Redox-active cysteines in TGACG-BINDING FACTOR 1 (TGA1) do not play a role in salicylic acid or pathogen-induced expression of TGA1-regulated target genes in Arabidopsis thaliana

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Introduction

Redox reactions drive all energy-converting processes in living organisms. To adjust metabolic and regulatory processes to the prevailing redox state, proteins possess reactive cysteines that can be subject to various oxidative modifications. Prominent examples of proteins regulated by these so-called thiol switches are enzymes of the Calvin cycle, which become inactivated during the night when less reducing power is available in the chloroplast (Michellet et al., 2013). Conversely, oxidation of yeast transcription factor yAP1 leads to its accumulation in the nucleus, where it activates genes of the antioxidative system (Delaunay et al., 2000).

Plant immune responses are associated with complex changes in the cellular redox state. The defense hormone salicylic acid (SA), for instance, promotes the production of reactive oxygen or nitrogen species, while on the other hand inducing genes of the anti-oxidative system; for example, oxidoreductases or glutathione biosynthesis genes (Herrera-Vasquez et al., 2015). Redox signals affect the activity of the important regulatory protein NONEXPRESSOR OF PATHOGENESIS-RELATED GENE1 (NPR1). NPR1 controls many processes that are induced by elevated SA levels. In SA-treated tissues, NPR1 becomes first nitrosylated, which is a prerequisite for the formation of intermolecular disulfide bonds. These force the protein into the inactive oligomeric form, which resides in the cytosol (Mou et al., 2003; Tada et al., 2008). On the other hand, transcription of the small oxidoreductase THIOREDOXIN h5 is activated, which in turn reduces the disulfide bonds, resulting in monomerization and nuclear translocation of NPR1 (Spoel et al., 2009). In the nucleus, NPR1 protein levels are regulated by NPR3 and NPR4 in an SA-dependent manner (Fu et al., 2012). All three NPR1 proteins bind SA, which is essential for their regulatory function (Fu et al., 2012; Ding et al., 2018). NPR1 interacts with TGACG-binding (TGA) transcription factors TGA2, TGA3, TGA5, and TGA6 to induce the expression of defense genes (Zhang et al., 2003; Saleh et al., 2015), whereas NPR3 and NPR4 function as repressors (Ding et al., 2018).

TGA factors form a family of 10 members that are grouped into five clades (Gatz, 2013). The partially redundant clade-II TGAs (TGA2, TGA5, and TGA6) function together with NPR1 in the context of the immune response ‘systemic acquired resistance’ (SAR) (Zhang et al., 2003). TGA3, like NPR1, is required for basal resistance against the bacterial pathogen Pseudomonas
Pseudomonas syringae pv. maculicola ES4356 (Psm; Kesarwani et al., 2007). Since NPR1 is sumoylated after SA treatment and since TGA3 only interacts with sumoylated NPR1, it has been concluded that TGA3 and NPR1 functionally interact in vivo (Saleh et al., 2015). The SA marker gene PATHOGENESIS-RELATED 1 (PRI) has been used as an example to provide evidence that the NPR1–TGA interactions described occur at TGA binding sites in SA-responsive promoters.

Initial studies also suggested that NPR1 and clade-I TGAs (TGA1 and TGA4) act in the same pathway. First, TGA1/TGA4 and NPR1 are required for basal resistance against Psm (Kesarwani et al., 2007; Shearer et al., 2012); second, TGA1 interacts with NPR1 only if an inhibitory internal disulfide bridge between cysteine residues 260 and 266 of TGA1 is not formed; third, the interaction between NPR1 and TGA1 promotes its binding to DNA (Despres et al., 2003; Lindermayr et al., 2010); and fourth, TGA1 is partially oxidized in untreated leaves and becomes reduced after SA treatment (Despres et al., 2003). Based on these circumstantial pieces of evidence, models presenting redox-modulated TGA1 interacting with NPR1 at SA-responsive promoters were published in numerous reviews and book chapters (Eckardt, 2003; Pieterse & Van Loon, 2004; Li & Zachgo, 2009; Moore et al., 2011; Chi et al., 2013; Li & Loake, 2016; Gullner et al., 2017).

However, the functional significance of TGA1 for the expression of SA/NPR1-regulated genes and the role of the often-cited redox modulation has not yet been conclusively demonstrated. Using microarray analysis of SA-treated plants, Shearer et al. (2012) observed that expression of 584 of the 629 SA-induced NPR1-dependent genes were independent from TGA1/TGA4 and that basal levels of the remaining 45 genes including PRI were upregulated in tga1 tga4. This implicated that oxidized TGA1/TGA4, which have a low DNA binding activity, at least in vitro, would repress these genes and repression would be released upon the interaction of reduced TGA1/TGA4 with NPR1. To explain the susceptibility of the tga1 tga4 mutant, an NPR1-independent defense mechanism was postulated and confirmed by the higher susceptibility of the npr1 tga1 tga4 mutant compared with npr1 and tga1 tga4 mutants. A very recent study explained the susceptibility of the tga1 tga4 mutant by lower SA and picopoic acid levels after Psm infections. These are due to the reduced expression of the master regulator of SA and picopoic acid biosynthesis, SAR DEFICIENT 1 (SARD1) in Psm-infected tga1 tga4 plants. Chromatin immunoprecipitation (ChIP) experiments confirmed SARD1 as a direct target gene of TGA1/TGA4 (Sun et al., 2018). We decided to readress the question of whether the redox-regulated cysteines in TGA1 play a regulatory role. Since, in our hands, basal expression of PRI was not enhanced in tga1 tga4, we again performed transcriptome analysis to identify TGA1/TGA4-regulated genes. RNA sequencing (RNAseq) analysis provided a number of SA-induced NPR1-dependent genes that were less expressed in tga1 tga4, with SA-3-HYDROXYLASE (S3H)/DOWNY MILDEW RESISTANT 6-LIKE OXYGENASE1 (DLO1) (Zhang et al., 2013; Zeilmaier et al., 2015) being the most robust TGA1/TGA-dependent gene. Under the conditions tested so far, no evidence for a function of the previously postulated redox switch of TGA1 for the regulation of DLO1 and other genes was obtained.

Materials and Methods

Plant material and cultivation

All plants used in this study are in the Arabidopsis thaliana Columbia background. Plants were cultivated in individual pots containing steamed soil (Archut, Fruhstorfer Erde, T25, Strifein, soaked twice with 0.2% Wuxal Super; Manna, Ammerbuch-Pfaffingen, Germany) in a growth cabinet at 22°C with a 12 h : 12 h, day : night cycle and a photon flux density of 100–120 μmol m⁻² s⁻¹ and 60% relative humidity. Genotypes used in the study and corresponding references are npr1-1 (Cao et al., 1997), sard1-1 cbp60g-1 (Zhang et al., 2010), SA induction-deficient 2-2 (sid2-2) (Wildermuth et al., 2001), tga1 tga4 (Kesarwani et al., 2007), and tga2 tga5 tga6 (Zhang et al., 2003). The sid2 tga1 tga4 triple mutant and the tga1 tga2 tga4 tga5 tga6 pentuple mutants were generated through crossings of the respective aforementioned genotypes. Mutants were either obtained from the Nottingham Arabidopsis Stock Centre or from Professor Dr Yuelin Zhang (UBC Vancouver, Canada; sard1 cbp60g tga1 tga4; tga2 tga5 tga6).

Salicylic acid treatment of soil-grown plants

Four week old plants were sprayed either with water or with a freshly prepared 1 mM solution of sodium salicylate (Sigma) until the whole rosette was equally moist. Treatment was conducted 1 h after the subjective dawn, and samples were collected at 8 h after treatment.

Pathogen infection assays

Pseudomonas syringae pv. maculicola ES4356 was cultivated at 28°C in King’s B medium. Overnight cultures were diluted in 10 mM magnesium chloride (MgCl₂) to the final optical density at 600 nm (OD₆₀₀) of 0.005. The 10 mM MgCl₂ solution (mock) or the diluted bacteria were hand infiltrated into three leaves of 5-wk-old plants. Then, 2 d after this primary infection, three younger upper leaves were infiltrated again with a Psm solution (OD₆₀₀ of 0.005) in 10 mM MgCl₂. These leaves were harvested for RNA extraction at 8 h postinfection. Pathogen infiltrations were generally conducted at 1 h after the subjective dawn.

Other methods

Construction of recombinant plasmids, selection of complementation lines, transcriptome analysis, quantitative reverse transcription PCR, transient expression analysis in Arabidopsis protoplasts, Western blot analysis, and accession numbers can be found in Supporting information Methods S1. Primer sequences are given in Table S1. Maps and sequences of plasmids can be found in Notes S1.
Results

TGA1/TGA4 positively regulate a subgroup of salicylic acid induced genes

In order to address the question of whether the disulfide bridge-forming cysteines of TGA1 that become reduced in SA-treated plants indeed play a role for accurate transcription of SA-responsive genes, we first tested SA-induced expression of SARD1, which has been recently identified as a target gene of TGA1/TGA4 (Sun et al., 2018). However, in contrast to Pm3 infections, spraying with 1 mM SA resulted in TGA1/TGA4-independent SARD1 expression (Fig. 1). Under these conditions, SARD1 was controlled by the well-established SA-responsive regulatory module that consists of NPR1 and clade-II TGAs TGA2/TGA5/TGA6. Still, it has to be noted that basal levels of SARD1 were lower in the tga1 tga4 and the npr1 mutants than in wild-type plants, suggesting that residual basal levels of TGA1/TGA4 and NPR1 stimulate basal SARD1 expression.

In contrast to previously published observations (Lindermayr et al., 2010; Shearer et al., 2012), basal PRI expression was not enhanced in the tga1 tga4 mutant, and SA-induced PRI transcript levels were only slightly affected (see ‘NPR1 and clade-II TGA factors are required for expression of selected TGA1/TGA4-dependent genes’ and Fig. 4). Therefore, we performed transcriptome analysis of RNA harvested from leaves of mock and SA-treated plants. We compared the expression pattern of sid2 and sid2 tga1 tga4 rather than that of wild-type and tga1 tga4 because we wanted to avoid any possible influence of TGA1/TGA4 on endogenous SA biosynthesis. Moreover, we aimed to reduce fluctuations in gene expression due to environmental factors affecting endogenous SA levels in different experiments. Four-week-old plants were sprayed either with water or with 1 mM SA. Eight hours after treatment, three leaves of five individual plants were collected and total RNA was isolated. The experiment was repeated four times with batches of independently grown plants. Thus, the RNA from 15 leaves of five plants served as one replicate, and replicates originated from four independent experiments.

Principal component analysis results in clusters of samples with a similar expression pattern, and thus yields a first impression of the global structure of the data set. The clusters from sid2 and sid2 tga1 tga4 plants treated with water showed a clear separation (Fig. S1) indicating that the transcriptomes of both genotypes are different even in the absence of ISOCHORISMATE SYNTHASE 1 (ICS1)-derived metabolites. The clusters from SA-treated plants indicate that both genotypes respond to SA. Since our aim was to identify target genes of TGA1/TGA4 after SA treatment, we focused on those 2090 genes that were induced (log2(fold change) > 1) by SA in sid2 (Table S2).

Forty-one percent (864 genes) of the 2090 SA-induced genes showed a differential expression pattern in sid2 tga1 tga4. These 864 genes fall in two major groups (Fig. 2a). Genes with lower expression values in the sid2 tga1 tga4 plants compared with sid2 establish the ‘green’ group (346 genes). Three major subgroups were identified based on reduced gene expression in sid2 tga1 tga4 either after SA treatment (119), mock and SA treatment (71), or only after mock treatment (153). Only three genes were less expressed in SA-treated leaves while background levels were elevated. The two major subgroups of the ‘red’ group, which comprises 518 genes that are higher expressed in sid2 tga1 tga4 compared with sid2, contain genes that have higher expression values only in the mock situation (401) and genes that had elevated transcript levels in mock and SA-treated plants (114). Three genes were hyper-induced upon SA treatment and have wild-type transcript levels upon mock treatment. The 1226 SA-induced genes that were not affected by TGA1/TGA4 are represented by the ‘yellow’ group.

Fig. 2(b) shows relative expression levels of representative genes of the green and the red groups. DLO1 encodes for an SA hydroxylase that is involved in dampening the immune response by inactivating SA (Zhang et al., 2013; Zeilmaker et al., 2015). Its expression responded strongly to SA (30-fold) and we observed a seven-fold reduction of expression in the sid2 tga1 tga4 mutant, both under basal conditions and after SA treatment. Thus, the induction factor after SA treatment was not changed, suggesting that TGA1/TGA4 act as amplifiers under both conditions. By contrast, induction factors were lower for other genes in sid2 tga1 tga4 compared with sid2 (Table S3). β-1,3 GLUCANASE (BGL2), for example, was induced by a factor of 4.3 in SA-treated sid2, and by a factor of 1.4 in SA-treated sid2 tga1 tga4. Another example is
GLUTATHIONE S-TRANSFERASE F6 (GSTF6), which was induced by a factor of 52 in sid2 and by a factor of 4.3 in sid2 tga1 tga4. The well-known gene of the NPR1-dependent SA response gene PR1 barely missed the cut-off for being differentially expressed in sid2 tga1 tga4 vs sid2 in the RNAseq analysis. Two genes with elevated expression levels (ATAF1 and WRKY6) in sid2 tga1 tga4 compared with sid2 are also displayed. These genes code for transcription factors.

The TGACGTCA motif is specifically enriched in salicylic acid induced genes that are positively regulated by TGA1/TGA4.

The ideal binding site for TGA factors is the palindromic sequence TGACGTCA, which is an extended C-box (GACGTC) (Izawa et al., 1993; Qin et al., 1994). However, the pentamer TGACG is sufficient for binding. Moreover, at
least TGA1 binds to the A-box (TACGTA) in vivo (Wang et al., 2019), and tobacco TGA1a binds to A and G (CAGCTG) boxes in vitro (Izawa et al., 1993). Therefore, we tested whether any of these potential binding sites is specifically enriched in promoters that are either lower or higher expressed in sid2 tga1 tga4 compared with sid2. To this end, the 1 kb sequences upstream of the predicted transcriptional start sites were scanned using the Motif Mapper cis element analysis tool (Berendzen et al., 2012). As displayed in Fig. 3, all potential TGA1 binding sites are enriched in the 2090 SA-inducible promoters compared with promoters arbitrarily selected from the whole genome. At least the enrichment of the extended C-boxes was expected, since most of the 2090 genes are likely to be regulated by NPR1 acting in concert with TGA2/TGA5/TGA6 or TGA3 (Wang et al., 2006). However, when comparing the relative frequency of these motifs in TGA1/TGA4-regulated promoters with their relative frequency in the 2090 SA-regulated genes, an enrichment of the TGACGTCA motif was detected in the group of those 346 genes that required TGA1/TGA4 for maximal expression. DLO1, for instance, contains the TGACGTCA palindrome at position −72 bp with respect to the transcriptional start site. Likewise, SARD1, which was slightly but significantly activated by TGA1/TGA4 in the absence of SA (Fig. 1) and after Psm infections (Sun et al., 2018), contains a TGACGTCA motif at position −212 bp. The G-box is significantly depleted in promoters being less activated in sid2 tga1 tga4 plants. Conversely, the G-box and the A-box are enriched in promoters being derepressed in this mutant. It might well be that more efficient transcriptional activators compete with TGA1 for A and G-boxes.

In conclusion, the enrichment analysis indicates that promoters containing the TGACGTCA motif might be preferred targets of TGA1/TGA4.

NPR1 and clade-II TGA factors are required for expression of selected TGA1/TGA4-dependent genes

Since the SA-induced redox modification of TGA1 alters the interaction with NPR1, we tested whether expression of the selected TGA1/TGA4-dependent genes (Fig. 2b) requires NPR1. We also included the tga2 tga5 tga6 mutant, since NPR1 has been functionally associated with clade-II TGAs (Zhang et al., 2003). Col-0 wild-type and tga1 tga4 mutant plants were included in the experiment. As observed before for sid2 tga1 tga4 vs sid2 (Fig. 2b), the three marker genes that are positively regulated by TGA1/TGA4 were less expressed in tga1 tga4 vs Col-0 after SA treatment (Fig. 4). In addition, expression of PRI was significantly reduced, but the elevated background levels of WRKY6 and ATAF1 (Fig. 2b) were not observed in the presence of a functional SID2 allele (Fig. 4). Expression of all genes with the exception of ATAF1 was significantly regulated by NPR1 (Fig. 4). Since expression of the four TGA1/TGA4-dependent genes also requires clade-II TGAs, a functional connection between TGA1/TGA4 and NPR1 cannot be excluded nor inferred.

The TGACGTCA motif in the DLO1 promoter is a target site for TGA1 and for TGA2

The DLO1 promoter contains only one of the typical TGA binding sites (TGACGTCA) and one A-box within 2000 bp upstream of the transcriptional start site. In order to investigate, whether representatives of both clades of TGAs might be accommodated at the promoter, we analyzed the effect of transiently expressed TGA1 and TGA2 on DLO1 promoter activity. The DLO1 regulatory region from −1777 bp (with respect to the transcriptional start site) to the ATG start codon was fused to the open reading frame of the firefly luciferase gene (FLUC), whereas

| SA-induced in sid2 (2090) | ext. C-box TGACG | ext. C-box TGACGT | ext. C-box TGACGTCA | A-box TACGTA | G-box CACGTG |
|---------------------------|------------------|------------------|-------------------|-------------|-------------|
| Background: whole genome  | 2178/1800.1      | 169/564.9        | 48/28.2           | 445/376.6   | 511/382.6   |

| TGA1/TGA4-dependent       | Lower in sid2 tga1 tga4 (446) | 300/358.2 | 143/135.0 | 148.0 | 75/72.6 | 50/84.4 |
|                          | Higher in sid2 tga1 tga4 (521) | 651/543.7 | 221/204.3 | 8/12.1 | 136/111.5 | 171/127.9 |
| TGA1/TGA4-independent     | (1226)                     | 1268/1277.5 | 456/480.2 | 26/28.2 | 234/261.1 | 290/299.9 |

| Color code | Depleted | Enriched |
|------------|----------|----------|
| p-values   |          |          |
|           | −0.05    | −0.01    | −0.001   | −0.0001  |
|           | 0.05     | 0.01     | 0.007    | 0.001    |

Fig. 3 TGACG-BINDING FACTOR 1/4 (TGA1/TGA4)-modulated salicylic acid (SA)-induced genes are characterized by a higher incidence of the TGACGTCA motif. The occurrence of enriched motifs was determined in the 1 kb sequences upstream of the 5′-untranslated regions. Numbers before the slash represent the total number of occurrences of the given motif within the indicated set. Numbers after the slash represent the expected number of occurrences in a set of randomly chosen promoters from either the whole Arabidopsis thaliana genome (upper panel) or the set of 2090 SA-induced genes (lower panel). The corresponding enrichment p-values are color coded (gray, not significant; green, significantly depleted; red, significantly enriched; according to the P-values indicated in italics); ext., extended. Note that three genes fall into the green group and the red group (see Fig. 2a), because they are lower expressed in sid2 tga1 tga4 vs sid2 after SA treatment and higher expressed in sid2 tga1 tga4 vs sid2 after mock treatment. Therefore, the total number of genes subjected to the analysis is 2093 and not 2090. sid2, SA induction-deficient 2.
TGA1 and TGA2 were expressed under the control of the UBIQUITIN10 (UBQ10) promoter. Proteins were tagged at their C-terminal ends with a triple hemagglutinin (HA) and a streptavidin tag. An ‘empty’ vector expressing only the triple HA tag under the control of the UBQ10 promoter was used as a control of background promoter activity and adjustment of equal amounts of DNA in the transfection mixture. Renilla luciferase (rLUC) served to normalize for transfection efficiency.

In order to avoid background activation by endogenous clade-I and clade-II TGAs, we used protoplasts of the tga1 tga2 tga4 tga5 tga6 mutant that was previously obtained by crossing the respective genotypes. In this assay, only TGA1 activated the promoter. Co-expression of TGA1 with TGA2 slightly enhanced promoter activity (Figs 5a, S2). Since TGA2, in contrast to TGA1, does not contain an extended N-terminal domain with transactivation capacities, a potential binding of TGA2 to the promoter might have been missed in this assay. SA treatment, which leads to association of the transcriptional co-activator NPR1 with clade-II TGAs in differentiated leaf cells, did not specifically increase TGA-enhanced expression of the PromDLO1: fLUC construct in protoplasts (Fig. S2). Thus, the SA signal transduction chain does not operate in the same manner in...
The promoter might be explained by a heterodimer being active at this promoter. This scenario seems feasible, since heterodimerization between in vitro co-translated tobacco TGA1a and TGA2 has been shown before (Niggeweg et al., 2000). Alternatively, they might bind as homodimers, not only at the final target gene but also at genes encoding for regulators acting upstream in the SA-dependent signaling cascade. This hypothesis fits to the expression pattern of SARD1, which is regulated by the well-established NPR1-TGA2/TGA5/TGA6 module, but not by TGA1/TGA4 (Fig. 1). As shown in Fig. 6, expression of DLO1 and BGL2 was strongly reduced in the sard1 cay60g double mutant, which lacks not only SARD1 but also the related and sometimes redundantly acting factor CALCIUM BINDING PROTEIN 60g (CBP60g; Wang et al., 2011). This effect was less pronounced for PR1 and absent for GSTF6 expression. At least for DLO1 and BGL2, which are more affected by TGA1/TGA4 than PR1 and GSTF6, are, the concept of indirect regulation by clade-II TGA-activated SARD1 and direct regulation by clade-I TGAs seems plausible. Consistently, both promoters contain SARD1 binding sites. No SARD1 binding sites were found in the other two promoters, which might be regulated by other transcription factors that are under the control of the NPR1-TGA2/TGA5/TGA6 regulatory module.

Mutation of redox-active cysteines does not alter the expression pattern of selected marker genes in salicylic acid treated leaves

As already mentioned, a disulfide bridge between C260 and C266 was detected in 50% of the TGA1 proteins in untreated tissue, whereas 100% of the TGA1 pool is reduced in SA-treated tissue. This results in a larger amount of TGA1 being able to interact with NPR1, which in turn leads to increased DNA binding (Despres et al., 2003). Having identified SA-induced TGA1/TGA4-dependent genes, we were now able address the importance of these ‘SA-switchable’ cysteines. Since the two flanking cysteines C172 and C287 are also prone to redox modifications (Lindermayr et al., 2010), all four cysteines were mutated (C172N C260N C266S C287S). The first three cysteines were changed into residues found in TGA2 at the corresponding positions, whereas the last cysteine was changed to serine, which is found at the corresponding positions in TGA3, TGA4, TGA7 and TGA9.Mutations were introduced into a genomic clone that contained the SARD1 promoter, which lacks not only SARD1 but also the related and sometimes redundantly acting factor CALCIUM BINDING PROTEIN 60g (CBP60g; Wang et al., 2011). This effect was less pronounced for PR1 and absent for GSTF6 expression. At least for DLO1 and BGL2, which are more affected by TGA1/TGA4 than PR1 and GSTF6, the concept of indirect regulation by clade-II TGA-activated SARD1 and direct regulation by clade-I TGAs seems plausible. Consistently, both promoters contain SARD1 binding sites. No SARD1 binding sites were found in the other two promoters, which might be regulated by other transcription factors that are under the control of the NPR1-TGA2/TGA5/TGA6 regulatory module.
was reduced to approximately the same levels in tga1 tga4 NPR1 and SA. We tested under these conditions, TGA1/TGA4 might act in concert with SARD1 Since et al. pathogen infection (Sun et al., 2018), we questioned whether, TGA1/TGA4-dependent target genes at 8 h after mock or SA treatments. Moreover, background levels were not differentially affected in plants expressing either TGA1 or mutated TGA1 (Figs 7, S3). Since the nonmutated and the mutated proteins were equally effective, it is concluded that the SA-mediated redox switch in TGA1 does not contribute to the proper expression of TGA1/TGA4-dependent target genes at 8 h after mock or SA treatments.

TGA1/TGA4 might act in concert with NPR1 when regulating SARD1 after pathogen infection

Since SARD1 expression is modulated by TGA1/TGA4 upon pathogen infection (Sun et al., 2018), we questioned whether, under these conditions, TGA1/TGA4 might act in concert with NPR1 and SA. We tested SARD1 expression 8 h after infection with Psm in the tga1 tga4, tga2 tga5 tga6, npr1, and the sid2 mutants (Fig. 8a). Indeed, Psm-induced expression of SARD1 was reduced to approximately the same levels in sid2, npr1, and tga1 tga4 (Fig. 8a). Importantly, expression levels were unaffected in tga2 tga5 tga6. This suggests that an SA-mediated feedforward loop using NPR1 and TGA1/TGA4 enhances SARD1 expression independently from TGA2/TGA5/TGA6. Likewise, in Psm-infected SAR leaves, NPR1 and TGA1/TGA4, but not TGA2/TGA5/TGA6, were important for SARD1 expression (Fig. 8b).

As observed in SA-treated tissue, SARD1/CBP60g, NPR1, and clade-I and clade-II TGAs were important for DLO1 and BGL2 expression in Psm-infected leaves, independent of whether they had been pretreated with Psm or with MgCl2. Since clade-II TGAs were not required for SARD1 expression and thus did not influence ICS1 transcript levels (Fig. S4), we consider it likely that SA levels were not reduced in tga2 tga5 tga6. It is concluded that these factors activate DLO1 directly, whereas the effect of the clade-I TGAs and NPR1 can be partially explained by reduced SA levels due to reduced SARD1 and ICS1 expression.

BGL2 transcript levels followed a similar trend. However, it has to be noted that, in Psm-infected SAR leaves, SARD1 and BGL2 were not as stringently dependent on SA as in Psm-infected leaves from plants that had been pretreated with MgCl2. NPR1 remained necessary even when SA levels were not as critical for induction. This suggests that a signaling molecule different from SA can activate the NPR1-TGA1/TGA4 regulatory module in Psm-infected SAR leaves. A similar phenomenon has been observed very recently in the autoimmune mutant camta123 showing that SARD1 transcript levels were higher in sid2 compared with in npr1 (Kim et al., 2019).

Mutation of redox-active cysteines of TGA1 does not change expression pattern of selected marker genes after pathogen infection

Having identified SARD1 as an SA/NPR1/TGA1/TGA4-dependent target gene after pathogen infection, we analyzed its...
expression in the complementation lines of pair #1 (Fig. 9). As expected, Psm-induced SARD1 expression was reduced in tga1 tga4 plants transformed with the ‘empty vector’. Importantly, both TGA1 constructs (i.e. TGA1 and TGA1red) complemented the phenotype to the same extent. Elevated expression after Psm pre-infections compared with mock pretreatments and the contribution of TGA1/TGA4 to gene expression was more pronounced for DLO1 and BGL2. Again, TGA1 lacking all four cysteines complemented the phenotype to a similar extent as the wild-type protein, supporting the notion that the lack of potential oxidative modifications does not alter the regulatory properties of the protein under these conditions.

**Discussion**

Arabidopsis TGA transcription factors TGA1 and TGA4 interact with the SA-activated transcriptional co-activator NPR1 in a redox-dependent manner (Despres et al., 2003; Lindermayr et al., 2010). Here, using TGA1 mutants with point mutations in all four cysteines, we show that these cysteines do not play a role in SA or pathogen-induced NPR1-dependent expression of TGA1/TGA4-regulated marker genes. We identified TGA1/TGA4 as a positive regulator of the SA catabolizing gene DLO1. Finally, we found that the relative influence of clade-I and clade-II TGAs on SARD1 expression depends on whether plants are treated with SA or with Psm.

In order to address the functional importance of redox-modulated cysteines in TGA1, we first identified SA-induced TGA1/TGA4-dependent genes by RNAseq analysis. Since it was known that SA synthesis is controlled by TGA1/TGA4 (Sun et al., 2018), we performed the analysis in the SA biosynthesis mutant sid2. This strategy guaranteed that genes affected in the SA-treated sid2 tga1 tga4 mutant compared with sid2 would require TGA1/TGA4 downstream of SA, whereas any effects upstream of SA were excluded. Only 193 out of the 2090 genes that were more highly expressed at 8 h after SA treatment compared with mock treatment showed reduced expression in SA-treated sid2 tga1 tga4 plants. It is likely that this number would be even lower
in the wild-type background, since we observed more fluctuations in the presence of endogenous amounts of SA. The low frequency might be due to the expression pattern of TGA1/TGA4, the promoters of which are mainly active in the vascular tissue (Song et al., 2008; Wang et al., 2019). This correlates well with the expression pattern of the two robustly regulated target genes: DLO1 is expressed near the vascular tissue in leaves infected with *Hyaloperonospora arabidopsidis* (Zeilmaker et al., 2015), whereas BGL2 is expressed near the vascular tissue in SA-treated leaves (Spoel et al., 2009). We assume that the other regulatory components influencing DLO1 and BGL2 expression (NPR1, clade-II TGAs, SARD1) are also present in this tissue.

Still, the discrepancy with previously published gene expression patterns of the SA-treated *tga1 tga4* mutant has to be pointed out. Shearer et al. (2012) performed a similar study by analyzing the transcriptomes of soil-grown *tga1 tga4* and *npr1* mutants 1 h and 8 h after SA treatment. DLO1, BGL2, and PRI, which were robustly less expressed in *tga1 tga4* in this study, showed increased basal levels in untreated *tga1 tga4*, whereas expression values were similar to wild-type levels after SA treatment. Likewise, Lindermayr et al. (2010) reported enhanced PRI transcript levels in the *tga1 tga4* mutant. We did not detect increased transcript levels of our marker genes in the *tga1 tga4* mutant after mock treatment. In the presence of SA, expression levels were clearly lower in the *tga1 tga4* mutant in our hands. Whatever the reason for these differences is, we were able to identify SA-induced NPR1-dependent genes that required TGA1/TGA4 for maximal expression. Owing to the limited

Fig. 8 Clade-II TGACG-BINDING FACTORS (TGAs) are not important for SYSTEMIC ACQUIRED RESISTANCE DEFICIENT 1 (SARD1) but are for DOWNY MILDEW RESISTANT 6-LIKE OXYGENASE1 (DLO1) and β-1,3 GLUCANASE (BGL2) expression after infection with *Pseudomonas syringae pv maculicola* ES4356 (Psm). Quantitative reverse transcription PCR analysis of transcript levels of SARD1 and DLO1 in wild-type (Col-0), *tga1 tga4*, *tga2 tga5 tga6*, *npr1*, *sid2*, and *sard1 cbp60g* plants. *cbp60g*, calcium binding protein 60g; *npr1*, nonexpressor of pathogenesis-related genes 1; *sid2*, salicylic acid induction-deficient 2. Three leaves of 5-wk-old *Arabidopsis thaliana* plants were either (a) magnesium chloride (mock)-infiltrated or (b) infiltrated with *Psm* (optical density at 600 nm (OD 600) of 0.005) at 1 h after the subjective dawn. Two days later, three younger upper leaves were infiltrated with *Psm* (OD 600 of 0.005). After 8 h, these were harvested for RNA extraction. Transcrip­tion levels were normalized to transcript levels of UBQ5 (UBIQUITIN5) and *SARD1*. Expression levels were clearly lower in the *tga1 tga4* mutant after mock treatment. In the presence of SA, expression levels were clearly lower in the *tga1 tga4* mutant in our hands. Whatever the reason for these differences is, we were able to identify SA-induced NPR1-dependent genes that required TGA1/TGA4 for maximal expression. Owing to the limited
expression domain of TGA1/TGA4, we failed to prove direct binding to, for example, the promoters of DLO1 or BGL2 by ChIP experiments. Similar problems were encountered before: binding of TGA1/TGA4 to the SARD1 promoter was only shown in protoplasts ectopically expressing TGA1 under the Cauliflower Mosaic Virus 35S promoter (Sun et al., 2018), whereas binding of clade-II TGAs could be documented by ChIP in wild-type plants (Ding et al., 2018).

To answer our primary research question, of whether the redox-modulated NPR1-dependent DNA-binding activity of TGA1 influences the expression of SA-dependent target genes, we had to make sure that expression of the identified target genes are regulated by the interplay between SA, TGA1 and NPR1. However, the analysis was complicated, since SA-induced expression of all four TGA1/TGA4-dependent target genes tested also depended on clade-II TGAs, which can recognize the same binding site as TGA1/TGA4. Given the fact that at least the DLO1 promoter contains only one TGA binding site, we postulate for SA-treated tissues that SA activates NPR1 to stimulate expression of SARD1 in concert with clade-II TGAs. Subsequently, SARD1 acts at the DLO1 and the BGL2 promoters, the expression of which is further enhanced by TGA1/TGA4. Thus, in SA-treated tissues, we could not clearly establish that DLO1 or BGL2 is regulated by a mechanism that is controlled by TGA1/TGA4 interacting with NPR1.

Interestingly, the functions of clade-I and clade-II TGAs in the SA-dependent regulatory network were changed in Psm-infected leaves. Here, the SARD1 promoter remained responsive to NPR1 but was regulated by TGA1/TGA4, whereas TGA2/TGA5/TGA6 became dispensable. Thus, in this tissue, at least SARD1 was the candidate gene we were looking for to address the functional importance of the redox-regulated cysteine. However, the redox-regulated cysteine did not play a role for SARD1 expression, at least at 8 h after pathogen infection of naive or SAR leaves. Under these conditions, endogenous SA levels might have already led to full reduction of the wild-type protein.

According to previously published data, interfering with the internal disulfide bridge formation should lead to a protein that constitutively interacts with NPR1 and subsequently binds to DNA with a higher affinity (Despres et al., 2003; Lindermayr et al., 2010). Thus, higher background activity of at least SARD1, and thus its downstream genes, might have been the expected consequence of the mutations. This was not observed, most likely due to other inhibitory mechanisms, including the repressive effects of NPR3 and NPR4 (Ding et al., 2018). A phenotype might be expected if oxidation, and thus inactivation, of TGA1 were to happen under certain conditions. Our complementation lines in combination with the TGA1/TGA4-dependent marker genes might provide useful tools to analyze whether potential antagonistic effects (e.g. abiotic stresses generating reactive oxygen species that then interfere with the SA pathway) are less pronounced in plants expressing a mutant TGA1 protein that cannot be oxidized.

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References

Berendsen KW, Weiste C, Wanke D, Kilian J, Harter K, Droge-Laser W. 2012. Bioinformatic cis-element analyses performed in Arabidopsis and rice disclose bZIP and MYB-related binding sites as potential AuxRE-coupling elements in auxin-mediated transcription. BMC Plant Biology 12: e125.

Cao H, Glazebrook J, Clarke JD, Volkos S, Dong X. 1997. The Arabidopsis NPR1 gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. Cell 88: 57–63.

Chi YH, Paeng SK, Kim MJ, Hwang GY, Melencion SM, Oh HT, Lee SY. 2013. Redox-dependent functional switching of plant proteins accompanying with their structural changes. Frontiers in Plant Science 4: e277.

Delauanay A, Isnard AD, Toledano MB. 2000. H2O2 sensing through oxidation of the Yap1 transcription factor. EMBO Journal 19: 5157–5166.

Despres C, Chubak C, Rochon A, Clark R, Desveaux D, Fobert PR. 2003. The Arabidopsis NPR1 disease resistance protein is a novel cofactor that confers redox regulation of DNA binding activity to the basic domain/leucine zipper transcription factor TGA1. Plant Cell 15: 2181–2191.

Ding Y, Sun T, Ao K, Peng Y, Zhang Y, Li X, Zhang Y. 2018. Opposite roles of salicylic acid receptors NPR1 and NPR3/NPR4 in transregulation of plant immunity. Cell 173: 1454–1467.

Eckardt NA. 2003. A new twist on systemic acquired resistance: redox control of the NPR1–TGA1 regulatory module by salicylic acid. Plant Cell 15: 1947–1949.

Fu ZQ, Yan S, Saleh A, Wang W, Ruble J, Oka N, Mohan R, Spool SH, Tada Y, Zheng N et al. 2012. NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. Nature 486: 228–232.

Gatz C. 2013. From pioneers to team players: TGA transcription factors provide a molecular link between different stress pathways. Molecular Plant–Microbe Interactions 26: 151–159.

Gullner G, Zechmann B, Küstler A, Kiraly L. 2017. The signaling roles of glutathione in plant disease resistance. In: Hossain MA, Mostofa MG, Vivancos PD, Burritt DJ, Fujita M, Tran LSP, eds. Glutathione in plant growth, development, and stress tolerance. Cham, Switzerland: Springer International, 331–357.

Herrera-Vasquez A, Salinas P, Holuigue L. 2015. Salicylic acid and reactive oxygen species interplay in the transcriptional control of defense genes expression. Frontiers in Plant Science 6: e171.

Izawa T, Foster R, Chua NH. 1993. Plant bZIP protein DNA binding specificity. Journal of Molecular Biology 230: 1131–1144.

Kesarwani M, Yoo J, Dong X. 2007. Genetic interactions of TGA transcription factors in the regulation of pathogenesis-related genes and disease resistance in Arabidopsis. Plant Physiology 144: 336–346.

Kim Y, Gilmour SJ, Chao L, Park S, Thomashow MF. 2019. Arabidopsis CAMTA transcription factors regulate piperolic acid biosynthesis and priming of immunity genes. Molecular Plant 13: 157–168.

Li S, Zachgo S. 2009. Glutaredoxins in development and stress responses. In: Jacquot J-P, ed. Advances in botanical research. Cambridge, MA, USA: Academic Press, 333–361.

Li Y, Spool SH. 2016. Redox-regulated plant transcription factors. In: Gonzales DH, ed. Plant transcription factors: evolutionary, structural and functional aspects. Cambridge, MA, USA: Academic Press, 373–384.

Lindermayr C, Sell S, Muller B, Leister D, Durner J. 2010. Redox regulation of the NPR1–TGA1 system of Arabidopsis thaliana by nitric oxide. Plant Cell 22: 2894–2907.

Michtelet L, Zaffagnini M, Morisse S, Sparla F, Perez-Perez ME, Francia F, Danon A, Marchand CH, Fermani S, Trost P et al. 2013. Redox regulation of the Calvin–Benson cycle: something old, something new. Frontiers in Plant Science 4: e470.

Moore JW, Spool SH, Spool SH. 2011. Transcription dynamics in plant immunity. Plant Cell 23: 2809–2820.

Mou Z, Fan W, Dong X. 2003. Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. Cell 113: 935–944.

Niggegeweg R, Thurow C, Weigel R, Pfitzner U, Gatz C. 2000. Tobacco TGA factors differ with respect to interaction with NPR1, activation potential and DNA-binding properties. Plant Molecular Biology 42: 775–788.

Pieterse CM, Van Loon LC. 2004. NPR1: the spider in the web of induced resistance signaling pathways. Current Opinion in Plant Biology 7: 456–464.

Qin XF, Holuigue L, Horvath DM, Chua NH. 1994. Immediate early transcription activation by salicylic acid via the cauliflower mosaic virus 35S element. Plant Cell 6: 863–874.

Saleh A, Withers J, Mohan R, Marques J, Gu Y, Yan S, Zavaliev R, Nomoto M, Tada Y, Dong X. 2015. Posttranslational modifications of the master transcriptional regulator NPR1 enable dynamic but tight control of plant immune responses. Cell Host & Microbe 18: 169–182.

Shearer HL, Cheng YT, Wang L, Liu J, Boyle P, Despres C, Zhang Y, Li X, Fobert PR. 2012. Arabidopsis clade I TGA transcription factors regulate plant defenses in an NPR1-independent fashion. Molecular Plant–Microbe Interactions 25: 1459–1468.

Song YH, Song NY, Shin SY, Kim HJ, Yun DJ, Lim CO, Lee SY, Kang KY, Hong JC. 2008. Isolation of CONSTANS as a TGA4/OBFI4 interacting protein. Molecules and Cells 25: 559–565.

Spool SH, Mou Z, Tada Y, Spool SH, Spool SH, Spool SH, Spool SH. 2009. Protein–mediated turnover of the transcription coactivator NPR1 plays dual roles in regulating plant immunity. Cell 137: 860–872.

Sun T, Busta L, Zhang Q, Ding P, Jetter R, Zhang Y. 2018. TGACG-BINDING FACTOR 1 (TGA1) and TGA4 regulate salicylic acid and picollic acid biosynthesis by modulating the expression of SYSTEMIC ACQUIRED RESISTANCE DEFICIENT 1 (SARD1) and CALMODULIN-BINDING PROTEIN 60g (CBP60g). New Phytologist 217: 344–354.

Tada Y, Spool SH, Pajeroswka-Mukhtar K, Mou Z, Song J, Wang C, Zuo J, Dong X. 2008. Plant immunity requires conformational charges of NPR1 via S-nitrosylation and thioredoxins. Science 321: 952–956.

Wang D, Amornrirapanich N, Dong X. 2006. A genomic approach to identify regulatory nodes in the transcriptional network of systemic acquired resistance in plants. PLoS Pathogens 2: e123.

Wang L, Tsuchida K, Truman W, Sato M, Le Nguyen V, Katagiri F, Glazebrook J. 2011. CBP60g and SARD1 play partially redundant critical roles in salicylic acid signaling. The Plant Journal 67: 1029–1041.

Wang Y, Salasini BC, Khan M, Devi B, Bush M, Subramaniam R, Hepworth SR. 2019. Clade I TGACG-motif binding basic leucine zipper transcription factors mediate BLADE-ON-PETIOLE-dependent regulation of development. Plant Physiology 180: 937–951.

Wildermuth MC, Dewdney J, Wu G, Ausubel FM. 2001. Isochorismate synthase is required to synthesize salicylic acid for plant defence. Nature 414: 562–565.

Zeilmaker M, Ludwig NR, Elberse J, Seifd MF, Berke L, Van Doorn A, Schuurink RC, Snel B, Van den Ackerveken G. 2015. DOWNY MILDEW RESISTANT 6 and DMR6-LIKE OXYGENASE 1 are partially redundant but
distinct suppressors of immunity in Arabidopsis. *The Plant Journal* **81**: 210–222.
Zhang K, Halitschke R, Yin C, Liu CJ, Gan SS. 2013. Salicylic acid 3-hydroxylase regulates Arabidopsis leaf longevity by mediating salicylic acid catabolism. *Proceedings of the National Academy of Sciences, USA* **110**: 14807–14812.
Zhang Y, Tessaro MJ, Lasner M, Li X. 2003. Knockout analysis of Arabidopsis transcription factors TGA2, TGA5, and TGA6 reveals their redundant and essential roles in systemic acquired resistance. *Plant Cell* **15**: 2647–2653.
Zhang Y, Xu S, Ding P, Wang D, Cheng YT, He J, Gao M, Xu F, Li Y, Zhu Z et al. 2010. Control of salicylic acid synthesis and systemic acquired resistance by two members of a plant-specific family of transcription factors. *Proceedings of the National Academy of Sciences, USA* **107**: 18220–18225.

Supporting Information

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**Fig. S1** Principal component analysis of the normalized transcriptome data obtained from RNAseq analysis.

**Fig. S2** Salicylic acid (SA) treatment does not increase TGA-dependent activation of the *DLO1* promoter in mesophyll protoplasts.

**Fig. S3** Cysteines in TGA1 are not important for wild-type-like salicylic acid (SA)-induced target gene expression.

**Fig. S4** Clade-II TGAs are not important for *ICS1* expression after infection with *Pseudomonas syringae* pv. *maculicola ES4356* (*Psm*).

**Methods S1** Detailed description of methods.

**Notes S1** Maps and sequences of plasmids used in this work.

**Table S1** Primers used for qRT-PCR.

**Table S2** Expression Data of 2090 salicylic acid-inducible genes.

**Table S3** Fold change in selected transcripts as identified by RNAseq analysis of 4-wk-old Arabidopsis *sid2* and *sid2 tga1 tga4* treated with water (mock) or 1 mM salicylic acid (SA) for 8 h.

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