TGA5 acts as a positive and TGA4 acts as a negative regulator of ocs element activity in Arabidopsis roots in response to defence signals

Rhonda C. Foley, Karam B. Singh*

CSIRO Plant Industry, Private Bag, #5, Wembley, WA 6913, Australia

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Abstract TGA/OBF family members are bZIP transcription factors that bind to the octopine synthase (ocs) element, a plant promoter sequence that has been strongly linked to defence/stress responses. Intron-containing hairpin (ihp) constructs were used to generate Arabidopsis lines with reduced expression of TGA4 and TGA5. No visible phenotypic differences were observed between ihpTGA and wild-type (WT) plants. However, the ihpTGA4 and ihpTGA5 plants had opposite affects on ocs element activity, with the ihpTGA4 lines enhancing, and the ihpTGA5 lines reducing, the response of an ocs element construct to the key defence signals, salicylic acid (SA) and H_2O_2, in roots.

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Key words: TGA; Activation sequence-1; Salicylic acid; Hydrogen peroxide; RNA silencing

1. Introduction

Octopine synthase (ocs) elements are among the best characterised plant promoter sequences. First found in the promoter of the Agrobacterium *ocs* gene [1], ocs elements were subsequently found to regulate the expression of a number of other plant pathogen genes [2], including the cauliflower mosaic virus (CaMV) 35S promoter [2,3] where they are also referred to as activation sequence-1 (*as-1*) [3]. In Arabidopsis, ocs element-like sequences are important for the expression of specific glutathione *S*-transferase (GST) and pathogenesis-related genes [4,5]. A large number of studies have shown that the ocs element is induced by specific xenobiotics, some phytohormones and plant defence signals. The ability to respond to such a range of treatments may be due in part to common conditions of oxidative stress generated by some of these treatments. Another contributing factor may be because multiple closely related bZIP proteins, called TGA or OBF, bind to ocs elements [6]. In Arabidopsis, studies have shown that individual TGA proteins vary in their DNA binding specificity, expression patterns, protein–protein interaction properties and posttranscriptional regulation [7–11].

The link between ocs elements and plant defence responses was strengthened by the discovery that Arabidopsis TGA family members interact with NPR1, a key component in the salicylic acid (SA) defence signalling pathway [8–10, 12,13]. Reverse genetic approaches to generate loss-of-function mutants have been employed to determine the function of TGA proteins. Individual knockout mutants in three Arabidopsis TGA proteins TGA2, TGA3 and TGA6 did not result in a detectable phenotype [14,15]. However, a triple mutant where TGA2, TGA5 and TGA6 were knocked out revealed that these three proteins had redundant and essential roles in plant defence [15]. In addition, overexpression of wild-type (WT) or dominant negative TGA factors resulted in a range of phenotypes, including in some cases altered expression of genes containing functional ocs elements in their promoters [14,16–18].

In this study we used intron-containing hairpin (ihp) constructs to explore the role of two TGA proteins, TGA4 and TGA5 (previously called OBF4 and OBF5 [19]) in the regulation of different *GSTF8* promoter constructs. We found that the ihpTGA lines had opposite effects on ocs element activity with the ihpTGA4 lines enhancing and the ihpTGA5 lines reducing the response to both SA and H_2O_2.

2. Materials and methods

2.1. Plant material

The transgenic lines containing the −783 *GSTF8* and 4xocs element promoter constructs fused to the luciferase reporter gene in the Columbia ecotype background are described in [5,20] respectively. For each construct, all the presented data are from a single T4 line that was homozygous for the transgene. WT plants of ecotype Columbia were used as controls.

To produce the ihpTGA4 construct, a 509-bp TGA4 fragment was amplified using the following primers: GCCATCGATGGTACCGTGCTATGGAGAGCAGCCGG and GGGGTCTAGACTCGAGTACCATGGTTATAAATC. To produce the ihpTGA5 construct, a 455-bp TGA5 fragment was amplified using the following primers: GGGGTCTAGACTCGAGCAACACAAAACAGTATA and GGGATCGATGATTCAAGCTTATAGCGTGTCAGTTG. The polymerase chain reaction (PCR) product was digested with the appropriate restriction enzymes, purified and ligated into the *Xba*I/*Cla*I site and either the *Xho*I/*Kpn*I (for TGA4 constructs) or the *Xho*I/*Eco*RI (for TGA5 constructs) site of pHANNIBAL [21]. The *Not*I cassette was then transferred to the binary vector pART27 and transformed into *Arabidopsis thaliana* (Columbia) using standard infiltration techniques. Transgenic plants were selected for kanamycin resistance. T3 homozygous single-insertion plants were identified using kanamycin segregation analysis.

2.2. Growth of Arabidopsis on agar plates

Growth of Arabidopsis on agar plates was as described in [20]. Seedlings used for reverse transcription (RT)-PCR were grown on 11 cm diameter agar plates. For bioluminescence assays, approximately 200 seeds were plated on square 10 cm plates, sealed with

*Corresponding author. Fax: (61)-8-93878991.
E-mail address: karam.singh@csiro.au (K.B. Singh).

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Micropore tape and incubated vertically in a growth room for 4 days (22°C, 16 h light:8 h dark photoperiod).

2.3. RT-PCR

8-day-old plants were harvested from MS agar plates. Methods for RNA isolation, cDNA and quantitative RT-PCR were as described by [22]. The following are the specific primer pairs used for the RT-PCR: TGA1: 5′-GGACTATGAACACACCAAAC and 5′-CGAAAAACCGAGAGAATTAG; TGA2: 5′-CCTGATCTTGGGTCGGAGGG and 5′-CTGTGCCTGAAATGAAGACG; TGA3: 5′-TCAGGACGAAGACCGGATCA and 5′-TCAGGACGAAGACCGGATCA; TGA4: 5′-GAGACATGTATACGCCTGGC and 5′-TGAGAAACTAAGAGCATTGG; TGA5: 5′-CAAGAACATCAGTCTCAACA and 5′-TAAAGACACCCTGTTGCCGT; UBQ: 5′-CTCCGGACCAGCAGCGTCTC and 5′-AGAACACTTATTCATCAGGG.

2.4. Plant treatment

12 ml of 1 mM SA or 1 mM H_2O_2 was pipetted onto 10 cm square plates containing 4-day-old seedlings. The solution was drained off after 40 min. The plates were left with the lids off in the dark chamber of the EG&G Berthold Molecular Light Imager during the bioluminescence assay.

2.5. Bioluminescence assay

4xocs::LUC or *GSTF8*::LUC T4 homozygous plants were crossed with WT, ihpTGA4, or ihpTGA5 homozygous T4 lines. Approximately 20 4-day seedlings from the F1 cross were used in each experiment. For each experiment, F1 seedlings from a separate cross were compared to F1 seedlings from WT crosses. Bioluminescence was measured as described in [20]. The average bioluminescence from the ihpTGA4 and ihpTGA5 lines was graphed with standard errors. Each line contains approximately 20 F1 plants. The WT values from 20–40 F1 plants were also graphed with standard errors.

3. Results

3.1. Generation of ihpTGA4 and ihpTGA5 silenced lines

We initiated loss-of-function experiments using gene constructs encoding ihpRNA [21] against specific members of the Arabidopsis TGA family. We targeted the TGA4 and TGA5 proteins, which were isolated in our group [19] and which belong to distinct TGA subfamilies. We designed ihp constructs of ~500 bp for each gene based on regions with the least homology to other TGA members and generated 22 TGA4 and 20 TGA5 lines. None of the ihpTGA lines had any visible phenotype. We focused on the lines that contained single T-DNA insertion sites as determined by segregation analysis.

We analysed the RNA levels of TGA4 and TGA5 in four independent ihp lines. As some members of the TGA family are highly conserved, it was possible that other TGA family members were also affected in the ihp lines. We used RT-PCR to analyse the levels of TGA1, TGA2/aHBP1b, TGA3, TGA4 and TGA5 in the ihpTGA5 lines and WT plants. The RT-PCR was repeated using two sets of tissue with similar results. We also tried to examine the levels of TGA6, but could not detect any transcript for this gene.

As shown in Fig. 1A, TGA4 RNA levels were on average 40% of WT in the four ihpTGA4 lines. TGA1 is the most closely related family member to TGA4 and its RNA levels were also reduced but to a lesser extent (64%). TGA2, TGA3 and TGA5 shared less sequence similarity with the ihpTGA4 construct and their RNA levels showed only small changes in the ihpTGA4 lines. As shown in Fig. 1B, TGA5 RNA levels were on average 23% of WT in the four ihpTGA5 lines. In contrast, TGA1, TGA2, TGA3 and TGA4 RNA levels showed only small changes in the ihpTGA5 lines.

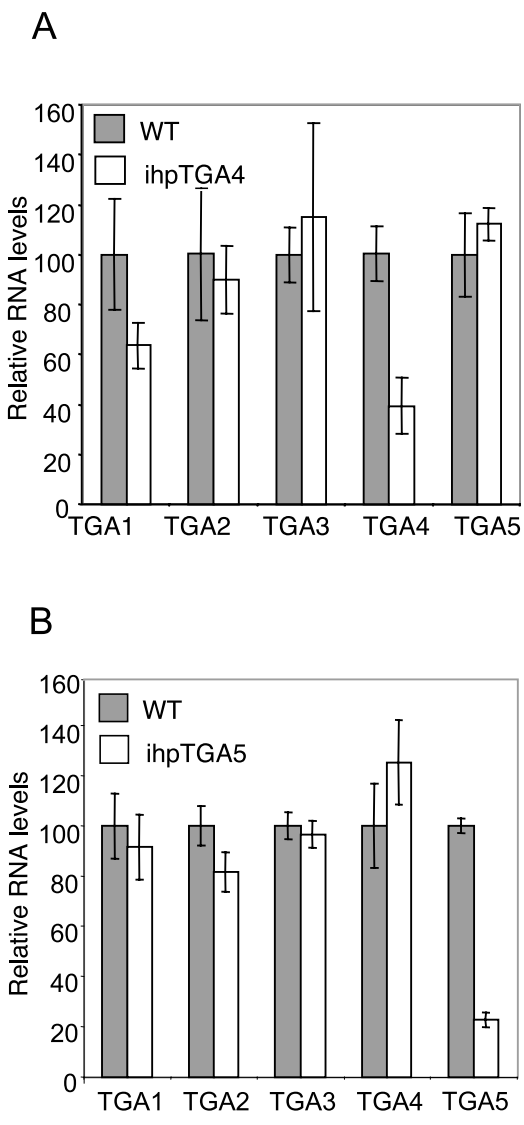

Fig. 1. TGA1, TGA2, TGA3, TGA4 and TGA5 RNA levels in ihpTGA4 and ihpTGA5 lines. Quantitative RT-PCR from RNA isolated from eight 8-day-old seedlings was performed on TGA1, TGA2, TGA3, TGA4 and TGA5. The average value and standard error for each gene are shown with WT set at 100 and the relative value of ihpTGA4 (A) and ihpTGA5 (B) lines compared to this value. A: Four ihpTGA4 lines (white columns) were compared to four WT lines (grey columns). B: Four ihpTGA5 lines (white columns) were compared to four WT lines (grey columns).

3.2. The ihpTGA4 and ihpTGA5 lines do not affect GSTF8 expression

Since we could not detect any visible phenotype with the ihpTGA4 or ihpTGA5 lines we looked to see if there were any changes in the expression of potential target genes. TGA4 and TGA5 have been shown to bind to the promoter of the *GSTF8* gene, previously called *GST6* [23] so we looked to see if *GSTF8* expression was altered. The endogenous *GSTF8* RNA levels were not affected in the ihpTGA4 and ihpTGA5 lines (data not shown). We have previously generated transgenic Arabidopsis plants containing a 791-bp *GSTF8* promoter fragment linked to the luciferase reporter gene and used an in vitro assay for luciferase activity to show that the *GSTF8* promoter was inducible by SA in roots [5]. We crossed a representative *GSTF8*::LUC line with either

WT plants or different ihpTGA4 and ihpTGA5 lines. The *GSTF8*::LUC activity in the F1 progeny was measured using a CCD camera following treatment with SA and these results are shown in Fig. 2A. The bioluminescence is depicted in blue colour and is superimposed over a white image which results from chlorophyll autofluorescence. With the F1 plants from the WT cross, bioluminescence was not detectable in untreated seedlings but significant bioluminescence could be detected at 12 h following SA treatment (Fig. 2A). The activity of the *GSTF8* promoter in the ihpTGA4 (Fig. 2B) and ihpTGA5 (Fig. 2C) silenced lines did not differ from WT.

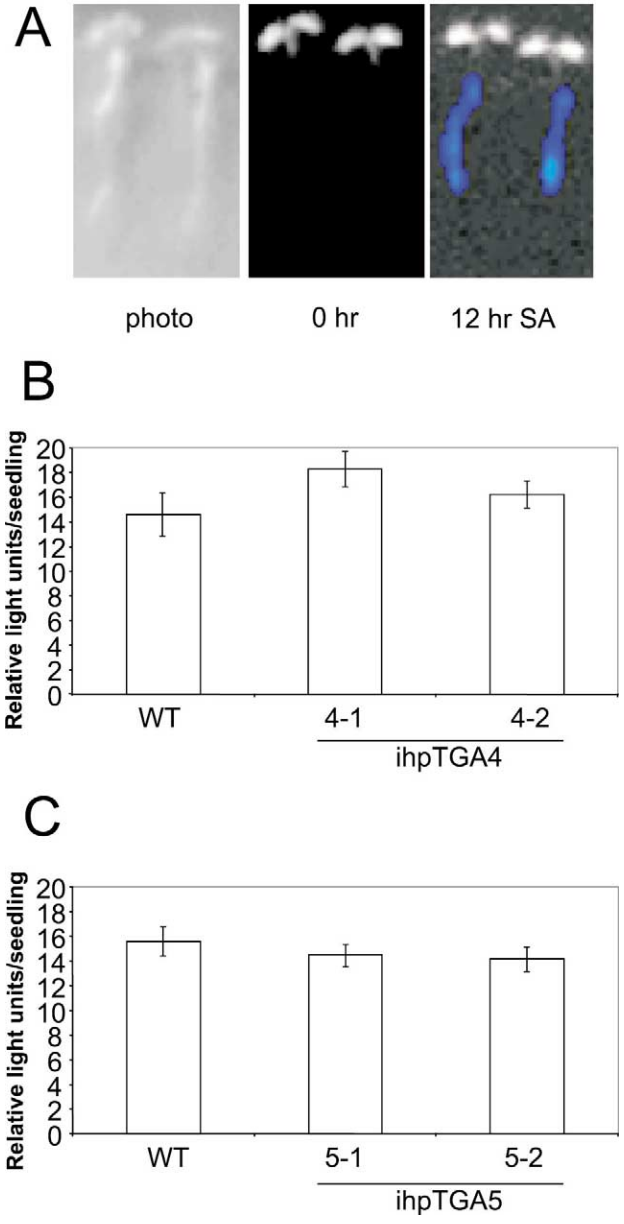

Fig. 2. GSTF8::LUC activity in ihpTGA4 and ihpTGA5 lines. A: In vivo luciferase assay of 4-day-old Arabidopsis *GSTF8*::LUC seedlings following treatment with 1 mM SA. The panel on the left is a photographic image of the seedlings. The middle (0 h) and right (12 h) panels show the autofluorescence of the leaves superimposed on the bioluminescence image. No bioluminescence was detectable at 0 h, but at 12 h bioluminescence can be observed from the roots. B and C: WT and two ihpTGA4 (B), or ihpTGA5 (C) lines were crossed with *GSTF8*::LUC and the bioluminescence of the F1 progeny was measured 12 h after 1 mM SA treatment using a CCD camera. The graph shows the average and standard errors from approximately 20 F1 plants.

There are a number of potential explanations for the lack of any affects on *GSTF8* expression in the ihpTGA4 and ihpTGA5 lines. One possibility is that *GSTF8* may not be regulated by TGA4 and TGA5. Alternatively, TGA4 and TGA5 expression may not have been reduced enough or redundancy in the TGA family could be a factor; perhaps multiple gene silencing is required. Another possibility is that other promoter elements in the *GSTF8* promoter may have masked the loss in ocs element activity in the ihpTGA lines. To test this last possibility we directly analysed ocs element activity in the silenced lines.

3.3. *The ihpTGA4 and ihpTGA5 lines have opposite affects on ocs element activity*

We have previously demonstrated that the ocs element, located at −423 in the *GSTF8* promoter, is responsive to SA and H_2O_2 when linked to a minimal promoter [5]. The in vivo imaging system was not able to detect the activity of the single ocs element::luciferase construct under any conditions tested (data not shown). However, bioluminescence from transgenic Arabidopsis plants containing a tetramer of the ocs element linked to a minimal promoter and the luciferase gene (4xocs::LUC) could be detected using the in vivo imaging system [20].

The four homozygous ihpTGA4 lines used in Fig. 1 were crossed to a representative 4xocs::LUC line and compared to WT plants crossed to the 4xocs::LUC line. Approximately 20 seeds were produced from a typical F1 cross and all of the progeny were used in a given experiment. Therefore, the F1 plants used for the SA versus H_2O_2 treatments were from independent crosses. Each experiment was repeated at least twice and independent crosses of the same lines gave similar results for a given treatment. The F1 plants were treated with SA or H_2O_2 and bioluminescence was measured at 0, 6, 9, and 12 h time points and the results presented in Fig. 3. In each experiment we found the WT/4xocs::LUC expression remains relatively constant, even though they were derived from different crosses. The basal activity of the ocs element was not changed in the ihpTGA4 background (Fig. 3A and B). However, the ihpTGA4 lines had about double the 4xocs::LUC activity compared to WT when treated with SA at each of the time points tested (Fig. 3A). In the case of H_2O_2, there was also an increase in activity in the ihpTGA4 background although this was not as marked as the change following SA treatment (Fig. 3B). These results indicate that TGA4 and/or TGA1 may act as a negative regulator(s) of ocs element activity in Arabidopsis roots.

We then tested the activity of the ocs element in the ihpTGA5 background by crossing the same 4xocs::LUC line with the four ihpTGA5 lines used in Fig. 1. The activity of the ocs element in the F1 progeny was then examined following treatment with SA and H_2O_2 and these results are shown in Fig. 4. The basal activity of the ocs element was reduced in the ihpTGA5 background compared to WT plants. Ocs element activity was also significantly reduced in the ihpTGA5 background compared to WT plants following treatment with SA or H_2O_2 at all time points tested. These results suggest that TGA5 is required for basal and inducible activity of the ocs element in Arabidopsis roots. Importantly, these results

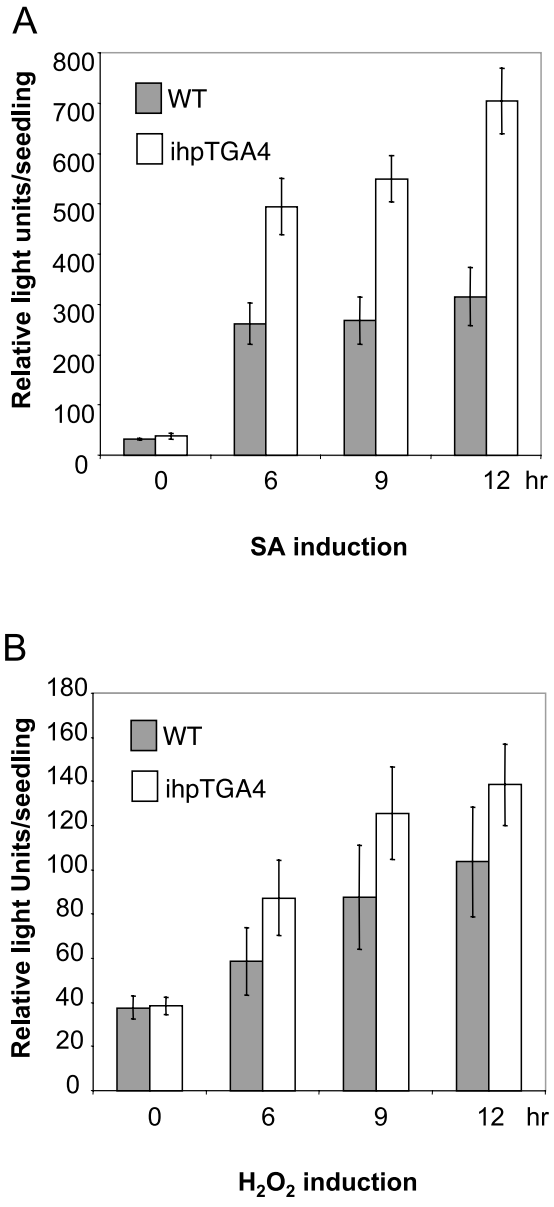

Fig. 3. ihpTGA4 lines have increased ocs element activity in planta. 4xocs::LUC plants were crossed to WT and four ihpTGA4 lines. Bioluminescence activity using a CCD camera was measured after A: 1 mM SA and B: 1 mM H_2O_2 treatment. The averages of approximately 20 F1 plants are graphed at each of the time points 0, 6, 9 and 12 h and shown with standard error. The results from the WT/4xocs plants and the ihpTGA4/4xocs plants are shown in the grey and white columns, respectively.

show that different members of the conserved TGA family can have quite opposite effects on the activity of the same ocs element construct.

4. Discussion

We have used an ihpRNA silencing strategy to analyse the roles of specific members of the Arabidopsis TGA family; important players in plant stress/defence gene expression. We were not able to observe phenotypic differences between ihpTGA and WT plants, similar to what has been observed with knockout mutants for TGA2, TGA3 and TGA6 [14,15] and plants containing an antisense construct against TGA2 [18]. However, by using a sensitive imaging system in which ocs element-containing promoters were linked to the luciferase reporter gene we were able to demonstrate that the ihpTGA4 and ihpTGA5 plants did affect ocs element activity, in planta. Interestingly, the different ihpTGA backgrounds had opposite effects on ocs element activity with the TGA4 lines enhancing and the TGA5 lines reducing the response to the key defence signals, SA and H_2O_2.

The Arabidopsis TGA family members have been divided into subclasses based on the degree of sequence identity between different members [24]. Of the family members analysed in this study, TGA4 and TGA1 belong to subclass 1, TGA5 and TGA2 belong to subclass 2 and TGA3 belongs to subclass 3. Evidence that different TGA members may be involved in different stress responses is emerging. Chromatin immunoprecipitation studies (ChIP) show that tobacco

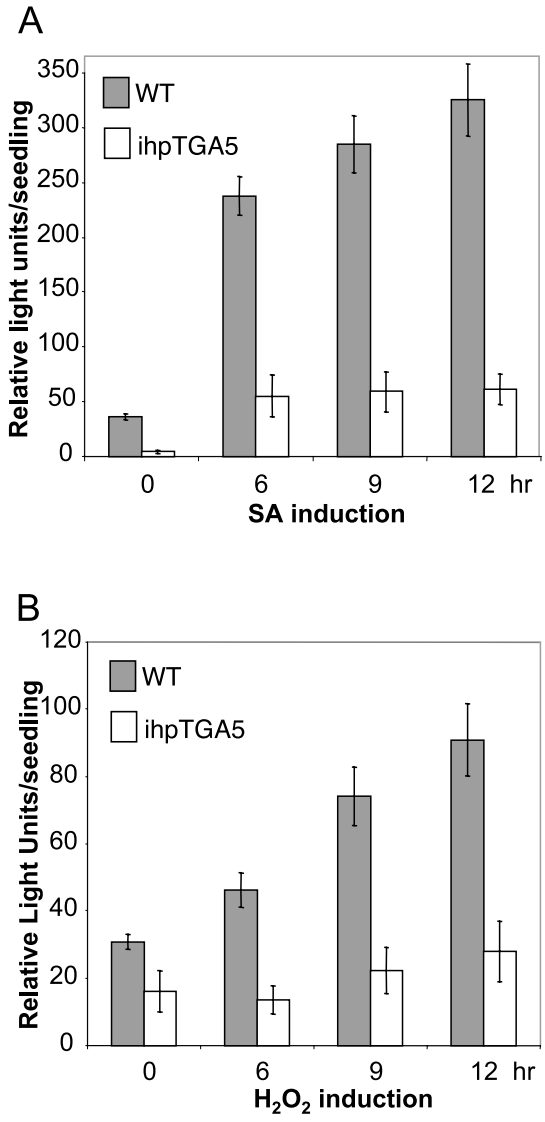

Fig. 4. ihpTGA5 lines have decreased ocs element activity in planta. 4xocs::LUC plants were crossed to WT and four ihpTGA5 lines. Bioluminescence activity using a CCD camera was measured after A: 1 mM SA and B: 1 mM H_2O_2 treatment. The averages of approximately 20 F1 plants are graphed at each of the time points 0, 6, 9 and 12 h and shown with standard error. The results from the WT/4xocs plants and the ihpTGA5/4xocs plants are shown in the grey and white columns, respectively.

TGA1a binds in vivo to xenobiotic responsive promoters but not to *PR* promoters [25], while the Arabidopsis TGA2 protein is responsive to SA but not to xenobiotic stresses [14]. When a trans-dominant mutant was used that specifically eliminated all detectable in vivo DNA binding activity for TGA proteins, both suppression of xenobiotic responsive genes and enhanced induction of *PR* genes by SA were observed [17]. Our studies add important new insight to this emerging picture by showing that family members from different TGA subclasses can have quite opposite affects on the activity of the same ocs element construct. Interestingly, while our results with TGA5 and the results of Zhang et al. [15] demonstrate a role for TGA5 in SA-mediated plant defence responses, overexpression of TGA5 resulted in SA-independent resistance to *Peronospora parasitica* [18] further underscoring the complex role of the TGA family in plant defence/stress responses.

Although the ihpTGA lines had a significant affect on the activity of the 4xocs promoter construct, they had no affect on *GSTF8* promoter activity. While the SA- and H_2O_2-induced expression of the *GSTF8* promoter is mediated in part by the ocs element, other promoter element(s) are critical for expression, since deletion of the ocs element did not abolish the response of the *GSTF8* promoter activity to these signals [5]. One or more of these other promoter elements may help to mask the loss of a specific TGA member(s) in the ihpTGA lines on *GSTF8* promoter activity, perhaps by facilitating the binding of other family members to the ocs element. Our results clearly highlight the importance of having an appropriate assay for reverse genetic studies to tease apart the roles of specific members in transcription factor gene families.

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