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SA-inducible Arabidopsis glutaredoxin interacts with TGA factors and suppresses JA-responsive *PDF1.2* transcription

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Summary

Salicylic acid (SA) is a plant signaling molecule that mediates the induction of defense responses upon attack by a variety of pathogens. Moreover, it antagonizes gene induction by the stress signaling molecule jasmonic acid (JA). Several SA-responsive genes are regulated by basic/leucine zipper-type transcription factors of the TGA family. TGA factors interact with NPR1, a central regulator of many SA-induced defense responses including SA/JA antagonism. In order to identify further regulatory proteins of SA-dependent signaling pathways, a yeast protein interaction screen with tobacco TGA2.2 as bait and an *Arabidopsis thaliana* cDNA prey library was performed and led to the identification of a member of the glutaredoxin family (GRX480, encoded by *At1g28480*). Glutaredoxins are candidates for mediating redox regulation of proteins because of their capacity to catalyze disulfide transitions. This agrees with previous findings that the redox state of both TGA1 and NPR1 changes under inducing conditions. Transgenic Arabidopsis plants ectopically expressing *GRX480* show near wild-type expression of standard marker genes for SA- and xenobiotic-inducible responses. In contrast, transcription of the JA-dependent defensin gene *PDF1.2* was antagonized by transgenic GRX480. This, together with the observation that *GRX480* transcription is SA-inducible and requires NPR1, suggests a role of GRX480 in SA/JA cross-talk. Suppression of *PDF1.2* by GRX480 depends on the presence of TGA factors, indicating that the GRX480/TGA interaction is effective *in planta*.

Keywords: jasmonic acid, glutaredoxin, TGA transcription factors, salicylic acid, NPR1, *PDF1.2*.

Introduction

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

Based on sequence similarities, TGA factors are grouped into various classes (Miao *et al.*, 1994). Loss-of-function studies have shown that class II TGA factors in particular play important roles in SA-mediated gene expression: Arabidopsis plants lacking TGA factors TGA2, TGA5 and TGA6 are severely compromised in *PR-1* expression (Zhang *et al.*, 2003) after treatment with the SA analog 2,6-dichloroisonicotinic acid (INA). Likewise, reduction of tobacco class II factors NtTGA2.2 and NtTGA2.1 in 2.2/2.1RNAi tobacco plants correlates with reduced expression of glutathione-*S*-transferase (*GST*) *Nt103* and *PR-1a* (Thurow *et al.*, 2005).

TGA factors interact with NPR1 (non-expressor of *PR* genes 1), a central regulatory protein of SA-dependent processes (Zhang *et al.*, 1999). As observed for the *tga2 tga5 tga6* triple mutant, the *npr1* mutant is unable to express *PR* genes after treatment of plants with INA. In addition, neither mutant mounts the SA-dependent global defense

program 'systemic acquired resistance' (SAR) (Cao *et al.*, 1997; Ryals *et al.*, 1997; Zhang *et al.*, 2003), indicating that class II TGA factors and NPR1 are components of the same signal transduction pathway.

NPR1 has also emerged as a critical modulator of the second important plant defense pathway, which is triggered by the signaling molecule jasmonic acid (JA; Beckers and Spoel, 2006). Whereas SAR is efficient against biotrophic pathogens that feed on living host cells, JA-induced responses protect the plant from insect infestation and necrotrophic pathogens. Cross-communication between these pathways, which includes both antagonistic and synergistic interactions, is necessary to provide an effective state of resistance to different invaders. NPR1 is involved in SA/JA antagonism by repressing genes of the JA biosynthesis pathway. Moreover, it interferes with the signal transduction chain leading from JA to the expression of JA-responsive genes such as *PDF1.2* and *VSP* (Spoel *et al.*, 2003). The impact of TGA factors on this cross-talk has not yet been addressed.

As TGA factors and NPR1 pre-exist under non-inducing conditions (Qin *et al.*, 1994), their activity must be tightly regulated. Whereas the biological significance of SA-inducible phosphorylation of TGA2 (Kang and Klessig, 2005) has remained elusive, redox modifications of both NPR1 and TGA1 have been shown to be functionally relevant. TGA1 interacts only with NPR1 when the intra-molecular disulfide bridge of TGA1 is reduced, a modification that is induced in SA-treated plant cells (Despres *et al.*, 2003). SA treatment also leads to the reduction of crucial cysteines of the NPR1 protein, resulting in transition from an oligomeric to a monomeric form (Mou *et al.*, 2003). The monomeric reduced form of NPR1 is subsequently translocated from the cytosol into the nucleus, where it promotes recruitment of TGA factors TGA2 and TGA3 to their target sequences (Fan and Dong, 2002; Johnson *et al.*, 2003). Whereas translocation of NPR1 into the nucleus is required for the induction of *PR-1*, suppression of JA-inducible responses can be mediated by SA-activated NPR1 even if it is retained in the cytosol (Spoel *et al.*, 2003).

In addition to the NPR1-dependent processes described above, TGA factors contribute to the activation of another subset of *as-1*-containing promoters even in the absence of NPR1 (Uquillas *et al.*, 2004). A well-characterized example of such genes is *GST6* from *Arabidopsis thaliana*, which responds to SA and xenobiotic or oxidative stress cues (Chen *et al.*, 1996; Garreton *et al.*, 2002; Uquillas *et al.*, 2004). It can be envisioned that, under these conditions, redox modifications might be also necessary for NPR1-independent TGA factor activation. In tobacco leaves, NtTGA2.2 is important for the induction of *PR-1a* and *GST Nt103*, indicating that the same TGA factor can be involved in NPR1- dependent and -independent processes. Thus, in addition to NPR1, further proteins regulating class II TGA factor activity are likely to exist. In previous protein interaction screens, members of the Dof (Zhang *et al.*, 1995) and ERF families (Buttner and Singh, 1997) of transcription factors have been isolated as TGA-interacting proteins. However, they may not be general regulators of TGA factor activity, but may instead contribute to their promoter specificity.

In order to further unravel mechanisms of TGA factor activation, we initiated a new yeast screen to search for proteins interacting with TGA factors. This screen resulted in the isolation of a glutaredoxin as a TGA-interacting protein. As glutaredoxins catalyze thiol disulfide reductions, they may be regarded as candidates for controlling the redox state of regulatory proteins (Lemaire, 2004). We demonstrate that the TGA-interacting glutaredoxin, which is transcriptionally activated under conditions of elevated SA levels, is a negative effector of JA-inducible expression of *PDF1.2*. The TGA-interacting glutaredoxin therefore represents a potential regulatory component of the SA/JA antagonism.

Results

Identification of TGA interacting protein GRX480 by a modified yeast two-hybrid screen

To identify proteins that interact with TGA factors, we designed a screening strategy that offers the authentic TGA dimer bound to its target sequence as bait (Serebriiskii *et al.*, 2001). *NtTGA2.2* was expressed under the control of the *MET25* promoter in a yeast strain containing three copies of the *as-1* element upstream of the *HIS3* selectable marker gene (Weigel *et al.*, 2005). Binding of NtTGA2.2 to its target sites does not lead to activation of the marker gene, which allows screening for interacting proteins. Subsequently an Arabidopsis cDNA library fused to the *GAL4* activation domain was used to isolate proteins recognizing *as-1*-bound NtTGA2.2.

After transformation of the Arabidopsis cDNA library (Weigel *et al.*, 2001) into the appropriate yeast strain (Weigel *et al.*, 2005), 28 clones out of 1×10^6 yeast transformants were able to grow on selective medium. Restriction analysis and sequencing of the inserts led to the classification of four groups of recombinant plasmids. The largest group (22 members) encoded cDNAs identical to *At1g28480*. The protein deduced from this sequence belongs to the family of glutaredoxins and was subsequently called GRX480. Recovered prey plasmids were re-transformed into *3x as-1::HIS3*-encoding yeast cells expressing or lacking NtTGA2.2. Histidine prototrophy was restored only in the presence of NtTGA2.2 (data not shown), indicating that the gene product of *At1g28480* is recruited to the *as-1* element only in the presence of NtTGA2.2.

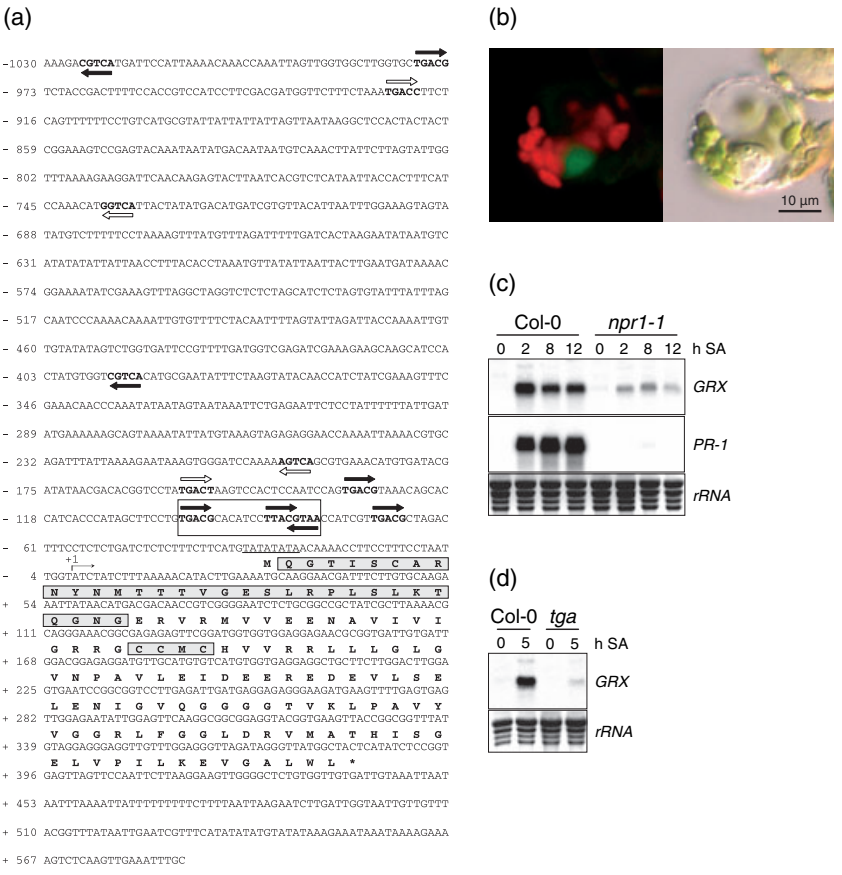

Figure 1. Sequence, BiFC analysis and expression of *GRX480*.
(a) Sequence of *At1g28480* from position −1030 to the 3′-end of the 3′ UTR. Black arrows indicate single TGAC/G sequences representing TGA binding sites; white arrows indicate binding sites for WRKY transcription factors. The boxed sequence marks the position of an *as-1* element consisting of two TGA binding sites. The putative TATA box is underlined. The angled arrow designates the transcription start site (+1). The open reading frame is given using the one-letter code; the shaded boxes highlight the GRX480-specific 31 amino acid N-terminal domain and the presumed active centre (CCMC).
(b) BiFC analysis of GRX480/TGA2 interaction *in planta*. Arabidopsis mesophyll protoplasts were transiently transformed with constructs encoding GRX480 and TGA2 fused to non-overlapping domains of YFP. Fluorescence results from reconstitution of the chromophore due to the interaction of GRX480 and TGA2. Red fluorescence of chloroplasts is visible. A bright-field image is shown on the right. The bar is included as a size standard.
(c, d) Expression analysis of *GRX480*. Three-week-old wild-type (Col-0), *npr1-1* (c) and *tga2 tga5 tga6* (d) plants were treated with 1 mM SA for the time periods (h) indicated above the lanes. The lanes were loaded with 20 μg of RNA, and the blots were hybridized with probes for *GRX480* and *PR-1*. Ethidium bromide-stained RNA (rRNA) is shown as evidence of equal loading.

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

Figure 1(a) depicts the genomic DNA and deduced protein sequence of *At1g28480*. Glutaredoxins (GRXs) are small redox proteins of approximately 12 kDa that are able to catalyze the reduction of disulfides or glutathione (GSH) mixed disulfides (Lemaire, 2004). The Arabidopsis genome encodes 31 *GRX* genes that have been classified into three major sub-groups according to the sequence of the active center: (i) the CPYC group that is also present in yeast, *Escherichia coli* and mammals; (ii) the CGFS group identified in higher plants, algae and yeast; and (iii) the higher plant-specific CC group. GRX480 encodes a CCMC sequence and thus belongs to the CC group with two potentially active cysteines (C). Twenty-five Arabidopsis glutaredoxin genes encode core proteins of only approximately 100 amino acids, whereas six glutaredoxin genes, including *GRX480*, encode specific N-terminal extensions with about 30–70 amino acids.

GRX480 interacts with TGA2 in planta

To confirm the TGA–GRX480 interaction *in planta*, a bimolecular fluorescence complementation (BiFC) assay was used. The N-terminus of the yellow fluorescent protein (YN) was fused in-frame to the *TGA2* coding region that is the Arabidopsis ortholog of *NtTGA2.2*. The YFP C-terminus (YC) was fused N-terminally to GRX480. The constructs were transiently expressed in Arabidopsis leaf mesophyll protoplasts. Reconstitution of a fluorescing YFP chromophore occurred only upon co-expression of both proteins (Figure 1b). Yellow fluorescence was observed in a defined compartment of the protoplast, which appeared to be the nucleus as judged from bright-field microscopy. No fluorescence was detected following co-expression of one of the fusion proteins with free non-fused YN or YC (data not shown). Further evidence that GRX480 interacts with TGA factors *in planta* comes from transient expression experiments in tobacco protoplasts, which were designed like two-hybrid systems: co-expression of GRX480 fused to the GAL4 DNA-binding domain together with NtTGA2.2 fused to the VP16 activation domain (Krawczyk *et al.*, 2002) resulted in activation of a GUS reporter construct containing four *GAL4* binding sites in its upstream regulatory region (Figure S1).

GRX480 transcription is induced by SA and depends on TGA factors and NPR1

The potential relevance of GRX480 in the biological context of SA-dependent defense responses was supported by Northern blot analysis that revealed increased steady-state

GRX480 transcript levels after SA treatment (Figure 1c). As described for *PR-1*, expression of *GRX480* is severely reduced in the *npr1-1* mutant (Cao *et al.*, 1994), although some background transcript levels remain. The *tga2 tga5 tga6* triple mutant (Zhang *et al.*, 2003) also showed compromised inducibility of *GRX480* transcript levels (Figure 1d). As expected, *GRX480* expression was induced after challenge of plants with *Pseudomonas syringae*, irrespective of whether a virulent or an avirulent strain was used (data not shown). Chemicals such as JA and 2,4-dichlorophenoxyacetic acid (2,4-D) that induce NPR1-independent glutathione-*S*-transferase promoters encoding an *as-1* element (Blanco *et al.*, 2005; Wagner *et al.*, 2002) did not efficiently induce *GRX480* (Figures 3a, 4a and 5a, and data not shown).

Consistent with the lack of SA-inducible expression of *GRX480* in the *npr1-1* and *tga2 tga5 tga6* triple mutants (Figure 1c,d), a number of TGA binding motifs were found in the *GRX480* promoter. A perfect binding site for TGA dimers is the palindromic sequence TGAC/GTCA (Qin *et al.*, 1994), but the first five base pairs (TGAC/G) are sufficient for recognition (Spoel *et al.*, 2003). Within 1030 bp of the *At1g28480* sequence upstream of the putative transcriptional start site, six TGAC/G motifs are found. Typical *as-1*-like elements are characterized by two binding sites with 12 bp between the palindromic centers. In this arrangement, the sequence requirement is less stringent (Krawczyk *et al.*, 2002). An *as-1*-like sequence (TGAC/GCACnnnnTTAC/GTAA) is located between positions −80 and −99 relative to the putative transcriptional start site, which is similar to its relative position within the CaMV 35S promoter. Also, four binding sites for WRKY transcription factors, which are often over-represented in pathogen-inducible promoters (Maleck *et al.*, 2000), are located in the *GRX480* promoter (Figure 1a).

GRX480 interacts with various TGA factors in the yeast two-hybrid system

In order to test whether GRX480 interacts with other TGA factors than TGA2 and NtTGA2.2, the classical yeast two-hybrid system was used. The prey plasmid isolated in the original screen (pGAD10-GRX480) was transformed into yeast HF7c cells containing either NtTGA2.2, TGA2 or TGA6 fused to the GAL4 DNA-binding domain (BD) and assayed for growth on histidine drop-out medium (Table 1). Prototrophic growth was detected whenever GRX480 was co-expressed with one of the TGA factors tested. Consistent with the BiFC assays, GRX480 was found to interact not only with NtTGA2.2 but also with the Arabidopsis orthologs TGA2 and TGA6. Interaction with NtTGA2.2 was confirmed by a domain swap experiment that used yeast cells expressing GRX480 fused to the GAL4 DNA-binding domain and NtTGA2.2 fused to the GAL4 activation domain (AD). Using this experimental set-up, interaction with other TGA factors encoding an activation domain could be tested. TGA factors of distinct classes such as NtTGA1a and NtTGA10 interact with GRX480 in this assay.

Table 1 Yeast two-hybrid assay revealing interactions between GRX480 and various TGA transcription factors

Bait	Prey	Growth
BD	AD:GRX480	−
BD:NtTGA2.2	AD	−
BD:NtTGA2.2	AD:GRX480	+
BD:TGA2	AD	−
BD:TGA2	AD:GRX480	+
BD:TGA6	AD	−
BD:TGA6	AD:GRX480	+
BD:GRX480	AD	−
BD	AD:NtTGA2.2	−
BD:GRX480	AD:NtTGA2.2	+
BD	AD:NtTGA2.1	−
BD:GRX480	AD:NtTGA2.1	+
BD	AD:NtTGA1a	−
BD:GRX480	AD:NtTGA1a	+
BD	AD:NtTGA10	−
BD:GRX480	AD:NtTGA10	+

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

Yeast cells (HF7c) containing the indicated fusion proteins were grown for 2 days at 30°C on drop-out medium lacking histidine, leucine and tryptophan. +, histidine prototrophy; −, no growth. BD, GAL4 DNA-binding domain; AD, GAL4 activation domain.

As NPR1 is a redox-regulated protein (Mou *et al.*, 2003), we asked the question whether the interaction with TGA factors can recruit GRX480 into the vicinity of NPR1. Therefore, a yeast 'bridge assay' was carried out with BD–GRX480 and AD–NPR1 fusion proteins expressed together with NtTGA2.2. Growth under selective conditions occurred only when NtTGA2.2 was provided as a bridging component (Table 1). Thus, it is possible that these three proteins form a ternary complex *in planta*, as soon as sufficient amounts of NPR1 accumulate in the nucleus.

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

As outlined above, Arabidopsis encodes 31 related *GRX* genes, raising the probability of functional redundancy. A unique feature of GRX480 is its specific 31 amino acid N-terminal domain (Figure 1a). To test whether this domain contributes to the interaction with TGA factors, an N-terminal deletion of GRX480 (GRX480ΔN) was tested in a quantitative yeast two-hybrid assay (Figure 2). In addition, GRX370 (*At5g40370*), a classical CPYC type glutaredoxin, which is highly represented in the EST databases, was challenged for its interaction with TGA2 (see Figure S2 for

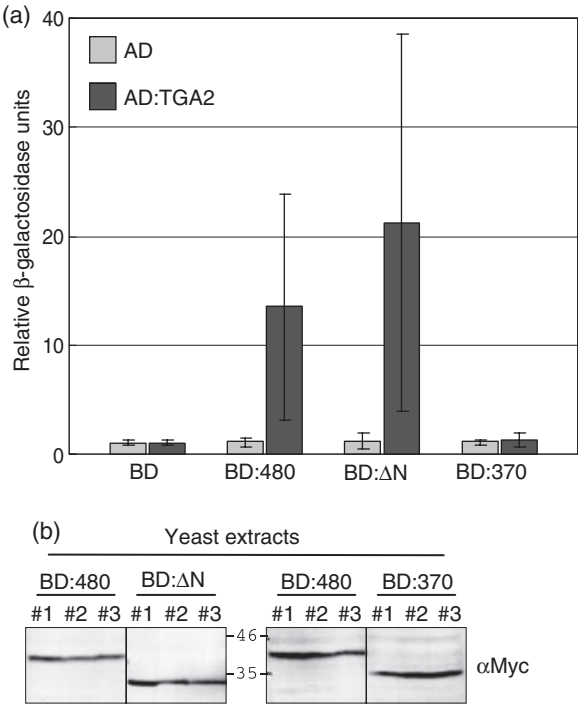

Figure 2. Analysis of the interaction of glutaredoxin variants with TGA2 in a quantitative yeast two-hybrid assay.
(a) Coding regions of GRX480, GRX480ΔN and GRX370 were fused to the GAL4 DNA-binding domain (BD:480, BD:ΔN, BD:370, respectively), and TGA2 was fused to the GAL4 activation domain (AD:TGA2). After transformation of the respective plasmids in yeast MAV203 cells, β-galactosidase activity was measured. Three to five independent clones were used for each construct in a single experiment. The results represent the average of five independent experiments. Values were normalized to the LacZ value of yeast cells transformed with the empty vectors, which was set to 1 relative β-galactosidase unit.
(b) Western blot analysis of extracts from three independent yeast clones transformed with vectors encoding BD:480, BD:ΔN and BD:370, respectively. Expression of the proteins was analyzed by immunodetection with the αMyc antibody.

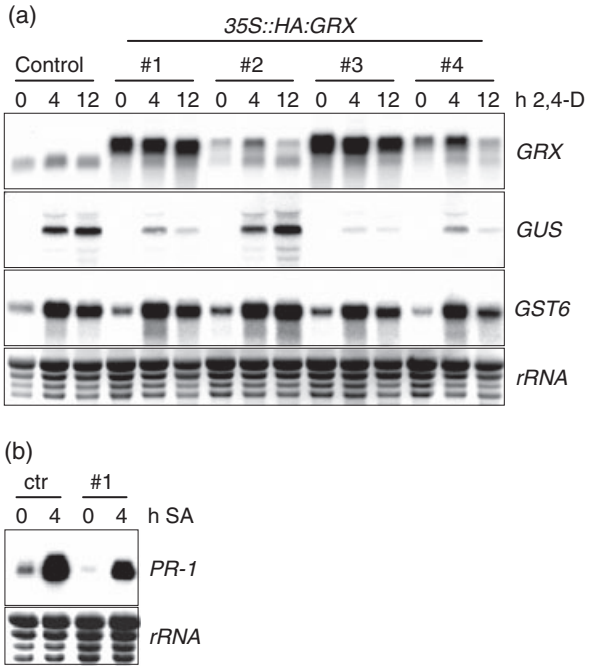

Figure 3. Expression of *as-1*::GUS, *GST6* and *PR-1* in independent transgenic Arabidopsis lines ectopically expressing HA_3-tagged GRX480.
Three-week-old plants were treated with (a) 50 μM 2,4-D or (b) 1 mM SA for the durations (h) indicated above the lanes. The lanes were loaded with 20 μg of RNA, and the blots were hybridized with the indicated probes. Ethidium bromide-stained RNA (rRNA) is shown as evidence of equal loading. As a control (ctr) line, *as-1*::GUS plants were used.

the alignment of GRX480 with GRX370). Individual yeast clones expressing either GRX480 or GRX480ΔN varied dramatically with regard to their interaction with TGA factors, independently from the amounts of proteins synthesized as analyzed by Western blot analysis. However, it can be concluded that TGA2 interacts with GRX480 and GRX480ΔN but not with GRX370 (Figure 2).

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

The effect of GRX480 on *as-1*-mediated gene expression was first analyzed in transgenic Arabidopsis lines encoding the ß-glucuronidase gene (GUS) under the control of the 'truncated' CaMV 35S promoter (*as-1*::GUS), encoding the sequences +1 to −90. The *as-1* element is the only upstream regulatory element of this promoter fragment and confers responsiveness to 2,4-D and SA (Redman *et al.*, 2002). The *GRX480* coding sequence under the control of the CaMV 35S promoter was transformed into Arabidopsis lines that had been previously transformed with *as-1*::GUS. *GRX480*-expressing transgenic Arabidopsis lines were selected by Western blot analyses using an αHA antibody that detects the HA_3-tagged transgenic protein (data not shown). As shown in Figure 3(a), 2,4-D-induced GUS transcript levels decreased in two lines with high *HA_3:GRX480* expression, whereas transgenic lines with only slightly enhanced *HA_3:GRX480* levels showed a response almost identical to that of the control plants. Thus, GRX480 negatively affects *as-1*-mediated gene expression. Interestingly, SA inducibility of the *as-1*::GUS reporter construct was found to be lower than 2,4-D inducibility (Redman *et al.*, 2002), which might be due to a negative feedback regulation after accumulation of the endogenous GRX480 protein upon SA induction. This mechanism might be saturating, as only marginal effects of HA_3:GRX480 were observed on *as-1*::GUS after SA induction (data not shown). Also, SA-induced expression of *PR-1* was only twofold reduced in line 1 (Figure 3b). A negative effect of HA_3:GRX480 was not observed for 2,4-D-induced *GST6*, which contains a functional *as-1*-like element in its promoter (Chen and Singh, 1999).

Ectopic expression of HA_3:GRX370, which does not interact with TGA factors, did not reveal any negative influence on the expression of the reporter gene, although the protein was clearly detectable by Western blot analysis (Figure S3a,b).

Constitutive expression of GRX480 interferes with induction of PDF1.2

The repressive effect of GRX480 on the activity of the 'truncated' CaMV 35S promoter prompted us to test whether GRX480 might be involved in the down-regulation of genes after SA treatment. One well-known example of such a gene is the JA-responsive gene *PDF1.2* (Spoel *et al.*, 2003), the induction of which is antagonized by SA. In order to analyze whether GRX480 affects *PDF1.2* induction, *HA_3:GRX480* plants (line 3) and control plants were treated with JA and analyzed for *PDF1.2* expression in a time-course experiment. *PDF1.2* expression was strongly impaired (Figure 4a), indicating that GRX480 might be involved in the down-regulation of JA responses. *LOX2* and *VSP*, two other JA-inducible genes that were shown previously to be negatively regulated by SA, were repressed to a much lesser extent (twofold) in *HA_3:GRX480* line 3.

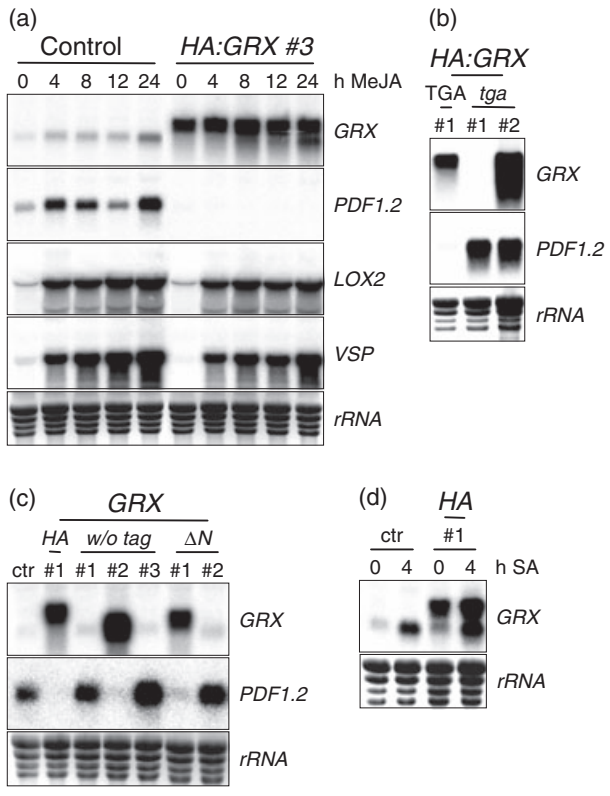

Figure 4. Expression of JA-responsive genes in transgenic Arabidopsis lines transformed with various *GRX480* constructs.
Three-week-old plants were treated with 20 μM MeJA for (a) the durations (h) indicated above the lanes, or (b) 5 h or (c) 6 h. (d) Plants were incubated with 1 mM SA. The lanes were loaded with 20 μg of RNA, and the blots were hybridized with the indicated probes. Ethidium bromide-stained RNA (rRNA) is shown as evidence of equal loading. As a control (ctr) line, *as-1*::GUS plants were used. Genetic backgrounds are abbreviated as *tga* (*tga2 tga5 tga6* triple mutant) and TGA (wild-type *TGA* alleles), and transgenic lines as *HA* (HA_3-tagged GRX480), *w/o tag* (GRX480) and *ΔN* (HA_3-tagged GRX480 lacking its 31 N-terminal amino acids).

To analyze whether the GRX480-mediated effect on the *PDF1.2* promoter depends on the presence of interacting TGA factors, *HA_3:GRX480* was expressed in the *tga2 tga5 tga6* triple mutant. Figure 4(b) shows that a transgenic line that expresses *HA_3:GRX480* even more strongly than *HA_3:GRX480* line 1 does showed induced *PDF1.2* transcript levels that are similar to those for a sister line that does not express the transgene. Thus GRX480 requires the TGA2 sub-class to negatively affect transcription. Impairment of JA-induced *PDF1.2* transcription was not observed in transgenic plants ectopically expressing *HA_3:GRX370*, which does not interact with TGA factors (Figure S3c).

To ensure that the negative effect of GRX480 on *PDF1.2* expression is not an artefact created by the HA_3 tag, a second construct leading to the expression of an untagged GRX480 was generated and transformed into Arabidopsis plants. As shown in Figure 4(c), expression of the untagged derivative also suppressed *PDF1.2* expression. Likewise, the GRX480-specific N-terminal domain, which was shown in Figure 2 to be dispensable for the interaction with TGA factors, is also dispensable for the repressive activity. In order to estimate whether *PDF1.2* suppression can be caused by the amounts of GRX480 that occur in wild-type plants after SA induction, we compared the endogenous and transgenic transcript levels. In the absence of SA, the transcript levels of line 1, which were sufficient to confer suppression of *PDF1.2* induction (Figure 4b,c), were only twofold higher than endogenous transcript levels after SA induction (Figure 4d). Assuming that both transcripts are translated with comparable efficiency, we conclude that the negative effect of GRX480 is unlikely to be caused by abnormally high levels of the transgenic protein.

The negative effect of HA_3:GRX480 on PDF1.2 *expression is mediated independently of NPR1*

It has been described previously that SA-mediated suppression of JA-responsive genes requires the presence of NPR1 (Spoel *et al.*, 2003). In order to analyze whether NPR1 is necessary for the GRX480-mediated repression of *PDF1.2* transcription, *HA_3:GRX480* was ectopically expressed in the *npr1-1* mutant. Transformants and the appropriate control plants (wild-type, *npr1-1*) were subjected to a 'cross-talk' experiment that included treatment with SA, JA, SA/JA and the solvent (0.01% ethanol) alone. The influence of SA/JA antagonism is clearly observed for *PDF1.2* in Col-0 wild-type plants. As described previously (Spoel *et al.*, 2003), this negative effect was partially compromised in the *npr1-1* mutant (Figure 5a). However, HA_3:GRX480 is functional in

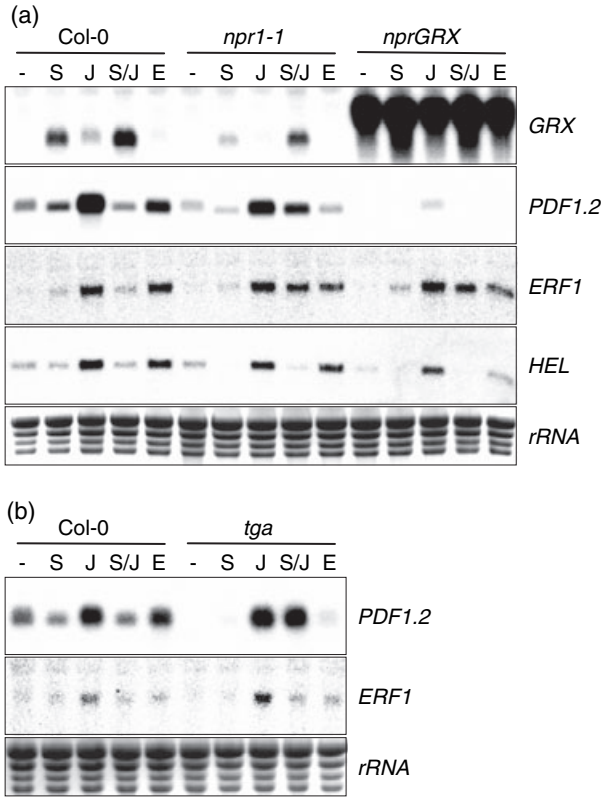

Figure 5. SA/JA antagonism of JA-responsive genes.
Three-week-old plants were treated with SA (S), MeJA (J), SA/MeJA (S/J) or solvent 0.01% ethanol (E) for 4 h. The lanes were loaded with 20 μg of RNA, and the blots were hybridized with the indicated probes. Ethidium bromide-stained RNA (rRNA) is shown as evidence of equal loading.
(a) Analysis of *npr1-1* mutant plants ectopically expressing *HA$_3$:GRX480* (*nprGRX*) along with control plants (Col-0 and *npr1-1*) as indicated above the lanes.
(b) Analysis of the *tga2 tga5 tga6* triple mutant (*tga*) in comparison with Col-0 control plants.

the absence of NPR1 as shown for the respective transgenic line in the *npr1-1* mutant background. Taking the NPR1 dependence of *GRX480* transcription into account, GRX480 must be positioned downstream of NPR1 in the SA-mediated suppression of *PDF1.2*.

ERF1 is a transcriptional regulator that induces expression of *PDF1.2* and other JA-responsive genes when overexpressed in Arabidopsis (Berrocal-Lobo *et al.*, 2002). As shown in Figure 5(a), this global regulator of JA-related defense responses is also under the control of the NPR1-mediated SA/JA antagonism. However, *ERF1* expression is not subject to negative regulation by GRX480. Thus, although *ERF1* and *PDF1.2* are both down-regulated in an NPR1-dependent manner, this regulation may be exerted through various mechanisms. This is supported by the different responses of *ERF1* and *PDF1.2* in the *tga2 tga5 tga6* mutant. In this mutant, SA/JA antagonism is working for *ERF1* but not for *PDF1.2* (Figure 5b).

This finding that different mechanisms for SA/JA cross-talk exist in Arabidopsis is extended by the expression pattern of the *PR* gene *HEL*. JA-induced transcription of *HEL* is also subject to the negative effect exerted by SA, but this effect is independent of NPR1 (Figure 5a).

TGA factors bind directly to the PDF1.2 *promoter*

Suppression of 2,4-D-induced *as-1::*GUS transcription by GRX480 is likely to be caused by a direct effect of TGA factor activity, as their binding sites are the only regulatory modules in this construct. Such a mechanism might also act on the *PDF1.2* promoter, which contains a TGAC/G motif at positions −399 to −395 relative to the predicted transcriptional start site (Spoel *et al.*, 2003). Chromatin immunoprecipitation experiments with an antiserum directed against the C-terminal regions of TGA2 and TGA5 confirmed the assumption that TGA factors bind to the *PDF1.2* promoter *in vivo*. The PCR signal was lacking in the *tga2 tga5 tga6* triple mutant, supporting the specificity of the assay (Figure 6). Thus it is possible that – within certain promoter contexts – DNA-bound TGA factors are directly involved in SA- and GRX480-mediated repression.

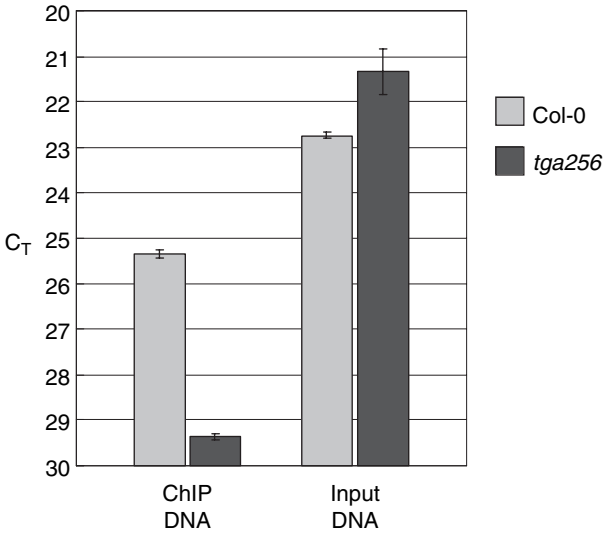

Figure 6. *In vivo* TGA factor binding to the *PDF1.2* promoter as revealed by chromatin immunoprecipitation analysis.
Leaves from Col-0 and *tga2 tga5 tga6* (*tga256*) plants were incubated in 1% formaldehyde before chromatin preparation. Chromatin samples were subjected to immunoprecipitation using 5 μl of the αTGA2,5-C antiserum. The (ChIP) DNA was recovered after reversal of the cross-links and analyzed for the enrichment of *PDF1.2* promoter sequences by quantitative real-time PCR. C_T values are given as a parameter to quantify the amount of PCR product. C_T values obtained from chromatin preparations before immunoprecipitation (input DNA) are shown on the right. C_T values above 30 cycles were not taken into account, as PCR performed without specific templates yielded C_T values in this range. Error bars indicate the variability of the PCR (one repetition).

Discussion

The family of TGA transcription factors is involved in the regulation of *PR* genes and the establishment of systemic acquired resistance (Zhang *et al.*, 2003). Moreover, they activate detoxification genes in response to xenobiotic stress (Johnson *et al.*, 2001). TGA factors are primary transcription factors whose function must be controlled at the protein level. Therefore, identification of TGA-interacting proteins constitutes an important step in understanding the mechanism of their activation. Using a yeast screen set up to identify proteins that interact with TGA factors, a clone encoding a glutaredoxin was isolated. The interaction was confirmed *in planta* using a bimolecular fluorescence complementation (BiFC) assay (Figure 1b) and a two-hybrid system in transiently transformed tobacco protoplasts (Figure S1).

GRX480 might be involved in the alteration of the redox state of regulatory proteins

The isolation of a glutaredoxin as a TGA-interacting factor might be biologically significant given that conditions that activate TGA-dependent transcription also alter the redox status of the cell. Furthermore, critical cysteines of TGA1 and NPR1 are reduced *in planta* after SA treatment (Despres *et al.*, 2003; Mou *et al.*, 2003). The promiscuous interaction of GRX480 with TGA factors from several sub-groups (Table 1) opens the possibility that it might be involved in various TGA-mediated functions. Although ectopic expression of *GRX480* reduced the expression of 2,4-D-inducible *as-1::*GUS and JA-inducible *PDF1.2* (Figures 3 and 4), the SAR gene *PR-1* and the xenobiotic stress-responsive gene *GST6* were not significantly affected. In the course of this study, we tried several *in vitro* approaches to analyze whether GRX480 catalyses the reduction of TGA1. So far, we have been unable to obtain any evidence for this. In addition, analysis of the *in vitro* interaction between NtTGA2.2 and GRX480 by pull-down assays, South-western analysis and electrophoretic mobility shift experiments was not successful, raising the question as to whether the TGA/GRX480 interaction requires additional factors that are lacking *in vitro*. Alternatively, the interaction might be too transient to be detected by these assays.

Glutaredoxins can act as negative regulators of gene expression

A strong and reproducible effect of the ectopic *GRX480* expression is the inhibition of 2,4-D-induced *as-1::*GUS and JA-induced *PDF1.2* expression. Inhibition of *PDF1.2* induction is also mediated by SA (Spoel *et al.*, 2003), representing an example of the cross-connection between the two stress signaling molecules SA and JA. As *GRX480* expression is induced by SA (Figure 1c,d) and further enhanced by JA (Figure 5a), we suggest a functional role of GRX480 in SA/JA antagonism. Negative effects of glutaredoxins on gene expression have been reported previously. Yeast GRX3 and GRX4 interact with the transcription factors ScAFT1 and ScAFT2, which are involved in inducing genes under iron deficiency. ScAFT target genes are constitutively expressed in the *grx3 grx4* double mutant, indicating that these glutaredoxins inhibit ScAFT1 and ScAFT2 activity in the presence of iron (Ojeda *et al.*, 2006). In human T cells, overexpression of the human glutaredoxin PICOT results in inhibition of the transcription factors AP-1 and NF-κB (Witte *et al.*, 2000). The plant glutaredoxin ROXY1 is necessary to repress expression of the homeotic gene AGAMOUS in the outer whorls of the Arabidopsis flower (Xing *et al.*, 2005). Interestingly, the *roxy1* mutant and the TGA factor mutant *perianthia* are disturbed in establishing the correct number of petals, implicating that TGA factors and glutaredoxins might also functionally interact in the flower development pathway.

The mechanism of GRX480-mediated gene repression remains to be elucidated. Direct recruitment of GRX480 to the affected plant promoters seems possible as *as-1*-bound TGA factors and GRX480 interact in yeast. In *HA_3:GRX* plants, no enrichment of target promoters (*as-1::*GUS, *PDF1.2*) was obtained when the αHA antibody was used for chromatin immunoprecipitation. Thus, the complex is either non-existent or very short-lived or hard to cross-link. Alternatively, GRX480 might exert its function by modifying unbound TGA factors or TGA/protein complexes that are recruited afterwards to the target promoter. This seems plausible for the *as-1::*GUS construct, whose activity relies exclusively on TGA factors. However, although TGA factors are recruited to the *PDF1.2* promoter (Figure 6), data from Spoel *et al.* (2003) argue against such a mechanism. A −316 *PDF1.2* promoter deletion derivative lacking the TGA binding site still shows JA inducibility and JA/SA antagonism. This contradiction can be reconciled by assuming that GRX480 and TGA factors regulate another gene acting upstream of *PDF1.2*. Whether redox reactions or removal of glutathione moieties from thiol groups of TGA factors or other target proteins are involved in these processes remains to be explored.

A *grx480* knock-out mutant obtained from the RIKEN Arabidopsis transposon mutant collection reacted like the wild-type with respect to the SA-mediated repression of *PDF1.2* expression (data not shown). Indeed, functional redundancy of GRX480 with one or several of the other 30 glutaredoxins seems likely given that the GRX480-specific N-terminus neither contributes to the interaction with TGA2 (Figure 2) nor to its function as a repressor of *PDF1.2* (Figure 4c). According to public databases, a number of glutaredoxins are activated by SA, and preliminary data indicate that several of these interact with

TGA2. The potential redundancy within the glutaredoxin family must be addressed before initiating further analysis of loss-of-function mutants that might unravel additional phenotypes that cannot be observed in gain-of-function approaches.

Various mechanisms regulate the cross-talk between the two stress signaling molecules SA and JA

In general, the SA/JA antagonism affects a variety of genes and may result in reduced resistance to herbivore attack in plants displaying SAR (Preston *et al.*, 1999). NPR1, a central regulator of many SA-regulated processes, clearly contributes to this SA/JA antagonism as demonstrated for *PDF1.2*, *VSP*, *LOX2* (Spoel *et al.*, 2003) and *ERF1* (Figure 5a). Although NPR1 acts in the nucleus to induce SA-activated genes such as *PR-1* (Kinkema *et al.*, 2000), SA-activated NPR1 interferes with *PDF1.2* expression even when it is retained in the cytosol by the ligand-binding domain of the rat glucocorticoid receptor (NPR1:GR). To explain the cytosolic action of NPR1, it has been hypothesized that NPR1 interferes with the function of the F-box protein COI1 (coronatin insensitive 1) (Xie *et al.*, 1998), which is suggested to be responsible for the degradation of repressors of JA signaling (Beckers and Spoel, 2006).

However attractive this model, it is hard to reconcile with our findings that suppression of *PDF1.2* requires the nuclear factors TGA2, TGA5 or TGA6 (Figure 5b). Instead we propose that NPR1 is required for *PDF1.2* suppression because it is required for the transcriptional activation of *GRX480* (Figure 1c). GRX480 in turn forms a possibly transient complex with TGA factors and inhibits *PDF1.2* transcription. This scenario may be reconciled with the data obtained by Spoel *et al.* (2003) by assuming that, even in the absence of the ligand dexamethasone, the NPR1:GR fusion protein might exhibit some residual nuclear localization after SA treatment that enables it to act as a positive regulator of *GRX480* transcription.

In contrast to *PDF1.2*, repression of *ERF1* is independent of the TGA/GRX480 complex (Figure 5b), and might therefore be mediated by the mechanism proposed by Beckers and Spoel (2006). Reduced *ERF1* transcript levels do not lead to reduced *PDF1.2* transcript levels in SA/JA-treated *tga2 tga5 tga6* plants, indicating that the mechanisms regulating *ERF1* transcription do not act upstream of the *PDF1.2* promoter (Figure 5b). Instead, two independent modes of NPR1 action seem to mediate the negative effects of SA on *ERF1* or *PDF1.2* expression, respectively.

In addition to the two different pathways requiring NPR1, an NPR1-independent route was detected for the negative control of *HEL* transcription by SA (Figure 5a). Interestingly, induction of *HEL* expression does not require the F-box protein COI1, which might be the target of NPR1 in the cytosol (Beckers and Spoel, 2006). The various SA/JA cross-talk pathways uncovered in this study are summarized in Figure 7.

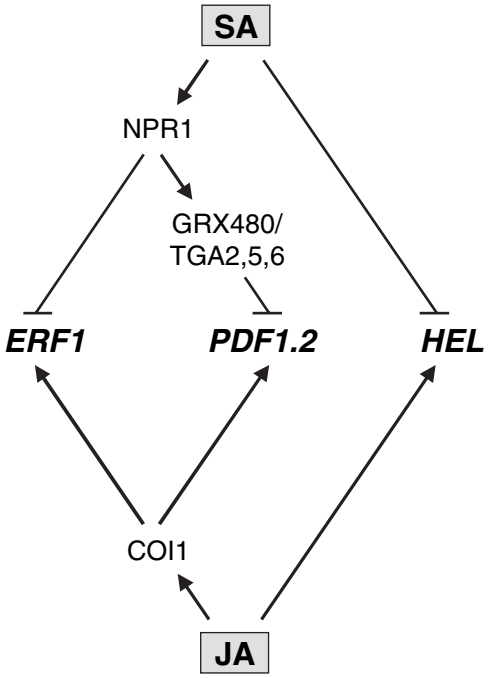

Figure 7. Schematic representation of various mechanisms mediating the negative effect of SA on various JA-regulated genes.
Two different NPR1-dependent pathways regulate SA-mediated suppression of COI1-dependent JA-inducible genes: *ERF1* is repressed by a mechanism that does not require TGA2, TGA5 or TGA6 (Figure 5b). In contrast, *PDF1.2* repression depends on TGA2, TGA5 or TGA6. TGA factors act upstream of GRX480 by regulating its expression after SA treatment (Figure 1d), as well as downstream by forming an inhibitory complex interfering with transcription of *PDF1.2* (Figure 4b). A third NPR1-independent mechanism affects expression of the COI1-independent gene *HEL*.

Experimental procedures

Plant growth conditions and chemical treatments

Arabidopsis plants (Col-0 background) containing an *as-1*::GUS reporter construct (Redman *et al.*, 2002) were provided by J. Arias (University of Maryland, Baltimore, MD, USA). Arabidopsis *npr1-1* and *tga2 tga 5tga6* mutants were obtained from the Nottingham Arabidopsis Stock Centre and Y. Zhang (University of British Columbia, Vancouver, Canada), respectively. Arabidopsis plants were grown in soil under controlled environmental conditions (21/19°C, 100 μmol photons m^{-2} sec^{-1}, 16 h light/8 h dark cycle, 60% relative humidity). The soil was heated for 10 min at 90°C before use. Before sowing the seeds, pots were irrigated with water containing a starter fertilizer (0.5 ml l^{-1} Wuxal liquid, AgNOVA Technologies; http://www.agnova.com.au) and an insecticide (CONFIDOR WG70, Bayer; http://www.bayercropscience.com.au). All seeds on moist soil were vernalized at 4°C for 2 days before placing them in the growth chamber. To maintain moderately high humidity, plant trays were covered with a lid with an air inlet for the first 3 weeks of growth. Plants were watered at 2-day intervals. For phytohormone treatments, 3-week-old plants were carefully uprooted. The roots were

washed twice in beakers containing tap water until the soil was completely removed. Plants were subsequently transferred to 30 ml of 50 mM sodium phosphate buffer containing the respective chemicals in 0.01% ethanol in Petri dishes and allowed to float for the indicated durations. Chemical treatment included 20 μM MeJA, 1 mM SA, 20 μM MeJA/1 mM SA or 0.1 mM 2,4-D. Chemicals were purchased from Sigma-Aldrich (http://www.sigmaaldrich.com/). For each time point and chemical treatment, material from 15 plants was collected. For each RNA preparation, 200 mg of the pooled samples was used.

Plant vectors and transformation

For transient expression of the YN:TGA2 and YC:GRX480 fusion proteins, GATEWAY-compatible vectors (Invitrogen; http://www.invitrogen.com/) were used (pE-SPYNE and pE-SPYCE, constructed by Caroline Mayer and Wolfgang Droege-Laser, University of Göttingen, Germany). Transformation of Arabidopsis mesophyll protoplasts was performed as described previously (Sheen, 2001). After an overnight incubation at 25°C in the dark, the transformed cells were subjected to microscopic analysis using a BX-60 fluorescence microscope (Olympus; http://www.olympus-global.com/).

In order to generate binary vectors for the expression of HA_3-tagged glutaredoxin proteins GRX480, GRX370 and GRX480ΔN (from which the first 31 amino acids were deleted), the respective pDONR vectors were incubated with binary destination vector pAlligator2 (Bensmihen *et al.*, 2004) that contains the GATEWAY recombination sites located downstream of a sequence encoding an HA_3 tag (http://www.psb.ugent.be/gateway/index.php). The final construct expresses GRX derivatives N-terminally fused to the HA_3 tag under the control of the CaMV 35S promoter. Using a cloning strategy involving overlapping PCR, the CaMV35S*::HA$_3$:GRX480* construct was modified so that the resulting GRX480 open reading frame starts directly at the methionine of the HA_3 tag. For generation of transgenic plants, binary plasmids were electroporated into *Agrobacterium tumefaciens* strain GV3101 (pMP90). The resulting agrobacteria were used to transform Col-0 (containing *as-1*::GUS) or *npr1-1* plants using the floral dipping method (Clough and Bent, 1998). Transgenic seeds were scored for the seed-specific GFP marker under a fluorescent microscope (BX51, Olympus) using light of wavelength 460 nm. If all of the seeds of an F_2 silique were GFP-positive, the plant was scored as homozygous.

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

To express the C-terminal domains of TGA2 and TGA5 as fusion proteins with glutathione-*S*-transferase (GST) for antigen production, the cDNA sequences encoding amino acids 64–329 were amplified by PCR and cloned into the pGEX4T-1 vector (Amersham Biosciences; http://www5.amershambiosciences.com/). Expression and purification of the antigens for generation of a polyclonal antiserum, and the generation of the antiserum were performed as described by Thurow *et al.* (2005).

Chromatin immunoprecipitations

Leaf material (5 g) from Arabidopsis plants grown for 6 weeks under a 8 h light/16 h dark cycle were used. Nuclei were isolated as described previously (Folta and Kaufman, 2000) with the following modifications. Briefly, the frozen tissue was ground with a pestle under liquid nitrogen, resuspended in 20 ml extraction buffer (1 M hexylene glycol, 50 mM PIPES KOH, pH 7.2, 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol), and homogenized for 5 min using a Miccra -D8 homogenizer (14 000 rev min^{-1}, ART Labortechnik; http://www.art-labortechnik.com). The homogenate was passed through a double layer of Miracloth (Calbiochem; http://www.merckbiosciences.com). Triton X-100 (25%) was added dropwise to the resulting liquid fraction with constant stirring to a final concentration of 1% to lyse organelle membranes. The lysate was gently layered on top of a 6 ml 35% Percoll cushion (Sigma-Aldrich) in gradient buffer (0.5 M hexylene glycol, 50 mM PIPES KOH, pH 7.2, 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 1% Triton X-100). After centrifugation at 2100 ***g*** for 30 min in a swinging bucket rotor, the nuclei were found as a pellet at the bottom of the tube. Nuclei were resuspended in 21 ml of gradient buffer and again gently layered on top of a 6 ml 35% Percoll cushion. After centrifugation as above, the pellet was resuspended in 1 ml of gradient buffer and centrifuged at 2100 ***g*** for 10 min. The pellet containing the nuclei was stored for no longer than a week at −80°C. For chromatin preparations, nuclei from 5 g of formaldehyde cross-linked leaf material were first resuspended in 1 ml sonication buffer (10 mM HEPES/NaOH, pH 7.4, 1 mM EDTA, 0.5% SDS) and diluted with 1 ml sonication buffer without SDS. Chromatin was sheared to an average size of 500 bp by repetitive sonication (Butterbrodt *et al.*, 2006). The final centrifugation was performed at 11 200 ***g*** for 20 min at 4°C. The supernatant contains the chromatin that is used for subsequent immunoprecipitations (Butterbrodt *et al.*, 2006). DNA was resuspended in 35 μl (ChIP DNA) or 175 μl (input control) of water for PCR analysis.

Quantitative real-time PCR analysis

Real-time PCR quantification was performed using the SYBR Green technology in a Mini Opticon Real PCR device from Bio-Rad (http://www.bio-rad.com/). PCR amplifications of 2.5 μl of the template DNA were performed in the presence of 0.25 μM of each primer, 100 μM deoxynucleotide triphosphate, 4 mM $MgCl_2$, a 10^{-5} dilution of the SYBR Green stock (Cambrex Bio Science Rockland Inc., http://www.cambrex.com) and 1.25 U Immolase DNA polymerase mix (Bioline; http://www.bioline.com) in buffer provided by the manufacturer. The PCR regime was: 95°C for 7 min, and 35 cycles of 95°C for 20 sec, 60°C for 20 sec and 72°C for 28 sec. Using the oligonucleotides PDFsense (5′-TTCAGTAATAGGTGTGTCCCAGG-3′) and PDFantisense (5′-GCGGCTGGTTAATCTGAATGG-3′), a 323 bp promoter fragment (−260 to −582) was amplified.

Methods for yeast protein extracts, Western blots, RNA gel blot analysis, yeast screen, assays, strains and plasmids are described in Appendix S1.

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. *In planta* two-hybrid system documenting the interaction between NtTGA2.2 and GRX480.

Figure S2. Alignment of GRX480 (*At1g28480*) and GRX370 (*At5g40370*).

Figure S3. Expression of *as-1::*GUS and *PDF1.2* in plants ectopically expressing either HA_3*:GRX480* or HA_3*:GRX370*.

Appendix S1. Additional experimental procedures.

This material is available as part of the online article from http://www.blackwell-synergy.com

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