The Rose (Rosa hybrida) NAC Transcription Factor 3 Gene, RhNAC3, Involved in ABA Signaling Pathway Both in Rose and Arabidopsis

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Abstract

Plant transcription factors involved in stress responses are generally classified by their involvement in either the abscisic acid (ABA)-dependent or the ABA-independent regulatory pathways. A stress-associated NAC gene from rose (Rosa hybrida), RhNAC3, was previously found to increase dehydration tolerance in both rose and Arabidopsis. However, the regulatory mechanism involved in RhNAC3 action is still not fully understood. In this study, we isolated and analyzed the upstream regulatory sequence of RhNAC3 and found many stress-related cis-elements to be present in the promoter, with five ABA-responsive element (ABRE) motifs being of particular interest. Characterization of Arabidopsis thaliana plants transformed with the putative RhNAC3 promoter sequence fused to the β-glucuronidase (GUS) reporter gene revealed that RhNAC3 is expressed at high basal levels in leaf guard cells and in vascular tissues. Moreover, the ABRE motifs in the RhNAC3 promoter were observed to have a cumulative effect on the transcriptional activity of this gene both in the presence and absence of exogenous ABA. Overexpression of RhNAC3 in A. thaliana resulted in ABA hypersensitivity during seed germination and promoted leaf closure after ABA or drought treatments. Additionally, the expression of 11 ABA-responsive genes was induced to a greater degree by dehydration in the transgenic plants overexpressing RhNAC3 than control lines transformed with the vector alone. Further analysis revealed that all these genes contain NAC binding cis-elements in their promoter regions, and RhNAC3 was found to partially bind to these putative NAC recognition sites. We further found that of 219 A. thaliana genes previously shown by microarray analysis to be regulated by heterologous overexpression RhNAC3, 85 are responsive to ABA. In rose, the expression of genes downstream of the ABA-signaling pathways was also repressed in RhNAC3-silenced petals. Taken together, we propose that the rose RhNAC3 protein could mediate ABA signaling both in rose and in A. thaliana.

Introduction

Drought, or dehydration, is one of the major limiting factors for plant growth, development, and productivity and plants have evolved a range of physiological, biochemical and molecular responses to promote drought stress tolerance [1]. One such response to drought stress is the production of the plant hormone abscisic acid (ABA), which mediates numerous downstream responses, including stomatal closure, thereby restricting water loss. Using genomic and transcriptomic analyses, the products of stress-associated NAC genes have identified many stress-related cis-elements. These include the G-box (CACGTG, a MYC recognition site), the dehydration-responsive element/C-repeat (DRE/CRT) and the ABA-responsive element [10,11]. Among these, the conserved ABA-responsive cis-element, PyACGTG/TGC, also named ABA-responsive element (ABRE), is a signature sequence for genes involved in the ABA signaling pathway [12,13], and is important for promoter activity under osmotic stress conditions, such as those resulting from dehydration and high salinity [12,14]. The functions of ABA-dependent TFs have also been investigated through overexpression in many plant species [11], which has...
been found to result in hypersensitivity to ABA during seed germination [5,15], constitutive stomatal closure [16] and severely inhibited root growth [17]. Microarray analysis further revealed that constitutive overexpression of ABA-related TF genes, such as ABO3 and MYB96, generally increases the expression of downstream ABA-responsive genes, and results in drought tolerance [18,19].

Another class of ABA-related TFs are NAC (NAM, ATAF1 and 2, and CUC2) proteins, plant-specific transcriptional regulators that contain conserved N-terminal NAC domains and divergent C-terminal regions [20]. NAC TFs play important roles in regulating numerous aspects of growth and development, including cell division, and senescence, as well as responses to environmental stress stimuli [21]. Many are involved in ABA mediated signaling during their response to abiotic stresses, such as A. thaliana ANAC019 and ANAC055, soybean GmNAC011 and GmNAC020 and rice OsNAC5 [22,23,24], and their overexpression can result in enhanced ABA sensitivity at both the germination and post-germination developmental stages [25].

Different members of the NAC family have also been shown to be responsive to dehydration in rose petals [7]. Of these, RhNAC2 has been found to promote petal cell expansion, in association with the regulation of cell wall-related genes [7], while RhNAC3 regulates osmotic stress-related genes when exposed to drought stress [26]. However, nothing has been reported to date regarding the mechanism by which RhNAC3 participates in the ABA regulatory pathway. In this study, we characterized the upstream regulatory sequence of RhNAC3 and found that ABREs in the RhNAC3 promoter are needed for gene activity in both the presence and absence of exogenous ABA. Transgenic A. thaliana overexpressing RhNAC3 showed enhanced ABA sensitivity during seed germination and during stomatal closure, and ABA-responsive genes were also up-regulated under dehydration conditions in both rose and A. thaliana overexpressing RhNAC lines. These data indicate that ectopically expressed RhNAC3 enhances ABA sensitivity in A. thaliana and is involved in an ABA-dependent signaling pathway, at least some components of which are likely conserved between rose and A. thaliana.

Materials and Methods

Regulatory cis-element analysis

The upstream regulatory sequence of RhNAC3 was isolated using PCR-based genome walking method [27]. (We state clearly that no specific permissions were required for these locations/activities and confirm that the field studies did not involve endangered or protected species). And the primers used are listed in Table S1. All amplified fragments were sub-cloned into the pGEM T-Easy Vector (Promega, Madison, WI, USA) and transformed into Escherichia coli DH5α cells after sequencing. The position of the translation start site was designated “0”. The cis-acting elements were analyzed and annotated using two software programs from the Plant Cis-acting Regulatory DNA Elements (PLACE) [28] (http://www.dna.afrc.go.jp/PLACE/) and Plant Cis-acting Regulatory Elements (PlantCARE) [29] (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) software programs. For NAC-binding site analysis of RhNAC3 upregulated genes in A. thaliana, a 1,000 bp regulatory sequence upstream of the genes was searched and analyzed by TAIR Loci Upstream Seq – 1,000 bp of Sequence Bulk Download and Analysis’ at www.arabidopsis.org.

Construction of plant expression vectors and Arabidopsis transformation

The 977 bp upstream regulatory sequence of RhNAC3 was amplified with 5’ ACCAAGCTTCTCCATCTGACTGCTCCATT-CTGAACCTG 3’ and 5’ GCTCTAGACGCTATCAGAGACATTCTACTTGTCCAAAT-CTGAACCTG 3’ and 5’ CCTCTAGACGCTATCAGAGACATTCTACTTGTCCAAAT-CTGAACCTG 3’ and 5’ GCTCTAGACGCTATCAGAGACATTCTACTTGTCCAAAT-CTGAACCTG 3’ (Table S1) and the product digested with Hind III and XbaI, and inserted into the pBI121 binary vector. The resulting ProRhNAC3-GUS plasmid was introduced into the Agrobacterium tumefaciens strain GV3101 and transformed into A. thaliana (Columbia) by the floral dip method [30]. Ten independent lines of kanamycin-resistant transgenic plants were obtained. The homozygous T3 generation seeds of the transgenic lines were used for subsequent experiments.

Histochemical staining and quantitative GUS activity assay

Histochemical staining for GUS activity was performed as described by Li et al. (2009) [31]. Plant samples exposed to different treatments were immersed in GUS staining buffer (0.5 mM 5-bromo-4-chloro-3-indoly-β-D-GlC, 0.5 M NaH2PO4, pH 7.0, 1 mM EDTA, 0.5 mM potassium ferricyanide and 0.5 mM potassium ferrocyanide). After staining at 37°C for 3–10 h, the samples were immersed in 95% (v/v) ethanol at 37°C to remove chlorophyll. For histochemical analysis of the ProRhNAC3:GUS transgenic A. thaliana plants in response to ABA treatment, 9-day-old seedlings were grown on MS medium supplemented with 100 μM ABA for 4 days, before being sampled for histochemical GUS staining. The GUS staining patterns were examined under a microscope (BX51; Olympus) and analyzed using Photoshop CS6 software (Adobe, McLean, VA). Quantitative assays of GUS activity were performed as described by Jefferson et al. (1987) [32]. All experiments were performed three times to give three independent biological replicates.

Figure 1. Schematic representation of the RhNAC3 promoter. The major stress-related cis-acting elements in the 1447 bp promoter of RhNAC3 are shown. The position and putative sequences of ABRE elements are listed. doi:10.1371/journal.pone.0109415.g001
Construction of the truncated *RhNAC3* promoter-GUS fusion and transient expression assays

Three truncated *RhNAC3* promoter fragments, N0 (−1447 to −160 bp), N1 (−707 to −160 bp) and N2 (−377 to −160 bp) were amplified from rose genomic DNA and cloned into a modified pUC19 plasmid containing the GUS reporter gene, as described by Dai et al. [2012] [7]. To mutate the ABRE cis-element, we replaced the ACGT of the ABRE core sequence with TTTA using overlap PCR methods [33]. Mutation fragments of N0 (mN0, five ABREs mutated) and N1 (mN1, three ABREs mutated) were amplified, and cloned into the modified pUC19 as described for N0 and N1. *A. thaliana* mesophyll protoplasts were transformed with the resulting vectors: N0, mN0, N1, mN1 and N2, and an empty (normal) vector control (NC). For the ABA treatment experiments, *Arabidopsis* mesophyll protoplasts harboring the different constructs (N0, N1, N2 and NC) were exposed to 10 μM ABA (Sigma, St. Louis, MO). GUS activity was measured in protoplast extracts after 24 h of incubation with ABA. Isolation of *A. thaliana* mesophyll protoplasts, transformation of protoplasts and GUS activity assays were carried out as previously described [32,34]. The primers are listed in Table S1, and the experiments were performed in triplicate.

Seed germination assay and root growth measurements

The *RhNAC3*-overexpressing plants (overexpressor OE###3, OE###6 and OE###12) had previously been generated [26], and wild type (WT) and vector (VC) plants were used as controls. Approximately 50 seeds were plated onto solid MS medium supplemented with either 0, 0.2, 0.4 or 0.8 μM ABA. After vernalization at 4°C for 3 days, the seeds were moved to a temperature controlled room at 23±1°C under long-day conditions (16 h light/8 h dark cycle), with a light intensity of 80–100 μmol/m²/s and 40–60% relative humidity. The rates of radicle emergence and cotyledon greening were measured after 7 days. All experiments were performed in triplicate. To measure seedling root growth, 5-day old seedlings of OE###3, OE###6 and OE###12 were transferred to plates of MS medium containing 5, 10, or 30 μM ABA respectively, and grown vertically. After growth for 10 d, primary root length and lateral root number was measured and analyzed using the Image J software (http://rsbweb.nih.gov/ij/). The WT and VC plants were used as controls.

Stomatal aperture measurements

For ABA-induced stomatal closure, mature leaves from light-grown 3-week-old control and *RhNAC3* transgenic plants were detached and incubated in stomatal opening solution (10 mM KCl, 100 μM CaCl₂ and 10 mM MES, pH 6.1) for 2 h at 22°C [16], before being transferred to fresh stomatal opening solution containing 0 μM or 10 μM ABA. Stomata on abaxial surfaces were photographed through a light microscope (BX51; Olympus), and the stomatal aperture (the ratio of width to length) was measured (n = 20). For drought-induced stomatal closure, 3-week-old seedlings of WT, VC and *RhNAC3*-overexpressing lines were grown for 10 d without water. Plants grown under normal conditions were used as control. Leaves in the same position on the plant were sampled, and the stomata on the leaf abaxial surfaces were immediately photographed. Stomatal aperture was measured (n = 20) and all experiments were repeated three times.

Quantitative reverse transcription PCR analysis

The detached leaves of 3-week-old vector (VC) and *RhNAC3* overexpressor (OE###3, 6 and 12) *A. thaliana* plants were dehydrated for 3 h at 23–25°C, 40–50% relative humidity, and 100 μmol m⁻²s⁻¹ light intensity, then sampled for quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis. Total RNAs were isolated from the leaf samples using the Trizol agent (Invitrogen, Carlsbad, CA). DNase-treated RNA (1 μg) was used for first-strand cDNA synthesis (Invitrogen, Carlsbad, CA) and the cDNA (2 μL) was used as the template in a 20 μL qRT-PCR using a qPCR Kit (Kapa Biosystems, Woburn, MA). The *A. thaliana Actin2* gene (GenBank accession no. NM_112764) was used as an internal control. The 11 selected genes and gene specific primers used for the qRT-PCR analysis are listed in Table S1. Each qRT-PCR evaluation was performed with three biological replicates.

For qRT-PCR analysis of downstream genes of *RhNAC3* action in rose, nine putative ABA signaling and downstream rose genes from the ABA-signaling pathways were selected from our rose transcriptome databases [7] (Table S2). The rose cDNAs from Tobacco Rattle Virus (TRV) and *RhNAC3*-silenced petals were obtained in our previous study [26]. *RhUbil* (accession no. JK622648) was used as the internal control. The gene specific primers for qRT-PCR are listed in Table S1. Each qRT-PCR analysis was performed with three biological replicates.
Electrophoretic mobility-shift assay

The electrophoretic mobility-shift assay (EMSA) was performed according as previously described [26] with minor modifications. To construct the GST-RhNAC3 fusion protein, the N-terminal of RhNAC3 (RhNAC3N1–162) was amplified by PCR (primers are listed in Table S1) and the PCR product was ligated into the pGEX-2T vector (Pharmacia LKB Biotechnology, Piscataway, NJ) via the BamHI and SacI sites and the recombinant vector was expressed in Escherichia coli BL21 cells. The fusion protein was induced by 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and the cells E. coli incubated at 28°C for a further 6 h. The recombinant protein was purified by GST-agarose affinity chromatography (GE Healthcare, http://www.gehealthcare.com/). Biotin-labeled DNA fragments used in the EMSA contain one or two putative NAC binding sequences [21]. The probes were incubated with the fusion protein at room temperature for 25 min in binding buffer (10 mM concentration: 100 mM Tris, 500 mM KCl, 10 mM dithiothreitol; pH 7.5). Each 20 μL binding reaction contained 0.2 pmol biotin probe and 2 μg fusion protein, and 1 μg Poly (dI-dC) was added to the reaction to minimize nonspecific interactions. The reaction products were analyzed using 5% native polyacrylamide gel electrophoresis and 0.5 Tris-borate/EDTA buffer. After electrophoresis, the DNA fragments on the gel were transferred to a nitrocellulose membrane using 0.5 Tris-borate/EDTA at 380 mA (100 V) for 30 min at 4°C. After UV cross-linking, the membrane was

Figure 3. Deletion analysis and ABA dose dependent response of RhNAC3 promoter activity. A, Assays of GUS activity in A. thaliana protoplasts containing RhNAC3 promoter deletion and ABRE mutation constructs. The numbers on top represent the positions of the ABRE cis-elements and mutations of ABREs in the RhNAC3 promoter region. Relative GUS activity in transient expression experiments using five different constructs (N0, mN0, N1, mN1 and N2) and the vector control (NC) is shown at the bottom. GUS activity was determined after 24 h of incubation. Error bars represent standard error (n = 3). **: P < 0.01, *: P < 0.05, t test. B, Effects of exogenous ABA on GUS activity in A. thaliana protoplasts containing RhNAC3 promoter deletion constructs. The truncated RhNAC3 promoter constructs (N0, N1 and N2) and vector control (NC) were transformed into A. thaliana protoplasts, which were then exposed to 0 and 10 μM exogenous ABA. GUS activity in protoplast extracts was measured after 24 h of incubation with ABA. Error bars represent standard error (n = 5). C, Histochemical analysis of RhNAC3 promoter::GUS expression in response to ABA. 9-day-old transgenic seedlings were grown on MS medium only or MS medium plus ABA (transferred to MS medium plus 100 μM ABA for 4 days) before being subjected to histochemical GUS staining. Scale bar = 1 mm.
Figure 4. Effect of ABA concentration on seed germination and root growth in WT and *RhNAC3* overexpressing A. *thaliana* plants. 

A, Seed germination phenotypes. The homozygous T3 seeds of *RhNAC3*-overexpressing lines (OE#3, OE#6 and OE#12), wild type (WT) and vector plants were plated on MS supplemented with 0, 0.2 or 0.4 μM ABA. Images were obtained 7 days after planting. 

B, Seed germination rates. The germination rates were measured 7 days after planting. Error bars represent standard error (n = 3). **: P<0.01, *: P<0.05, t test. 

C, Root growth phenotypes. Five-day-old seedlings of WT, vector only and three *RhNAC3*-overexpressing A. *thaliana* lines (OE#3, #6 and #12) were transferred to MS plates supplemented with 0, 5, 10 and 30 μM ABA. Root phenotypes were visualized 10 days after planting. 

D, Primary root length analysis. **: P<0.01, *: P<0.05, t test. 

E, Lateral root number analysis. Both primary root length and lateral root number were measured after 10 days of growth. Three independent experiments were performed using 15 plants in each experiment in D and E. Error bars represent standard error (n = 3). 

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transferred to conjugate/blocking buffer by mixing 16.75 μL stabilized streptavidin-horseradish peroxidase conjugate with 5 mL blocking buffer. After washing, biotin-labeled DNA was detected by chemiluminescence according to the manufacturer’s protocol (Pierce, http://www.piercenet.com/).

Results

Structure and sequence analysis of the RhNAC3 promoter

To elucidate the regulation of RhNAC3 transcription, a 1,447 bp fragment corresponding to the sequence immediately upstream of its translational start site (TSS) (GenBank accession number: KJ000025) was isolated by PCR-based genome walking. Subsequent sequence analysis of this region revealed a number of putative cis-elements, including elements associated with ABA, cold, pathogen and wounding responses (Figure 1, Figure S1 and Table S2). The TATA box (TTATTT) was found 2104 bp upstream of the TSS and a CAAT-box sequence (CAAT) was found 2 bp downstream of the TATA-box sequence. Five ABRE-related sequence motifs (ACGTG), located at positions 21140 to 21149, were identified (Figure S2).

Table 1. Analysis of putative NAC binding cis-elements in the promoter regions of genes downstream from RhNAC3 action.

| Gene name | NAC protein binding sites |
|-----------|---------------------------|
|           | CACG | CGTG | GTGC | CATGTG |
| RD29A     | 4    | 1    | 1    | 1      |
| RD29B     | 2    | 4    | 2    |        |
| RD20      | 7    | 4    | 2    | 2      |
| RD26      | 5    | 6    | 3    |        |
| COR15A    | 1    | 1    | 1    |        |
| COR15A    | 4    | 1    |      |
| KIN2      | 6    | 3    |      |
| ABI1      | 2    |      |
| ABI3      | 1    | 2    | 1    |
| ABI4      | 3    | 1    |      |
| ABA3      | 4    | 4    |      |

Table 1 Data Source: "Rose RhNAC3 Involved in ABA Signaling Pathway" 6 October 2014 | Volume 9 | Issue 10 | e109415

Figure 5. Stomatal aperture of the RhNAC3 overexpressing A. thaliana plants in response to ABA and drought treatments. A, Stomatal aperture in response to ABA. Mature leaves from three-week old wild type (WT), vector control and independent RhNAC3-overexpressing plants (OE#3, OE#6 and OE#12) were treated with a stomatal opening solution for 2 h (0 μM) and incubated with 10 μM ABA for 2 h (10 μM). Stomata on the abaxial surfaces were imaged by light microscopy. Stomatal aperture (the ratio of width to length) was quantified using at least 20 guard cells from each sample. Bar 10 μm. B, Stomatal aperture of RhNAC3 overexpressing lines in response to drought stress. Three-week old seedlings of WT, vector control and independent RhNAC3-overexpressing plants (OE#3, OE#6 and OE#12) were subjected to 10 days without water. Plants grown under normal well watered conditions were used as a control. The leaves were harvested and the stomata on the leaf abaxial surfaces were immediately photographed. Stomatal apertures were then quantified (n = 20). Bar 10 μm. **: P<0.01, *: P<0.05, t test.

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were identified, which we hypothesized might be involved in an ABA response. Other potential regulatory elements that were found include two CBF sequences (RYCGAC) at positions 29493 to 29488 and 29404 to 29399, and a Myb-type TF recognition sequence (GGATA) at position 2291 to 2287. There are also two Myc-type TF recognition sequences (CACATG) at positions 9985 to 9980 and 9951 to 9946, and two W-box sequences (TGACT) at positions 12930 to 12926 and 12924 to 12927, relative to the TSS. Collectively, the presence of these cis-elements suggests that RhNAC3 may play a role in responses to a variety of stresses, such as cold, pathogen challenge or wounding, particularly via the ABA-dependent pathway.

Figure 6: RhNAC3 binding to the regulatory sequences of ABA-related A. thaliana genes. A, ABA-related gene expression in RhNAC3 overexpressing A. thaliana lines. The aerial parts of light-grown, 3-week old vector control and three independent RhNAC3 overexpressing A. thaliana lines (OE#3, OE#6 and OE#12) were dehydrated for 3 h and sampled (23–25 °C, 40–50% relative humidity). The expression patterns of 11 ABA-responsive genes were analyzed by qPCR and the data represents the fold induction of each gene by dehydration relative to the control treatment. Mean values from three independent biological replicates were normalized to the levels of the internal control gene Actin2. B, Sequences and positions of putative RhNAC3 binding elements used in an electrophoretic mobility shift assay (EMSA). Probes were derived from the regulatory sequences of 4 selected ABA-responsive A. thaliana genes. Underlined letters indicate the core sequences of NAC protein targeted promoters. The sense strands of the oligonucleotide probes corresponding to the predicted RhNAC3 binding sites are shown. C, Interaction between GST-RhNAC3N1–162 and biotin-labeled probes indicated in (B). D, DNA-binding specificity for RhNAC3 with interacting probes. The arrows indicate the positions of protein/DNA complexes and the free probes. Purified protein (2 μg) was incubated with 0.2 pmol of the biotin probe. GST incubated with the P1 probe was used as a control, and a 10 or 100 fold excess of the unlabeled P1, P2, or P3 probes was used for competitive binding. doi:10.1371/journal.pone.0109415.g006

Activity of the RhNAC3 promoter in ProRhNAC3::GUS transgenic A. thaliana lines

We next examined the spatial expression pattern of the RhNAC3 gene in 10 independent A. thaliana lines (ProRhNAC3::GUS) that had been transformed with a construct containing the RhNAC3 promoter fused to the GUS reporter gene. The T3 generation homozygotes of six lines were selected to analyze by GUS staining. Histochemical GUS staining revealed that ProRhNAC3::GUS was expressed almost throughout the entire plant.
### Table 2. Up-regulated genes involved in the ABA response in *A. thaliana* lines overexpressing RhNAC3.

| Affy ID | Description | AGI Code | Fold change | P-value |
|---------|-------------|----------|-------------|---------|
| **Signal transduction (21)** | | | | |
| 267069_at | Calmodulin (CAM)-binding protein of 25 kDa | At2g41010 | 6.04 | 0.025 |
| 258947_at | Calcium-binding EF-hand family protein | At3g01830 | 2.58 | 0.045 |
| 255844_at | Protein kinase family protein / peptidoglycan-binding LysM domain-containing protein | At2g33580 | 2.48 | 0 |
| 255503_at | Concanaulin A-like lectin protein kinase family protein | At4g02420 | 2.39 | 0.045 |
| 251054_at | Lectin receptor kinase a4.3 | At5g01540 | 2.39 | 0.02 |
| 261662_at | MAP kinase kinase 7 | At1g18350 | 2.19 | 0.019 |
| 266371_at | Calcium-binding EF-hand family protein | At3g1410 | 2.14 | 0.006 |
| 257751_at | MAP kinase substrate 1 | At3g18690 | 2.09 | 0.011 |
| 266037_at | Protein kinase superfamily protein | At2g05940 | 2 | 0.001 |
| **Transcriptional regulation (32)** | | | | |
| 261648_at | Salt tolerance zinc finger | At1g27730 | 10.1 | 0.011 |
| 257022_at | Zinc-finger protein 2 | At3g19590 | 7.56 | 0.004 |
| 248448_at | Integrase-type DNA-binding superfamily protein | At4g51190 | 4.41 | 0.04 |
| 257053_at | Ethylene responsive element binding factor 4 | At3g15210 | 3.69 | 0.022 |
| 266719_at | Circadian clock associated 1 | At2g6830 | 3.44 | 0.049 |
| 246932_at | Integrase-type DNA-binding superfamily protein | At5g25190 | 3.04 | 0.044 |
| 252659_at | Integrase-type DNA-binding superfamily protein | At4g9780 | 2.76 | 0.032 |
| 266656_at | Zinc finger C-x8-C-x5-C-x3-H type family protein | At2g25900 | 2.41 | 0.018 |
| 258436_at | RING/U-box superfamily protein | At3g16720 | 2.4 | 0.001 |
| 245051_at | WRKY DNA-binding protein 15 | At2g23320 | 2.34 | 0.003 |
| 259626_at | Basic region/leucine zipper motif 60 | At1g42990 | 2.26 | 0.007 |
| 256426_at | RING/FYVE/PHD zinc finger superfamily protein | At1g33420 | 2.05 | 0.002 |
| 252009_at | A20/AN1-like zinc finger family protein | At3g52800 | 2.01 | 0.047 |
| **Stress responsive (19)** | | | | |
| 247708_at | Zinc finger (C3HC4-type RING finger) family protein | At5g59550 | 4.75 | 0.034 |
| 257763_s_at | Receptor like protein 38 | At3g23110 | 3.9 | 0.001 |
| 262911_s_at | HSP20-like chaperones superfamily protein | At1g59860 | 2.45 | 0.03 |
| 262383_at | Toll-Interleukin-Resistance (TIR) domain-containing protein | At1g72940 | 2.34 | 0.043 |
| 259105_s_at | Rubber elongation factor protein (REF) | At3g05500 | 2.22 | 0.044 |
| 253046_at | Cytochrome P450, family 81, subfamily D, polypeptide 8 | At4g37370 | 2.2 | 0 |
| **Enzymes and metabolism (55)** | | | | |
| 254975_at | 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein | At4g10500 | 7.62 | 0.003 |
| 256933_at | Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein | At3g26000 | 4.07 | 0.005 |
| 266993_at | Major facilitator superfamily protein | At2g39210 | 3.58 | 0.005 |
| 263852_at | Nudix hydrolase homolog 6 | At2g04450 | 3.52 | 0.021 |
| 252908_at | Glycolipid transfer protein (GLTP) family protein | At4g36970 | 3.45 | 0 |
| 248330_at | NAD(P)-binding Rossmann-fold superfamily protein | At5g52810 | 3.33 | 0.003 |
| 248970_at | Solute:sodium symporter; urea transmembrane transporters | At5g45380 | 3.12 | 0.003 |
| 255630_at | C2 calcium/lipid-binding plant phosphoribosyltransferase family protein | At4g00700 | 2.94 | 0.048 |
| 253332_at | Peroxidase superfamily protein | At4g33420 | 2.86 | 0.028 |
| 249188_at | HXXXD-type acyl-transferase family protein | At5g42830 | 2.78 | 0.006 |
| 266761_at | NAD(P)-binding Rossmann-fold superfamily protein | At2g47130 | 2.73 | 0.001 |
| 252098_at | Eukaryotic aspartyl protease family protein | At3g51330 | 2.67 | 0.019 |
| 253806_at | RING membrane-anchor 2 | At4g28270 | 2.61 | 0.037 |
| 249910_at | Arogenate dehydratase 2 | At5g26300 | 2.58 | 0.013 |
| 245035_at | Acireductone dioxygenase 3 | At2g26400 | 2.5 | 0.018 |
| 251422_at | Preprotein translocase Sec, Sec61-beta subunit protein | At3g60540 | 2.48 | 0.032 |
| 267337_at | HXXXD-type acyl-transferase family protein | At2g39890 | 2.38 | 0.02 |
| Affy ID   | Description                                                                 | AGI Code   | Fold change | P-value  |
|----------|------------------------------------------------------------------------------|------------|-------------|----------|
| 247604_at| COBRA-like protein 5 precursor                                               | At5g60950  | 2.38        | 0.05     |
| 262237_at| Thioesterase superfamily protein                                             | At1g48320  | 2.32        | 0        |
| 264843_at| 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein     | At1g03400  | 2.31        | 0.005    |
| 267300_at| UDP-Glycosyltransferase superfamily protein                                  | At2g30140  | 2.24        | 0.006    |
| 253238_at| O-Glycosyl hydrolases family 17 protein                                      | At4g34480  | 2.23        | 0.019    |

**Cell expansion related (4)**

| Affy ID   | Description                                                                 | AGI Code   | Fold change | P-value  |
|----------|------------------------------------------------------------------------------|------------|-------------|----------|
| 247866_at| Xyloglucan endotransglucosylase/hydrolase 25                                | At5g57550  | 2.29        | 0.04     |
| 248263_at| Plant invertase/pectin methyltransferase inhibitor superfamily              | At5g53370  | 2.12        | 0.024    |

**Others (61)**

| Affy ID   | Description                                                                 | AGI Code   | Fold change | P-value  |
|----------|------------------------------------------------------------------------------|------------|-------------|----------|
| 244966_at| Polyketide cyclase/dehydrase and lipid transport superfamily protein        | At1g02470  | 5.58        | 0.041    |
| 256337_at| Serine-type endopeptidase inhibitors                                         | At1g72060  | 4.84        | 0.008    |
| 257264_at| Receptor-like protein kinase-related family protein                          | At3g22060  | 3.77        | 0.015    |
| 258792_at| Glycine-rich protein                                                         | At3g06460  | 3.73        | 0.031    |
| 247193_at| MATE efflux family protein                                                   | At5g65380  | 3.2         | 0.049    |
| 250942_at| Legume lectin family protein                                                 | At5g03350  | 3.18        | 0.003    |
| 254832_at| Bifunctional inhibitor/lipid-transfer protein/seed storage 25 albumin family| At4g12490  | 3.13        | 0.026    |
| 266097_at| SOUL heme-binding family protein                                             | At2g37970  | 3.1         | 0.033    |
| 246289_at| VQ motif-containing protein                                                  | At3g58800  | 3.02        | 0.019    |
| 246495_at| Unknown protein                                                              | At5g16200  | 2.87        | 0.038    |
| 257690_at| SAUR-like auxin-responsive protein family                                    | At3g12830  | 2.83        | 0.009    |
| 249769_at| Sigma factor E                                                               | At5g24120  | 2.75        | 0.027    |
| 263948_at| Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family    | At2g35980  | 2.46        | 0.016    |
| 259502_at| Galactose oxidase/kecl repeat superfamily protein                            | At1g15670  | 2.32        | 0.002    |
| 248592_at| hydroxyproline-rich glycoprotein family protein                              | At5g49280  | 2.21        | 0.025    |
| 259410_at| Regulator of Vps4 activity in the MVB pathway                                 | At1g13340  | 2.2         | 0.002    |
| 266247_at| Cysteine/Histidine-rich C1 domain family protein                             | At2g27660  | 2.15        | 0.019    |
| 252053_at| Syntaxin of plants 122                                                       | At3g24000  | 2.13        | 0.039    |
| 259507_at| P-loop containing nucleoside triphosphate hydrolases superfamily protein    | At1g43910  | 2.11        | 0.03     |
| 264951_at| Target of Myb protein 1                                                       | At1g67970  | 2.11        | 0       |
| 263703_at| SAUR-like auxin-responsive protein family                                    | At1g15610  | 2.1         | 0.025    |
| 258501_at| Glycine-rich protein                                                         | At3g06780  | 2.08        | 0.02     |
| 262571_at| Protein of unknown function (DUF1644)                                        | At1g15430  | 2.08        | 0.024    |
| 251859_at| Proteophosphoglycan-related                                                  | At3g54680  | 2.03        | 0.02     |

**Unknown (27)**

| Affy ID   | Description                                                                 | AGI Code   | Fold change | P-value  |
|----------|------------------------------------------------------------------------------|------------|-------------|----------|
| 253859_at| unknown protein                                                              | At4g27657  | 8.71        | 0.017    |
| 256891_at| unknown protein                                                              | At3g19030  | 3.98        | 0.031    |
| 260656_at| unknown protein                                                              | At1g19380  | 3.95        | 0.022    |
| 266017_at| unknown protein                                                              | At2g18690  | 3.53        | 0.008    |
| 265276_at| unknown protein                                                              | At2g28400  | 3.05        | 0.011    |
| 258188_at| unknown protein                                                              | At3g17800  | 2.57        | 0.021    |
| 268275_at| unknown protein                                                              | At3g15760  | 2.47        | 0.031    |
| 266259_at| unknown protein                                                              | At2g27830  | 2.19        | 0.009    |
| 252057_at| unknown protein                                                              | At3g52480  | 2.04        | 0.03     |

Genes derived from the RHNAC3 up-regulated genes identified by the ATH1 microarray analysis in our previous study [26], classified to be responsive to ABA treatment according to the AtGenExpress global stress expression dataset [34].

a Affymetrix identification codes for the probes.
b Description as given by the Munich Information Center for Protein Sequences (MIPS) database.
c Represents a hyperlink to TAIR (www.arabidopsis.org) for more information.
d The ratio of three independent transgenic lines compared with the ratio of vector control plants. Genes expressed in RHNAC3 overexpressing transgenic plants with an up-regulation ratio higher than 2.0 are shown.
e Indicates one-way ANOVA of the differences in mean transcript expression levels between the transgenic and vector control plants at the 0.05 significance level.

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Figure 7. ABA-related gene expression in \textit{RhNAC3}-silenced rose petals. \textbf{A}, The putative ABA signaling and downstream rose genes from the ABA-signaling pathway in rose. a, The clone ID from the rose transcriptome database [7]. b, Description of the \textit{A. thaliana} homolog given by The Arabidopsis Information Resource (TAIR, http://www.arabidopsis.org). B, qRT-PCR analysis of \textit{RhNAC3}-silenced rose petals. The rose cDNAs of TRV and \textit{RhNAC3}-silenced (TRV-\textit{RhNAC3}) petals were described in our previous report [26]. Data represent the fold change of each gene by TRV-\textit{RhNAC3} relative to the TRV control. \textit{RhUbi1} was used as the internal control. Error bars indicate SE (\(n = 3\)). C, Sequences and positions of putative \textit{RhNAC3} binding elements used for the EMSA. Probes were derived from the regulatory sequence of three selected ABA-related rose genes. Underlined letters indicate the core sequences of putative NAC protein-binding sites. The sense strands of oligonucleotide probes corresponding to the predicted \textit{RhNAC3} binding sites are shown. \textbf{D}, DNA-binding specificity for \textit{RhNAC3} with the probes indicated in \textbf{C}. The arrows indicate the positions of protein/
during the seedling stage of development (Figure 2a and b), and particularly strong staining was observed in the vascular system and leaf stomatal guard cells (Figure 2c and d). In addition, GUS staining was detected in the flower petals (Figure 2e and f), stigma (Figure 2g) and apical stem of the inflorescence (Figure 2h), while in mature siliques, staining was primarily localized to the stigma and immature seeds (Figure 2i, j and k). We conclude that the RhNAC3 gene was expressed ubiquitously in a number of different plant tissues, with higher basal expression levels in leaf guard cells and areas of the vascular system.

**ABREs are important for RhNAC3 promoter activity**

To assess the potential role of ABREs in the transcriptional activity of the RhNAC3 promoter, we made three truncated promoter fragments containing five (N0), three (N1) or no (N2) ABREs, and two fragments, mN0 and mN1 with replacement of ACGT by TTTA in ABRE core sequence. These fragments were fused to the GUS reporter gene in the plant transient expression vector pUC19 (Figure 3A, top). The resulting constructs, as well as the vector control (NC), were transformed into A. thaliana protoplasts and relative GUS activity was measured. We observed that extracts from the protoplasts transformed with constructs containing a higher number of ABRE copies had higher GUS activity than control transformant extracts. Specifically, the N0 and N1 construct extracts had 7.2-fold and 3.1-fold greater GUS activity, respectively, than the NC extract, while constructs with ABRE mutations (mN0 and mN1) conferred only slight GUS activity and minimal activity was detected in the N2 construct extract (Figure 3A, bottom). We also investigated the transcriptional activity of three truncated RhNAC3 promoter fragments in A. thaliana protoplasts exposed to exogenous ABA. Transcription of the RhNAC3 promoter (N0 and N1) was found to be induced with a higher GUS activity for the N0 fragment (five ABREs) than for N1 (three ABREs), while no difference was seen for N2 (no ABRE) when ABA was added (Figure 3B). The potential role of the ABREs in the RhNAC3 promoter in ABA induced transcription was also evaluated using the ProRhNAC3::GUS transgenic A. thaliana lines. Seedlings of three lines had stronger GUS staining after treatment with ABA than those without ABA treatment (Figure 3C). Taken together these data indicate that ABREs in the RhNAC3 promoter have a cumulative effect on the transcription activity of RhNAC3 both in the presence and absence of ABA, and that ABA significantly induces RhNAC3 transcription.

**A. thaliana plants overexpressing RhNAC3 show hypersensitivity to ABA during germination**

Our previous study showed that three representative RhNAC3-overexpressing A. thaliana lines (OE#3, OE#6 and OE#12) had enhanced drought tolerance, with a higher water-retaining ability [26]. To understand the roles of RhNAC3 in the ABA signaling pathway, we investigated the seed germination rates of RhNAC3-overexpressing lines following ABA treatment. More than 98% of the seeds sown on control MS medium germinated well, while the germination rate of both the control and transgenic seeds decreased when grown for 7 days on MS medium plus ABA. In the presence of 0.2 μM ABA, the germination rates of WT and Vector (VC) seeds were 63% and 59%, while the rates for OE#3, OE#6 and OE#12 were 49%, 40% and 36%, respectively. A higher concentration of ABA (0.4 μM) resulted in lower germination rates for both the control and RhNAC3-overexpressing transgenic plants, and the latter showed a greater decrease (Figure 4A and 4B). We also compared the effects of ABA on the root architectures of the RhNAC3-overexpressing and control plants. Primary root growth of RhNAC3-overexpressors was inhibited more by 30 μM ABA treatment than that of the control plants, while no significant differences in the number of lateral roots was observed (Figure 4C–E). We conclude from these results that RhNAC3 overexpression in A. thaliana results in ABA hypersensitivity at the seed germination stage.

**RhNAC3 participated positively in ABA- and drought-induced stomatal closure**

Since the leaves of RhNAC3-overexpressing A. thaliana plants have greater water-retaining capacity than those of WT plants [26], we examined ABA-dependent stomatal movement phenotypes. Expanded leaves of 3-week-old plants (12 h day/12 h night) were submerged in stomatal opening solution, treated with 10 μM ABA for 2 h, and then the stomatal apertures of the guard cells were measured in the focal planes of the outer edge in epidermal strips. None of the plants exhibited altered stomatal movement in the absence of ABA, and most of the guard cells examined were fully opened. No obvious difference in stomatal aperture (the ratio of width to length) were observed between the controls (WT and VC) and RhNAC3 overexpressors (OE#3, OE#6 and OE#12). However, in the presence of 10 μM ABA, stomatal closure in the leaves of RhNAC3 overexpressing transgenic plants was substantially enhanced compared with that of control plant leaves. The stomatal aperture ratios in the OE#3, OE#6, and OE#12 lines were approximately 0.16, 0.22 and 0.25, respectively, compared with 0.30 and 0.29 for the WT and VC plants (Figure 5A).

We also investigated stomatal movement in the leaves of RhNAC3-overexpressing A. thaliana plants grown under drought conditions. Water was withheld for 10 days from three-week-old plants that had previously been grown under normal conditions, after which the stomatal apertures of the expanded leaves were measured. Under normal growth conditions (0 days), both the controls (WT and VC) and overexpressor lines (OE#3, OE#6 and OE#12) showed no obvious difference, with stomatal apertures of 0.29, 0.20, 0.29, 0.27 and 0.28, respectively. However, after 10 days of exposure to drought conditions, the stomatal aperture ratios had decreased to 0.17, 0.15 and 0.12 in OE#3, OE#6 and OE#12 lines, respectively, but only 0.24 in WT and 0.23 in VC plants (Figure 5B). These results indicate that RhNAC3 is involved in stress responses in an ABA-dependent manner.

**RhNAC3 activates ABA-responsive gene expression in rose and Arabidopsis**

Given that the expression of RhNAC3 showed an association with both ABA sensitivity and drought tolerance, we investigated the expression profiles of drought-induced ABA-responsive genes. Eleven representative genes were selected, including ABA and stress-induced downstream marker genes (RD29A, RD29B, RD20, RD26, COR47, COR15A, and KIN2), the ABA-responsive protein phosphatase 2C gene (ABI1), the B3-domain transcription factor ABA-insensitive 3 (ABI3), the ABA-activated basic Leu zipper TF gene ABF4, and the ABA-biosynthesis gene.
ABA3 (Table 1 and Figure 6). Under dehydrating conditions, RD26, ABA3 and ABA3 showed a slightly higher level of expression in RhNAC3 overexpressing plants than in VC plants, but all the other tested genes showed more than a two-fold greater expression (Figure 6A). The promoters of these genes were then screened for putative NAC binding cis-elements and indeed they were identified in all the tested genes (Table 1). We then used an electrophoretic mobility shift assay (EMSA) to investigate whether the RhNAC3 protein directly binds to 4 of the selected genes: RD29A, RD20, COR47 and COR15A. We observed that RhNAC3 bound to the putative NAC recognition sites of RD29A, RD20 and COR47, but no binding signal was detected for COR15A, which may therefore be an indirect target of RhNAC3 following its overexpression in A. thaliana (Figure 6B–D). In our previous study, we used the ATH1 microarray to identify 219 RhNAC3-up-regulated genes [26] and in this current study, we further analyzed the response to ABA of these genes using the AtGenExpress global stress expression dataset [35]. In total, 85 of the 219 genes were found to be ABA responsive, including those encoding proteins involved in signal transduction (e.g. calmodulin-binding protein) and TFs (e.g. zinc-finger protein) (Table 2). In rose, we found 9 putative signaling and downstream genes of the ABA-signaling pathway from our rose transcriptome databases [7] (Figure 7A). qRT-PCR analysis revealed that the expression levels of 6 of these genes were substantially repressed in RhNAC3-silenced rose petals (with a fold change <0.8) (Figure 7B). Three ABA-responsive genes with putative NAC binding cis-elements (RU25535, RU04740 and RU03861) were selected for RhNAC3 binding assays (Table S3). EMSA revealed that RhNAC3 could bind to the promoter region of RU03861, a rose homolog of ABF4, whereas no binding signals were detected for RU25535 and RU04740 (Figure 7C–E), which may therefore be indirect targets of RhNAC3 in rose. Collectively, these data suggest that RhNAC3 positively activates ABA-responsive gene expression and is involved in the ABA signaling pathway in rose and A. thaliana.

Discussion

RhNAC3 is involved in the ABA-dependent signaling pathway

Plants respond and adapt to drought stresses through a broad range of molecular and biochemical processes that result in cellular physiological changes [1]. Many drought-inducible TFs with various functions, including a number of TFs that regulate stress-inducible gene expression, have been identified by molecular and genomic analyses of A. thaliana, rice and other plants, [9,18,23]. These TFs have been classified as being involved in one of two signal transduction pathways: ABA-independent or ABA-dependent [2,36]. ABA-dependent gene induction is controlled by at least five different classes of TFs at the transcriptional level: AREB (bZIPs), NACs, MYB/MYC and DREB1D [2,37]. Many ABA-inducible genes contain a conserved ABRE motif in their promoter regions [38], which functions as a cis-element in ABA-regulated gene expression. In a previous study, we determined that RhNAC3 expression is induced by exogenous ABA [26], implying RhNAC3 is involved in the ABA signaling pathway during stress responses. In this current study, we further analyzed the RhNAC3 promoter, which was found to contain five ABRE motifs, as well as other stress-responsive elements (Figure 1, Figure S1 and Table S2). Promoter activity was detected ubiquitously in a number of tissues upon transformation into A. thaliana, and was highest in leaf guard cells and some areas of the vascular system (Figure 2). In rose, RhNAC3 expression was also detected in sepal, petals, gynoecia, stamens and receptacles [26]. ABREs were found to be important for the activity of the RhNAC3 promoter, and the multiple copies had a cumulative effect on transcriptional activity in both the presence and absence of exogenous ABA in A. thaliana protoplasts (Figure 3). It has been shown that multiple ABRE elements can collectively confer ABA responsiveness to a minimal promoter, whereas a single copy of ABRE is insufficient for the full ABA response of AREB1 and AREB2, two basic leucine zipper TFs [14]. ABREs are also regarded as one of the major types of cis-acting elements in the promoter regions of stress-inducible genes during osmotic stress-responsive transcriptional regulation [12,39]. This is consistent with our previous findings that RhNAC3 confers dehydration tolerance to rose petals, mainly through the regulation of osmotic adjustment-associated genes [26].

RhNAC3 overexpression in A. thaliana enhances ABA sensitivity

Many ABA-responsive TFs have been isolated and characterized from different plant species, including A. thaliana [16], rice [5] soybean [22], maize [40] and Citrus reshni [41]. Overexpression of these ABA-responsive TF genes has been reported to result in a range of phenotypic changes, including dwarfing [42,43], ABA hypersensitivity [44], lateral root formation [22] and stomatal closure [45]. In our study, RhNAC3 overexpression in A. thaliana lead to ABA hypersensitivity during seedling germination and primary root growth (Figure 4), and promoted stomatal closure after exogenous ABA or drought treatments (Figure 5). We note that this differs from the effects of the soybean ABA-inducible gene GmNAC20, which promotes lateral root formation enhances salt and freezing tolerance when overexpressed in transgenic Arabidopsis [22].

RhNAC3 enhanced ABA-responsive gene expression in rose and A. thaliana

ABA-inducible TFs involved in ABA signaling pathways typically up-regulate ABA-responsive genes or stress-responsive genes [13,18] and such downstream genes have studied using qRT-PCR [16], cDNA microarrays [13] and other transcriptomic analyses [23]. Among these genes, AREB TFs play a primary role in the ABA-dependent signaling pathway [13,46], while other TFs (NAC, MYB/MYC and AZF/STZ TFs) can play additional direct or indirect regulatory roles. The diverse cis-elements in the promoter regions of these TFs suggest additional potential mechanisms of transcriptional regulation for ABA-signaling downstream genes as a consequence of abiotic stresses [47]. In the current study, 11 representative ABA-induced genes were investigated, all of which were found to be up-regulated in RhNAC3 overexpressing A. thaliana plants (Figure 6A). Further analysis showed that NAC binding cis-elements were present in the upstream regulatory sequences of these genes (Table 1) and that RhNAC3 was able to bind to the putative NAC recognition sites of some of the tested genes (Figure 6B–D). We conclude that RhNAC3 may therefore directly or indirectly regulate their expression at the transcriptional level. These genes were selected based on their involvement in the ABA-dependent signaling pathway and the fact that their overexpression in A. thaliana has been shown to result in an increased ABA sensitivity [25,48]. In a previous microarray study we found that the expression of 219 genes was up-regulated in RhNAC3 overexpressing plants [26], of which 85 responded to ABA treatment in the current study (Table 2). These results suggest that these genes may contribute to ABA sensitivity in the RhNAC3 overexpressing A. thaliana plants,
an idea that is supported by previous experimental evidence. For example, *CYP81D8* expression has been suggested to be regulated by the ABA-dependent pathway under osmotic stress conditions [49], and loss-of-function mutations of *atrdufs* (*Atg59550*) resulted in hyposensitivity to ABA and reduced tolerance to drought stress [50]. Moreover, the expression of downstream genes in the ABA-signaling pathway was also repressed in *RhNAC3*-silenced rose petals (Figure 7). In our previous study, *RhNAC3* was shown to bind to the promoter of *RU23063*, a rose homolog of *ABIZ* [26], which encodes a protein phosphatase 2C involved in ABA signal transduction [51]. Here *RhNAC3* was observed to bind to the promoter region of the ABA-responsive rose gene *ABF4*, which encodes the ABRE binding factor 4 (Figure 7C–E). Taken together, the data suggest that *RhNAC3* regulated genes that were responsive to osmotic stress, are also involved in the ABA-dependent signaling pathway in both rose and *A. thaliana*.

In conclusion, we found that: (1) ABRE elements in the *RhNAC3* promoter were necessary for, and had a cumulative effect on, its transcription activity in both the presence and absence of exogenous ABA; (2) *RhNAC3*-overexpressing *A. thaliana* lines showed ABA hypersensitivity during seed germination and constitutive leaf stomatal closure under ABA or drought treatment; and (3) *RhNAC3* up-regulated the expression level of ABA-responsive genes, which were responsive to osmotic stress. These findings provide new evidence that *RhNAC3* is a positive mediator of ABA signaling in the regulation of drought stress tolerance in rose and at least some components of the associated signaling pathways are conserved between rose and *A. thaliana*.

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