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Functional analysis of a TGA factor-binding site located in the promoter region controlling salicylic acid-induced *NIMIN-1* expression in *Arabidopsis*

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ABSTRACT. TGA factors play a key role in plant defense by binding to the promoter region of defense genes, inducing expression. Salicylic acid (SA) induces the expression of the gene encoding NIMIN-1, which interacts with NPR1/NIM1, a key regulator of systemic acquired resistance. We investigated whether the TGA2-binding motif TGACG located upstream of the *NIMIN-1* gene is necessary for SA induction of *NIMIN-1* expression. A mutated version of the *NIMIN-1* promoter was created by site-directed mutagenesis. We generated T-DNA constructs in which native *NIMIN-1* and mutated promoters were fused to green fluorescent protein and β-glucuronidase reporters. We produced transgenic *Arabidopsis* plants and observed *NIMIN-1* promoter-driven green fluorescent protein expression in the roots, petiole and leaves. Constructs were agroinfiltrated into the leaves for transient quantitative assays of gene expression. Using quantitative real-time RT-PCR, we characterized the normal gene response to SA and compared it to the response of the mutant version of the *NIMIN-1* promoter. Both the native *NIMIN-1* construct and an endogenous copy of *NIMIN-1* were induced by SA. However, the mutated promoter construct was much less sensitive to SA than the native *NIMIN-1* promoter, indicating that

this TGA2-binding motif is directly involved in the modulation of SA-induced *NIMIN-1* expression in *Arabidopsis*.

Key words: NIMIN-1; TGA; Defense; Salicylic acid; *Arabidopsis*; Transient assays

INTRODUCTION

Systemic acquired resistance (SAR) has been defined as a general plant defense response triggered by pathogen attack, which occurs in distal, non-infected parts of the plant (Glazebrook et al., 1996; Durrant and Dong, 2004). The signaling pathway involved in SAR begins with the synthesis and accumulation of the signal molecule salicylic acid (SA) in both infected and non-infected tissues of the plant in response to the pathogen. SA is required for the induction of pathogenesis-related (PR) genes and pathogen resistance during SAR (Zhang et al., 2003). SA accumulation activates an intermediate cytosolic protein NPR1 (NIM1), which in its monomeric form migrates to the nucleus (Mou et al., 2003), where it binds and activates TGA transcription factors. In turn, these factors bind to and regulate the expression of target PR genes (Johnson et al., 2003). Indeed, it has been shown that the treatment of plants with SA leads to induction of PR genes and establishment of SAR (Ward et al., 1991).

TGA factors are a conserved plant subfamily of bZIPs and were first described in tobacco by their ability to selectively bind to the *as-1* element of the CaMV 35S promoter and to promote transcription (Lam et al., 1989). The involvement of TGA factors in SA response is supported by the presence of the TGA-binding site in the PR-1 promoter (Lebel et al., 1998; Despres et al., 2000). *In vitro* experiments have shown that the motif TGACG is sufficient for TGA factor binding (Lam et al., 1989). In *Arabidopsis*, the TGA family comprises 10 members, and so far, 2 members (TGA2 and TGA3) were shown by chromatin immunoprecipitation (ChIP) to bind to the PR-1 gene promoter *in vivo* (Johnson et al., 2003).

In *Arabidopsis*, 51 putative binding sites for TGA2 were identified by hybridization of immunoprecipitated chromatin to ChIP-chip whole-genome arrays (Thibaud-Nissen et al., 2006). One of these sites lies upstream of *NIMIN-1*, an SA-induced gene from *Arabidopsis* (Weigel et al., 2001; Glocova et al., 2005; Thibaud-Nissen et al., 2006).

The *NIMIN* (NIM1 interacting) genes were first identified for their interaction with NPR1 (NIM1) in a yeast 2-hybrid screen (Weigel et al., 2001). NIMIN-1 was shown to interact with NPR1 *in planta*, and transgenic plants overexpressing *NIMIN-1* show reduced SA-mediated PR gene induction and reduced amounts of NPR1, demonstrating that NIMIN-1 acts like a negative regulator of NPR1 (Cao et al., 1998; Weigel et al., 2005).

Although *NIMIN-1* response to SA had been previously described (Weigel et al., 2001; Glocova et al., 2005), the element(s) regulating transcriptional activation of SA induced *NIMIN-1* expression at its promoter region remains unknown. NIMIN-1 promoter contains two cis-acting TGACG motifs 70 bp apart. Here, we evaluated the role of mutation in one of them, located in the -360-bp region upstream of the *NIMIN-1* gene, in the regulation of SA-induced expression of *NIMIN-1*. In contrast with the high induction in response to SA observed for the native *NIMIN-1* promoter, SA-mediated induction of gene expression was much lower when 2 bp in the TGA binding site in the *NIMIN-1* promoter were altered by site-directed mutagenesis. These data indicate that this promoter element is involved in the regulation of SA-induced *NIMIN-1* expression.

MATERIAL AND METHODS

Plant material

Arabidopsis wild-type plants (Columbia-0) were grown on Murashige and Skoog medium and agar (0.8%), at 22°C under a 16-h daytime period for long-day growth and 8-h daytime for short-day growth. Plants used for transient assays were raised directly on soil in a growth chamber under short-day conditions for a period of 4 to 6 weeks. Plants used for transient assays were sprayed with 1 mM salicylic acid (Sigma), harvested after 2 h and stored at -80°C for RNA extraction.

Gateway cloning of *NIMIN-1* promoter region

Primers used to polymerase chain reaction (PCR) amplify and Gateway clone the *NIMIN-1* (-804 to +7) promoter region into pDONR207 were as follows: *NIMIN-1* forward (*att*B1) 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTGGAATCCAATTGTTCCA CAC-3'; *NIMIN-1* reverse (*att*B2) 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTAG GATACATTTAGAGAAAGTGATTGAT-3'.

Amplification was done using Hot Start Phusion DNA polymerase (New England Biolabs). The PCR product obtained (812 bp) was cloned into pDONR207 by the BP reaction (recombination and insertion of a PCR product flanked by attB sites into a Gateway entry clone catalyzed by the enzyme BP clonase). The product of the BP reaction was transformed into *Escherichia coli* TOP10 cells (Invitrogen). Inserts were validated by sequencing with pDONR207 forward 5'-TCGCGTTAACGCTAGCATGGATCTC-3' and reverse 5'- GTAACATCAGAGA TTTTGAGACAC-3' primers. The mutagenized promoter was obtained from the cloned native promoter by site-directed mutagenesis using the Stratagene QuickChange Site Directed Mutagenesis kit. pDONR207 containing *NIMIN-1* promoter was then transferred by the LR reaction (recombination and insertion of an entry clone flanked by attL sites into a Gateway destination vector catalyzed by the enzyme LR clonase) to pYXT1 (β-glucuronidase, GUS) and pYXT2 (green fluorescente protein, GFP) binary reporter constructs. Both BP and LR reactions were stopped with proteinase K treatment. The resulting constructs contain the *NIMIN-1* promoter, 5'UTR and the first two amino acids of the native gene as an in-frame fusion to the each reporter construct connected via the Gateway *att*B-encoded peptide. pWTNIM::GFP and pWTNIM::GUS contain the wild-type NIMIN-1 promoter fused to the coding regions from GFP and GUS, respectively, while pMUTNIM::GUS contains the mutagenized NIMIN-1 promoter fused to the GUS gene.

Plasmid transfer to *Agrobacterium* by triparental mating

The recombinant binary plasmids pYXT1 and pYXT2 (Xiao et al., 2005) used in this study containing the different promoters were transformed into *E. coli* TOP10 cells by chemical transformation after the Gateway LR reaction. Recombinant plasmids were then transferred to *Agrobacterium tumefaciens* strain GV3101 by triparental mating. *Agrobacterium* strain GV3101 was grown overnight in liquid LB medium containing 50 mg/mL rifampicin and 50 mg/mL gentamicin at 28°C on a shaker at 250 rpm. *E. coli* cultures containing the plasmid of interest and *E. coli* helper strain (pRK 2013) were also grown overnight in

separate flasks with LB medium containing 50 mg/mL kanamycin at 37°C at 250 rpm. On the next day, 50 µL of each of the three cultures were plated together on an LB agar plate for the mating and incubated for 2 days at 28°C to produce colonies. Further confirmation of agro-transformations was done by PCR analysis with gene-specific primers.

Stable transformation of *Arabidopsis*

Arabidopsis thaliana transformation with the pWTNIM::GFP construct was done according to the floral dip method (Clough and Bent, 1998). *A. tumefaciens* carrying the promoter reporter construct was grown in LB medium containing 50 µg/mL each of rifampicin, gentamicin and kanamycin at 28°C for 2 days. The culture was transferred to a 50-mL plastic tube and centrifuged at 6000 rpm, 4°C for 5 min. We resuspended the pellet in 50 mL *Agrobacterium* suspension (5% sucrose solution with surfactant Silwet L-77 at a final concentration of 0.05%). We dipped the inflorescences into *Agrobacterium* suspension for 10 s and covered the plants with a transparent film. Seeds were harvested and transformants were screened on Murashige and Skoog medium containing 50 mg/mL kanamycin and then transferred to soil.

Transient assay by agroinfiltration

We agroinfiltrated *Arabidopsis* leaves (SA-treated *vs* -non-treated) with a needleless syringe containing *Agrobacterium* suspension carrying constructs pMUTNIM::GUS (mutagenized promoter) and pWTNIM::GUS (native promoter) according to the method described by Wroblewski et al. (2005), with modifications. After infiltration with the promoter reporter constructs, the plants were placed back in the growth chamber for a minimum of 2 days to allow expression of the reporter constructs after which leaves were stained for visual inspection. Cultures of *Agrobacterium* were grown overnight in YEP liquid medium with antibiotic selection at 29°C on a shaker. The culture was adjusted to OD_{600} = 0.3-0.6 prior to infiltration. We used only soil-grown *Arabidopsis* plants in the vegetative stage for agroinfiltration experiment since plants develop larger leaves than those from early flowering plants. Leaves were taken on the third day post-infiltration and stored at -80°C until gene expression analysis by quantitative real-time RT-PCR (qRT-PCR).

RNA extraction and cDNA synthesis

Total RNA was isolated from 50 mg *Arabidopsis* leaves using RNeasy Plant Mini Kit (Qiagen) with additional RNase-free DNase (Qiagen) treatment of RNA samples for qRT-PCR. RNA concentration was measured using a NanoDrop ND-1000 spectrophotometer. The purity of RNA (A260/A280) was above 1.9. RNA integrity was checked on a 1% (w/v) agarose gel after RNA extractions. Each cDNA sample was synthesized from 2 µg RNA using SuperScript™ III First Strand Synthesis for RT-PCR (Invitrogen) according to the manufacturer protocol.

Quantitative real-time RT-PCR

Sequences of specific primers for *GUS*, *NPTII*, *NIMIN-1*, and endogenous gene invariant controls (*AT4G26410*, *AT5G15710*, *AT4G27960*, *AT5G46630*) used for qRT-PCR are listed in Table 1. All primer sequences used in this study were blasted against TAIR's *A. thaliana* cDNA database to

Table 1. Primers used for quantitative real-time RT-PCR.

Gene	Forward	Reverse
GUS	TAATGTTCTGCGACGCTCAC	TTCTCTGCCGTTTCCAAAT
NPTII	CGTTGGCTACCCGTGATATT	CTCGTCAAGAAGGCGATAGAA
NIMIN-1	ATCTAACGGCGGAGAAAGGT	TGTGATCCGAAACAAACATCA
AT4G26410	CCTGGAAGGGATGCTATCAA	GTCCGACATACCCATGATCC
AT5G15710	GCACTTCTGAGACTTTCGGC	ATGACTGAAGAGCACAACCG
AT4G27960	TAACCATCCATTTCCCTCCA	TGGAAATTGTGAGAGCAGGA
AT5G46630	GTGCCAATGTTCACAGCATC	TGATCTCGTAAGATCCCGCT

All primers are shown in the 5'-3' direction.

check for amplicon specificity and avoid nonspecific amplification. Short amplicons were used (100-150 bp) for increased assay performance (Marino et al., 2003).

qRT-PCR were performed in an ABI PRISM® 7900HT instrument (Applied Biosystems), using SYBR® Green to monitor dsDNA synthesis according to the following parameters: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C, and 1 min at 60°C. Melting curves were generated to confirm the presence of a single PCR product specific peak and for detection of primer-dimer formation using incremental temperatures 95°C for 15 s plus 60°C for 30 s. No template control samples were included in each run as a control for possible contaminations. Final reaction volume was 10 µL. A master mix containing 85 µL 2X SYBR® Green (Applied Biosystems) and 45 µL of previously diluted cDNA was prepared prior to dispensing in individual wells to reduce pipetting errors. An electronic adjustable automatic multipipette was used to pipette the master mix (7.5 µL per well) and 2.4 µL of primer mix was dispensed per well with an Eppendorf 12-channel pipette. Data were analyzed using the SDS 2.0 software (Applied Biosystems). Relative quantitation values were calculated using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001; Czechowski et al., 2004) by measuring the difference in C_T values of target genes treated with SA (T_2) and non-treated (T_1), normalized to C_T values of housekeeping genes (internal control), also treated with SA (C_2) and non-treated (C_1): $\Delta\Delta C_T = (T_{1(sa-)} - T_{2(sa+)}) - (C_{1(sa-)} - C_{2(sa+)})$. This reports the logarithm to base 2 of the relative expression values.

RESULTS AND DISCUSSION

In order to functionally validate the TGA2-binding site TGACG located 360 bp upstream of the start site of the *NIMIN-1* coding region, 2 bp in the motif were altered by site-directed mutagenesis resulting in a "mutagenized" version of the *NIMIN-1* promoter. This mutagenized promoter version was sequenced and aligned to native promoter region using ClustalW (Figure 1).

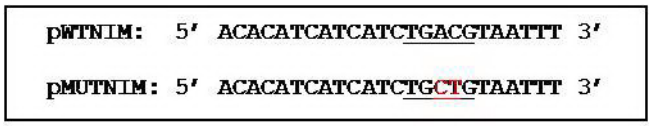

Figure 1. The wild-type *NIMIN-1* promoter region with the TGA2-binding motif (top, pWTNIM) and mutagenized motif (bottom, pMUTNIM). A 2-bp alteration (in red) generated by site-directed mutagenesis of TGA2-binding motif (underlined) located -360 bp upstream of the translational start site of the *NIMIN-1* gene.

Native and mutagenized *NIMIN-1* promoter regions encompassing position +7 in the CDS until -805 bp upstream of the *NIMIN-1* start site were isolated using PCR from pre-existing Gateway entry clones containing native and mutagenized promoters. Using the Gateway system, the amplified promoter regions for NIMIN-1 were cloned into the entry vector pDONR207 (BP reaction) and then into the reporter binary vectors pYXT1 (GUS) and pYXT2 (GFP) by the Gateway LR reaction. We transformed all BP and LR reactions into *E. coli* and validated cloning after BP reactions by sequencing the insert with pDONR207 flanking primers before proceeding to LR reaction. LR reactions were checked by colony PCR screening of transformed *E. coli* colonies using promoter-specific primers. In total, three constructs were generated for analysis of the *NIMIN-1* promoter: the two native versions of the promoter region being pWTNIM::GFP and pWTNIM::GUS, and one mutagenized: pMUTNIM::GUS.

We generated more than 50 transgenic *Arabidopsis* plants expressing the pWTNIM::GFP promoter reporter construct, and T1 plants were observed under the microscope for the pattern of reporter gene expression (Figure 2). The independent segregating lines expressing pWTNIM::GFP showed GFP expression in the shoot apex, roots, midvein, secondary veins, hydathodes, and at the petiole. This expression pattern (e.g., GFP expression in roots and leaves of young *Arabidopsis* rosette) is in agreement with the data from the *Arabidopsis* eFP Browser tool (Winter et al., 2007), which depicts the endogenous *NIMIN-1* expression data from Affymetrix ATH1 GeneChip (data not shown).

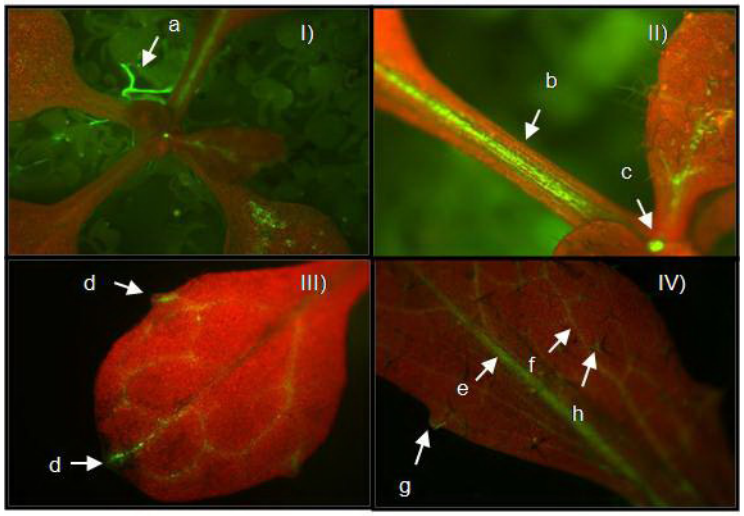

Figure 2. Green fluorescent protein (GFP) expression patterns in T1 transgenic *Arabidopsis* lines containing the pWTNIM::GFP construct. I) GFP expression in roots (a) from a 3-week-old rosette; II) GFP expression at petiole (b) and shoot apex (c); III) GFP expression at primary and secondary veins and hydathodes (d) from a 3-week-old rosette leaf; IV) GFP expression in midvein (e), secondary vein (f), hydathodes (g), and trichomes (h) of a 6-week-old plant.

To determine if the TGA2-binding motif located in the promoter region of *NIMIN-1* is involved in the transcriptional regulation in response to SA of *NIMIN-1* expression, we performed comparative functional analysis of the native and mutagenized version of *NIMIN-1* promoter re-

gions. We compared levels of gene expression by qRT-PCR from native and mutant *NIMIN-1* promoters in relation to endogenous copy of *NIMIN-1* using promoter regions fused to the GUS reporter gene. qRT-PCR was the method of choice for measuring SA-induced *NIMIN-1* expression because we aimed to better estimate the response at the transcriptional level in a short period of time after SA exposure (2 h). Reporter gene expression was measured by qRT-PCR from agroinfiltrated leaves on the third day post-infiltration, to allow expression of the reporter gene (Figure 3).

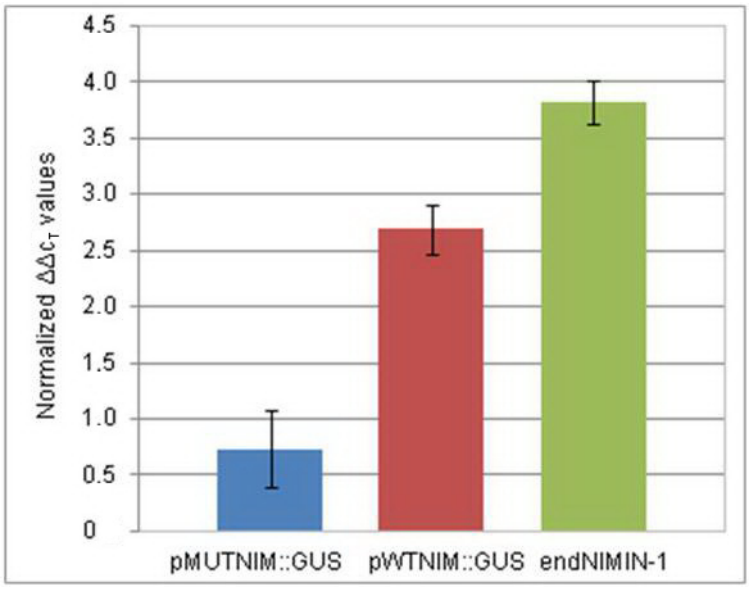

Figure 3. Effect of mutation in the TGA2-binding site in the *NIMIN-1* promoter. Bars represent the change in expression of the wild-type (pWTNIM::GUS) and mutated (pMUTNIM::GUS) promoters and in the endogenous *NIMIN-1* gene (endNIMIN-*1*) relative to the internal controls *NPTII* (for promoter reporter constructs) and *At5g15710* (endogenous *NIMIN-1*) in SA-treated *vs* -untreated samples. Data are reported as means ± SD of three independent biological replicates. GUS = β-glucuronidase.

We measured transcript levels from leaves sprayed with 1 mM SA at 2 h (SA+) and non-treated leaves at 0 h (SA-). C_T values for endogenous *NIMIN-1* were normalized to the C_T values of the internal control gene *At4g26410*, which was one of the most constant of the four housekeeping genes included in each qRT-PCR run. The average C_T value for *At4g26410* was 20.44 ± 0.32 at a threshold of 0.1 for all three biological replicates. C_T values for agroinfiltrated promoter reporter constructs pWTNIM::GUS and pMUTNIM::GUS were normalized to the internal control *NPTII*. The average C_T value for NPTII was 26.46 ± 0.34 for all three biological replicas. The changes in expression levels in response do SA was calculated using the the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001; Czechowski et al., 2004), as described in Methods.

We found that the pWTNIM::GUS construct containing the native promoter and the endogenous copy of *NIMIN-1* were highly responsive to SA (Figure 3), in agreement with previous study (Weigel et al., 2001; Glocova et al., 2005). qRT-PCR analysis also showed that

the SA-mediated induction of the native promoter was more than 3-fold higher compared to that observed in the mutagenized promoter.

This difference in reporter gene expression between mutant and native promoters indicates that TGA2 binding to the TGACG *cis*-element is relevant to the *NIMIN-1* response to SA. It is worth noting that the weak induction of the mutant promoter could suggest that the mutation in the 2 bp does not completely abolish the binding of the TGA2 factor or that the other TGACG motif present in the promoter region also plays a minor role in mediating SA induction of *NIMIN-1*.

In summary, our data on the mutagenized and wild-type *NIMIN-1* promoter indicate that the TGACG *cis*-element is involved in regulation of SA-induced expression.

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