2012). In fact, involvement of TGA class II factors in the canonic pathway that controls expression of SA- and NPR1-dependent genes containing TGA motifs in their promoters, has been extensively reported using the *Arabidopsis PR-1* gene as a model (Lebel et al., 1998; Kesarwani et al., 2007). In contrast, the pathway that activates SA-dependent and NPR1-independent genes has been far less explored. It has been reported that the transcriptional activation of *GRXC9* by SA requires the transcription factors

TGA class II (TGA2, TGA5 and TGA6), nevertheless there is no information about the dependence of the other bZIP transcription factors belonging to the TGA family (Ndamukong et al., 2007; Blanco et al., 2009).

TGA class III includes the TGA3 and TGA7 transcription factors. TGA3 has been described as a positive regulator in defense response (Kesarwani et al., 2007) and in the cytokinin-enhanced resistance against virulent *Pseudomonas syringae* pv. Tomato (Choi et al., 2010). There are no reports that show the effect of TGA7 in any biological process. The only available information is the expression pattern; TGA7 is highly expressed in xylem or under conditions that induce xylem differentiation (Zimmermann et al., 2008; Gatz, 2013).

In sum, there is a group of genes early induced by SA which mechanism is poorly characterized. Under the conditions tested in our laboratory, the mRNA of those genes is increased upon SA treatments. In the promoter of those genes, the *as-1* element is over-represented. The *GRXC9* gene is the most induced gene in this group and it possess two *as-1*-like elements in its upstream intergenic region. In contrast to our results, Ndamukong and colleagues show that this gene is induced through an NPR1-dependent mechanism (Ndamukong et al., 2007).

Supported by data presented above, the working hypothesis of this thesis is "Stress induces *GRXC9* expression in Arabidopsis through a salicylic acid-dependent transcriptional mechanism" and according to that, the main objective is to determine the transcriptional activation mechanism of *GRXC9* by SA.

To evaluate this hypothesis we will set the conditions for induction of *GRXC9*, evaluating the dependence of NPR1 upon SA treatments and under stress conditions where SA act as a signal. These stress conditions and the use of *ics1* (*sid2*) and *npr1* mutant plants will allow us to determine if effectively this gene is dependent of SA and

NPR1 for its activation. In a second objective, we will evaluate the dependence of TGA transcription factors for the *GRXC9* transcriptional induction and the participation of the *as-1*-like elements present in the *GRXC9* promoter. In a third objective, we will evaluate the participation of the protein GRXC9 over its own expression.

1.5 OBJECTIVES AND HYPOTHESIS

Main Objective

To elucidate the mechanism of transcriptional activation of *GRXC9* by SA.

Hypothesis

Stress induces *GRXC9* expression in Arabidopsis through a salicylic acid-dependent transcriptional mechanism.

Specific Objectives

1. To determine the expression pattern of *GRXC9* under basal and stress conditions and to evaluate the dependence of SA and NPR1.
2. To determine the participation of *as-1*-like elements and TGA transcription factors in the transcriptional induction of *GRXC9*.
3. To identify genes with co-regulatory activities that participate in the modulation of *GRXC9* gene expression.

2. METHODS

2.1 Plant growth conditions and treatments

Arabidopsis thaliana wild-type (WT), *npr1-1* (Cao et al., 1994), *tga1-1/tga4-1*, *tga3-1*, *tga2-1/tga3-1/tga5-1/tga6-1* (Kesarwani et al., 2007) *tga-7-1*, *tga2-1/tga5-1/tga6-1* (Zhang et al., 2003), and *sid2-2* (Wildermuth et al., 2001) plants were in Columbia (Col-0) background. Seedlings were grown *in vitro* in 0.5X MS medium supplemented with 10 g/l sucrose and 2.6 g/l Phytagel (Sigma) under controlled conditions (16 h light, 80 µmoles m^{-2} s^{-1}, 22 ± 2ºC). For ChIP and gene expression assays, 15-day-old seedlings were floated on 0.5 mM SA (treatment) or 0.5X MS medium as a control, and incubated for the indicated periods of time under continuous light (80 µmoles m^{-2} s^{-1}). For gene expression assays, whole seedlings were immediately frozen in liquid nitrogen and stored at -70ºC until RNA isolation. For ChIP assays, whole seedlings were processed immediately as described below. For UV-B irradiation assays, 15-day-old seedlings were exposed to UV-B light (0.07 mW/cm^2) in a chamber equipped with two USHIO UVB F8T5.UB-V, UVP 3400401 fluorescent tubes (λ= 306 nm). As a control we used non irradiated seedlings.

2.2 Genetic constructs and plant transformation

Genetic constructs were generated using the Gateway technology following the manufacturer's instructions (Invitrogen). The *GRXC9* promoter regions including the 5'UTR: -1849 to +26; -168 to +26; -112 to +26 and -61 to +26, called in the text as pC9 WT, pC9-168, pC9-112 and pC9-61, respectively, were obtained by amplification from genomic DNA, using the oligonucleotides indicated in TABLE 1 . PCR fragments were cloned into the pENTR/SD/D-TOPO vector (Invitrogen) and then recombined into the

pKGWFS7 vector to generate transcriptional fusions with *eGFP* and β-glucuronidase *GUS* reporter genes (Karimi et al., 2002). Site directed mutation of the distal and the proximal *as-1*-like element were performed on the pC9 WT promoter fragment cloned into the pENTR/SD/TOPO vector, as previously described (Weiner et al., 1994). The site directed mutations generated were performed with the oligonucleotides listed in the TABLE1. The purified PCR products were recombined into the pKGWFS7 vector. In order to generate *GRXC9* over-expressor lines, the *GRXC9* coding region was amplified from cDNA using the primers described in the TABLE 1. The PCR product was cloned into the pENTR/SD/D-TOPO vector and then recombined into the pBADcMyc vector to express the GRXC9 protein fused to a c-Myc tag controlled by the 35S CaMV promoter. Final constructs were verified by sequencing and introduced into the *Agrobacterium tumefaciens* C58 strain. *Arabidopsis* plants were transformed by floral dip method (Zhang et al., 2006a). Transgenic seeds were selected in 0.5X MS solid medium supplemented with 50 µg/ml kanamycin for the GUS reporter lines or 15 µg/ml glufosinate-ammonium for the over-expressor lines. Stable homozygous transgenic lines were used for further analyses.

2.3 GUS assays

GUS activity was determined in control- and SA-treated seedlings from each transgenic line carrying the *GRXC9* promoter-driven GUS constructs described above. The 4-methylumbelliferyl-D-glucuronide was used as substrate and the fluorescent product 4-methylumbelliferone was quantified, as previously described (Jefferson et al., 1987). Treatments were done by triplicate for each line and the measurements were normalized with total protein content quantified using the Bradford assay (BioRad).

2.4 Western Blot assays

Total protein extracts from WT and *35S:GRXC9-6xMYC* transgenic lines were separated in a SDS-PAGE in a 12% acrylamide/bisacrylamide gel. For western blotting detection, c-Myc polyclonal antibody (A-14, sc-789, Santa Cruz) was used in a 1:2000 dilution as the primary antibody and Goat anti-Rabbit IgG (H+L) HRP conjugate (1:10000, #65-6120, Invitrogen) as the secondary antibody. Thermo Scientific Pierce chemiluminiscent Western Blotting Substrate (#32109, Pierce) was used for detection.

2.5Chromatin immunoprecipitation assays

ChIP assays were performed as described (Saleh et al., 2008). Five µl of the following antibodies were used for immunoprecipitation assays: RNAPII polyclonal antibody (sc-33754, Santa Cruz Biotechnology), TGA1, TGA2 and TGA3 polyclonal antibodies (Lam and Lam, 1995), c-Myc polyclonal antibody (A-14, sc-789, Santa Cruz) and normal purified IgG (A2609, Santa Cruz Biotechnology) used as control of a non-specific antibody. The concentration of DNA in each sample (input chromatin and chromatin immunoprecipitated with either specific or non-specific antibodies) was quantified by qPCR, using the Stratagene MX3000P® equipment and the Sensimix Plus SYBR Green Reagents (Quantece). Primers used to amplify the *GRXC9* promoter region containing the *as-1*-like elements (-212 to +78) are listed in TABLE 1.

2.6 Gene expression analysis

Total RNA was obtained from frozen samples using the TRIzol® Reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized from each sample (2 µg of total RNA) with an ImProm II Kit (Promega). qPCR was performed with the Stratagene MX3000P® equipment. The expression levels of *GRXC9* and *PR-1* were calculated relative to the *YLS8* (AT5G08290) or *Clathrin adaptor*

complex subunit (AT4G24550) genes. Primers used for each gene are listed in TABLE 1.

2.7 Yeast two-hybrid assays

The coding regions of TGA factors (TGA2, TGA5, and TGA6 and TGA3) were cloned into the pDONR201 vector (Jakoby et al., 2002). These coding regions were then recombined into the pDEST22 vector, to produce a fusion protein with the Gal4 DNA binding domain; and into the pDEST32 vector, to produce a fusion protein with the transactivation domain of the Gal4 factor. Different combinations of two constructs were used to transform the SFY526 yeast strain (harboring the *Gal4RE::β-Gal* reporter construct) and qualitative assays for β-Gal activity were performed as described (Gietz and Schiestl, 2007). Interaction between NPR1 and TGA2 was assayed as a positive control and a combination of the pDEST32 and pDEST22 empty vectors was used as a negative control.

Table 1: Primers used for cloning genetic constructs, ChIP and RT-qPCR assays.

Target sequence	Forward Primer	Reverse Primer
	Cloning *GRXC9* promoter-GUS constructs	
pC9 Wt	5' CACCAAAACGCATCACCTGC 3'	5' TTTCAAGTATGTTTTTAAAGATAG 3'
pC9 -168	5' CACCGACACGGTCCTATG 3'	5' TTTCAAGTATGTTTTTAAAGATAG 3'
pC9 -112	5' CACCCCATAGCTTCCTGTG 3'	5' TTTCAAGTATGTTTTTAAAGATAG 3'
pC9 -61	5' CACCTTTCCTCTCTGATCTC 3'	5' TTTCAAGTATGTTTTTAAAGATAG 3'
	Cloning *GRXC9* CDS	
OX GRXC9	5' CACCATGCCAAGGAACGATTTC 3'	5' CAACCACAGAGCCCCAACTTCCT 3'
	Site directed *CRXC9* promoter mutations	
pC9 MD	5' TCCAATCCAGTTTTGTAAATAGC ACTATCACCCATAGCTTC 3'	5' GAAGCTATGGGTGATAGTGCTATTT ACAAAACTGGATTGGA 3'
pC9 MP	5' ATAGCTTCCTGTGTTTCACATCCTT ATTTAACCATCGTTGACG 3'	5' CGTCAACGATGGTTAAATAAGGATG TGAAACACAGGAAGCTAT 3'
	ChIP assays	
GRXC9 proximal promoter -212 to +78	5′ GTGGGATCCAAAAAGTCAGC 3′	5′ CGACGGTTGTCGTCATGTTA 3′
	RT-qPCR	
***Total GRXC9* (At1g28480)**	5' CACTCCAAGTCCAAGAAGCAG 3'	5' AGAGAGTTCGGATGGTGGTG 3'
***Endogenous GRXC9* (At1g28480)**	5′ TTAAGGAAGTTGGGGCTCTG 3′	5' CCGTAAACAACAATTACCAATCA 3′
PR-1(At2g14610)	5' GTGGGTTAGCGAGAAGGCTA 3'	5' ACTTTGGCACATCCGAGTCT 3'
***CLATHRIN ADAPTOR COMPLEX* (At5g46630)**	5' AATACGCGCTGAGTTCCCTT 3'	5' AGCACCGGGTTCTAACTCAA 3'
YLS8 (At5g08290)	5′ TTACTGTTTCGGTTGTTCTCCATTT 3'	5′ CACTGAATCATGTTCGAAGCAAGT 3'

3. RESULTS

3.1 *Objective 1:* To determine the expression pattern of *GRXC9* under basal and stress conditions and to evaluate the dependence of SA and NPR1.

3.1.1 Determination of the *GRXC9* transcript levels during the development of *Arabidopsis thaliana.*

SA plays an important role in the development of *Arabidopsis thaliana* (Rivas-San Vicente and Plasencia, 2011). Considering this, we test if *GRXC9* transcript levels are modulated during the development, evaluating different tissues and developmental ages, using RT-qPCR. Arabidopsis seeds were stratified and germinated in vermiculite and plants were grown by two months. Samples from different tissues were frozen for further analysis. The whole seedlings were collected every week until the third week. After the 3rd week, the roots and shoots were collected separately to evaluate the expression of *GRXC9*. In the 7th week, the roots, shoots, stems, caulline leaves and flowers were independently collected. In the 8th week, siliques and seeds were also independently collected.

The results show no significant differences in *GRXC9* transcript level among all tissues and ages tested (FIGURE 4), indicating that the *GRXC9* gene expression is not specifically regulated by tissue or developmental signals. As a positive control, two-weeks-old seedlings treated with 0,5 mM SA during two hours were used.

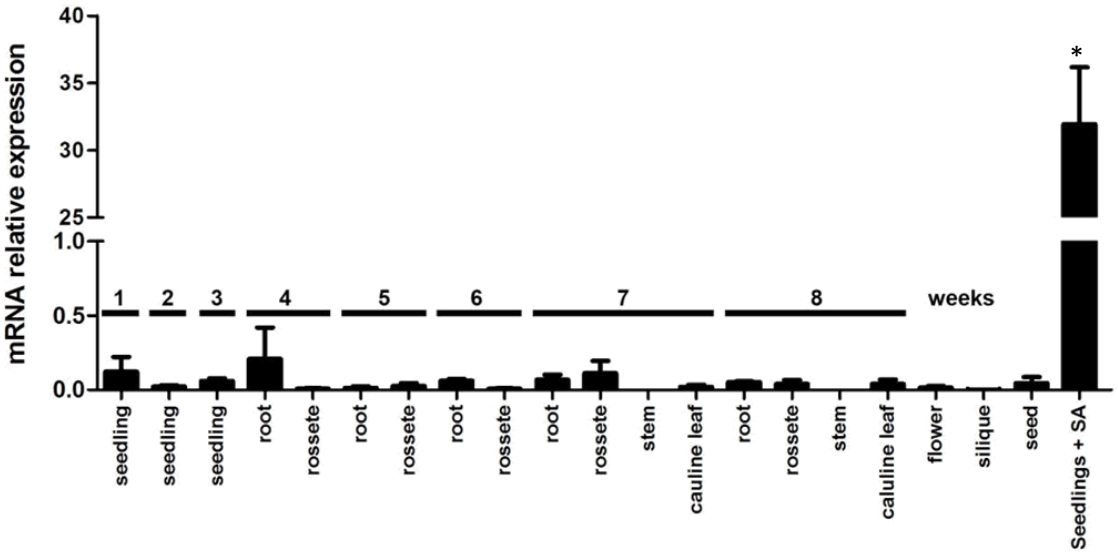

FIGURE 4. *GRXC9* transcript levels at different developmental stages of *Arabidopsis plants.*

Arabidopsis plants were grown on vermiculite substrate for 8 weeks and samples were collected every week. After the 3rd week the plants were collected separating shoot and root to test differences in tissue basal expression. Flowers, siliques and seeds were collected after the 6th week. As an induced control we used 2-weeks-old Arabidopsis plants treated with SA 0,5 mM by 2.5 h. The transcript levels of *GRXC9* gene was quantified by RT-qPCR, using *YLS8* as the housekeeping gene. Data represent mean values of 3 biological replicates ± standard error. (*p<0,05 in an one-way ANOVA and Bonferroni's multiple comparison test)

3.1.2 Evaluation of the NPR1-dependence of *GRXC9* gene expression induced by SA.

Previous and contradictory reports claim that *GRXC9* expression induced by SA is dependent on NPR1 (Ndamukong et al., 2007) and is independent of this co-activator (Blanco et al., 2009). To assess this point, we measure the *GRXC9* transcript levels in wild type (WT) and *npr1-1* mutant seedlings treated with 0,5 mM SA, using RT-qPCR. The result shows that a significant increase in *GRXC9* transcript levels occurs after 2.5 h of SA treatment in both, WT and *npr1-1* mutant plants (FIGURE 5). After 24 hours of SA treatment the transcript levels decrease to basal levels. These results are consistent with previous published results (Blanco et al., 2009). To confirm the NPR1 independence of *GRXC9* induction, we performed assays with the *npr3-1 npr4-3* double mutant plants. NPR3 and NPR4 proteins are required to control NPR1 degradation (Fu et al., 2012). The *npr3-1 npr4-3* mutant shows increased basal levels of NPR1 protein and, as a consequence, higher basal expression levels of NPR1-dependent *PR-1, PR-2* and *PR-5* genes than the WT plants (Zhang et al., 2006b; Fu et al., 2012). In the case of *GRXC9* expression, we detected an early and transient increase of transcript levels in the *npr3 npr4* double mutants (FIGURE 5), while the basal levels remained unchanged when compared to WT or *npr1-1* plants (FIGURE 5). This evidence confirms that *GRXC9* gene expression induced by SA treatment does not depend on NPR1 levels.

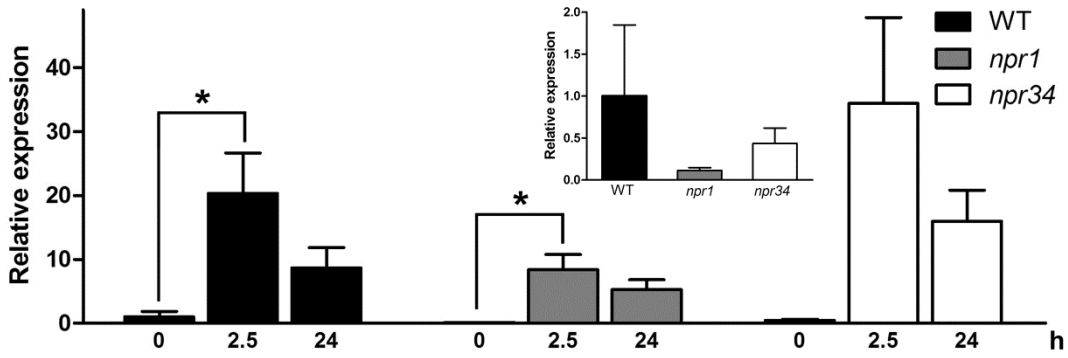

FIGURE 5. *GRXC9* expression levels upon SA treatment in wild type, *npr1-1*, and *npr3-1/npr4-3* backgrounds.

Fifteen-days-old wild type plants (WT), *npr1-1* mutant plants (*npr1*) and *npr3-1/npr3-4* double mutant plants (*npr34*) were treated with SA 0,5mM (SA) for the periods of time indicated in the graph. The samples were collected and used to prepare RNA and perform RT-qPCR assays. The basal levels of *GRXC9* expression in the analyzed genotypes are shown in the insert. The *GRXC9* relative expression was calculated by normalizing the expression level of *GRXC9* to the expression level of *YLS8* gene used as housekeeping and to the wild type basal level. Bars represent the average value +/- standard error of three biological replicates. * $p<0,05$, based on one-way ANOVA and Bonferroni multiple comparison test. There no statically significant differences in the basal expression levels of *GRXC9* among the different backgrounds in a two-ways ANOVA test.

3.1.3 Determination of *GRXC9* gene expression under stress conditions and evaluation of the dependence of SA and NPR1.

3.1.2.1 *GRXC9* induction profile upon abiotic stress.

To further validate the expression of GRXC9 under a stressor condition sensed naturally by the plant, we used UV-B radiation as a stress condition since SA has been identified as a signaling molecule in this defense response (Surplus et al., 1998). To evaluate the *GRXC9* gene expression dependence on SA, we used *sid2-2* mutant plants that are deficient in SA biosynthesis (Wildermuth et al., 2001). Then, *GRXC9* transcript levels were measured by RT-qPCR in WT, *npr1-1* and *sid2-2 Arabidopsis* plants exposed for 2.5 and 24 h to UV-B light. Mean values from 3-6 replicates are shown in FIGURE 6. We detected a significant increase in *GRXC9* transcript levels after 24 h of stress exposure in WT and *npr1-1* plants. This response was almost completely abolished in the *sid2-2* mutant plants (FIGURE 6a). *PR-1* gene expression after UV-B treatment was evaluated as a control for the SA- and NPR1-dependent pathway (FIGURE 6b).

Concerning the basal levels of *GRXC9* and *PR-1* expression, we detected a reduction in *npr1-1* and *sid2-2* mutants compared to WT plants, but these differences were not statistically significant (FIGURE 6a, b).

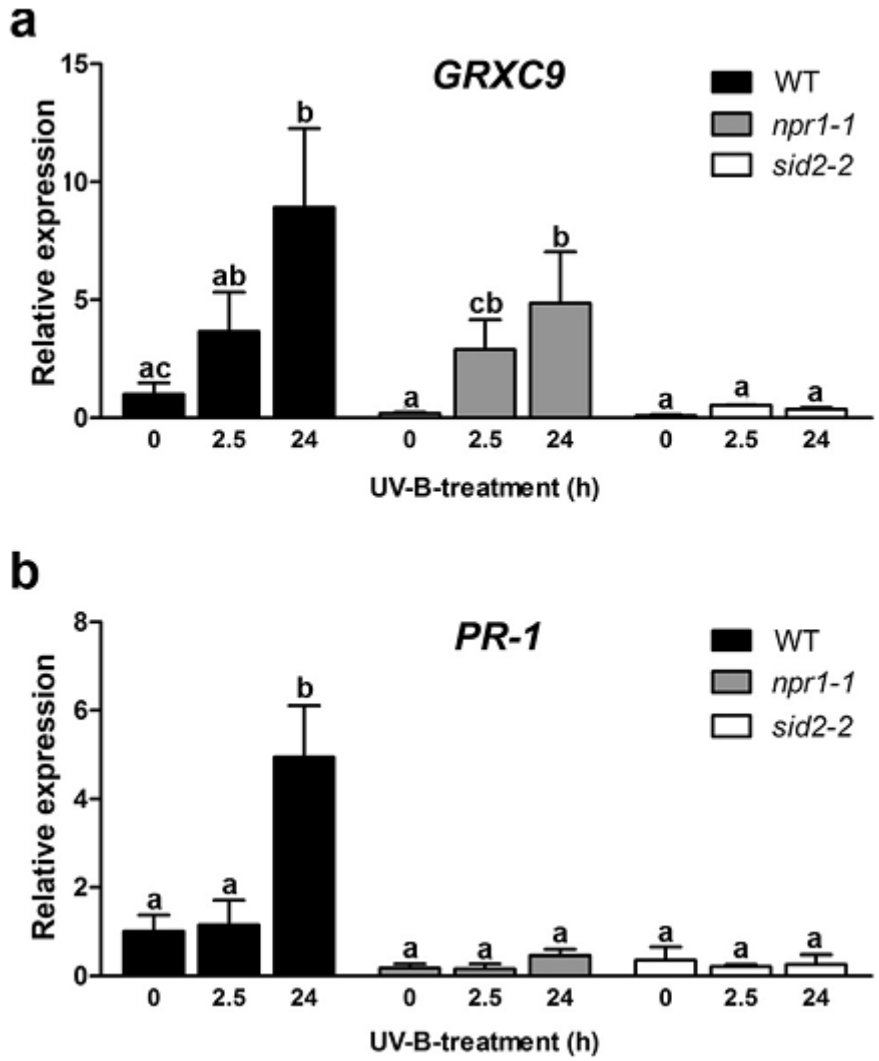

FIGURE 6. *GRXC9* expression levels in WT, *sid2-2* and *npr1-1* seedlings upon UV-B chronic exposure.

Expression levels of *GRXC9* (**a**) and *PR-1* (**b**) genes in fifteen-day-old seedlings from WT (black bars), *npr1-1* (grey bars) and *sid2-2* (white bars) plants exposed to UV-B light. The transcript levels for each gene were quantified by RT-qPCR from samples collected after 0, 2.5 and 24 h of UV-B exposure. The relative expression was calculated by normalizing the *GRXC9* and *PR-1* transcript levels to that of the *YLS8* gene and to the WT basal levels. Error bars represent the mean ± standard error from 3-6 replicates. Letters above the bars indicate significant differences based on a two-way ANOVA analysis and Bonferroni post test.

3.3.2.2 *GRXC9* induction profile upon biotic stress.

Some biotic stresses also are able to induce SA accumulation (Enyedi et al., 1992; Tsuda et al., 2008). In this context, the infection of Arabidopsis plants with an avirulent strain of the pathogenic bacteria *Pseudomonas syringae* is a very used model to induce the SA-mediated pathway. To evaluate the *GRXC9* activation and the NPR1 and SA-dependence in the defense response to biotic stress, 4-weeks-old WT, *npr1-1* and *sid2-2* plants were infected with the avirulent *Pseudomonas syringae* AvrRpm1 strain ($1{,}0*10^8$ ufc/ml) or $MgCl_2$ 10 mM as a mock control. The results show an induction of *GRXC9* gene at 5 and 8 h post inoculation with *Pseudomonas* in WT and *npr1-1* plants. In *sid2-2* genotype the *GRXC9* gene is not induced at the analyzed times (FIGURE 7a). As a control we measured the *PR-1* transcript (FIGURE 7b). *PR-1* shows an induction at 24 and 48 h in WT plants but, as we expected, it is not induced in *npr1-1* and *sid2-2* mutant plants.

Together, these results show that *GRXC9* is responsive to UV-B and avirulent *Pseudomonas* strain, via a SA-dependent and NPR1-independent pathway.

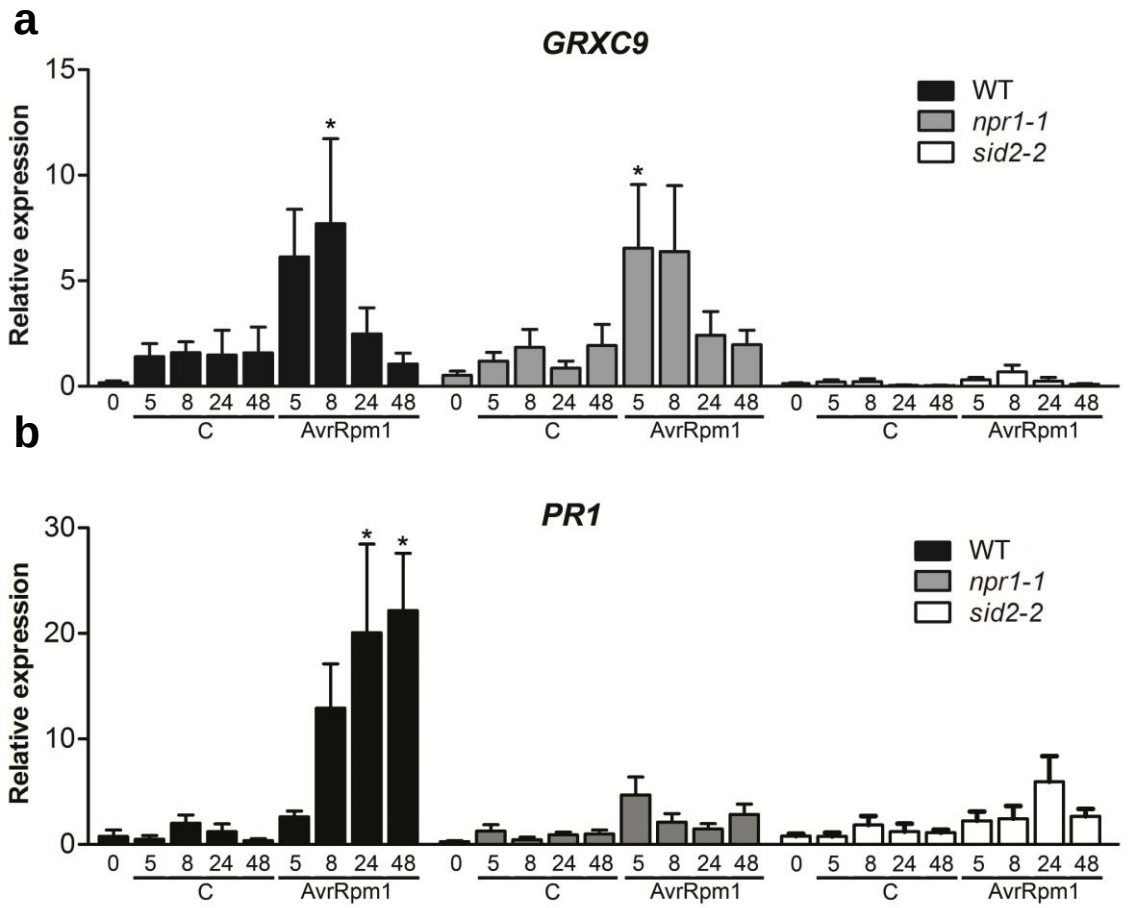

FIGURE 7. *GRXC9* expression levels in WT, *sid2-2* and *npr1-1* plants upon avirulent *Pseudomonas syringae* AvrRpm1 challenge.

Expression levels of *GRXC9* (**a**) and *PR-1* (**b**) genes in 4 to 5-weeks-old plants from WT (black bars), *npr1-1* (grey bars) and *sid2-2* (white bars) backgrounds. Plants were inoculated with *Pseudomonas syringae* pv. tomato AvrRpm1 $1{,}0*10^{8}$ ufc/ml (AvrRpm1) or with $MgCl_2$ as a control (C). The transcript levels for each gene were quantified by RT-qPCR from samples collected after 0, 5, 8, 24 and 48 h of inoculation. The relative expression was calculated by normalizing the *GRXC9* and - transcript levels to that of the *YLS8* gene and to the WT basal levels. Error bars represent the mean ± standard error from 3-5 biological replicates. * indicates $p<0{,}05$ in a one-way ANOVA and Bonferroni post test comparing the treatments in every genotype. No statically differences were found in *GRXC9* expression between WT and *npr1-1* in a two-way ANOVA analysis.

***3.2 Objective 2*: To determine the participation of *as-1*-like elements and TGA transcription factors in the transcriptional induction of *GRXC9*.**

3.2.1 Evaluation of the role of the *as-1*-elements in the transcriptional induction of *GRXC9*.

In silico analysis of the *GRXC9* promoter sequence revealed the presence of several putative SA-responsive elements including two *as-1*-like elements, several W boxes and isolated TGA boxes (FIGURE 3). The proximal *as-1*-like element is located between -80 and -99 bp, and the distal one is located between -114 and -133 bp upstream of the transcriptional start site (FIGURE 3, 8a). In order to evaluate whether these elements mediate the SA-dependent transcriptional activation of the gene, we generated *Arabidopsis* transgenic lines harboring different versions of the *GRXC9* promoter fused to the *GUS* reporter gene. The constructs contained either the complete intergenic region (-1849 to +26), considered as the full *GRXC9* promoter (*pC9 WT::GUS*), or truncated versions of this sequence that include: the two *as-1*-like elements up to -168 bp (*pC9-168::GUS*), only the proximal *as-1*-like element up to -112 bp (*pC9-112::GUS*), or a minimal promoter up to -61 that includes the putative TATA box (*pC9-61::GUS*) (FIGURE 8).

We treated seedlings (from six to 13 independent homozygous lines for each construct) with 0.5X MS as control or with 0.5 mM SA for 2.5 h. We then quantified the basal and SA-induced GUS activities in total protein extracts (FIGURE 8b). The responsiveness to SA of each construct was represented as the mean value of the GUS activity induction ratio (SA-induced/basal GUS activities) (FIGURE 8c). An average of six fold increase in GUS activity after SA treatment was recorded in lines that contain the full promoter (*pC9 WT::GUS*), which indicates that *GRXC9* gene expression is effectively activated by SA at the transcriptional level. Surprisingly, a very small

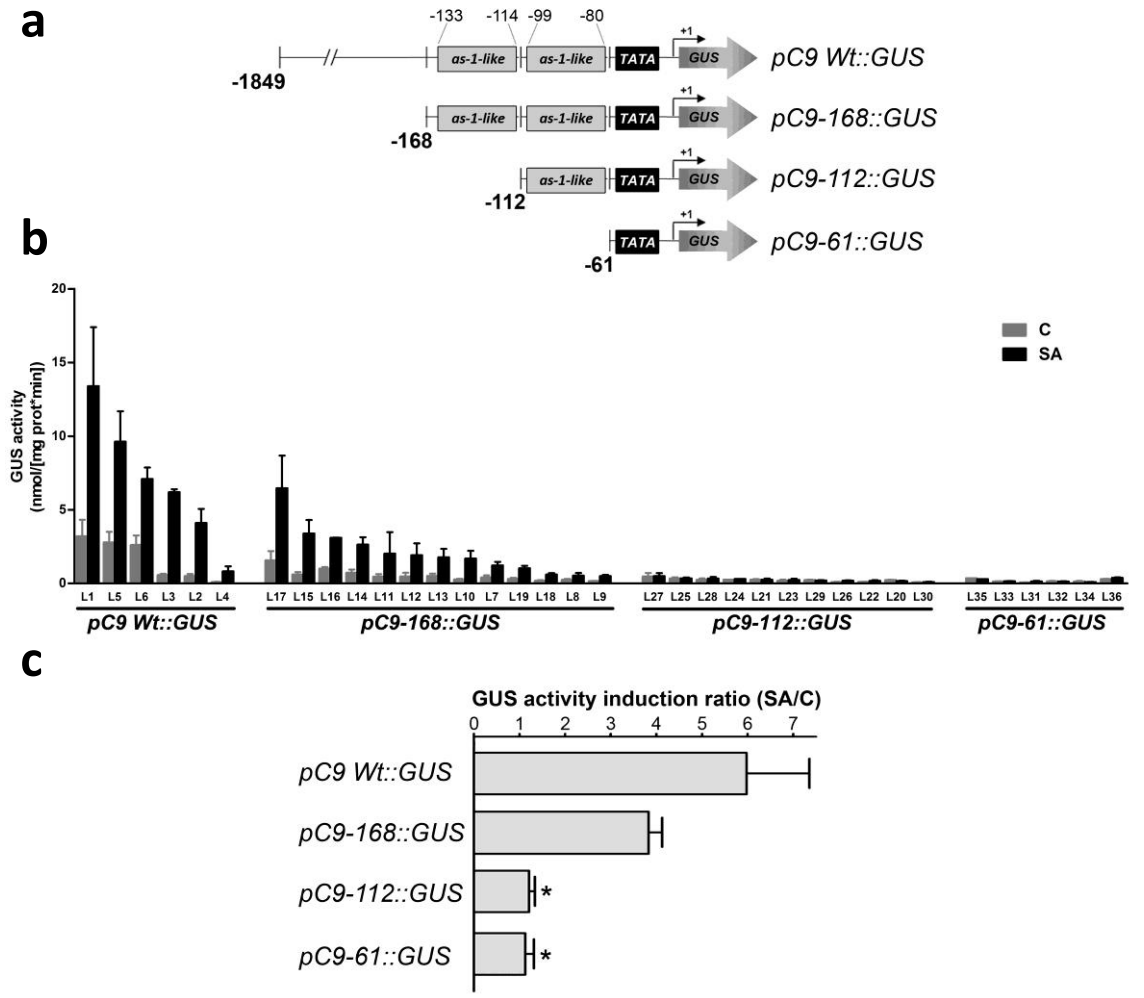

FIGURE 8. Analysis of *GRXC9* promoter using wild type and truncated versions of the promoter fused to the GUS reporter gene.

(**a**) Schematic representation of *GRXC9* promoter constructs used to generate the GUS reporter lines. The numbers on the left side indicate the size of the promoter region cloned to drive *GUS* expression and the numbers above the first construct indicate the position of the *as-1*-like elements from the transcriptional start site in the *GRXC9* promoter. *pC9 WT::GUS*: complete intergenic region for *GRXC9*; *pC9-168::GUS*: promoter region containing the two *as-1*-like elements; *pC9-112::GUS*: region containing the proximal *as-1*-like element and *pC9-61::GUS*: sequence of the promoter region containing the putative TATA box (**b**) The mentioned lines were treated with SA 0.5 mM (SA, black bars) or MS 0,5X as a control (C, grey bars). GUS activity was quantified from total protein extracts of each independent line. Bars represent the average value ± standard error of three biological replicates for each line. Every pair of bars (L1, L2, L3, etc.) represents an independent transgenic line (**c**) The ratio between SA treatment and its respective control were calculated and the graph represents the

average of GUS activity ratio obtained from the different lines analyzed in (**b**). Error bars represent the standard error. * indicate significant differences based on one-way ANOVA and Bonferroni test in the different lines compared to the pC9 WT::GUS lines ($p<0.05$).

promoter region up to -168 retains an important part of the SA responsiveness of the *GRXC9* full promoter (FIGURE 8). This region contains both *as-1*-like elements, the most proximal TGA box, and the most proximal W box, while lacking all the rest of the putative SA-responsive elements (FIGURE 3**)**. In contrast, lines expressing the *pC9-112::GUS* and the *pC9-61::GUS* constructs were completely insensitive to SA treatment (FIGURE 8b,c), suggesting that the loss of the distal *as-1*-like element, and/or the proximal W and TGA boxes, is enough to abolish SA-responsiveness. The basal expression is increased in the lines containing the complete intergenic region compared to the other constructions. This effect could be explained by functional elements located upstream the distal as-1-like element that confers a basal expression.

To further evaluate the importance of the two *as-1*-like elements in the SA-responsiveness of the promoter, we generated genetic constructs containing the full *GRXC9* promoter carrying point mutations in each *as-1*-like element (FIGURE 9). The nucleotides mutated in the *as-1* like sequences were chosen considering the most conserved ones in the consensus *as-1*-like element detected in the cluster of genes induced by SA in a NPR1-independent manner (Blanco et al., 2009). Four independent *Arabidopsis* reporter lines were selected for each construct, either having mutations in the proximal (*pC9 MP::GUS*) or in the distal (*pC9 MD::GUS*) *as-1*-like elements (FIGURE 9a). Seedlings from each line were treated with SA or 0.5X MS and the GUS activity was quantified. As shown in FIGURE 9b**,** lines that carry mutations in any of the two *as-1* elements no longer respond to SA treatment.

These results indicate that the increase in *GRXC9* transcript levels in response to SA is mainly due to transcriptional activation of the gene mediated by this hormone, and that the loss of any of the *as-1*-like elements is enough to abolish this activation.

Both elements are functional, essential and sufficient for SA responsiveness of the *GRXC9* promoter.

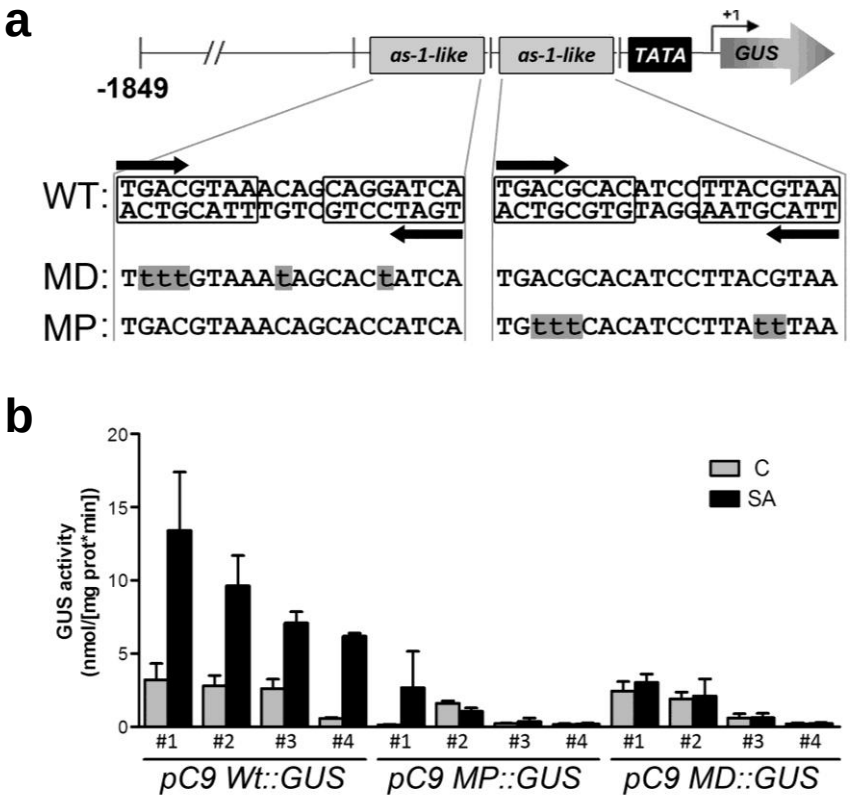

FIGURE 9. Analysis of *GRXC9* promoter using wild type *GRXC9* promoter or site directed mutations of *GRXC9* promoter fused to the GUS reporter gene.

(a) Schematic representation of genetic constructs containing site-directed mutations in the *as-1*-like elements, in the context of the full *GRXC9* promoter sequence, and fused to *GUS* coding region. *pC9 WT::GUS* (*WT* in the scheme) was used as a template to mutate the TGACG boxes (indicated by black arrows) from the two *as-1*-like elements (highlighted in black boxes). The mutated base pairs in distal *as-1*-like element (MD) and proximal *as-1*-like element (MP) are indicated in lowercase and highlighted in gray boxes. **(b)**. Four independent homozygous GUS reporter lines carrying the *pC9 WT::GUS* and the mutated version in the proximal and distal *as-1*-like elements, *pC9 MP::GUS* and *pC9 MD::GUS* respectively (described in a), were used to quantify GUS activity. Fifteen-day-old seedlings were treated with SA 0.5 mM (black bars) or 0.5X MS (gray bars) as control for 2.5 h; total proteins were prepared and GUS activity was quantified and normalized with total protein concentration. The graph shows the mean value of three biological replicates for each line. Error bars represent the ± standard error.

3.2.2 Evaluation of the participation of TGA factors in the transcriptional induction of *GRXC9.*

It has been reported that *GRXC9* induction by SA treatment is abolished in the TGA class II triple mutant (*tga2-1/tga5-1/tga6-1*) (Ndamukong et al., 2007; Blanco et al., 2009). Nevertheless, the possible participation of other members of the TGA family proteins, as well as the direct binding of TGA factors to the *GRXC9* promoter, has not been addressed. With this purpose in mind, we first analyzed the SA-induced expression of *GRXC9* by RT-qPCR in different *tga* mutant backgrounds (FIGURE 10). Considering that TGAs belonging to class I (TGA1 and TGA4) and class II (TGA2, TGA5 and TGA6) show different degrees of redundancy (Zhang et al., 2003; Kesarwani et al., 2007), we used the double and triple mutant, respectively. The redundancy of TGAs class III (TGA3 and TGA7) has not been demonstrated, thus we analyzed the single mutants for each gene (Kesarwani et al., 2007). We correlated the expression data with *in vivo* binding assays of TGA factors to the *GRXC9* promoter after SA treatment using chromatin immunoprecipitation (ChIP) assays (FIGURE 11). ChIP-qPCR assays were performed in WT plants treated with SA or 0.5X MS as control, using primers that amplify a 290 bp fragment (-212 to +78 region) that includes the basal promoter and the *as-1*-like elements (FIGURE 11a). Antibodies that specifically recognize TGA1, TGA2 and TGA3, raised against the divergent N terminal regions, were used (Lam and Lam, 1995).

In the double mutant of class I TGAs (*tga1-1/tga4-1*), *GRXC9* expression was early and transiently activated reaching its peak 2.5 h after SA treatment, as in WT plants (FIGURE 10). Accordingly, the *in vivo* binding of TGA1 to the *GRXC9* promoter, evaluated by ChIP-qPCR, was not detected either under control conditions or

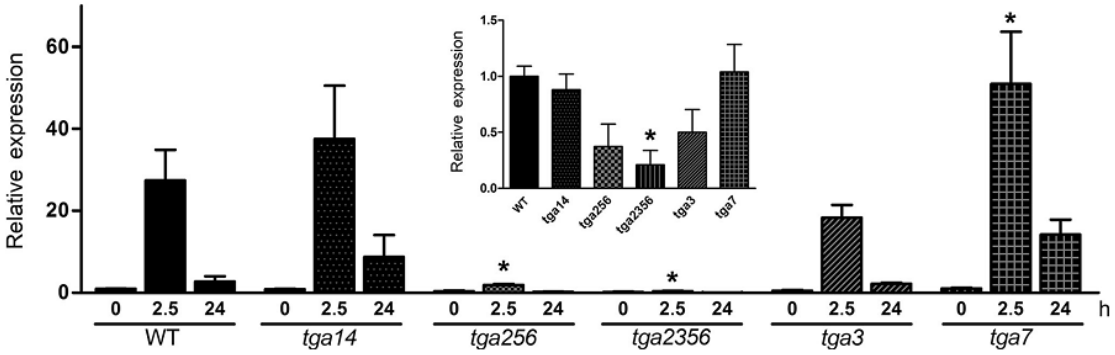

FIGURE 10. *GRXC9* gene expression in *tga* mutant backgrounds.

Expression analysis of the *GRXC9* gene was evaluated by RT-qPCR in fifteen-day-old seedlings of WT, *tga1-1tga4-1 (tga14), tga3-1 (tga3), tga7-1 (tga7), tga2-1tga5-1tga6-1 (tga256),* and *tga2-1tga3-1tga5-1tga6-1 (tga2356)* genotypes, under basal conditions (insert) and after treatment with SA 0.5 mM or 0.5X MS as a control for 2.5 and 24 h. The *GRXC9* relative expression was calculated by normalizing the expression level of *GRXC9* with the expression level of the housekeeping gene *Clathrin adaptor complex subunit,* and with the WT basal condition. Error bars represent the ± standard error from three biological replicates (* $p<0.05$, compared to the WT genotype in a two way ANOVA and Bonferroni post test).

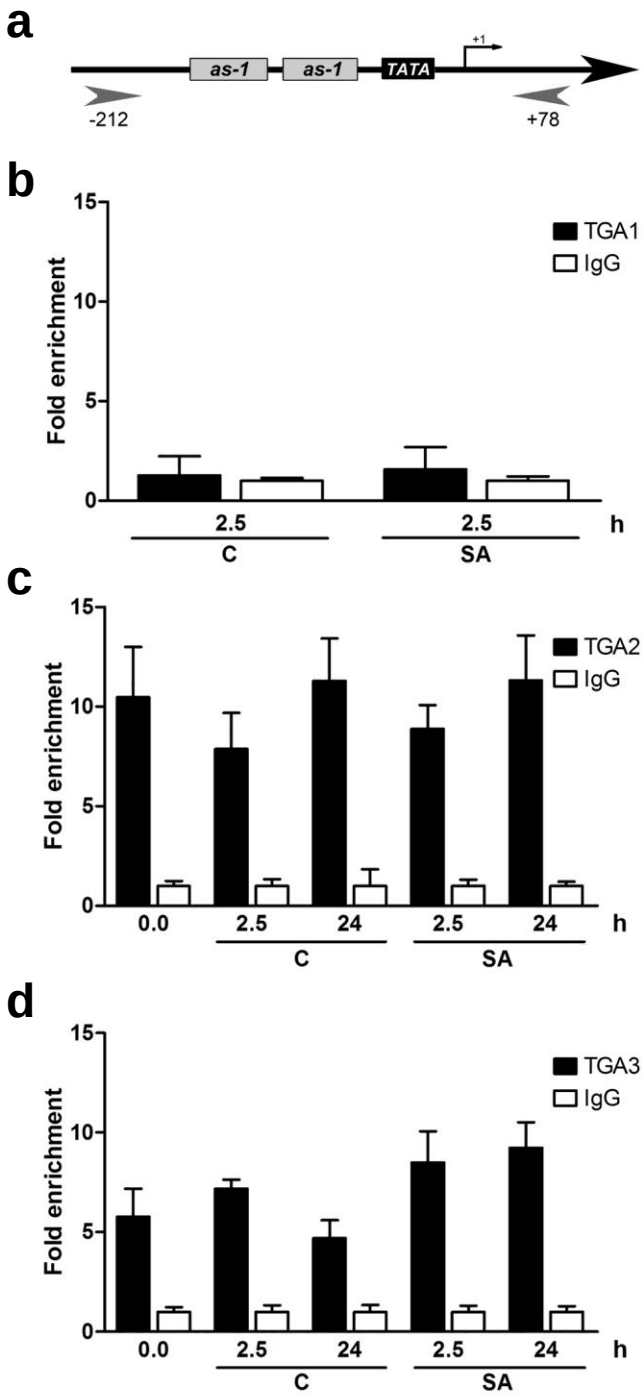

FIGURE 11. Analysis of TGAs binding to *as-1*- like elements of the *GRXC9* promoter by ChIP-qPCR assays.

(a) Diagram of the *GRXC9* promoter region amplified in the ChIP-qPCR assays. The arrow-heads indicate the location of the primers used to quantify the DNA of the *GRXC9* promoter bound to TGAs by qPCR**.** Fifteen-day-old plants treated with 0.5 mM SA (SA) or 0.5X MS as control (C) for 2.5 and 24 h, were used to perform ChIP-qPCR assays. Antibodies raised against TGA1 **(b)**, TGA2 **(c)** and TGA3 **(d)** transcription factors (in black bars) or IgG as a control (white bars) were used for the ChIP assays. qPCR analyses to quantify the DNA recovered from the ChIP were performed using the primers described in (**a**). The values for the immunoprecipitated DNA samples are expressed as fold enrichment with the specific antibody over a non-specific immunoprecipitation condition (IgG). Error bars represent ± standard error of three biological replicates

after 2.5 h of SA treatment (FIGURE 11b). These results indicate that class I TGAs are not involved in *GRXC9* induction by SA.

In contrast, *GRXC9* induction by SA was significantly reduced in the triple mutant of TGA class II (*tga2-1/tga5-1/tga6-1*), compared to WT plants (FIGURE 10); although a slight increase of the transcript after 2.5 h of SA was observed. On the other hand, the induction level by SA was 33.4% reduced in the *tga3-1* mutant compared to WT plants; albeit this difference was not statistically significant (FIGURE 10). To better evaluate the importance of TGA3 in *GRXC9* expression, we also assayed the quadruple *tga2-1/tga3-1/tga5-1/tga6-1* mutant background. Although the lack of TGA class II had a striking negative effect on SA-induced *GRXC9* transcription, the slight increase in mRNA levels after 2.5 h of SA treatment seen in the triple mutant, is no longer observed in the quadruple mutant (FIGURE 10). These results support that TGA class II, and to a lesser extent TGA3, are involved in *GRXC9* induction by SA. Interestingly, mutations in TGA class II and TGA3 factors also have an effect on the basal levels of *GRXC9* expression (FIGURE 10, insert). Compared to WT plants, basal *GRXC9* transcript levels are reduced in the single *tga3-1* and in the triple *tga2-1/tga5-1/tga6-1* mutants, and this difference is higher and only statistically significant in the quadruple *tga2-1/tga3-1/tga5-1/tga6-1* mutant (FIGURE 10 insert). Supporting these expression results, ChIP-qPCR assays show that TGA2 and TGA3 are constitutively bound to the *GRXC9* promoter, either in the presence or in the absence of SA stimulus (FIGURE 11c-d).

Surprisingly, the lack of TGA7 produces a significant increase in *GRXC9* induction by SA (FIGURE 10), suggesting that TGA7 can play a negative role in this control mechanism. We did not detect involvement of TGA factors in repressing

GRXC9 expression under basal conditions (FIGURE 10, insert), in contrast to what was previously reported for *PR-1* expression (Kesarwani et al., 2007).

Together, these results indicate that constitutive binding of the TGA2 factor to the *GRXC9* promoter is essential for transcriptional activation mediated by SA. Even though TGA3 is also constitutively bound, its role is more important in the basal than in the SA-induced *GRXC9* expression.

3.2.3 Evaluation of the transactivation capacity and the protein-protein interaction profiles of TGA transcription factors.

Considering that the constitutive binding of TGA2 and TGA3 to the *GRXC9* promoter detected *in vivo* does not correlate with a constitutive expression of the gene, we propose that these TGA factors could bind- either as homo or heterodimers, without being directly able to transactivate transcription. In order to evaluate the potential homo and hetero-dimerization ability of TGA class II and TGA3 factors, as well as their transactivation activity, we performed yeast one and two hybrid assays. For this purpose, we cloned the CDS of TGA factors in frame with the DNA binding domain (BD) or the activation domain (AD) of the yeast Gal4 factor. Interactions between TGA factors were evaluated in yeast by qualitative assays of the β-galactosidase reporter gene, whose expression is controlled by four copies of the Gal4-responsive element. The transactivation ability of the TGA factors was evaluated in assays using the TGA factors fused to the BD-Gal4 and the empty pDEST22 vector. Our results indicate that TGA2, TGA3, TGA5 and TGA6 cannot transactivate the β-galactosidase gene in a yeast system, reflected by the null activity of the reporter enzyme (FIGURE 12, first lane). Furthermore, the two-hybrid assays show that TGA class II and TGA3 factors are able to homo- and also hetero-dimerize among them (FIGURE 12). The interaction

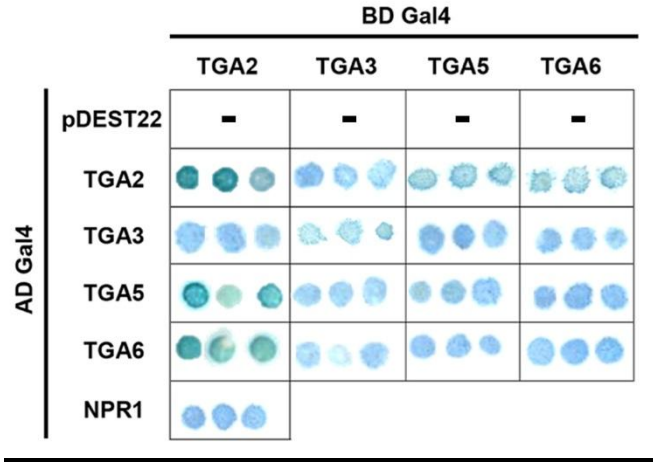

FIGURE 12. Interaction of TGA transcription factors by yeast two hybrid assays.
TGA2, TGA3, TGA5 and TGA6 were expressed as fusion protein either to the Gal4 DNA binding domain (BD Gal4) or to the Gal4 transactivation domain (AD Gal4), by cloning the respective CDS into pDEST32 and pDEST22 vectors. Three colonies of SFY526 yeast strain (harboring the *Gal4RE::β-Gal* reporter construct) expressing different combinations of TGA fusion proteins, as indicated in the figure, were qualitatively assayed for β-Galactosidase activity (blue: positive colonies, - : white negative colonies). The transactivation activity of TGA factors was addressed by co-expressing the TGA-BD Gal4 constructs with the empty pDEST22 vector (first line). Interaction between NPR1 and TGA2 was used as a positive control.

between NPR1 and TGA2 proteins was used as a positive control in these assays (Fan and Dong, 2002).

These results support the idea that TGA2 and TGA3 bind to the *GRXC9* promoter, most probably through the SA-responsive *as-1*-like elements, both as homo or heterodimers, without acting directly in transactivation.

3.2.4 Evaluation of the RNA Polymerase II recruitment to the *GRXC9* promoter in SA treatments and the dependence of TGA factors.

We evaluated whether, independently of the constitutive binding of TGA2 and TGA3 to the *GRXC9* promoter, the transient increase in *GRXC9* transcript levels correlates with a transient recruitment of RNA polymerase II (RNAPII) to the *GRXC9* promoter, induced by SA. In order to test this, *Arabidopsis* seedlings were treated with 0.5 mM SA or 0.5X MS as a control, and ChIP assays were performed at different times. We used antibodies that recognize the N-terminal domain of RNAPII from *Arabidopsis* and the set of primers previously described (FIGURE 9a). Interestingly, we found a good correlation between the increment in *GRXC9* mRNA levels (FIGURE 10), and the recruitment of RNAPII to the *GRXC9* promoter triggered by SA (FIGURE 13). Similarly, the recruitment of RNAPII, as well as *GRXC9* expression was abolished in the *tga2-1/tga5-1/tga6-1* triple mutant (FIGURE 13b and FIGURE 10).

Taken together, these results indicate that SA triggers the transient recruitment of RNAPII to the basal *GRXC9* promoter, which explain the transient increase in *GRXC9* transcript levels triggered by SA. On the other hand, the fact that the increase in *GRXC9* transcript levels and the RNAPII recruitment are impaired in the *tga2-1/tga5-1/tga6-1* triple mutant, suggest that the constitutive binding of TGA factors is required for differential RNAPII recruitment to the promoter.

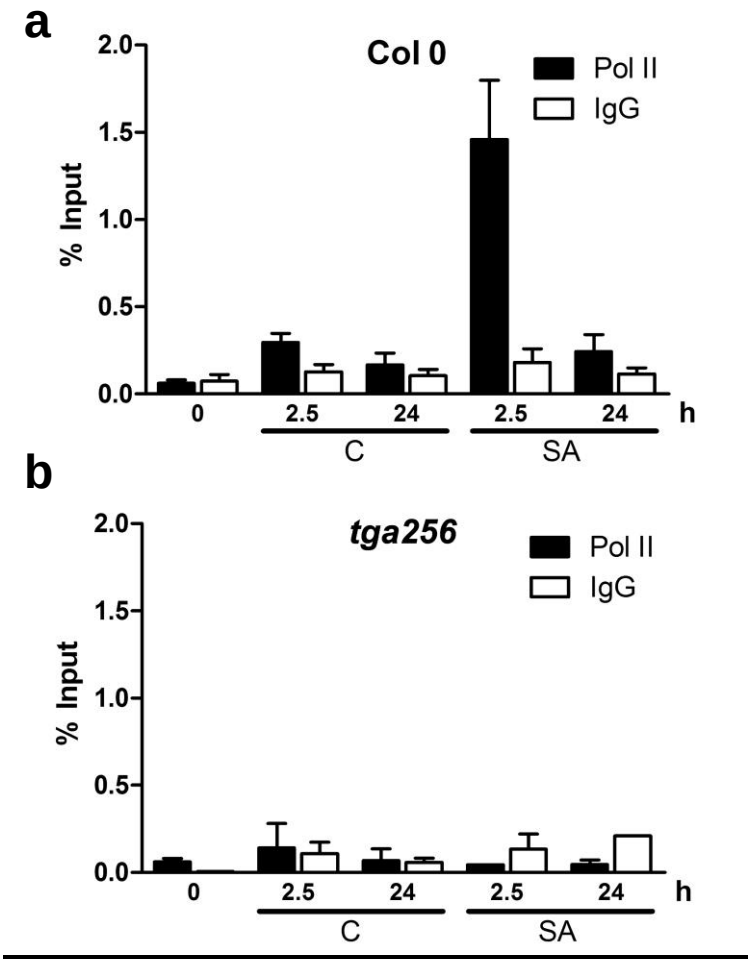

FIGURE 13. Recruitment of the RNA Polymerase II to the *GRXC9* promoter region by ChIP-qPCR assays.

WT (**a**) and *tga2-1 tga 5-1 tga 6-1* (**b**) plants treated with 0.5 mM SA (SA) or 0.5X MS as control (C) for 0, 2.5 and 24 h were used to perform ChIP-qPCR assays**.** The ChIP assays were performed with a polyclonal antibody raised against the RNAPII (black bars) or with a purified IgG (white bars) as a control. The qPCR analyses to quantify the DNA recovered from the ChIP were performed using the primers described in FIGURE 11a. The values for the immunoprecipitated DNA samples were expressed as fold enrichment with the specific antibody over a non-specific immunoprecipitation condition (IgG). Error bars represent ± standard error of three biological replicates.

3.3 *Objective 3*: To identify genes with co-regulatory activities that participate in the modulation of *GRXC9* gene expression.

It has been shown that TGA2 and GRXC9 are able to interact *in vivo* and that this interaction may have a role in controlling the expression of genes involved in the defense response triggered by jasmonic acid (Ndamukong et al., 2007). However, the significance of this interaction in the SA response has not been addressed. Our results indicate that TGA2 is a key factor in the transcriptional induction of *GRXC9*, thus we evaluated whether the over-expression of *GRXC9* has an effect in its own transient SA-dependent transcriptional induction.

3.3.1. Evaluation of GRXC9 overexpressor lines.

In order to evaluate the effect of the protein GRXC9 on the transcriptional expression of its own gene controlled by *as-1*-like elements and TGA transcription factors, we evaluate transgenic lines harboring the *CaMV35S:GRXC9-6xMYC* construct previously developed in our laboratory (Paula Salinas, unpublished results). This construct allows the constitutive expression of *GRXC9* coding region fused to the immunological 6xMYC tag. We chose two homozygous transgenic lines (L3 and L7).

The total *GRXC9* transcripts level (endogenous *GRXC9* and transgenic *GRXC9-6xMYC* mRNAs) was measured by RT-qPCR under basal conditions in WT plants and in the L3 and L7 overexpressor lines (FIGURE 14a). Both transgenic lines show a high constitutive expression levels of *GRXC9* compared to the WT plants.

As the transgenic construct also include a 6xMYC tag, we also evaluate the GRXC9 protein levels in nuclear extracts by western blot. As a control we used untransformed WT plants (FIGURE 14b). The GRXC9-6xMYC protein (40Kda) was only detected in transgenic plants from both lines. Despite the differences in mRNA levels between L3 and L7 transgenic lines (FIGURE 14a), no quantifiable differences

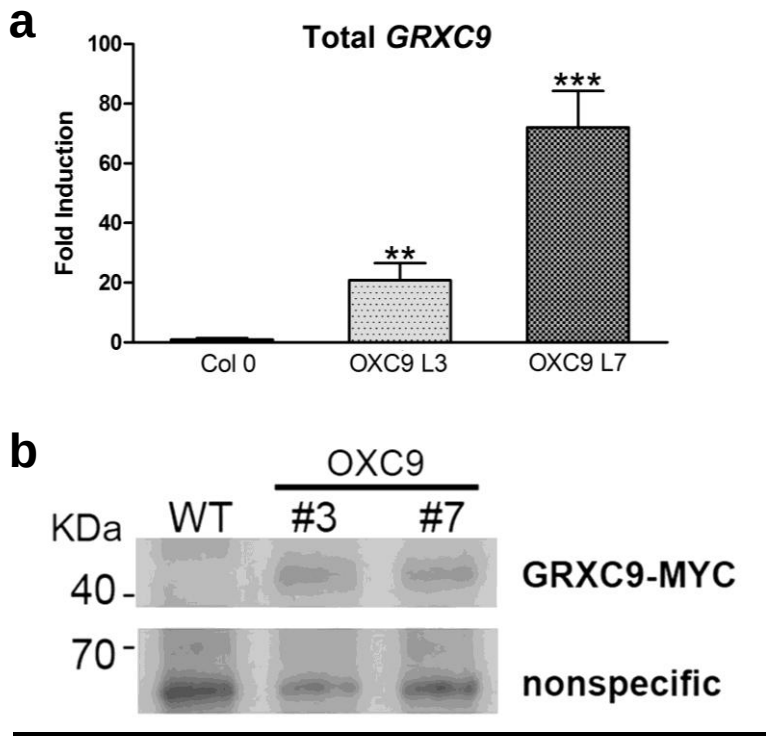

FIGURE 14. Characterization of *GRXC9* overexpressor plants.

Wild type plants were transformed by floral dip protocol with *Agrobacterium tumefaciens* carrying the *GRXC9* coding sequence fused to the *6xMyc* tag controlled by the CaMV35S promoter. Two independent lines were isolated. **(a)** The levels of total *GRXC9* transcripts were detected by RT-qPCR in fifteen-day-old seedlings of WT and *GRXC9* overexpressor lines (OXC9 L3 and OXC9 L7). Error bars represent the ± standard error. * above the bars indicate significant differences based on one way ANOVA and Bonferroni test (n=3, $p<0.05$). **(b)** The GRXC9-6xMYC protein (40 KDa) was detected by western blot using 6xMYC antibodies. A nonspecific signal is showed as a loading control.

were detected in protein levels by ImageJ analysis between the L3 and L7 transgenic lines (data not shown).

3.3.2. Evaluation of the endogenous *GRXC9* expression in GRXC9-6xMYC overexpressor lines.

In order to evaluate the influence of GRXC9 protein over the transcriptional control of its own gene, we evaluated the levels of the endogenous *GRXC9* transcript by RT-qPCR in WT and overexpressor lines treated with SA or 0.5X MS as control for 2.5 h (FIGURE 15). SA induction of the endogenous *GRXC9* gene was significantly reduced in both overexpressor lines to less than 50% of the SA induction observed in WT plants. Although basal levels of *GRXC9* expression are reduced in both overexpressor lines compared to WT, these differences were not statistically significant (FIGURE 15a). As a control, we show that over-expression of *GRXC9* does not affect basal or SA-induced *PR-1* gene expression (FIGURE 15b). These results suggest that GRXC9 negatively regulates the expression of its own gene.

3.3.3 Evaluation of the binding of GRXC9-6xMYC protein to the endogenous *GRXC9* gene promoter.

To further investigate the role of GRXC9 in the regulation of its own gene, we used ChIP-qPCR assays in the overexpressor lines to evaluate whether the GRXC9-6xMYC protein is associated to the *GRXC9* promoter. Interestingly, we detected that GRXC9-6xMYC protein effectively forms part of the protein complex bound to the promoter (FIGURe 16), strongly suggesting that GRXC9 regulates its own gene expression through binding to TGA factors while they are bound to the DNA.

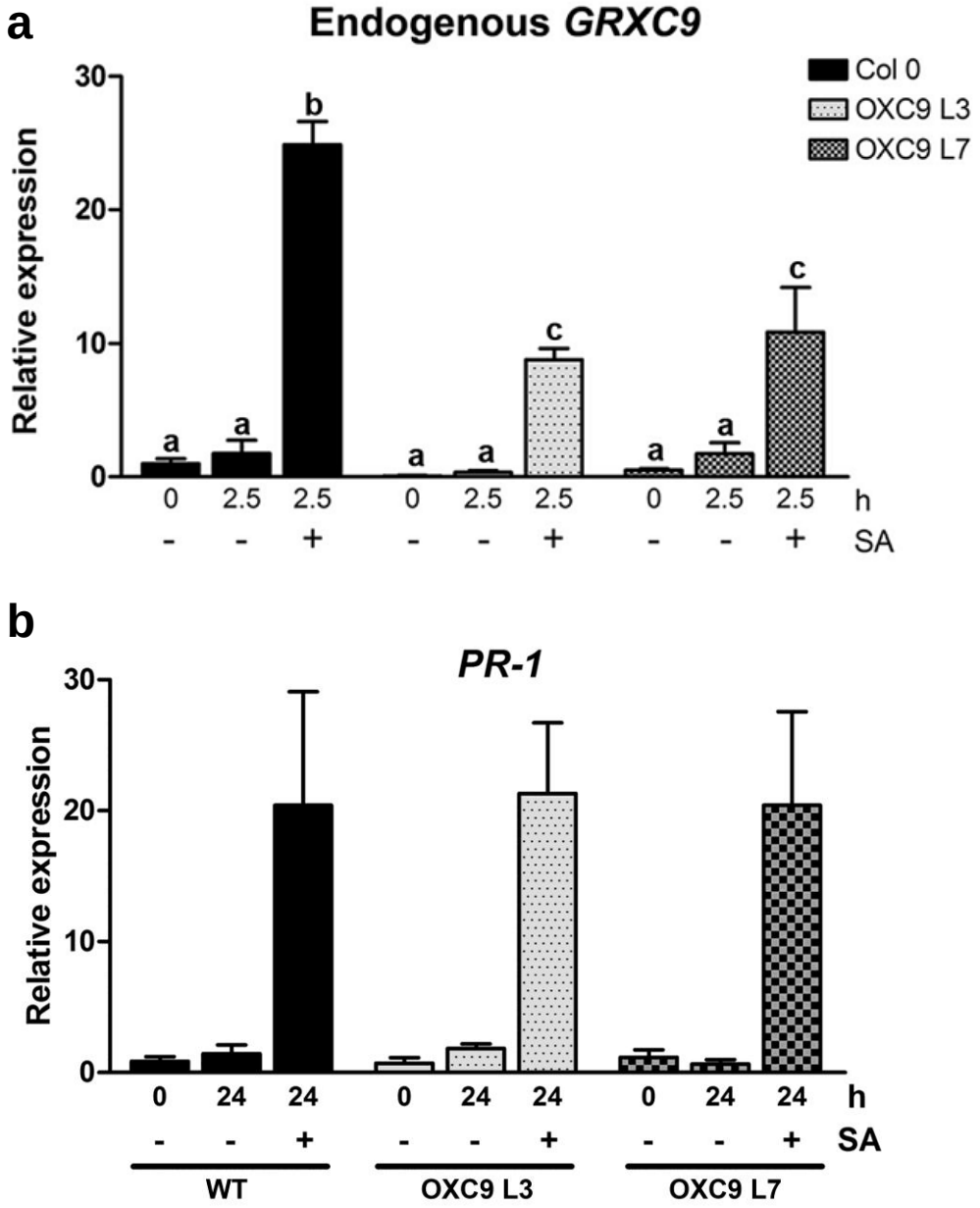

FIGURE 15. Effect of *GRXC9-6xMYC* overexpression on *GRXC9* endogenous expression.

The transcript levels for the endogenous *GRXC9* (**a**) and *PR-1* (**b**) genes were detected by RT-qPCR in fifteen-day-old seedlings of WT and *GRXC9* OE lines (L3 and L7) treated with SA 0.5 mM or 0.5X MS as a control for 2.5 h. The relative gene expression was calculated by normalizing the expression level of *GRXC9* or *PR-1* with the expression level of the housekeeping gene *YLS8*, and to the WT basal condition. Error bars represent the ± standard error. Letters above the bars indicate significant differences based on a two-way ANOVA analysis and Bonferroni post test (n=3, $p<0.05$).

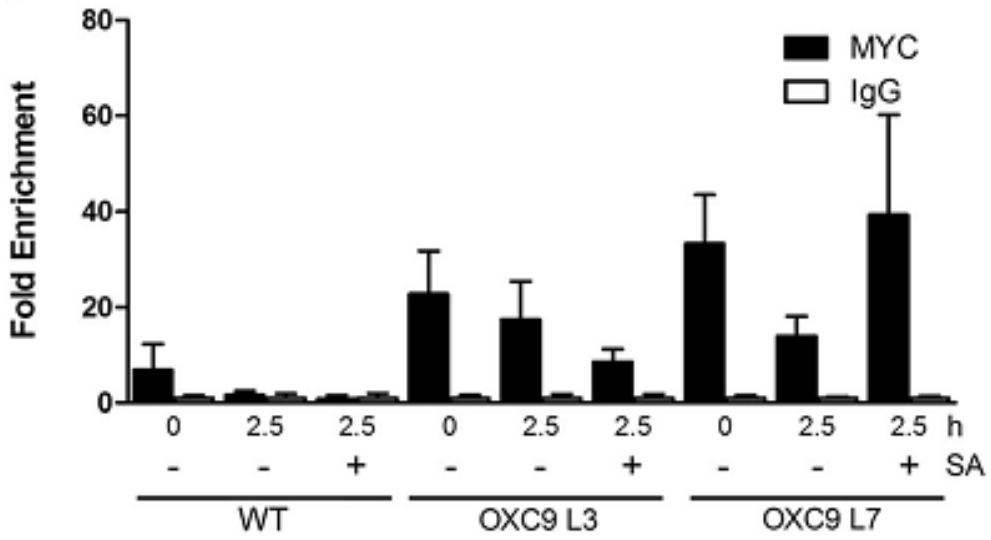

FIGURE 16. Binding of GRXC9-6xMYC protein to the endogenous *GRXC9* promoter. The binding capacity of GRXC9-6xMYC to the GRXC9 promoter was evaluated by ChIP-qPCR. WT plants and the two overexpressor lines (OXC9 L3 and OXC9 L7) were evaluated in plants treated with SA 0.5 mM or 0.5X MS as a control for 2.5 h. The immunoprecipitation was performed with commercial antibodies raised against the MYC-tag (black bars) and a non-specific IgG (white bars) as control. The qPCR analyses to quantify the DNA recovered from the ChIP were performed using the primers described in Fig. 4a. The values for the immunoprecipitated DNA samples were expressed as fold enrichment of the specific antibody over a non-specific immunoprecipitation condition (IgG). Error bars represent ± standard error of three biological replicates.

4. DISCUSSION

In this study, we explore the mechanism of control of the *GRXC9* gene. GRXC9 is a SA-responsive gene induced early and transiently by a NPR1-independent pathway. Those characteristics are shared by a group of genes induced by SA with a potential antioxidant function (Blanco et al., 2009). Despite the importance of redox changes that occurs in response to SA, the molecular mechanism that controls the induction of this particular group of genes is still unknown. In this work, we assess this mechanism using *GRXC9* as a model for the SA-dependent and NPR1-independent pathway that controls defense gene expression.

We showed that the mRNA level of *GRXC9* gene does not change in the development of Arabidopsis under basal conditions (FIGURE 4). On the other hand, in SA treatments, GRXC9 is induced by a NPR1-independent pathway (FIGURE 5). Also, we showed evidence for the induction of *GRXC9* expression in a SA dependent and NPR1-independent pathway in response to UV-B exposure and to inoculation with an avirulent bacterium, validating its activation in the context of a defense response to abiotic and biotic stress (FIGURE 6 and FIGURE 7). By assaying *in planta* GRXC9:GUS reporter activity (FIGURE 8b) and *in vivo* binding of RNAPII to the *GRXC9* promoter (FIGURE 13), we showed that the control of *GRXC9* gene expression by SA is exerted at the initiation of transcription. Accordingly, we established that TGA class II factors (FIGURE 10 and 13), as well as the two *as-1*-like elements located in the *GRXC9* proximal promoter (FIGURE 9), are essential for the induction of *GRXC9* expression by SA. The constitutive binding of TGA2 and TGA3 factors to the *GRXC9* promoter, as detected by *in vivo* ChIP assays (FIGURE 11), indicates that the inducing effect of SA is not due to an increase in binding of TGA factors to the *as-1*-like

elements. TGA class II and TGA3 factors have the capacity to interact with each other, as detected in yeast by two-hybrid assays (FIGURE 12), suggesting that TGA2 and TGA3 can bind to the *GRXC9* promoter as homo or heterodimers. Furthermore, TGA class II and TGA3 factors did not show transactivation capacity in yeast one-hybrid assays (FIGURE 12). Therefore, even though TGA class II factors are essential for recruiting RNAPII to the *GRXC9* promoter (FIGURE 13), additional co-regulators are required for transactivation, because as we already mentioned, TGA class II and TGA3 did not show transactivation capacity. Finally, we showed that over-expressed GRXC9-6xMYC protein binds to the TGA-containing complex at the *GRXC9* promoter and inhibits SA-mediated induction of the gene (FIGURE 15). This result, together with previous evidence showing that the GRXC9 protein can interact with TGA2 in the nucleus (Ndamukong et al., 2007), suggests that GRXC9 negatively controls its own gene through binding to TGA factors while they are bound to the DNA, turning off gene expression.

4.1 Role of the SA-dependent and NPR1-independent pathway in the stress defense response

The existence of a SA-dependent and NPR1-independent pathway for the expression of genes with a putative antioxidant and/or detoxifying roles in defense, such as *GRXs*, *GSTs* and *UGTs* (coding for UDP-glucosyl transferases), has been reported by our group and by others (Lieberherr et al., 2003; Uquillas et al., 2004; Blanco et al., 2005; Langlois-Meurinne et al., 2005; Fode et al., 2008; Blanco et al., 2009). The induction of two *GST* (Lieberherr et al., 2003) and three *UGT* genes (Langlois-Meurinne et al., 2005), through a SA-dependent and NPR1-independent mechanism, was reported in *Arabidopsis* plants inoculated with avirulent strains of *Pseudomonas*

syringae pv. *tomato* (*Pst*). There is no available evidence about the mechanism of transcriptional induction of these genes. Here, we validate the activation of the *GRXC9* gene by this pathway under an abiotic stress condition such as UV-B radiation that, like the immune reaction induced by avirulent *Pst* strains, triggers a SA-mediated defense response (Surplus et al., 1998).

On the other hand, members of *GST* and *UGT* gene families have also been found to be responsive to treatments with oxilipins (including JA) and xenobiotic chemicals like 2,4-D, in the context of the chemical detoxification process. Based on the overlap of some *GST* and *UGT* (early NPR1-independent SAIGs (Blanco et al., 2009)) that are also responsive to oxilipins/xenobiotics (Baerson et al., 2005; Fode et al., 2008; Mueller et al., 2008), as well as on the involvement of common TGA factors and *as-1*-like elements in their transcriptional control (as discussed in the next section), it was assumed that exogenous treatments SA unspecifically induced the chemical detoxification process (Fode et al., 2008; Gatz, 2012). Results shown in this work for *GRXC9*, and in other works for the *GSTF2*, *GSTF6*, *UGT73B3*, *UGT73B5* and *UGT73D1* genes (Lieberherr et al., 2003; Langlois-Meurinne et al., 2005), clearly argue against this idea, indicating that these antioxidant/detoxifying genes are activated by an endogenous stress-driven and SA-mediated pathway, which is distinct from the NPR1-dependent pathway that activates defense genes such as *PR-1*. The rapid and transient expression of genes with antioxidant and detoxifying roles could be important to restrict the oxidative burst produced in the infected/damaged tissues, avoiding the oxidative damage of systemic tissues.

4.2 Mechanistic aspects of the transcriptional control of *GRXC9* expression via a SA-dependent and NPR1-independent pathway. Involvement of TGA class II factors and *as-1*-like promoter elements.

Evidence provided in this thesis further supports the idea that, even though *as-1*-like and isolated TGACG boxes bind the same class of TGA factors, they are functionally different. These factors respond to different pathways to control the expression of distinct groups of genes that are activated at different times during the defense response, using different mechanism for promoter recognition and activation.

In *Arabidopsis*, *as-1*-like elements are over-represented in promoters of genes that code for enzymes with antioxidant or detoxifying activity that are responsive to exogenous application of SA, xenobiotics and oxilipins (Fode et al., 2008; Blanco et al., 2009; Köster et al., 2012). Functional requirement for *as-1*-like elements has been previously reported for only a couple of these *Arabidopsis* genes: *GST6* coding for a glutathione *S*-transferase inducible by xenobiotics, H_2O_2 and SA (Chen and Singh, 1999) and *CYP81D11* coding for a cytochrome P450 inducible by xenobiotics (Köster et al., 2012). For these genes, the NPR1 dependence has not been addressed in the context of the SA response. The functional analysis of the *as-1*-like elements from the *GRXC9* promoter described in this work represents the first functional promoter analysis performed in an early SA-dependent and NPR1-independent gene activated under an abiotic stress.

The *GRXC9* gene has two contiguous *as-1*-like elements in its promoter sequence (FIGURE 3 and FIGURE 8). In both cases, the second TGACG box is less conserved than the first one, as previously described for other *as-1*-like elements (Ellis et al., 1993). The functional analysis of the *GRXC9* gene promoter clearly indicates that both *as-1*-like elements are essential for SA-mediated expression of the gene (FIGURE

8 and FIGURE 9). Interestingly, the *all or none* effect of mutating any *as-1*-like element, instead of additive or synergistic effects, indicates that both elements must work together in the formation of transcriptional complexes.

In contrast, isolated TGACG boxes are enriched in promoters of SA-inducible genes by an NPR1-dependent pathway (Maleck et al., 2000). Functional requirement of a TGACG box for SA-mediated expression was demonstrated for *PR-1* and *NIMIN1* promoters, which are known to be induced by SA via an NPR1-dependent mechanism (Lebel et al., 1998; Fonseca et al., 2010). In the case of the *PR-1* gene, isolated TGACG boxes control its transcriptional activation by SA and its repression under basal conditions (Lebel et al., 1998).

Interestingly, this work shows that common TGA factors (TGA class II and TGA3) recognize *as-1*-like elements and TGACG boxes, being therefore involved in different transcriptional control processes. In *Arabidopsis*, TGA class II factors have been reported to be essential in several processes: the basal repression and the SA-mediated and NPR1-dependent induction of plant TGACG box-containing genes, such as *PR-1* gene (Zhang et al., 2003; Rochon et al., 2006; Kesarwani et al., 2007); the induction of *as-1*-like-containing plant genes belonging to the chemical detoxification process, in response to treatments with xenobiotics and oxilipins (including JA) (Fode et al., 2008; Mueller et al., 2008; Stotz et al., 2013); the induction of JA/ethylene-inducible genes like *PDF1.2* and its negative modulation by SA (Ndamukong et al., 2007; Zander et al., 2010); and the SA-mediated induction of early and NPR1-independent SAIGs (Blanco et al., 2009), the last one supported by the results of this work.

One interesting conclusion of this work is that TGA2 is involved in different signaling pathways that operate at different times in the response to stress (UV-B in this case). One of these pathways leads to the expression of early SA-dependent and NPR1-

independent genes, such as *GRXC9;* the other pathway leads to the expression of late SA- and NPR1-dependent genes, such as *PR-1* (FIGURE 6). Furthermore, in the case of *GRXC9*, TGA2 is bound to the promoter during all the phases of its expression profile (FIGURE 11), being essential for the transient increase in transcript levels (FIGURE 10) and recruitment of the RNAPII (FIGURE 13). The question is: how is the activity of TGA factors that are involved in different mechanisms of transcriptional control and that act at different times after stress controlled?

Results showed in this work prompt us to propose a model (FIGURE 17) to explain how SA controls the transcription process of *GRXC9* in the context of the defense response to stress. According to our model, TGA2 and TGA3 (forming homo- or heterodimers, T2/3) are constitutively bound to the two *as-1*-like elements of the *GRXC9* promoter. Under basal conditions, we propose that an inactive form of a co-regulator complex (Co-R_I) is bound to the TGA2-3/*as-1*-like complex, forming a transcriptionally inactive complex. Upon a stress condition, SA levels increase producing the activation of the co-regulator complex (switch from Co-R_I to Co-R_A). According to our results, Co-R_A must provide the transactivation activity for recruitment of the RNAPII basal machinery (RNAPII complex) to initiate transcription. Based on our results with the *GRXC9* over-expressor lines (FIGURE 14), we propose that the GRXC9 protein is involved in turning off the SA-mediated activation of its own gene through a direct interaction with the TGA2-3/*as-1*-like complex. So, once *GRXC9* gene expression is induced, the GRXC9 protein produced and translocated to the nucleus could bind the TGA2-3/*as-1*-like complex, as indicated by (Ndamukong et al., 2007). We propose that GRXC9 bound to the complex promotes the inactivation of the co-regulator complex (Co-R_A to Co-R_I), switching from a transcriptionally active to a transcriptionally inactive complex. *GRXC9* expression is turned off because Co-R_I does

not have the ability to recruit RNAPII basal machinery. This mechanism would allow a rapid transcriptional response to stress signals, through transient changes in the activity of a co-regulator complex bound to the TGA2-3/*as-1*-like platform complex preformed at the *GRXC9* promoter.

Results indicating that GRXC9 and TGA2 interact in the nucleus and that *GRXC9* over-expression reduces the expression of the 2,4-D-inducible CaMV*as-1*::GUS transgene and the JA-inducible *PDF1.2* gene (Ndamukong et al., 2007), supports the idea that GRXC9 could play a more general role in controlling the expression of TGA class II-target genes. Interestingly, the binding of other CC-type GRX (ROXY1 and ROXY2) to TGA9/10 factors and their role in anthers development (Murmu et al., 2010), supports a role for CC-type GRXs in the control of gene expression. Considering that ROXY1 must be expressed in the nucleus to complement *roxy1* mutant, and that GRXC9 can also complement the *roxy1* mutant (Li et al., 2009), it can be inferred that nuclear GRXC9 is required to exert its activity. Our results indicating that GRXC9 binds to the TGA2-3/*as-1*-like complex support this idea.

One of the key questions raised by this model is what is the nature of the proteins that form the co-regulator complex, either in its active (Co-R_A) or inactive (Co-R_I) forms. The existence of a co-repressor complex that binds the TGA2-3/*as-1*-like complex under basal conditions (FIGURE 17) was previously proposed for the 35SCaMV *as-1* in tobacco (Johnson et al., 2001; Butterbrodt et al., 2006). In the case of *GRXC9*, previous evidence showing that treatment with the protein inhibitor cycloheximide highly increases the basal and SA-induced levels of *GRXC9* transcripts,

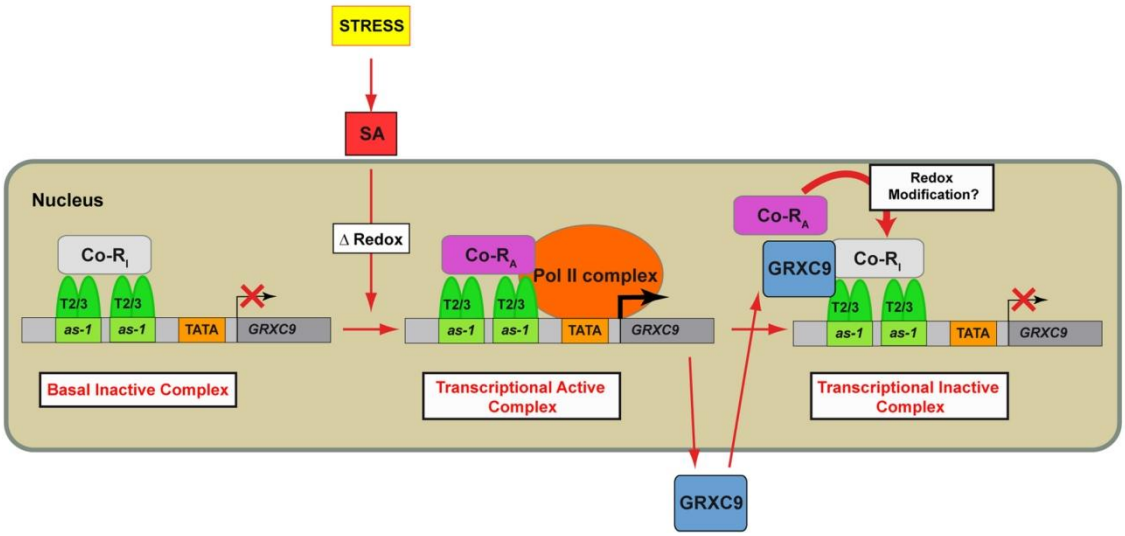

FIGURE 17. Mechanistic model for the transcriptional control of *GRXC9* expression by stress, via a SA-dependent and NPR1-independent pathway in *Arabidopsis*.

Homo- or heterodimers of TGA2 and TGA3 (T2/3) are constitutively bound to the two *as-1*-like elements of the *GRXC9* promoter, acting as a platform for the formation of transcriptionally inactive and active complexes. Under basal conditions, an inactive form of a co-regulator (Co-R_I) is bound to the TGA2-3/*as-1*-like complex forming a basal complex that impairs recruitment of the RNAPII to the *GRXC9* promoter. Upon stress, SA is rapidly accumulated promoting the activation of the co-regulator complex (switch from Co-R_I to Co-R_A) that binds to the TGA2-3/*as-1*-like complex, allowing the formation of a transcriptionally active complex that recruits the RNAPII basal machinery (RNAPII complex) to the *GRXC9* basal promoter. Transcription of *GRXC9* leads to the accumulation of the GRXC9 protein in the nucleus, were it binds to the TGA2-3/*as-1*-like complex producing the inactivation of the co-regulator complex (switch from Co-R_A to Co-R_I) and therefore turning off *GRXC9* transcription. We speculate that the switch from Co-R_I to Co-R_A promoted by SA is produced by the oxidative modification of one of the proteins involved in the promoter complex, while the switch from Co-R_A to Co-R_I is produced by the protein's reduction catalyzed by GRXC9

suggests the existence of a repressor with a high turnover rate (Blanco et al., 2009). Recently MED18 and its interacting protein Ying Yang 1 (YY1) have been described as co-regulators in plant immunity (Lai et al., 2014). Mutant plants on those genes are more susceptible to the pathogen *B. cinerea*. MED18 controls the expression of 3 antioxidant genes described above: *TRXh5*, associated to the NPR1 monomerization (Tada et al., 2008; Kneeshaw et al., 2014), *GRXS13*, associated to tolerance to photooxidative stress and an enhancing factor in susceptibility to *B. cinerea* infections (La Camera et al., 2011; Laporte et al., 2012) and the model gene in this thesis, *GRXC9*. MED18 is a subunit of the mediator complex and has a dual effect over the expression of those genes: in a basal condition it acts as an activator of *GRXC9* and *GRXS13*. On the other hand, upon *B. cinerea* challenge, MED18 acts as a transcriptional repressor in the mentioned genes (Lai et al., 2014) suggesting that this subunit forms part of the Co-R_I complex in the *GRXC9* transcriptional induction. Here we show evidence that although TGA2 and TGA3 are basally bound to the *GRXC9* promoter, their binding is not required for turning off the gene in the absence of stress. In fact, knock out mutants for TGA class II and TGA3 factors do not show increased basal levels of *GRXC9* transcripts (FIGURE 10). Together, this evidence indicates that even though TGA2/3 and Co-R_I (probably containing MED18) forms part of the basal inactive complex, the presence of MED18 but not of TGA2/3 factors is essential to repress *GRXC9* transcription.

Concerning proteins with co-activator function that could be part of the Co-R_A complex, we discard NPR1 and SCL14 proteins. In fact, the induction of *GRXC9* by SA is not only independent of the NPR1 protein ((Blanco et al., 2009) and FIGURE 5), but it is also independent of the SCL14 protein (Fode et al., 2008), which was previously identified as an interactor of TGA2 factor essential for the expression of a group of *as*-

1-like containing genes. Previously, a Dof protein named OBP1 was found to interact with TGA4 and TGA5 to enhance binding to the *as-1* element (Zhang et al., 1995); whether this kind of protein forms part of the Co-R_A complex, remains to be elucidated. Therefore, further efforts are required for the identification of the protein(s) that form part of the Co-R_A complex that binds to the TGA2-3/*as-1*-like complex in *GRXC9* promoter.

4.3 Possible candidates for the Co-R protein

In order to find possible candidates for the Co-R proteins in the active or inactive form, we used the protein-protein interaction database Arabidopsis Network Analysis Pipeline (ANAP) that integrates 11 Arabidopsis protein interactions databases (Wang et al., 2012). As input, we used the TGA2 and TGA3 proteins. The database shows 14 TGA2/3-interacting proteins validated experimentally by two hybrid screening, directed two hybrid, pull down, bimolecular fluorescence complementation, coimmunoprecipitation or pull down assays (TABLE 2). As we expect, the protein NPR1 and its orthologs NPR3 and NPR4 are shown as TGA2 or TGA3 interactors. Nevertheless, we show that the expression of *GRXC9* is independent of those genes (FIGURE 5).

The NPR1 Interacting-1 (NIMIN1) protein has been described as an NPR1 interacting protein that participates in the SA and defense responses, down-regulating the *PRs* expression (Weigel et al., 2005; Hermann et al., 2013). The NIMIN1 structure includes an EAR motif described as a transcriptional repressor domain (Ohta et al., 2001; Weigel et al., 2005). On the other hand, the Glabrous-Enhancer-Binding Protein-Like-1 GPL1 protein has been described as a member of the 3 GeBP-like proteins with a non-canonic leucine zipper motif. It locates into the nucleus and acts repressing the cytokinin responsive genes Arabidopsis Response Regulator 5 (ARR5) and ARR7

Table 2: TGA2 and TGA3 interacting proteins

AGI	TGA-Interacting protein	TGA Protein	Interaction Detection Method	Reference
AT1G32230	RCD1	TGA2	two hybrid	(Jaspers et al., 2009)
AT1G64280	NPR1	TGA2	affinity chromatography	(Johnson et al., 2003)
			anti tag CoIP	(Spoel, 2003)
			biochemical	(Zhang et al., 1999)
			pull down	(Zhang et al., 1999)
			two hybrid	(Zhang et al., 1999)
			two hybrid array	(Consortium, 2011)l
			reconstituted complex	(Kim and Delaney, 2002)
			3 hybrid method	(Weigel et al., 2005)
		TGA3	two hybrid array	(Consortium, 2011)
			two hybrid	(Liu et al., 2005)
AT2G47880	GRXC13	TGA2	two hybrid array	(Consortium, 2011)
AT3G02000	ROXY1	TGA2	BiFC	(Li et al., 2009)
			two hybrid	(Murmu et al., 2010)
		TGA3	two hybrid	(Li et al., 2009)
			BiFC	(Li et al., 2009)
AT4G19660	NPR4	TGA2	two hybrid	(Zhang et al., 2006b)
		TGA3	two hybrid	(Zhang et al., 2006b)
AT5G06780	EMSY-LIKE2	TGA2	two hybrid array	(Consortium, 2011)
AT1G02450	NIMIN-1	TGA2	3 hybrid method	(Weigel et al., 2005)
			two hybrid	(Weigel et al., 2005)
AT1G28480	GRXC9	TGA2	BiFC	(Ndamukong et al., 2007)
			two hybrid	(Ndamukong et al., 2007)
			two hybrid array	(Consortium, 2011)
AT2G25650	GEBP-LIKE PROTEIN 1	TGA2	two hybrid array	(Consortium, 2011)
AT2G30540	Thioredoxin	TGA2	two hybrid array	(Consortium, 2011)
AT4G33040	Thioredoxin	TGA2	two hybrid array	(Consortium, 2011)
AT5G14070	ROXY2	TGA2	pull down	(Consortium, 2011)
			two hybrid	(Murmu et al., 2010)
			two hybrid	(Murmu et al., 2010)
			two hybrid array	(Consortium, 2011)
AT5G45110	NPR3	TGA2	protein complementation assay	(Zhang et al., 2006b)
			two hybrid	(Zhang et al., 2006b)
			two hybrid array	(Consortium, 2011)
		TGA3	two hybrid	(Zhang et al., 2006b)

(Chevalier et al., 2008). Consistently, GPL1 acts redundantly with GPL2 and GEBP conferring sensitivity to Cytokinin (Chevalier et al., 2008). Given the data described for NIMIN1 and GPL1 and its tested interaction with TGA2, we could speculate that those proteins could act as Co-R_I. Further analyses are required to determine the participation of these proteins in the transcriptional regulation of *GRXC9* in the SA-mediated defense response.

The Radical-induced Cell Death-1 (RCD1) is a protein that protects plants against oxidative stress (Belles-Boix et al., 2000) and saline stress (Katiyar-Agarwal et al., 2006). Under basal conditions, it is located at the nucleus and under oxidative stress conditions migrates to the cytoplasm (Katiyar-Agarwal et al., 2006). The *rcd1* mutant plants are more susceptible to NaCl and H_2O_2 than the WT plants (Katiyar-Agarwal et al., 2006). The domain analyses of RCD1 protein suggest that it does not bind to DNA, but it has the capacity to interact with transcription factors such as STO, DREB2A and TGA2 (Belles-Boix et al., 2000; Jaspers et al., 2009), suggesting a possible function as a transcriptional co-regulator. Transcriptomic experiments have been performed comparing *rcd1* mutant to WT plants under basal conditions (Ahlfors et al., 2004) and after ozone treatment (Brosche et al., 2014). In none of these conditions *GRXC9* induction is shown. For this reason we cannot conclude respect to the dependence of RCD1 for the *GRXC9* induction.

The Emsy-like 2 (EML2) protein is located at the nucleus and it is an Enhanced Downy Mildew-2 (EDM2)-interacting protein. *EDM2* is required for RPP7-disease resistance against *Hyaloperonospora arabidopsidis* and some developmental processes such as flowering (Tsuchiya and Eulgem, 2011). There are 4 *EML* genes in Arabidopsis and the quadruple *eml* mutant plants are weakly but significantly compromised in basal defense response against *H. arabidopsidis* (Tsuchiya and Eulgem, 2011). The EMLs

proteins have not been functionally characterized in Arabidopsis but given the architecture of the protein, a possible function as regulator of the chromatin state can be suggested (Tsuchiya and Eulgem, 2011). From this analysis of TGA2/3 interactors, we can conclude that RCD1 and EML2 are good candidates for Co-R_A as coactivators in *GRXC9* induction under UV-B or SA treatments. Experimental analyses are required to address this point.

Other protein that could act as Co-R_A is NPR2. This protein is highly similar to NPR1, sharing a 61.3% aminoacidic identity. Also, NPR1 and NPR2 shares key aminoacids to the NPR1 function as the Cysteine-82, necessary for the NPR1 oligomer formation and cytoplasmic localization (Mou et al., 2003), the Histidines-300 and 334 and the Cysteine-150 necessaries for the interaction with TGA2 and TGA3 (Despres et al., 2003). In spite its similarity to NPR1, NPR2 have been not studied in a defense response context. Nevertheless the *npr1/npr2* double mutant shows an exacerbated phenotype compared to *npr1* single mutant in response to applications of BTH (a SA functional analogue), suggesting a possible functional redundancy of those genes in this condition (Canet et al., 2010). For the same reason it is possible that NPR2 satisfies the need of a transcriptional coactivator in the SA-dependent and NPR1-independent *GRXC9* induction. The evaluation of *GRXC9* expression in the *npr1 npr2* double mutant plant should help us to test this hypothesis.

4.4 Post-translational redox modification of Co-R complexes

Another interesting question raised by this model is what is the mechanism by which SA promotes the activation of the co-regulator complex (switch from Co-R_I to Co-R_A), as well as how GRXC9 can promote the inactivation of the co-regulator complex (switch from Co-R_A to Co-R_I). We speculate in our model that a redox change

promoted by SA accumulation can be responsible for the activation of the co-regulator complex. This idea is supported by evidence indicating that SA promotes a biphasic change in the GSH/GSSG ratio, first an oxidative phase characterized by a decrease in GSH/GSSG ratio and then a reductive phase characterized by increase in GSH/GSSG ratio (Mou et al., 2003; Mateo et al., 2006). On the other hand and in agreement with the idea that a redox modification could affect the Co-R complex, 6 of the 14 TGA2-interacting proteins found in the ANAP are oxidoreductases enzymes (TABLE 2). Four of them are GRXs proteins: the already mentioned ROXY1, ROXY2, GRXC9 and the protein GRXC13 that has not been functionally described. The other two oxido-reductases are classified as thioredoxins: AT2G30540 and AT4G33040. The nature of these enzymes leads us to speculate that they could catalyze a redox reaction that could affect a component of the Co-R complex.

The potential proteins targets for a redox modification of the Co-R complex are the TGA transcription factors, in particular TGA2. We already know that this protein is necessary for *GRXC9* transcriptional induction and by other hand we speculate that the effect of GRXC9 overexpression, on its own genic expression is mediated by the interaction with TGA2. The idea of a post translational modification of TGA2 have been also raised by other authors (Gatz, 2013; Gutsche et al., 2015; Waszczak et al., 2015), and supported by previous works that show the redox modification of Cys residues of the TGA family members TGA1 and TGA4 modulating their binding to NPR1 and to DNA (Despres et al., 2003; Lindermayr et al., 2010).

Considering this background and the data shown, we speculate that GRXC9, through its oxidoreductase activity, can catalyze the reduction of a protein that forms part of the co-regulator complex (maybe TGA2), producing its inactivation. In this way

GRXC9 could be a key piece in the redox control of the expression of genes controlled by TGA class II factors.

5. CONCLUSIONS

- The transcriptional induction of *GRXC9* in SA treatments is independent of the master coactivator NPR1.
- The *GRXC9* gene is responsive to UV-B and avirulent *Pseudomonas* strain, via a SA-dependent and NPR1-independent pathway.
- The increase in *GRXC9* transcript levels in response to SA is mainly due to transcriptional activation of the gene mediated by this hormone.
- The two *as-1*-like elements located in the *GRXC9* promoter are necessaries for its transcriptional activation in SA treatments. Both elements are functional, essential and sufficient for the SA responsiveness of the gene.
- The TGA transcription factors class II are necessaries for the SA-mediated *GRXC9* induction.
- The TGA2 and TGA3 transcription factors are constitutively bound to the *GRXC9* promoter. Nevertheless, TGA3 contribution in the *GRXC9* transcriptional induction is less important that the TGA2 contribution.
- The TGA2 and TGA3 are able to form homo- and heterodimers. They do not have transactivation activity.
- SA triggers the transient recruitment of RNAPII to the basal *GRXC9* promoter. This recruitment is TGA class II-dependent.
- The GRXC9 protein bounds to the endogenous *GRXC9* promoter repressing its SA-dependent transcriptional induction.

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