An NAC Transcription Factor Controls Ethylene-Regulated Cell Expansion in Flower Petals

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Cell expansion is crucial for plant growth. It is well known that the phytohormone ethylene functions in plant development as a key modulator of cell expansion. However, the role of ethylene in the regulation of this process remains unclear. In this study, 2,189 ethylene-responsive transcripts were identified in rose (Rosa hybrida) petals using transcriptome sequencing and microarray analysis. Among these transcripts, an NAC (no apical meristem [NAM], Arabidopsis transcription activation factor [ATAF], and cup-shaped cotyledon [CUC])-domain transcription factor gene, RhNAC100, was rapidly and dramatically induced by ethylene in the petals. Interestingly, accumulation of the RhNAC100 transcript was modulated by ethylene via microRNA164-dependent posttranscriptional regulation. Overexpression of RhNAC100 in Arabidopsis (Arabidopsis thaliana) substantially reduced the petal size by repressing petal cell expansion. By contrast, silencing of RhNAC100 in rose petals using virus-induced gene silencing significantly increased petal size and promoted cell expansion in the petal abaxial subepidermis (P < 0.05). Expression analysis showed that 22 out of the 29 cell expansion-related genes tested exhibited changes in expression in RhNAC100-silenced rose petals. Moreover, of those genes, one cellulose synthase and two aquaporin genes (Rosa hybrida Cellulose Synthase2 and R. hybrida Plasma Membrane Intrinsic Protein1;1/2;1) were identified as targets of RhNAC100. Our results suggest that ethylene regulates cell expansion by fine-tuning the microRNA164/RhNAC100 module and also provide new insights into the function of NAC transcription factors.

Rose (Rosa hybrida) is one of the most important ornamental plants worldwide, and it has been cultivated since 500 BC. The morphological changes occurring during flower opening typically consist of petal outgrowth and unfurling, processes that are also important horticulturally for rose flowers. Petal growth is a complex process that integrates cell division and cell expansion. In Arabidopsis (Arabidopsis thaliana), cell division is typically phased out in petals prior to bud opening and flower development, and cell expansion predominantly contributes to petal growth at later stages (Irish, 2008). Similarly, in rose, petal growth also largely depends upon cell expansion at later stages of flower opening (Yamada et al., 2009).

Ethylene, an important plant hormone, regulates various plant developmental processes including seed germination, organ elongation, flowering, fruit ripening, organ senescence, and abscission (Abeles et al., 1992). Although several reports have demonstrated that ethylene stimulates cell division (Ortega-Martinez et al., 2007; Love et al., 2009), it typically functions in plant development as a key modulator of cell expansion. In Arabidopsis, dark-grown seedlings treated with ethylene exhibit a triple response, which consists of radial swelling of the hypocotyl, exaggeration of the apical hook, and inhibition of hypocotyl and root elongation (Guzmán and Ecker, 1990). The constitutive ethylene-responsive mutant constitutive triple response1 (ctr1) shows severe dwarfishm and much smaller leaf cell sizes compared with wild-type plants (Kieber et al., 1993). It has been reported that ethylene can inhibit elongation of leaves in Poa species and roots in Rumex palustris and Cucumis sativus (Pierik et al., 2006). In rose, we previously showed that ethylene accelerates the flower-opening process but inhibits the expansion of petal abaxial subepidermis (AbsE) cells, thereby further inhibiting petal expansion (Ma et al., 2008). To date, the ethylene-responsive regulatory mechanism that arises during cell expansion remains unclear, although several reports have demonstrated that ethylene regulates the expression of genes associated with cell expansion, such as cell wall metabolism.
and transporter genes (Zhong and Burns, 2003; De Paeppe et al., 2004; Alba et al., 2005).

As regulators of gene expression, transcription factors (TFs) play an important role in plant development and in the response to hormone signals (Riechmann et al., 2000). NAC (for no apical meristem [NAM], Arabidopsis transcription activation factor [ATAF], and cup-shaped cotyledon [CUC])-domain proteins comprise one of the largest families of plant-specific TFs. Many NAC-domain genes have been identified from a range of plant species, including Arabidopsis (Riechmann et al., 2000), rice (Oryza sativa; Xiong et al., 2005), tobacco (Nicotiana tabacum; Rushton et al., 2008), poplar (Populus spp.; Hu et al., 2010), and soybean (Glycine max; Pinheiro et al., 2009). The NAC superfamily can be divided into at least seven subfamilies (Rushton et al., 2008), and the function of NAC genes is typically subfamily specific. In Arabidopsis, members of the NAM subfamily CUC1/CUC2/CUC3 are well known as regulators involved in shoot apical meristem formation and cotyledon separation during embryogenesis (Aida et al., 1997; Olsen et al., 2005), whereas members of the Vascular-related NAC-Domain protein/NAC Secondary wall Thickening promoting factor/Secondary wall-associated NAC-Domain protein (VND/NST/SND1) subfamily are typically involved in regulating secondary wall formation (Yamaguchi and Demura, 2010). Previous reports have indicated that several NAC genes may be regulated by ethylene. In a tomato (Solanum lycopersicum) plant, at least one NAC gene is influenced by the mutation of Never Ripe, which is a gene encoding an ethylene receptor (Alba et al., 2005). In Arabidopsis, mutation of ETHYLENE INSENSITIVE2 (EIN2) blocked the induction of Arabidopsis NAC092 (ANAC092)/AtNAC2/ORESARA1 (ORE1) expression under salt conditions (He et al., 2005). Microarray analysis has revealed that approximately one-third of NAC genes are regulated by application of 1-aminocyclopropane-1-carboxylate, which is a precursor of ethylene, in Arabidopsis (Jensen et al., 2010). In addition to transcriptional regulation, several NAC genes, including NAC1 and a few members of the NAM subfamily are also posttranscriptionally regulated by microRNA164 (miR164; Sieber et al., 2007). Auxin can increase the abundance of miR164 and thus decrease transcript accumulation of NAC1, which is a target of miR164, thereby promoting lateral root initiation in Arabidopsis (Guo et al., 2005). Increased miR164 levels have also been reported in ein2-1, which is an ethylene-insensitive mutant. However, the question of whether NAC genes are involved in ethylene-regulated floral organ growth remains unclear.

In this study, potential key regulators that mediate ethylene-regulated cell expansion in rose petals were screened using microarray analysis. One hundredfive ethylene-responsive TFs and regulators were identified from rose petals. RhNAC100, a homolog of ANAC092/AtNAC2/ORE1 in Arabidopsis, was found to function as a negative regulator of cell expansion in petals. Accumulation of RhNAC100 transcripts is regulated by miR164 in vivo in an ethylene-dependent manner, and RhNAC100 is capable of binding to promoters of the cell expansion-related genes R. hybrida Cellulose Synthase2 (RCh CesA2) and R. hybrida Plasma Membrane Intrinsic Protein1/2/1 (RhPIP1/2/2):1. Taken together, these data reveal an ethylene-mediated miR164/RhNAC100 module and identify a set of cell expansion-related genes that are regulated by this module during petal cell expansion in rose and transgenic Arabidopsis.

RESULTS

Transcriptome Sequencing of Rose Flowers

To investigate the molecular mechanism of ethylene-regulated flower opening, firstly, large scale transcriptome sequencing of ethylene-treated and control flowers were performed using 454 massive parallel pyrosequencing technology. (Primers for library construction are listed in Supplemental Table S1.) We obtained 60,944 assembled transcripts with an average length of 302 bp and a total length of approximately 18.4 million base pair (Mb; Supplemental Fig. S1; see Supplemental Text S1 for detailed analysis). The assembled transcript sequences were compared against National Center for Biotechnology Information non-redundant, UniProt, and the TAIR9 release of The Arabidopsis Information Resource protein databases, respectively, using blast with the e-value cutoff set to 1e−5. The results showed that 33,763 transcripts (55.4%) had significant matches in the databases. The annotated assembled transcripts were classified into different functional categories according to a set of plant-specific gene ontology (GO) slims. A total of 26,462 were assigned with at least one GO term, and 21,331, 23,868, and 19,229 were assigned with GO term in biological process, molecular function, and cellular component categories, respectively (Supplemental Table S2). All these analyses supported that the sequences generated in this study provide a valuable resource for rose gene discovery and functional analyses. The raw sequence data were deposited into the National Center for Biotechnology Information Sequence Read Archive database under accession number SRA045958, and the processed sequence reads and assembled transcript sequences are also available at the rose transcriptome database (http://bioinfo.bti.cornell.edu/rose).

Identification of Ethylene-Responsive Genes in Rose Petals Using Microarray Analysis

To investigate the molecular mechanism underlying ethylene-regulated flower opening, we first designed and constructed an oligonucleotide microarray containing probes representing 14,997 rose assembled transcripts, which were extracted from a rose floral transcriptome database (http://bioinfo.bti.cornell.edu/rose).
This custom microarray was used to investigate gene expression profiles in rose petals following ethylene treatment. A total of 2,189 assembled transcripts in petals were significantly regulated by ethylene (false discovery rate [FDR] < 0.05 and fold changes ≥ 2), and these transcripts were divided into three groups based on their expression patterns (Fig. 1A; Supplemental Table S3). Interestingly, 386 ethylene-responsive transcripts exhibited rapid responses to ethylene (within 1 h), including 90 up-regulated and 296 down-regulated transcripts. Notably, the ethylene receptor and CTR1 were among the up-regulated genes, which is consistent with our previous report (Tan et al., 2006). Many genes involved in cell expansion-related processes, such as cell wall catabolism, water transportation, and hexose transport, were responsive to ethylene. For example, the expression of 22 out of the 26 water transporter genes on the microarray showed significant repression, including two that had been previously shown to be down-regulated following ethylene treatment (Ma et al., 2008; Xue et al., 2009). In addition, 105 ethylene-responsive transcripts were identified as TFs and transcriptional regulators (TRs), which were grouped into 23 families (Fig. 1B; Supplemental Table S4). The major families included myeloblastosis (14 transcripts), APETALA2/ethylene-responsive element-binding protein (12 transcripts), homeobox domain (11 transcripts), WRKY (10 transcripts), basic helix-loop-helix (bHLH; 10 transcripts), GIBBERELLIC-ACID INSENSITIVE, REPRESSOR OF GA1-3 SCARECROW (10 transcripts), and NAC-domain family (eight transcripts). Interestingly, 14 TFs and TRs were involved in the signaling cascades of auxin (two auxin response factors [ARFs] and four Auxin/Indole-3-acetic acid [AUX/IAA] inducible genes), gibberellin (five DELLAs), or brassinosteroid (three BR Enhanced Expressions [BEEs]).

**RhNAC100 Encodes an Ethylene-Inducible NAC-Domain TF**

To identify potential key regulators involved in ethylene-regulated petal expansion in rose, we focused primarily on an analysis of expression profiles of TFs

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**Figure 1.** Screening of ethylene-responsive genes in rose petals and characterization of RhNAC100. A, Expression profiles of genes following ethylene treatment of rose petals. Log-of-fold changes in each probe set responding to ethylene at 1-, 6-, 12-, 18-, and 24-h ethylene treatment were used to generate the cluster. B, Ethylene-responsive TFs and TRs. The numbers in the parentheses after each TF or TR family indicate the number of changed TFs or TRs (first number) and the number of tested TFs or TRs on the microarray (second number). C, Subcellular localization of RhNAC100 in onion epidermal cells. Bar = 100 μm. D, qRT-PCR analysis of RhNAC100 in response to ethylene in petals. The results show the mean ± SD (error bars) of qRT-PCR experiments and were generated from three biological replicates, each with three technical replicates. *RhACT5* was used as the internal control.
and TRs in response to ethylene. The expression of RU02822, a putative NAC-domain TF, was rapidly and robustly induced by ethylene (Supplemental Table S4). The coding region of RU02822 contains 1,020 nucleotides, and it encodes a putative protein containing 339 amino acids. Sequence analysis indicated that RU02822 contains a typical NAC-domain consisting of five conserved amino acid motifs (N1–N5; Olsen et al., 2005) in its N terminus, whereas its C-terminal domain is quite specific (Supplemental Fig. S2A). RU02822 was named as RhNAC100 (JQ001774) because its closest homolog is ANAC100 in Arabidopsis. Phylogenetic analysis indicated that RhNAC100 belongs to the NAM subfamily of the NAC-domain TF family (Supplemental Fig. S2B), which has been reported to be involved in cotyledon, leaf, and petal development in Arabidopsis (Laufs et al., 2004; Baker et al., 2005; Kim et al., 2009; Hasson et al., 2011). Quantitative reverse transcription (qRT)-PCR showed that expression of RhNAC100 was rapidly and dramatically increased by approximately 40-fold following 1-MCP treatment. 1-MCP is a strong inhibitor of ethylene (Supplemental Fig. S2C). Interestingly, N terminus of RhNAC100 also contains an NARD (NAC repression domain) domain (Supplemental Fig. S2A), which was recently reported to be a conserved and active repression domain in NAC proteins (Hao et al., 2010), suggesting that RhNAC100 may function as a transcription repressor. In addition, we performed transactivation analyses in leaf protoplast of Arabidopsis using VP16 (viral protein16 from herpes simplex) transcriptional activation domain as the positive control (Hao et al., 2010). The results showed that RhNAC100 could clearly repress the transcription activity of VP16, implying that RhNAC100 could function as a repressor (Supplemental Fig. S2D).

A transient expression assay in onion (Allium cepa) epidermal cells showed that RhNAC100-Enhanced GFP fusion protein is localized to the nucleus, thereby supporting the theory that RhNAC100 is a TF (Fig. 1C). qRT-PCR showed that the expression of RhNAC100 rapidly and dramatically increased by approximately 6-fold following ethylene treatment (1 h) and was clearly inhibited following 1-methylocyclop propane (1-MCP) treatment. 1-MCP is a strong inhibitor of ethylene function that is capable of binding to the ethylene receptor and preventing the physiological action of ethylene (Sisler and Serek, 1997). These results confirmed our microarray analysis (Fig. 1D). In addition, RhNAC100 expression was suppressed by auxin and GA treatments, whereas it was not altered following treatment with brassinosteroid (Supplemental Fig. S3).

**Ethylene Fine-Tunes the Accumulation of RhNAC100 Transcripts by Repressing miR164**

To understand how ethylene regulates RhNAC100 expression, we first isolated the promoter of RhNAC100 and analyzed the sequence using the PLACE database (http://www.dna.affrc.go.jp/PLACE/). Unexpectedly, no ethylene-responsive elements were found (Supplemental Fig. S4A). In Arabidopsis, three members of the NAM subfamily, ANAC092/AtNAC2/ORE1 (AT5G39610), CUC1 (AT3G15170), and ANAC100 (AT5G61430), are ethylene-inducible, although they do not contain any ethylene-responsive elements in their promoter regions (Jensen et al., 2010). However, all of these genes are targets of miR164, a conserved family of plant microRNAs (miRNAs; Sieber et al., 2007). Here, we also found that RhNAC100 possesses a potential miR164 binding site located in the third exon (Fig. 2A). This miR164 cleavage site was verified using modified RNA ligase-mediated 5′-RACE, and sequencing analysis revealed that six out of the 10 independent complementary DNA (cDNA) clones were cleaved at the position between the tenth and eleventh nucleotide from the 5′ end of miR164 (Fig. 2A), which is identical to the cleavage position noted in Arabidopsis (Mallory et al., 2004; Guo et al., 2005; Nikovics et al., 2006; Sieber et al., 2007; Kim et al., 2009). These results indicate that RhNAC100 is a potential target of miR164 in rose.

To verify whether the accumulation of RhNAC100 transcripts is posttranscriptionally regulated by miR164, miR164 was obtained from rose petals and named rhy-miR164. The sequence of rhy-miR164 was identical to the miR164 sequence in Arabidopsis (Fig. 2A) and was consistent with a recent report in rose (Kim et al., 2012). The rhy-miR164 abundance in ethylene-treated rose petals was reduced to 48.8% of that of control petals. Conversely, 1-MCP treatment increased the abundance of rhy-miR164 in rose petals (Fig. 2B). The abundance of RhNAC100 transcripts in rose petals was substantially increased following ethylene treatment and the expression pattern of RhNAC100 was inversely correlated to that of rhy-miR164 (Figs. 1D and 2B). To test whether the ethylene-induced accumulation of RhNAC100 transcripts resulted from the decreased level of rhy-miR164 in rose petals, we generated RhNAC100m, an miR164-resistant version of RhNAC100, by substituting six nucleotides in the miR164/RhNAC100 complementary region to produce mismatches without changing the amino acid sequence (Fig. 2A). MiR164 is a well-conserved miRNA in plants, and the mature sequence of rhy-miR164 is identical to miR164 in Arabidopsis. According to the principles of target reorganization and cleavage by miRNA (Bartel, 2009), RhNAC100 could be targeted and cleaved properly in Arabidopsis. Thus, we generated transgenic Arabidopsis overexpressing RhNAC100 and RhNAC100m (an miR164-resistant version of RhNAC100) in wild-type Arabidopsis (ecotype Columbia [Col-0]) and in ein2-1 mutant to evaluate the cleaving activity of Arabidopsis mir164 on rose RhNAC100 with and without ethylene.

In plants, miRNA-guided cleavage of target mRNAs produces both the 5′ and 3′ cleavage fragments. Although both 5′ and 3′ cleavage fragments are degraded, in many instances, the 3′ cleavage fragment is
more stable and thus detectable (Llave et al., 2002; Dunoyer et al., 2004; Souret et al., 2004). In RhNAC100-ox/Col transgenic plants, both the full-length transcript and the 3’ cleavage fragment of RhNAC100 were detected. Ethylene treatment dramatically increased the levels of full-length RhNAC100 transcript, whereas it decreased the levels of the RhNAC100 3’ cleavage fragment in RhNAC100-ox/Col plants. By contrast, the amount of the full-length RhNAC100 was lower and that of the cleaved 3’ fragment was higher in RhNAC100-ox/ein2 plants, indicating that the cleavage of RhNAC100 was enhanced in the ein2-1 mutant. In addition, ethylene treatment was unable to change the abundance of either intact transcripts or 3’ cleavage fragments of RhNAC100 in RhNAC100-ox/ein2 plants. In both RhNAC100m-ox/Col and RhNAC100m-ox/ein2 transgenic plants, full-length RhNAC100 transcripts were highly accumulated, whereas the 3’ cleavage fragment was undetectable. Moreover, the abundance of intact RhNAC100m transcripts was almost identical in RhNAC100m-ox/Col and RhNAC100m-ox/ein2 plants with or without ethylene treatment (Fig. 2C), indicating that ethylene-regulated accumulation of the RhNAC100 transcript was mediated via a suppression of miR164-guided cleavage of RhNAC100. Consistent with a previous report (Kim et al., 2009), levels of miR164 were greater in the ein2-1 mutant than in the Col-0 control plants. Furthermore, levels of miR164 decreased following ethylene treatment in the Col-0 plants, whereas the level was not influenced by ethylene in the ein2-1 mutants (Fig. 2D). On the basis of these results, it was hypothesized that ethylene could fine-tune the accumulation of RhNAC100 transcripts by repressing miR164 and that this regulatory process requires EIN2. To test this hypothesis during the natural opening process of rose flowers, we determined

Figure 2. Cleavage of RhNAC100 mRNA by miR164. A, A schematic representation of RhNAC100 and alignment of rhy-miR164 with RhNAC100 (normal) and RhNAC100 mutated (RhNAC100m) transcripts. Short vertical lines represent Watson-Crick pairing, and circles indicate G:U wobble pairing. The vertical arrow indicates the 5’ termini of miRNA-guided cleavage products, and the frequencies of the clones are also shown. The mutant nucleotides are underlined. rhy-miR164 and ath-miR164 are miR164 sequences in rose and Arabidopsis, respectively. B, qRT-PCR of rhy-miR164 in rose petals. Flowers (stage 2) were exposed to air (–) or 10 μL L⁻¹ ethylene (+) for 24 h (left). Flowers (stage 2) were exposed to air (C24), 10 μL L⁻¹ ethylene (E24), or 2 μL L⁻¹ 1-MCP (M24) for 24 h (right). qRT-PCR was performed on at least three biological replicates. Bars indicate the sd (Student’s t test, *P < 0.05). The internal control used was 5S rRNA. C, Effect of ethylene on miR164-directed cleavage of RhNAC100 in Arabidopsis. The full-length RhNAC100 transcript and its 3’ fragments cleaved by miR164 are indicated. rRNA visualized by ethidium bromide staining was used as a loading control. Six biological replicates were used, and representative results are shown. D, qRT-PCR of ath-miR164 from Arabidopsis leaves. The qRT-PCR was performed using at least three biological replicates. The internal control used was 5S rRNA. Bars indicate the sd (Student’s t test, *P < 0.05). [See online article for color version of this figure.]
the petal AbsE cell density, ethylene production, rhy-miR164 abundance, and RhNAC100 expression in rose petals. In rose flowers, stages 0, 2, and 4 represent unopened buds, completely opened buds, and opened flowers, respectively (Ma et al., 2005). The petal AbsE cell density decreased continuously from stage 0 to 4, but the decrease was more pronounced during the earlier stages (stages 0–2) than during the later stages (stages 2–4), indicating that the petal growth rate declined as the flower opened (Fig. 3A). Petal ethylene production was relatively low during stage 0, showed a substantial increase during stage 2, and exhibited a further increase during stage 4. The abundance of rhy-miR164 was relatively high in the petals at stage 0, whereas it decreased dramatically during stage 2 and decreased further during stage 4. By contrast, the expression of RhNAC100 was low during stage 0 and increased substantially upon flower opening in rose petals (Fig. 3B), indicating that the expression pattern of RhNAC100 was negatively correlated with rhy-miR164 during flower opening. The rhy-miR164-directed cleavage of RhNAC100 mRNA was monitored in rose petals during flower opening using regional amplification-PCR (Vazquez et al., 2004; Oh et al., 2008). The results demonstrated that the level of intact RhNAC100 transcripts increased substantially upon flower opening, whereas the abundance of the RhNAC100 3’ fragment remained relatively stable during flower opening (Supplemental Fig. 5A), which further supports the theory that rhy-miR164 cleaves RhNAC100 during the natural flower-opening process.

RhNAC100 Is Involved in Cell Expansion

The phenotype of transgenic Arabidopsis plants was observed. Compared with transgenic plants expressing an empty vector, the petal size decreased by 33% to 36% in RhNAC100-ox/Col and by 49% to 52% in RhNAC100m-ox/Col plants (Fig. 4, A and B). Microscopic analysis revealed that the density of abaxial epidermal cells in the petals increased by 20% to 23% and 37% to 41% in RhNAC100-ox/Col and RhNAC100m-ox/Col plants, respectively, compared with vector/Col-0 plants (P < 0.01, Tukey’s test; Fig. 4C). Vector/ein2-expressing plants produced larger petals approximately 1.4-fold) than the vector/Col-0 plants, while the abaxial epidermal cell density in the former plants was approximately 70% of the latter, indicating that blockage of the ethylene signaling pathway led to enhanced cell expansion. Compared with the vector/ein2 line, the petal size was reduced by 21% to 22% and 49% to 57%, respectively, whereas the abaxial epidermal cell density increased by 14% to 25% and 39% to 47%, respectively, in the RhNAC100-ox/ein2 and RhNAC100m-ox/ein2 plants (P < 0.01, Tukey’s test).

Because the mutation of miR164-binding site did not alter the deduced amino acid sequence, the function of RhNAC100m was supposed to be identical to

Figure 3. Changes in petal cell density, ethylene production, and expression of rhy-miR164 and RhNAC100 in rose petals during flower opening. A, Petal cell density of AbsE. Each data point represents the mean ± so (n = 30). Different letters indicate significant differences (P < 0.05) calculated using one-way ANOVA followed by Tukey’s honestly significant difference test. 0, Unopened buds; 2, completely opened buds; 4, opened flowers. Bar = 200 μm. The cells were counted in visual fields of 1,024 × 1,024 μm. B, Ethylene production (top) and expression profiles of rhy-miR164 (middle) and RhNAC100 (bottom) in rose petals during natural flower opening. The qRT-PCR was performed using at least three biological replicates. The internal controls used were 5S rRNA and RhACT5 for rhy-miR164 and RhNAC100, respectively. [See online article for color version of this figure.]
RhNAC100. Therefore, we analyzed the expression level of transgene RhNAC100/RhNAC100m to confirm whether it is related to the phenotype. RhNAC100 and RhNAC100m were detected by qRT-PCR using the same primers that were designed across the miR164-binding site.

qRT-PCR demonstrated that, compared with RhNAC100/Col (#18) plants, expression of the RhNAC100 transgene was 33.8% to 47.2% in RhNAC100/ein2, while expression of the RhNAC100m was 23.0- to 33.5-fold and 21.5- to 29.7-fold in RhNAC100m/Col and RhNAC100m/ein2 plants, respectively (Fig. 4D). Interestingly, although the expression level of RhNAC100 was much lower in the RhNAC100/ein2 lines than in RhNAC100/Col, expression level of RhNAC100m was similar between the RhNAC100m/ein2 and RhNAC100m/Col lines, which is consistent with the results presented in Figure 2C.

These results indicated that expression level of the RhNAC100 transgene is inversely correlated with petal size but positively correlated with abaxial epidermal cell density in transgenic Arabidopsis. The overexpression of RhNAC100 was also observed, and RhNAC100m significantly decreased the length of hypocotyls and roots, as well as leaf size in transgenic plants (P < 0.05, Tukey’s test), particularly in RhNAC100m-ox lines (Supplemental Fig. S5). Surprisingly, overaccumulation of RhNAC100 transcripts repressed the elongation of hypocotyls in transgenic plants with Col-0 and ethylene-insensitive ein2 backgrounds. These results further indicate that RhNAC100 may function as a TF downstream of the ethylene signal.

The role of RhNAC100 was also investigated in rose petal expansion using a virus-induced gene silencing (VIGS) approach (Ma et al., 2008; Spitzer-Rimon et al., 2013).

Figure 4. Petal phenotype of transgenic Arabidopsis overexpressing RhNAC100 and RhNAC100m. A, Petal phenotype of RhNAC100-ox and RhNAC100m-ox with a Col-0 (left) and ein2-1 (right) background. Bar = 200 μm. B, Statistical analysis of petal size of transgenic plants. The bars indicate the so (n > 15 for each line). **Student’s t test, P < 0.01. C, Cell density in petals from transgenic Arabidopsis plants. Petals of stage-14 flowers were fixed using formaldehyde-acetic acid and cleared by ethanol. The distal portion of the petal epidermis was used to analyze the cell number because this portion contains cells that are diploid and uniform in size (Mizukami and Fischer, 2000). The images of abaxial epidermal cells were taken by using a Nikon IX-71. Cell numbers were counted per visual field using ImageJ software. At least 15 flowers were used for each line. Bar = 50 μm. **Student’s t test, P < 0.01. D, Expression of RhNAC100/RhNAC100m in transgenic plants. The qRT-PCR was performed using at least three biological replicates. ACTIN2 was used as the internal control. Bars indicate the so.
et al., 2010; Jia et al., 2011). The \( RhNAC100 \)-specific 3′-untranslated region was chosen to construct a TRV (Tobacco Rattle Virus)-\( RhNAC100 \) vector to specifically silence \( RhNAC100 \) in rose petals (Fig. 5A). Compared with TRV petals \((14.83 ± 1.46 \text{ cm}^2)\), \( RhNAC100 \)-silencing significantly increased petal size (by approximately 19.3%) to \( 17.68 ± 1.58 \text{ cm}^2 (P < 0.05; \text{Fig. 5B}) \). To determine whether the close homologs of \( RhNAC100 \) were also silenced, we developed gene-specific primers to test the transcript abundance of homologous NAC genes in \( RhNAC100 \)-silenced and TRV-infected petals, including RU20317, RU01707, RU04695, RU026684, and RU26219, which are close homologs of \( RhNAC100 \) (Supplemental Fig. S6A). No reductions in transcript abundance were detected (Supplemental Fig. S6B). Therefore, although the possibility cannot be completely excluded that some closely related homologs were down-regulated, these data verify a high degree of target specificity in our VIGS experiments.

In our previous research, we observed that ethylene inhibits cell expansion in the petal AbsE, particularly in the upper petal regions (Ma et al., 2008). Therefore, it was examined whether \( RhNAC100 \) influences cell expansion in the upper petal region. Silencing of \( RhNAC100 \) resulted in a significantly decreased number of AbsE cells per microscope visual field, from \( 181.9 ± 11.8 \) in TRV petals to \( 138.9 ± 10.8 \) in \( RhNAC100 \)-silenced petals \((P < 0.05, \text{Tukey's test}) \), which is a decrease of approximately 23.6% (Figs. 5, C and D). These results suggest that \( RhNAC100 \) plays an important role in ethylene-regulated inhibition of petal cell expansion.

**\( RhNAC100 \) Regulates a Subset of Genes Involved in Cell Expansion**

To investigate how \( RhNAC100 \) regulates cell expansion in transgenic Arabidopsis, the potential downstream target genes of \( RhNAC100 \) were explored by microarray analysis using the ATH1 Genome Array. Overaccumulation of \( RhNAC100 \) transcripts in transgenic Arabidopsis plants (\( RhNAC100m\text{-ox/Col} \)) resulted in significant changes in the expression of 549 genes (164 up-regulated and 385 down-regulated), including genes related to cell wall catabolism/organization, water and solute transportation, and cytoskeleton remodeling (Supplemental Table S5). We chose 29 cell expansion-related rose genes based on their homology with the changed Arabidopsis genes in the \( RhNAC100m\text{-ox} \) plant and quantified their expression in \( RhNAC100 \)-silenced petals. The expression levels of 22 out of the 29 genes tested were altered following \( RhNAC100 \) silencing, and 19 out of those 22 exhibited an inverse expression patterns compared with their homologs in the \( RhNAC100m\text{-ox/Col} \) Arabidopsis plants (Table I). In addition, the expression of these 29 genes were tested in the petals during flower opening. The result showed that most exhibited a substantial change in expression upon flower opening.

Figure 5. Silencing of \( RhNAC100 \) in rose petals by VIGS. A, The qRT-PCR analysis of \( RhNAC100 \) in \( RhNAC100 \)-silenced petals. \( RhACT5 \) was used as the internal control. TRV, Empty vector; TRV- \( RhNAC100 \), TRV containing a fragment of \( RhNAC100 \); control, petals without infiltration. B, Size of \( RhNAC100 \)-silenced petals. These photographs were taken 8 d after infiltration. Each data point indicates the mean ± SD. Six independent experiments were performed; at least 80 petals were used for each treatment per experiment. C, Cell density of AbsE of \( RhNAC100 \)-silenced petals. Bar = 200 \( \mu \text{m} \). Each data point represents the mean ± SD. Different letters indicate significant differences \((P < 0.05) \) calculated using one-way ANOVA followed by Tukey's honestly significant difference test. The cells were counted using visual fields of 1,024 × 1,024 \( \mu \text{m} \). D, The relative petal area and cell density. The petal area and cell density of TRV petals was set to 1.
However, whether these genes are involved in petal development needs to be further tested.

Of the cell wall-related genes, the genes encoding cellulose synthases were focused on because our pathway analysis showed that the cellulose synthetic pathway, which is primarily governed by cellulose synthases, changed significantly following ethylene treatment of rose petals (adjusted P value, 0.00277; Supplemental Table S7). In this pathway, one of the cellulose synthase genes (RU04718) was strongly repressed following ethylene treatment (Supplemental Table S8), whereas the levels of RU04718 transcript increased substantially in RhNAC100-silenced rose petals (Table I). The open reading frame of RU04718 is 3,285 bp in length and encodes a putative cellulose synthase of 1,094 amino acids. This gene was designated RhCesA2 because its closest homolog is CESA2 in Arabidopsis and has been deposited in GenBank with the accession number JQ001775. Phylogenetic analysis showed that RhCesA2 is closely related to the CESA2/CESA5/CESA6/CESA9 subclade of 10 CESA proteins from Arabidopsis (Supplemental Fig. S7A), and the expression of RhCesA2 was repressed by ethylene treatment of rose petals (Supplemental Fig. S7B). To test whether RhCesA2 is a direct downstream target gene of RhNAC100, we isolated the promoter of RhCesA2 and performed an electrophoretic mobility shift assay (EMSA). A previous report showed that NAC protein is capable of binding the core sequence CGT[G/A] or its reverse complementary sequence [C/T]ACG (Tran et al., 2004; Olsen et al., 2005; Balazadeh et al., 2010; Hao et al., 2011). Biotin-labeled DNA probes were designed based on the region –354 to –324 bp upstream of RhCesA2, which contains the core sequence CGTG. Because the N-terminal domains of NAC proteins possess DNA-binding activity, a GST (Glutathione S-transferase)-RhNAC100-N recombinant protein was generated containing the N terminus of RhNAC100 (1–171 amino acids) fused to a GST tag. As shown in Figure 6, two bands were detected using this assay, which may correspond to the RhNAC100 homodimer-DNA complex (top band) and the RhNAC100 monomer-DNA complex (bottom band) that is frequently associated with NAC proteins when they bind the promoter of a target gene (Yamaguchi et al., 2011). Shifted bands were detected when the GST-RhNAC100-N recombinant protein was combined with biotin-labeled DNA probes, whereas no shifted bands were detected when a GST control protein was combined with a biotin-labeled DNA probe. The protein/DNA-binding bands showed a decreasing

(Supplemental Table S6). However, whether these genes are involved in petal development needs to be further tested.

Of the cell wall-related genes, the genes encoding cellulose synthases were focused on because our pathway analysis showed that the cellulose synthetic pathway, which is primarily governed by cellulose synthases, changed significantly following ethylene treatment of rose petals (adjusted P value, 0.00277; Supplemental Table S7). In this pathway, one of the cellulose synthase genes (RU04718) was strongly repressed following ethylene treatment (Supplemental Table S8), whereas the levels of RU04718 transcript increased substantially in RhNAC100-silenced rose petals (Table I). The open reading frame of RU04718 is 3,285 bp in length and encodes a putative cellulose synthase of 1,094 amino acids. This gene was designated RhCesA2 because its closest homolog is CESA2 in Arabidopsis and has been deposited in GenBank with the accession number JQ001775. Phylogenetic analysis showed that RhCesA2 is closely related to the CESA2/CESA5/CESA6/CESA9 subclade of 10 CESA proteins from Arabidopsis (Supplemental Fig. S7A), and the expression of RhCesA2 was repressed by ethylene treatment of rose petals (Supplemental Fig. S7B). To test whether RhCesA2 is a direct downstream target gene of RhNAC100, we isolated the promoter of RhCesA2 and performed an electrophoretic mobility shift assay (EMSA). A previous report showed that NAC protein is capable of binding the core sequence CGT[G/A] or its reverse complementary sequence [C/T]ACG (Tran et al., 2004; Olsen et al., 2005; Balazadeh et al., 2010; Hao et al., 2011). Biotin-labeled DNA probes were designed based on the region –354 to –324 bp upstream of RhCesA2, which contains the core sequence CGTG. Because the N-terminal domains of NAC proteins possess DNA-binding activity, a GST (Glutathione S-transferase)-RhNAC100-N recombinant protein was generated containing the N terminus of RhNAC100 (1–171 amino acids) fused to a GST tag. As shown in Figure 6, two bands were detected using this assay, which may correspond to the RhNAC100 homodimer-DNA complex (top band) and the RhNAC100 monomer-DNA complex (bottom band) that is frequently associated with NAC proteins when they bind the promoter of a target gene (Yamaguchi et al., 2011). Shifted bands were detected when the GST-RhNAC100-N recombinant protein was combined with biotin-labeled DNA probes, whereas no shifted bands were detected when a GST control protein was combined with a biotin-labeled DNA probe. The protein/DNA-binding bands showed a decreasing

### Table I. qRT-PCR analysis of 29 putative downstream genes of RhNAC100 in RhNAC100-silenced rose petals comparing with TRV (petals infiltrated by empty vector)

<table>
<thead>
<tr>
<th>Gene Family ID</th>
<th>Expression Changed Fold</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAC domain TF</td>
<td>RhNAC100</td>
<td>0.08</td>
</tr>
<tr>
<td>Cellulose synthase-like protein</td>
<td>RU04718</td>
<td>8.01</td>
</tr>
<tr>
<td></td>
<td>RU23321</td>
<td>3.32</td>
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<tr>
<td></td>
<td>RU01568</td>
<td>2.41</td>
</tr>
<tr>
<td></td>
<td>RU05842</td>
<td>1.17</td>
</tr>
<tr>
<td>Cellulase</td>
<td>RU22926</td>
<td>4.51</td>
</tr>
<tr>
<td></td>
<td>RU04698</td>
<td>0.92</td>
</tr>
<tr>
<td>β-1,3-glucanase-like protein</td>
<td>RU05209</td>
<td>0.12</td>
</tr>
<tr>
<td>Endoxylloglucan transferase</td>
<td>RU00964</td>
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</tr>
<tr>
<td></td>
<td>RU04847</td>
<td>7.62</td>
</tr>
<tr>
<td></td>
<td>RU06454</td>
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<tr>
<td>Expansin</td>
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</tr>
<tr>
<td>Extensin</td>
<td>RU02382</td>
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</tr>
<tr>
<td>Pectinesterase</td>
<td>RU03736</td>
<td>3.44</td>
</tr>
<tr>
<td>Xyloglucan endotransglycosylase-related protein</td>
<td>RU00040</td>
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<tr>
<td></td>
<td>RU10722</td>
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<td></td>
<td>RU19414</td>
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</tr>
<tr>
<td></td>
<td>RU20002</td>
<td>11.66</td>
</tr>
<tr>
<td>Aquaporin</td>
<td>RHPI1P1;1</td>
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<tr>
<td></td>
<td>RHPI2P1;1</td>
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</tr>
<tr>
<td></td>
<td>RU34590</td>
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<tr>
<td>Sugar transporter</td>
<td>RU04906</td>
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<tr>
<td></td>
<td>RU08526</td>
<td>0.11</td>
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<tr>
<td></td>
<td>RU04803</td>
<td>1.51</td>
</tr>
<tr>
<td>Putative microtubule protein</td>
<td>RU20999</td>
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<tr>
<td>Actin depolymerizing-factor-like protein</td>
<td>RU03106</td>
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<td></td>
<td>RU06169</td>
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<td>Formin</td>
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<td>Kinesin</td>
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<tr>
<td>Profilin</td>
<td>RU22810</td>
<td>1.15</td>
</tr>
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</table>
Figure 6. Binding test of RhNAC100 to promoters of its downstream targets in vitro (A) and in vivo (B–D). A, Gel-shift analysis of RhNAC100 N terminus binding to RhCesA2, RhPIP1;1, and RhPIP2;1 promoters. Purified protein (1 μg) was incubated with 25 picomoles biotin-labeled probes or mutant probes (mP). For the competition test, nonlabeled probes at varying concentrations (from 10- to 1,000-fold excess) or labeled mutant probes (1,000-fold) were added to the above experiment. B, Schematics of the promoter sequences of RhCesA2, RhPIP1;1, and RhPIP2;1. The core sites and regions used for the ChIP assays are indicated. C, ChIP-qPCR analysis of RhCesA2, CESA7, DREB1A, RhPIP1;1, CESA7, DREB1A, RhPIP2;1, and CESA7 in mock and GFP control. D, Relative expression levels of CESA7, DREB1A, and RhPIP1;1 in mock and GFP control.
signal as the concentration of cold competitor DNA probes increased. However, the GST-RhNAC100-N protein did not bind to the biotin-labeled and mutated DNA probe, and a 1,000-fold excess of unlabeled and mutated probes was incapable of weakening the binding of the GST-RhNAC100-N protein to the labeled probes (Fig. 6A). These results indicate that RhNAC100 binds to the promoter of RhCesA2. In addition, the core sequence CGT[G/A] was noted in the promoter of two aquaporin genes, those of RhPIP1;1 (CGTG) and RhPIP2;1 (CGTG; ACE600220). RhNAC100 is capable of binding to the promoters of RhPIP1;1 and RhPIP2;1 (Fig. 6A). To confirm the binding of RhNAC100 to promoters of downstream genes in vivo, a chromatin immunoprecipitation (ChIP) assay was performed using mesophyll protoplasts of transgenic Arabidopsis plants expressing ProRhCesA2:GUS, ProRhPIP1;1:GUS, or ProRhPIP2;1:GUS. The protoplasts were transfected with 35S:RhNAC100-GFP. RhNAC100 binding fragments were enriched following immunoprecipitation with an anti-GFP antibody. Promoter fragments containing the core sequence of all three downstream genes, RhCesA2, RhPIP1;1, and RhPIP2;1, were enriched (Fig. 6, B–D), indicating that RhNAC100 can bind to the promoters of RhCesA2, RhPIP1;1, and RhPIP2;1 in vivo.

The silencing of RhPIP1;1 (Chen et al., 2013) or RhPIP2;1 (Ma et al., 2008) substantially inhibited abaxial epidermal cell expansion in rose petals. To investigate whether RhCesA2 is also involved in rose petal expansion, RhCesA2 was silenced in rose petals using a 377-bp RhCesA2-specific fragment using the VIGS approach. Silencing of RhCesA2 decreased the petal area by 13.6% and increased the AbsE cell density by 16.2% in the upper petal region compared with the TRV control (Supplemental Fig. S7, C–F; P < 0.05, Tukey’s test).

DISCUSSION

Floral Transcriptome and Ethylene-responsive Profiles in Rose Petals

Despite the economic and cultural importance of rose, genomic resources for this crop species are relatively limited. During the past decade, genomics approaches have been applied in rose, and several EST libraries from rose petals were reported (Chanelière et al., 2002; Guterman et al., 2002; Bendahmane et al., 2013). In 2008, approximately 10,000 ESTs were deposited in public databases, including GenBank and Genome Database for the Rosaceae (http://www. rosaceae.org/). Based on these data, expression profiles of 4,765 transcripts were detected in rose from floral transition to flower senescence using a newly developed Affymetrix microarray (Dubois et al., 2011). Moreover, application of next-generation sequencing technologies greatly promoted the study on rose genomics. A rose transcriptome database was reported recently, which contained more than 30,000 transcripts from three modern rose cultivars and Rosa rugosa (Kim et al., 2012). Lately, a transcriptome data set containing 80,714 transcript clusters was generated by using the RNA from various tissues of Rosa chinensis ‘Old Blush’ and in response to biotic and abiotic stresses (Dubois et al., 2012).

In this work, using 454 technology, approximately 300,000 high-quality rose transcript sequences were generated, which were further assembled into 60,944 unique transcripts, providing a valuable resource for rose functional genomic studies.

In Arabidopsis, the expression of approximately 4% of genes are significantly affected by ethylene, including genes involved in plant defense, ethylene biosynthesis and signaling, and cell wall biosynthesis and degradation (De Paepe et al., 2004). In tomato pericarp, Never ripe, an ethylene insensitive mutation, leads to significant changes in expression patterns of 607 genes, including those involved in protein synthesis and primary metabolic processes (Alba et al., 2005). Here, in rose petals, we obtained 2,189 ethylene-responsive transcripts. Annotation of these transcripts indicated that cell expansion-related processes, such as cell wall synthesis, cell turgidity, and cytoskeleton remodeling, were significantly affected by ethylene treatment.

Multiple hormones largely contribute to similar biological processes (Jaillais and Chory, 2010). Here, we demonstrated that ethylene influences the expression of several TFs and regulator genes that are involved in the signaling of auxin (e.g. ARF and AUX/IAA), gibberellin (e.g. DELLA), and brassinosteroid (e.g. BEE). Recently, a report showed that ARF8 interacted with BIGETALp, a bHLH TF, to regulate both cell proliferation and expansion, ultimately affecting petal size in Arabidopsis (Varaud et al., 2011). In addition, microarray analysis showed that expression of two AUX/IAA genes, one ARF gene, and one Brassinosteroid-regulated protein (Brassinosteroid Upregulated Protein1) gene were significantly altered during flower opening of Rosa spp. (Dubois et al., 2011). The integration of these hormonal signals into a model of petal growth requires further investigation.
The \textit{miR164/RhNAC100} Module Is Involved in Ethylene-Regulated Cell Expansion

In rose flowers, ethylene can reduce the petal size, which is associated with increased AbsE density (Ma et al., 2008). Here, an ethylene-inducible NAC TF gene, \textit{RhNAC100}, was identified in rose petals. Silencing of \textit{RhNAC100} in rose significantly increased the petal size and decreased the AbsE cell density, indicating that \textit{RhNAC100} plays an important role in mediating the ethylene signal to modulate cell expansion. However, our results also indicated that \textit{RhNAC100} might be involved in cell proliferation too, because the petal size change (approximately 50\%) in \textit{RhNAC100m-ox/Col} transgenic plant is larger than the change in density of abaxial epidermal cells (approximately 40\% increase). Conversely, qRT-PCR showed that the level of \textit{RhNAC100} transcript is low during the early stages of petal opening (stages 0 and 2), when growth is mainly driven by cell division (Yamada et al., 2009). Therefore, despite that \textit{RhNAC100} might be able to affect cell proliferation, it could not substantially affect the cell proliferation in natural developmental progress in rose petals.

Meanwhile, the possibility cannot be ruled out that other factors may act synergistically with \textit{RhNAC100} to regulate cell expansion. Given that at least 105 TFs are responsive to ethylene in rose petals, it is likely that other TFs may also be involved in ethylene-regulated cell expansion.

In Arabidopsis, a subset of NAC TFs is targeted by the well-conserved \textit{miR164} family (Jones-Rhoades and Bartel, 2004; Sieber et al., 2007). Although the \textit{miR164} targeted site and the mechanism of \textit{miR164}-mediated cleavage are conserved, \textit{miR164} can participate in various developmental processes by targeting different NAC genes. In Arabidopsis roots, the \textit{miR164/NAC1} module is involved in lateral root initiation (Guo et al., 2005). The \textit{miR164/CUC1/CUC2} module governs meristem initiation and organ separation and postembryonic boundary maintenance by primarily controlling the cell division process in shoots of Arabidopsis (Jones-Rhoades and Bartel, 2004; Laufs et al., 2004; Peaucelle et al., 2007; Raman et al., 2008). Over-accumulation of the \textit{miR164}-resistant version of \textit{CUC2} mRNA results in a longer period of cell proliferation (Peaucelle et al., 2007; Larue et al., 2009). Interestingly, the \textit{miR164/CUC2} module is also considered to influence cell elongation in internodes during inflorescence development. \textit{CUC2} can preserve cell proliferation but represses cell elongation during embryogenesis in Arabidopsis. \textit{MiR164} is thought to cleave \textit{CUC2} transcripts in internode cells to enable rapid growth of the internode at later stages of inflorescence development (Peaucelle et al., 2007). However, in rose petals, \textit{miR164/RhNAC100} regulates cell expansion differently. In early period of flower opening (stages 0–2), levels of \textit{miR164} are high, thereby preventing the accumulation of \textit{RhNAC100} transcripts in petals and ensuring rapid cell expansion and petal growth. Later (stages 2–4), \textit{miR164} abundance decreases as ethylene production increases, resulting in an accumulation of \textit{RhNAC100}, which restricts cell expansion and slows the rate of petal growth. These different modes of action indicate that temporal and/or spatial-specific expression patterns contribute to the diverse roles of \textit{miR164} during multiple developmental events. The possibility cannot be also ruled out that \textit{RhNAC100} may be regulated by multiple factors at both the transcriptional and posttranscriptional level. \textit{RhNAC100} also influences the organ size of hypocotyls, roots, and leaves in transgenic Arabidopsis, but further investigation is necessary to clarify whether the \textit{miR164}/\textit{RhNAC100} module is involved growth of other organs in rose.

Some miRNAs have been implicated in the regulatory network of several phytohormones, such as abscisic acid and GA (Rhoades et al., 2002; Achat et al., 2004; Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004; Khraiwesh et al., 2012). The \textit{miR164/ORE1} \textit{(ANAC092/AINAC2)} miRNA regulates aging-induced cell death in leaves of Arabidopsis and can be influenced by mutating \textit{EIN2} (Kim et al., 2009). In rose petals, ethylene greatly reduces the abundance of \textit{miR164} while increasing the levels of \textit{RhNAC100} (Fig. 2). It has been demonstrated that a loss-of-function mutation in \textit{EIN2} leads to an increase in \textit{miR164} abundance in Arabidopsis leaves (Kim et al., 2009). The \textit{miR164}-directed cleavage of \textit{RhNAC100} transcripts was substantially enhanced in transgenic Arabidopsis of an \textit{ein2-1} background, whereas cleavage of the \textit{miR164}-resistant version of \textit{RhNAC100} was undetected in both Col-0 and \textit{ein2-1} plants. All of these results indicate that ethylene may fine-tune the balance between \textit{miR164} and \textit{RhNAC100}.

Here, the abundance of \textit{miR164} was increased by auxin and GA, and the expression of \textit{RhNAC100} was suppressed by auxin and GA (Supplemental Fig. S3). Therefore, the balance between \textit{miR164} and \textit{RhNAC100} may be influenced by multiple hormones rather than solely by ethylene in rose petals. To date, auxin is known to regulate \textit{miR164}, while it is unclear whether GA can regulate \textit{miR164}. In Arabidopsis roots, auxin is capable of increasing the abundance of \textit{miR164}, which targets \textit{NAC1} and consequently inhibits lateral root initiation (Guo et al., 2005). In Arabidopsis leaves, a CINCINNATA-like TF, TEOSINTE BRANCHED1, CYCLOIDEA, and PCF3, directly activates the expression of \textit{MIR164}, ASYMMETRIC LEAVES1, and auxin response repressor gene \textit{IAA3/SHORT HYPOCOTYL2}, which cooperatively repress the accumulation of \textit{CUC} gene transcripts (Koyama et al., 2007). However, there is no evidence to show how ethylene and auxin interact to regulate \textit{miR164}. Interestingly, ethylene is known to work with auxin synergistically during plant development, although ethylene typically acts as an antagonist to auxin (Pierik et al., 2006). Ethylene stimulates auxin biosynthesis and basipetal auxin transport to zones of elongation (Ruzicka et al., 2007), and the effects of ethylene on the inhibition of
cell elongation in elongation zones are enhanced when auxin is present (Swarup et al., 2007). By contrast, the functions of auxin or ethylene on root inhibition are partially overlapped. In particular, some ethylene-mediated inhibition of root growth is auxin independent (Stepanova et al., 2007; Swarup et al., 2007). Meanwhile, ethylene is capable of modulating the bioactive level of GA and the protein stability of DELLA proteins, including GIBBERELLIC-ACID INSSENSITIVE and REPRESSOR OF GA1-3 in Arabidopsis (Achard et al., 2003; Achard et al., 2007; Vandebussche et al., 2007; Weiss and Ori, 2007). Here, miR164 abundance was increased by GA, whereas the expression of RhNAC100 was reduced in rose petals. It remains unclear whether the effects on the miR164/RhNAC100 module by GA are ethylene dependent. Notably, cross regulation by ethylene, auxin, and GA is highly tissue specific (Yoo et al., 2009; Muday et al., 2012). For example, ethylene and GA have antagonistic roles in the floral meristem to modulate floral transition (Achard et al., 2007), whereas ethylene-induced formation of the apical hook requires GA and auxin in etiolated seedlings (Vriessen et al., 2004; Stepanova et al., 2008). Therefore, the type of cross regulation by ethylene, auxin, and GA that occurs in rose petals and whether miR164 functions as a mediator to integrate these hormone signals require further study.

RhNAC100 Regulates the Expression of Cell Expansion-Related Genes

Cell expansion is a comprehensive biological process requiring a highly efficient coordination of cell wall growth, turgor modulation, and cytoskeleton remodeling. Here, we found that the expression level of 22 functional genes that may participate in the cell expansion was affected by RhNAC100 in rose petal. Three of these genes, two aquaporin (RhPIP1;1 and RhPIP2;1) and one cellulose synthase gene (RhCesA2), were identified as the possible direct downstream genes of RhNAC100 both in vitro and in vivo.

During the cell expansion process, enlargement in cell volume is driven by cellular water uptake, which results from turgor relaxation accompanied by a loosening of the cell wall. Aquaporin serves as the primary channel enabling water uptake across biological membranes. Previously, we observed that two plasma aquaporins, RhPIP2;1 and RhPIP1;1, are involved in cell expansion (Ma et al., 2008; Chen et al., 2013). Our current results imply that RhNAC100 is likely to be involved in turgor modulation.

However, the role of RhNAC100 in cell wall loosening and synthesis is unclear. Previously, several NAC genes from the VND/NSST/SND1 subfamily were reported to regulate cellulose syntheses involved in the cellulose synthesis of the secondary cell wall (Mitsuda et al., 2005; Zhong et al., 2006, 2010; Ko et al., 2007). A recent report showed that cell wall-related genes were highly enriched and up-regulated in the anac2/anac092/ore1 mutant, indicating that ANAC092/AtNAC2/ORE1 plays a role in the modification of the cell wall (Balazadeh et al., 2010). In addition, based on the current model, different CESAs proteins form a rosette complex to facilitate the synthesis of cellulose, and the cellulose in the primary and secondary cell walls is synthesized by different sets of cellulose synthases. Deposits of cellulose in the secondary cell wall but not in the primary cell wall typically arise when the cell stops expanding and results in a restriction of cell growth. In Arabidopsis, CESAs2, CESAs5, CESAs6, and CESAs9 contribute to cellulose synthesis for the primary cell wall, whereas CESAs4, CESAs7, and CESAs8 contribute to cellulose synthesis for the secondary cell wall (Taylor, 2008).

Here, RhNAC100 was able to bind to the promoter of RhCesA2 and probably suppressed the expression of RhCesA2, a homolog of Arabidopsis CESA2. The cesa2 mutant exhibits stunted growth of hypocotyls due to reduced cell expansion caused by a defect in cellulose synthesis (Chu et al., 2007).

Notably, our current data are insufficient to provide a complete view of the role of RhNAC100 during cell expansion. The 29 genes that were tested in this study represent only a small fraction of the total possible number of genes involved because many other genes could be involved in cell expansion. For instance, we noted that the expression of four xyloglucan endotransglycosylase/hydrolase (XTH) genes decreased in RhNAC100-silenced petals, whereas they showed different patterns and levels of expression during the flower opening (Table 1). XTH is a large gene family and is thought to be involved in varying, and in some cases opposing, functions, such as wall loosening, wall strengthening, the integration of new xyloglucans into the wall, and the hydrolysis of xyloglucans (Cosgrove, 2005). Thus, it will be necessary to confirm the actual XTH genes are downstream to RhNAC100 and to clarify their actual function in petal cell expansion. In addition, it would be interesting to investigate whether other cell wall growth-related proteins, such as expansins, pectin methylesterase, and endo-1,4-β-D-glucanase, are also involved in ethylene-regulated cell expansion. When rose genome becomes available, a chromatin-immunoprecipitation followed by massively parallel sequencing assay will assist in establishing the RhNAC100-dependent regulatory network involved in cell expansion.

miR164/NACs Might Be Conserved Module for Petal Development

Petal development is an important process for life cycle in most higher plants. In present work, an NAC TF gene, RhNAC100, was found to be able to regulate petal expansion in rose. In addition, overexpression of RhNAC100 also influenced petal size in transgenic Arabidopsis in a miR164-dependent manner. In
Arabidopsis, although the exact function of ANAC100 (AT5G61430), the closest homolog of RhNAC100, is still unknown, two homologs, CUC1 and CUC2, were found to be involved in petal initiation and petal number decision (Aida and Tasaka, 2006). Similarly, mutation of miR164 also resulted in abnormal petal number and phenotype (Mallory et al., 2004; Baker et al., 2005; Sieber et al., 2007). Considering that miR164 is highly conserved in plants, the miR164/NAC module might play a conserved role in petal development in plants, although different NAC members probably function in different petal development phases, such as petal initiation, petal expansion, etc.

In conclusion, we created a rose floral transcriptome and exploited it to identify the potential key regulators for ethylene-influenced cell expansion. We identified an NAC domain TF, RhNAC100, whose expression is highly inducible by ethylene. RhNAC100 is a negative regulator for petal cell expansion in rose and is able to influence petal growth when overexpressed in Arabidopsis. Detailed genetic and biochemical analysis revealed that RhNAC100 was a target of miR164 and that ethylene elevated the accumulation of RhNAC100 transcript by blocking miR164-guided cleavage of RhNAC100. RhNAC100 regulated genes related to cell expansion, such as cellulose synthase and water transporter (aquaporin). We provide evidence for a TF/miRNA-based genetic pathway in the control of cell expansion by ethylene.

MATERIALS AND METHODS

Plant Materials

Flowers were harvested from rose (Rosa hybrida) ‘Samantha’ at stage 2 of floral opening (Ma et al., 2005). The flowers were immediately placed in tap water after harvesting and transported to the laboratory within 1 h. After cutting to 25-cm lengths under water, the flowers were placed in deionized water. The flowers were treated with 10 μL L⁻¹ ethylene and 2 μL L⁻¹ 1-MCP in a sealed airtight chamber, and flowers exposed to air were used as a control. Treatments were conducted at 23°C to 25°C, and 1 mol L⁻¹ NaOH was included in the chamber to prevent the accumulation of CO₂ (Ma et al., 2006). Treatment time points were 0, 0.5, 1, 2, 6, 12, 18, 24, and 48 h for the 454 transcriptome sequencing and 0, 1, 6, 12, 18, and 24 h for microarray analysis.

Arabidopsis (Arabidopsis thaliana) seeds were surface sterilized, plated on Murashige and Skoog medium, and imbibed in the dark at 4°C for 2 d. The plates were transferred to a growth room maintained at 21°C to 22°C at 60% relative humidity under a 16-h-light/8-h-dark photoperiod. After 7 d, seedlings were transplanted in pots containing a 1:1 mixture of vermiculite and peat moss and grown under the same conditions. For ethylene treatment, 2-week-old Arabidopsis seedlings were exposed to 10 μL L⁻¹ ethylene for 24 h in an airtight chamber. For naphthaleneacetic acid (NAA), GA₃, and brassinosteroid treatments, roses at stage 2 were treated with 20 μM NAA, 25 μM or 10 μM brassinosteroid for 24 h at 23°C to 25°C. Mock samples were treated with dimethyl sulfoxide without any phytohormones.

Microarray Construction, Hybridization, and Data Analyses

The unisequences were selected for designing the microarray primarily based on their annotations, digital expression patterns, and abundance obtained from the corresponding EST counts. First, the unisequences were screened by digital expression patterns and corresponding EST counting. Digitally differential expression was computed by comparison of reads from ethylene-treated rose flowers and untreated controls using IDEG6 software (Romualdi et al., 2003). The results were tested using a Fisher exact test with Bonferroni correction, and 5,652 unisequences were selected. Second, all unisequences that were annotated as regulatory factors, such as TF, TR, kinase, and phosphatase, were selected, regardless of their digital expression patterns and corresponding EST counting, and 10,922 unisequences were selected. Based on the total number of selected unisequences (16,574), we chose the Agilent 8X15 K microarray format. All 16,574 unisequences were sent to Agilent to design probes using eArray (https://earray.chem.agilent.com/earray/). Probe designs failed for a total of 1,577 unisequences due to their short length and low sequence specificity, leaving a total of 14,997 unisequences in the final probe set.

Cy3-labeled cDNA samples were denatured independently and used for the microarray assay. All hybridizations used three biological replications with only one exception; the hybridization of the 12-h ethylene-treated sample was conducted for only two biological replicates. Fluorescence intensities on the scanned images were quantified. The resulting values were corrected for background nonspecific hybridization and normalized using the quantile normalization method (Bolstad et al., 2003). Differentially expressed genes between ethylene-treated and control flower petals at each time point were identified using the moderated t statistics in the LIMMA package (Bolstad et al., 2003). Raw P values were adjusted for multiple testing using the FDR (Benjamini and Hochberg, 1995). Genes with FDR of less than 0.05 and fold changes in expression of at least 2 were considered differentially expressed genes. Differentially expressed genes were clustered using Cluster 3.0 (de Hoon et al., 2004) using the average linkage method and, the Pearson correlation was used as the distance metric. The clustered gene expression profiles were visualized using Treeview (Eisen et al., 1998). GO, biochemical pathway, and differential expression data sets were imported into the Plant MetGenMAP system (Joung et al., 2009) to identify significantly changed pathways (adjusted P value < 0.05). The entire microarray data set has been deposited into the rose transcriptome database (http://bioinfo.bti.cornell.edu/rose).

For possible downstream gene screening of RhNAC100, total RNA from Arabidopsis wild-type Col-0 and transgenic plants overexpressing RhNAC100 were extracted and labeled, and hybridization was performed using the Affymetrix ATH1 Genome Array.

Sequence Analysis

Alignment of the amino acid sequence and phylogenetic analysis was performed using ClustalW and MEGA 4.0, respectively. The phylogenetic trees were computed using the neighbor-joining algorithm with 10,000 bootstrap replications.

qRT-PCR

For qRT-PCR, 1 μg of DNase-treated RNA was used to synthesize cDNA according to the manufacturer’s instruction for the reverse transcription system (Promega) using a 25-μL reaction volume. Two microfilters of cDNA was used as the template in a 20-μL PCR reaction, and the Bio-Rad CFX96 real-time PCR detection system was used in standard mode with the Bio-Rad iQ SYBR-Green Supermix. Each reaction was performed in triplicate, and products were verified by melting curve analysis. The abundance of mRNA and miRNA was analyzed using the relative standard curve method normalized to the R. hybrida actin5 and 5S ribosomal RNA, respectively. All reactions were performed with at least three biological replicates.

VIGS in Rose Petals

RhNAC100 silencing was performed as previously described (Ma et al., 2008). A 320-bp fragment at the 3′ end of RhNAC100 was used to generate the pTRV2-RhNAC100 construct. pTRV2-RhNAC100, pTRV1, and pTRV2 vectors were transformed into Agrobacterium tumefaciens GV3101. A. tumefaciens was grown in Luria-Bertani broth containing 30 μg mL⁻¹ kanamycin and 50 μg mL⁻¹ gentamycin sulfate at 28°C with shaking at 200 rpm overnight. These cultures were diluted 1.5 (v/v) in fresh Luria-Bertani broth containing 10 mM MES, 20 mM acetoxyringsone, 50 μg mL⁻¹ kanamycin, and 50 μg mL⁻¹ gentamycin sulfate and grown overnight as described above. A. tumefaciens cells were harvested by centrifugation at 4,000 rpm for 10 min and were suspended in infiltration buffer (10 mM MgCl₂, 150 mM acetoxyringsone, and 10 mM MES, pH 5.6) to a final optical density at 600 nm of approximately 1.5. A mixture of A. tumefaciens
cultures containing pTRV1 and pTRV2-RhNAC100 at a ratio of 1:1 (v/v) and a mixture containing pTRV1 and pTRV2 at a 1:1 ratio (the negative control) were stored at room temperature for 4 h in the dark prior to vacuum infiltration. For vacuum infiltration, three to six pieces of petals were picked from the second and third whorl of each stage 1.5 flower, and they were randomly assigned to different treatments. For each treatment, at least 80 petals with six replicates were used. The petals were immersed in the bacterial suspension solution and infiltrated at a vacuum of 0.7 atm. After releasing the vacuum, the petals were washed with deionized water, maintained vertically in deionized water for 3 d at 8°C, and then transferred to 23°C for 5 d. Photos of the petals were taken daily, and petals were picked for RNA isolation on the eighth day after infiltration. Petal size was measured from the photos using ImageJ software. Statistical testing was performed using SPSS software.

Microscopic Examination and Cell Counting

For rose, AbsE cell photography and cell counting were performed as described previously (Ma et al., 2008). Briefly, discs of 3-mm diameter petal at 25% of the petal length from the petal tip were taken. The discs were fixed in formaldehyde-acetic acid (3.7% [v/v] formaldehyde, 5% [v/v] glacial acetic acid, and 50% [v/v] ethanol) and cleaned using ethanol. AbsE cells from the discs were photographed using a Nikon IX-71 camera. For Arabidopsis, petals of stage-14 flowers were fixed using formaldehyde-acetic acid and cleared by ethanol. The distal portion of the petal epidermis was used to analyze the cell number because this portion contains cells that are diploid and uniform in size (Mizukami and Fischer, 2000). The images of abaxial epidermal cells were taken by using a Nikon IX-71 camera. Cell numbers were counted per visual field using ImageJ software.

Northern Hybridization

Northern blotting was performed as described previously (Ma et al., 2006). A gene-specific probe was generated using a PCR DIG Probe Synthesis Kit (Roche). Hybridization was performed overnight at 43°C. The chemiluminescent reaction was performed using CDP-Star according to the manufacturer’s protocol (Roche). All hybridizations were performed using at least three biological replicates.

EMSA

EMSA s were performed according to a recent report (Wang et al., 2011). Briefly, the N terminus of RhNAC100 was fused in frame to GST and expressed in Escherichia coli BL21. The fused protein was induced by adding isopropylthio-β-galactoside (0.2 mM), and the cells were incubated at 170 rpm for 6 h at 28°C. The recombinant protein was purified using Glutathione Sepharose 4B (GE Healthcare) according to the manufacturer’s instructions. EMSAs were performed using the LightShift chemiluminescent EMSA kit (Pierce) according to the manufacturer’s instructions.

ChIP Assay

ChIP assays were performed using Arabidopsis prooplasts as described previously (Hao et al., 2012). The mesophyll prooplasts were isolated from Arabidopsis leaves (Col-0) transformed with RhCesA2, RhPIP1;1, or RhPIP2;1 promoters. The plasmid 35S:RhNAC100-GFP was transfected into the prooplasts. After transfection and incubation at room temperature overnight, the prooplasts were fixed for 20 min using formaldehyde (final concentration, 1% [v/v]). The chromatin complex was fragmented by sonication and incubated with anti-GFP antibody-Protein A beads overnight. After washing, the beads were incubated at 65°C for 6 h for reverse cross linking, and the coprecipitated DNA was purified. The DNA was analyzed using semiquantitative reverse transcription-PCR and quantitative real-time PCR using the Bioline SYBR master mix (Bio-Rad). Primers for the ChIP-PCR are listed in Supplemental Table S1.

The GenBank accession numbers of the sequences are as follows: RhNAC100 (JQ001774), RhCesA2 (JQ001775), ATNAC2 (NP_198767), ATNAC3 (NP_189546), ANAC100 (NP_200951), ANAC080 (NP_568182), ANAC087 (NP_197328), ANAC046 (NP_187056), CUC2 (NP_200206), CUC1 (NP_188135), CUC3 (NP_377768), NAC1 (NP_175997), and ANAC074 (NP_567811).

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Generation and assembly of high-quality sequences from rose flowers.

**Supplemental Figure S2.** Alignment and phylogenetic tree of RhNAC100 with known NAC-domain proteins, expression in different organs, and transcription activity assay of RhNAC100.

**Supplemental Figure S3.** qRT-PCR of RhNAC100 and rhy-miR164 in petals treated by NAA, CA, or brassinosteroid.

**Supplemental Figure S4.** Promoter analysis of RhNAC100 and region amplification-PCR of RhNAC100 during flower opening.

**Supplemental Figure S5.** Phenotype of transgenic Arabidopsis plants overexpressing RhNAC100 and RhNAC100m.

**Supplemental Figure S6.** qRT-PCR of five RhNAC100 homologous genes in RhNAC100-silenced petals.

**Supplemental Figure S7.** Functional characterization of a RhNAC100 downstream gene, RhCesA2, in rose petals.

**Supplemental Table S1.** Oligonucleotide primer sequences used in this work.

**Supplemental Table S2.** GO slim classification of rose assembled transcripts.

**Supplemental Table S3.** List of 2,189 ethylene-responsive assembled transcripts in rose petals.

**Supplemental Table S4.** List of ethylene-responsive TFs and TRs.

**Supplemental Table S5.** Changes in gene expression in transgenic Arabidopsis plants overexpressing RhNAC100m.

**Supplemental Table S6.** qRT-PCR of 29 putative downstream genes of RhNAC100 in petals during natural flower opening.

**Supplemental Table S7.** List of biochemical pathways altered in rose petals in response to ethylene.

**Supplemental Table S8.** List of cellulose synthase genes significantly down-regulated by ethylene in rose petals.

**Supplemental Text S1.** Supplemental Materials and Methods.

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**LITERATURE CITED**


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